RÔLE DES MICROPARTICULES MEMBRANAIRES DANS LA PHYSIOPATOLOGIE DE LA MALADIE DE CROHN: EFFECTS SUR LA VASOMOTRICITE ET LES TISSUES CIBLES

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ROLE OF MICROPARTICLES IN THE PATHOPHYSIOLOGY OF CROHN'S DISEASE: EFFECTS ON VASOMOTRICITY AND TARGET TISSUES

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Abbreviations

AA: arachidonic acid

Ach: acetylcholine

ActD: actinomycin D

CDMPs: MPs isolated from plasma of Crohn's disease patients

CMH: 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin

COX: cyclooxygenase

COX-1: cyclooxygenase-1

COX-2: cyclooxygenase-2

DETC: Fe^{2+} diethyldithiocarbamate

EDHF: endothelium-derived hyperpolarizing factor

EMPs: endothelial microparticles

EPCs: progenitor cell cultures

EPR: electronic paramagnetic resonance

eNOS: endothelial nitric oxide synthase

GP: glycoprotein

HSMPs: MPs isolated from plasma of healthy subjects

5-HT: serotonin

HUVEC: human umbilical vein endothelial cells

IBD: inflammatory bowel disease

ICAM-1: intercellular adhesion molecule-1

Ig: immunoglobulin

iNOS: inducible nitric oxide synthase

IL-1α: interleukin-1 α

IL1-β: interleukin-1 β

IL6: interleukin-6

IL-8: interleukin-8

IL-12: interleukin-12

IL-17: interleukin-17

IL-18: interleukin-18

IL-21: interleukin-21

IL-23: interleukin-23

IL-23R: interleukin-23 receptor

IFN-γ: interferon-γ

L-Arg: L- Arginine

L-NA: N^G-nitro-L-arginine

MAP: *Mycobacteriu avium* subspecies *paratubercolosis*

M cells: microfold cells

MCP-1: monocyte chemoattractant protein-1

MnTMPyP: manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin

MPs: microparticles

NADPH: nicotinamide adenine dinucleotide phosphate

NO: nitric oxide

NS-398: *N*-2-cyclohexyloxy-4-nitrophenyl

OSA: obstructive sleep apnea

PAI-1: plasminogen activator inhibitor-1

PAF: platelet activating factor

PDE4: phosphodiesterase 4

PFP: platelet-free plasma

PGI_2: prostacyclin

PHA: phytohemagglutinin

PKC: protein kinase C

PMA: phorbol-myristate-acetate

PMPs: platelet-derived MPs

PPARγ: peroxisome proliferator activated receptor γ

PS: phosphatidylserine

ROCK: Rho-associated kinase

ROS: reactive oxygen species

SC-560: 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole

Shh: sonic hedgehog

SOD: superoxide dismutase

SNP: sodium nitroprusside

TAFI: thrombin-activatable fibrinolysis inhibitor

TGFbeta1: transforming growth factor beta1

TF: tissue factor

Th1: T helper 1 cells

Th2: T helper 2 cells

Th9: T helper 9 cells

Th17: T helper 17 cells

Th22: T helper 22 cells

TNF-α: tumour necrosis factor α

Treg: T regulatory cells

Publications

Publications in revision:

Review:

S. Tual-Chalot, D. Leonetti, R. Andriantsitohaina, MC. Martínez. Microvesicles: intercellular vectors of biological messages. Soumis à *Molecular Interventions*

Publications under submission:

D. Leonetti, AL. Bretagne, A. Tesse, MC. Martinez, S. Viennot, JM. Reimund, R. Andriantsitohaina. Microparticles are relevant markers of Crohn's disease activity and cause endothelial and vascular dysfunctions. Soumis à *Gastroenterology*

D. Leonetti, AL. Bretagne, M. Chalopin, MA. Panaro, MC. Martinez, JM. Reimund, R. Andriantsitohaina. Circulation microparticles from Crohn's disease patients exert differential effects on nitric oxide and superoxide anion productions, and inflammatory markers. Soumis à *The American Journal of Gastroenterology*

Part I

Crohn's disease

I. General aspects

Crohn's disease is, with ulcerative colitis, one of the major forms of inflammatory bowel disease (IBD). The cause of IBD is still unknown but several studies have shown that this pathology is characterised by an abnormal activation of the mucosal immune system in response to bacterial flora or infectious agents which is linked to alteration of barrier function of the intestinal epithelium.

This chronic inflammatory disorder is due to a transmural inflammation that extends through all layers of the bowel wall and can affect the entire gastrointestinal tract, with greater involvement of colon and terminal ileum (Fig 1a). Histopathological features include an aggregation of macrophages that frequently form, with giant cells and epithelioid cells, non-caseating granulomas (Fig 1b).

The clinical presentation is largely dependent on disease location and can include diarrhoea, abdominal pain, fever, clinical signs of bowel obstruction, as well as passage of blood or mucus or both. Crohn and coworkers described the first time the inflammation of the large intestine either localized or diffuse in 1952. Indeed, they designated a disease of the terminal ileum, affecting mainly young adults, characterized by a sub-acute or chronic necrotizing and cicatrizing inflammation (Crohn *et al*., 1952).

The definition of the disease has been expanded in next ten years as a granulomatous inflammation of the rest of the gastrointestinal tract (including the colon) and distinguishing them from ulcerative colitis on clinical and pathological aspects (Lockhart-Mummery *et al*., 1960).

Even now, the definition of the disease is not entirely clear because the patients show a clinical heterogeneity. The discovery of genetic and serological markers associated with phenotype in IBD and clinical data have revealed the existence of subtypes of Crohn's disease based on the location. These subtypes have been defined as terminal ileal (L1), colonic (L2), ileocolonic (L3) and upper GI (L4) during World Congress of Gastroenterology at Montreal (Silverberg *et al*., 2005).

a **b**

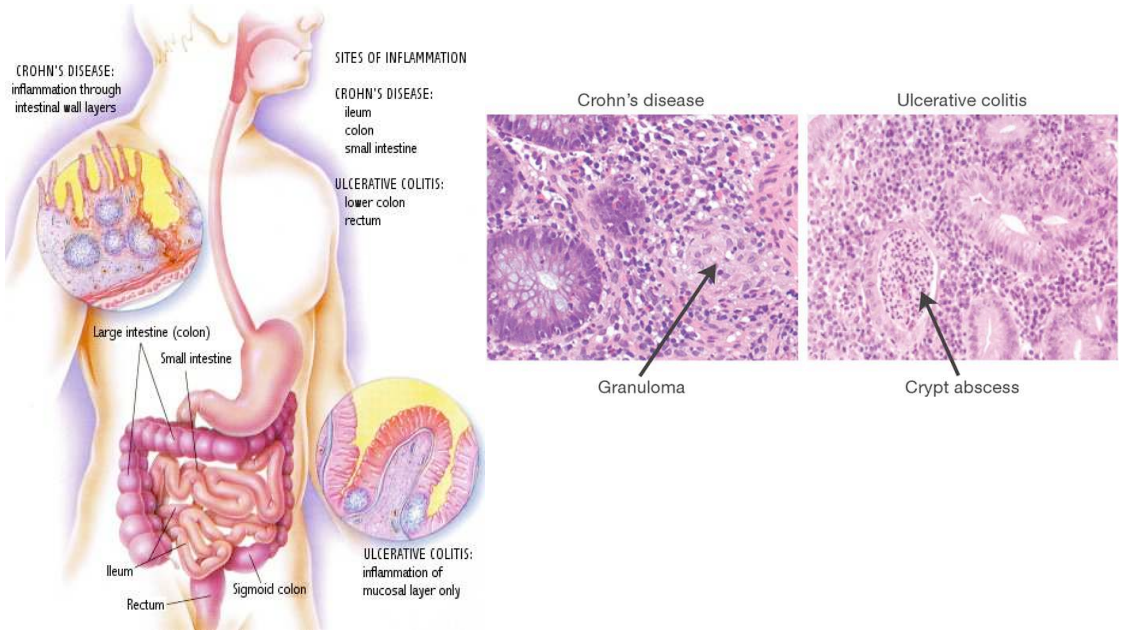

Fig 1: Crohn's disease: (a) Crohn's disease is a transmural inflammation that extends through all layers of the bowel wall and can affect the entire gastrointestinal tract, with greater involvement of colon and terminal ileum; compared to ulcerative colitis that is a diffuse mucosal inflammation. **(b)** Histopathological features of Crohn's disease include granuloma composed of compact macrophages, giant cells and epithelioid cells. Whereas, ulcerative colitis shown crypt abscess composed of transmigrated neutrophils and the surrounding epithelium exhibits features of acute mucosal injury (From Xavier et al., Nature Reviews 2007).

II. Epidemiology

Crohn's disease affects ≈ 0.1% of Western population. Epidemiology analysis showed a higher incidence in North America and Northern and Western Europe than Asia, Africa and South America (Logan and Bowlus, 2010).

Since the beginning of the 20th century, there has been a steady increase in reported cases of both Crohn's disease and ulcerative colitis. This increase in IBD mainly affects the developed countries, especially populations with high living standard and urban areas (Bernstein and Shanahan, 2008). Particularly, Europe has seen a change in the epidemiology of IBD over the past several decades. Recent data showed a stabilization of incidence rates in Northern countries of Europe and a significant increase in some Southern countries tied to an improvement in living standards. In addition, some regions of Europe have shown a particular increase of Crohn's disease. In Northern France, for example, it has been shown, over a 12 year period, a 23% increase in Crohn's disease with a 17% decrease in ulcerative colitis over the same period of time (Molinié *et al*., 2004).

Statistically the frequency of the disease correlates with the introduction of tap water, soap and improvement in the living conditions. The hygiene hypothesis, proposed by Strachan in 1989 (Strachan, 1989), argues therefore, that improved hygiene and a lack of exposure to microorganisms of various types have sensitized our immune system, leading to inadequate reaction to harmless bacteria in our environment (Koloski *et al*., 2008).

The environmental factors play a major role in the development of disease particularly in teenagers or young adults. There are many factors related to the pathogenesis of IBD such as smoking, prenatal events, breastfeeding, childhood infections, microbial agents, oral

contraceptives, diet, hygiene, occupation, education, tonsillectomy, appendectomy, blood transfusions, contact with animals and physical activities.

Among those one of the most studied factors is the smoking. Recent studies showed that smoking interferes with a shortage of zinc present in subjects with Crohn's disease, that may facilitate release of pro-inflammatory mediators and their activities and may cause exacerbates symptoms of disease (El-Tawil, 2010). An interesting study reports that emigrates from an area of low prevalence to one of high prevalence have a similar rate of IBD as before, but their children have an increased risk of IBD (Pinsk *et al*., 2007). This suggests that several factors related to life in a developed country can affect the development of the immune system during infancy.

Finally, Crohn's disease is a pathology that significantly impairs quality of life, requires expensive drugs, surgery or multidisciplinary care and represents a major burden on public health-care resources.

III. Pathogenesis of Crohn's Disease

The pathogenesis of Crohn's disease is still not completely clear but several evidences suggest that the balance between microbes (particularly commensal flora) and host defensive responses at the intestinal mucosal barrier may play an important role.

In healthy individuals, the mucosal immune system represents a complex of interacting mechanisms designed to interpret the environment, distinguish danger from harmless antigenic stimuli, and respond appropriately to maintain internal homeostasis. The maintenance of mucosal homeostasis involves constrained responsiveness to dietary antigens and resident microflora, while retaining the capacity for effective immune responsiveness

against episodic threats from pathogens. Errors in discrimination of danger signals from innocuous stimuli or in regulation of effector immune responses, disrupt mucosal homoeostasis, and predispose the individual to uncontrolled or pathological inflammation (Shanahan, 2000).

Therefore, depending on the genetic susceptibility of the host, the normal intestinal flora may become a liability. The pathogenesis of Crohn's disease is complex and mainly consists of the three interacting elements: genetic susceptibility factors, enteric microflora and immune-mediated tissue injury (Fig 2).

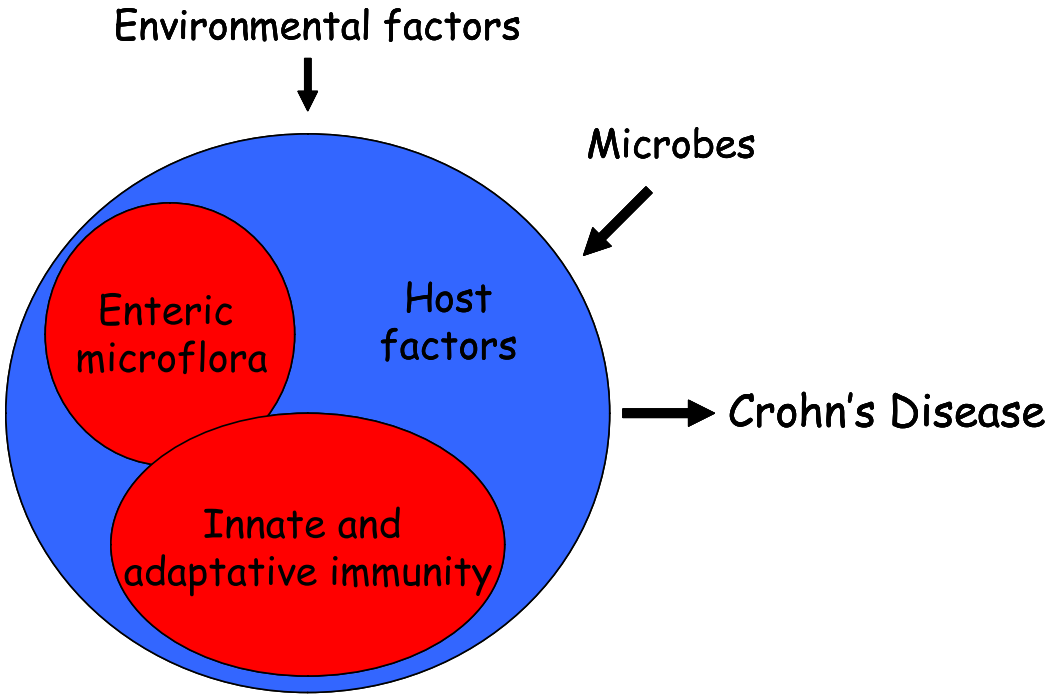

Fig 2: Pathogenesis of Crohn's disease: Intestinal inflammation in Crohn's disease results from alteration in the interaction between resident microbes and the mucosa. This can result from the influence of environmental factors and/ or host factors, which vary depending on genetic inheritance at several susceptibility loci. Genetic factors discovered to date affect barrier function, and innate and adaptive immunity (Modified from Xavier and Podolsky 2007). .

III.1. Genetic factors

Several clinical observation have pointed out that genetic factors contribute to pathogenesis of Crohn's disease:

- First, the ethnic differences in disease frequency have been shown since the incidence and prevalence of Crohn's disease varies widely among different populations.

- Second, a familial aggregation of IBD has been demonstrated. A study of familial occurrence of IBD has established that a risk of IBD in first-degree relatives of an affected patient is 4 to 20 times as high as that among the background population (Orholm *et al*., 1991).

- Third, in patients with Crohn's disease, a higher rate of disease concordance in monozygotic (44%) twins than in dizygotic (3-8%) twin has been observed (Tysk *et al*., 1988).

Also, a manifestation of syndrome resembling IBD in families with rare genetic disorders such as Hermansky-Pudlak syndrome and Turner's syndrome has been demonstrated (Kouklakis *et al*., 2007); (Triantafillidis *et al*., 2010) (Table 1).

All these data suggest a genetic complexity of Crohn's disease, and that the susceptibility of disease is inherited. In recent years, the genome-wide screening for susceptibility genes in Crohn's disease has identified several genes involved in disease. The first study of screening of DNA has identified a candidate susceptibility gene, in patients with Crohn's disease, located at the region of chromosome 16 named NOD2 or IBD1 (or also CARD15). This gene encodes a cytoplasmic protein which is expressed in monocytes and is involved in regulation

of macrophage apoptosis and NF-κB activation that is a transcriptional factor directly involved in activation of immunoinflammatory responses (Ogura *et al*., 2001b).

The patients with Crohn's disease display the mutations of NOD2 gene and this variant appear to be related of reduced macrophage activation of NF-κB in response to lipopolysaccharide (Hugot *et al*., 2001).

Over the years have been identified several other loci particularly, their association with genes coding for factors involved in the regulation of immune and inflammatory response or function of mucosal barrier, such as IBD2 (chromosome 12), IBD3 (chromosome6), IBD4 (chromosome14), IBD5 (chromosome 5q31), IL-23R and susceptibility locus identified on the X chromosome (Vermeire *et al*., 2001; Duerr *et al*., 2006).

The complexity of the genetic component of Crohn's disease is enhanced by a variable role that the different loci may have in different ethnic population. For example, Silverberg et al. (Silverberg *et al*., 2007) confirm the importance of IBD5 to Crohn's disease susceptibility and demonstrate that the locus may play a role in non-Jewish individuals only.

The identification of new genes, and understanding about their interaction, is important for the future because it might provide new information about the cause of the disease. At present, it have been identified more than 30 susceptibility genes and loci associated with disease which led to a better understanding of new pathways involved in the pathogenesis of the disease.

Evidence	Example/ comment
Ethnic differences in disease frequency	The incidence and prevalence of Crohn's disease varies widely among different populations (high prevalence in Jews)
Familial aggregation of IBD	Risk of IBD in first-degree relatives of an affected patient is 4 to 20 times as high as that among the background population
Concordance rate in monozygotic (44%) twins is higher than in dizygotic twins	Contribution of both genetic and enviromental factors
Syndrome resembling IBD in families with rare genetics disorders	Examples of rare genetic disorders: Hermansky-Pudlak syndrome and Turner's syndrome
Linkage with specifical chromosomal regions	Examples: IBD1, IBD2, IBD3, IBD4, IL-23R and locus on X chromosome
Variable role of different loci in different ethnic population.	Example: IBD5 play a role in non-Jewish individuals only

Table1: Evidence of Genetics contributions to Crohn's Disease.

II.2. Enteric microflora

The possibility that Crohn's disease is an inflammatory response against microbial agent has been evaluated in several studies. The development of immune reactivity to enteric bacteria is an entirely physiological phenomenon that starts immediately after birth when the immature intestinal immune system begins to be exposed to a variety of microbial antigens and creates a lifelong state of tolerance against them in order to avoid excessive or detrimental responses (Duerkop *et al*., 2009).

The intestinal mucosa plays a barrier function that physically impedes penetration of macromolecules and intact bacteria. The epithelium is in constant communication with intestinal flora and intestinal epithelial cells express Toll-like receptors, NOD1 and 2 and receptors for different chemokines. In particular, epithelial cells-specific NF-κB seems to play an important role in the suppression or activation of immune response in IBD. Some bacteria seem to be able to modify this process through the reduction of epithelial NF-κB activation by inhibition of IκBα degradation (Neish *et al*., 2000).

Specialized cells interspersed along the crypt villus axis that enhance protection against microbes and promote repair also form the epithelium. In the base of the crypt, the Paneth cells secrete antimicrobial peptides such as α-defensine. These antimicrobial peptides not only defend against pathogenic bacteria but also control the balance between various bacterial populations and contribute to local homeostasis (Menendez *et al*., 2010). Some observations suggest that a reduction of α-defensine production might contribute to the pathogenesis of terminal ileal Crohn's disease in patients with mutant NOD2 (Wehkamp *et al*., 2004; Wehkamp *et al*., 2005).

Globlet cells are another important component of epithelium that produces trefoil peptides involved in defence and repair of epithelium and mucosa, whereas Microfold cells (M cells) transport organisms and particles from the gut lumen to immune cells across the epithelial barrier and with dendritic cells sample intestinal contents (Fig 3).

In patients with Crohn's disease, modifications of luminal bacteria concentrations compared to control patients group have been found. For example, a recent study has shown that the neoterminal ileum of CD patients is heavily colonized by a colonic-like bacterial flora, with *E. coli* predominating (Barnich *et al*., 2007).

Evidence of involvement of intestinal microbiota in the pathogenesis of disease is provided by the therapeutic benefits of antibiotics' treatment in a group of patients with IBD (Gionchetti *et al*., 2003). In particular, two microorganisms have been associated with Crohn's disease: *Mycobacteriu avium* subspecies *paratubercolosis* (MAP) and *Escherichia coli*.

MAP has been identified in 1980 but its potential etiological role is cause of debate. Recently, it has been suggested that elimination of MAP in Crohn's disease does not significantly affect the clinical course of patients receiving three anti-mycobacterial antibiotics or placebo (Selby *et al*., 2007).

Escherichia coli has been isolated in 1990 from the ileal mucosa of Crohn's disease patients and its capacity to adhere and penetrate intestinal epithelial cells has been shown. However, it is not yet clear whether this microorganism acts as a pathogens or a commensal in Crohn's disease patients (Darfeuille-Michaud *et al*., 1998; Darfeuille-Michaud *et al*., 2004).

The importance of intestinal flora is supported by studies in murine model, in which, colitis is not observed when any of several of these lines are maintained in a gnotobiotic state, but rapidly emerges when they are reconstituted with bacteria that are considered normal constituents of luminal flora (Elson *et al*., 2005).

Based on several reports, it is now well established that essentially all animals raised under germ-free conditions, in the complete absence of commensal flora, do not develop experimental intestinal inflammation independently from strain and genetic background or method used to induce inflammation.

In patients with IBD, the existence of the immune reactivity against intestinal microbes is provided by series of serum antibodies against a variety of microorganisms such as *Saccharomyces cerevisiae,* anti-outer membrane protein C and anti-I2. In patients with Crohn's disease, these antibodies are currently used as biomarkers and a high level of detected antibodies is often associated with a complicated or rapidly progressive disease course (Mow *et al*., 2004; Dubinsky *et al*., 2008).

A limit on the understanding the microbial-host interrelationship is an incomplete knowledge of the composition of the normal human intestinal microbial flora (Gill *et al*., 2006). Furthermore, recent studies have shown that some bacteria do not always stimulate an intestinal immune response but can promote an anti-inflammatory status (Mazmanian *et al*., 2008).

Understanding the distribution, dynamics and responses to microbial flora in this disease could provide additional information on the pathogenesis and regional location of the disease.

III.3. Immune Response

The immune response is essential to functional integrity of the intestinal mucosa and health. Chronic intestinal inflammation appears to result from stimulation of the mucosal immune system by products of commensal bacteria in the lumen or antigens from dietary. The bacterial products may stimulate the surface epithelium, possibly through receptors that are components of the innate immune response system or can penetrate through the mucosal barrier, leading to their direct interaction with immune cells. The innate immunity is carried out mainly trough two types of cells: macrophages and dendritic cells.

In the normal intestine, macrophages are conditioned by the mucosal microenvironment to express a non-inflammatory phenotype that consists of a down-regulated expression of innate immunity receptors and a limited production of pro-inflammatory cytokines. In intestinal tissue affected by IBD, mucosal macrophages show an activated phenotype and are phenotypically heterogeneous. Moreover, macrophages recruited from the peripheral blood, $CD14^+$ pro-inflammatory macrophage activate the production of various pro-inflammatory cytokines such as IL-1α, IL1-β and TNF-α (Rugtveit *et al*., 1997). In Crohn's disease, the number of $CD14^+$ pro-inflammatory macrophages is increased and these macrophages produce mainly IL-23 and TNF-α and contribute to the production to IFN-γ by T cells (Kamada *et al*., 2008).

The dendritic cells are antigen-presenting cells involved in the initiation and regulation of local innate immune response but also have a role in adaptive immunity. These cells play a role of protection and defence and mediate inflammation. Their function depends on the location, state of maturation and stage of inflammation. In IBD, the dendritic cells are activated and produce elevated levels of pro-inflammatory cytokines such as IL-12 and IL6 (Rescigno and Di Sabatino, 2009; Hart *et al*., 2005). The innate immune cells are also able to

generate reactive oxygen species (ROS) that are directly involved in inflammation and tissue injury and increase epithelial permeability.

The adaptive immune response is produced by a combination of cell population: B cells and a complex variety of T cells. B cells produce immunoglobulin (Ig) M, IgG and IgA. In IBD patients, the synthesis and secretion of these Igs is altered both in peripheral blood and in mucosal mononuclear cells (MacDermott *et al*., 1981). In particular, in Crohn's disease patients, the production of IgG1, IgG2 and IgG3 is increased compared to control cells (Scott *et al*., 1986).

The two more important types of T cells involved in IBD are $CD4^+$ T helper 1 (Th1) and T helper 2 (Th2). In recent years, it has been identified a series of new types of T cells such as IL-17-producing Th17 cells, Th9 and Th22 cells (Weaver *et al*., 2007; Annunziato and Romagnani, 2009). In addition to Th cells, T regulatory (Treg) cells are appointed to monitor the immune response and prevent an excessive and potentially harmful immune activation (Izcue *et al*., 2006) (Fig 3).

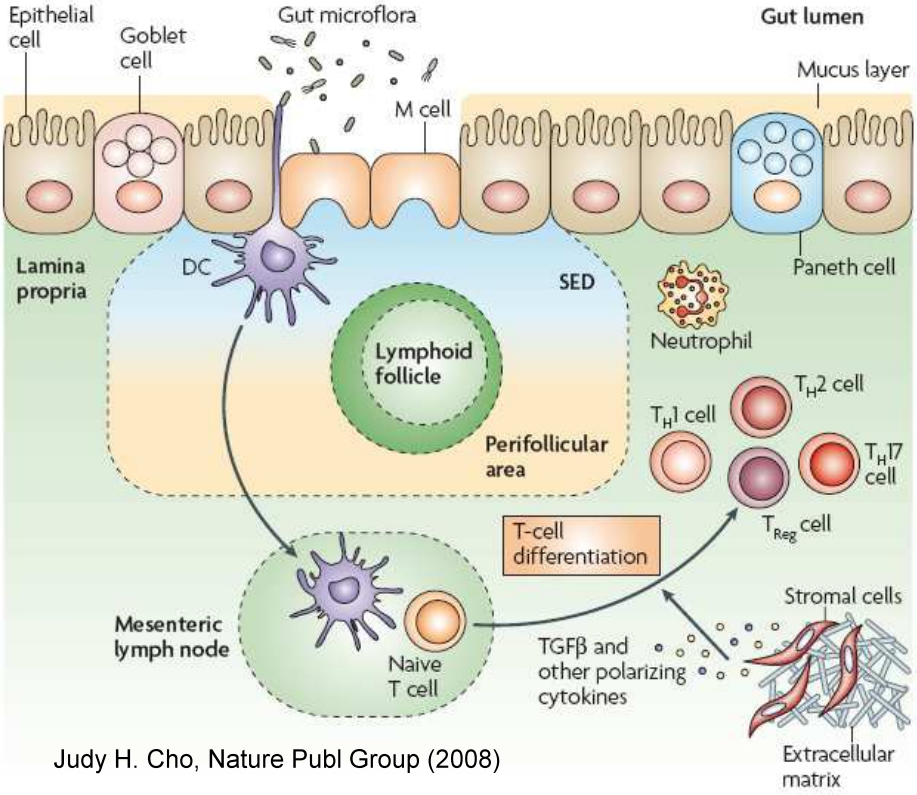

Fig 3: Intestinal immune system: The intestinal immune system is extensive and unique with respect to its close apposition to intraluminal bacteria, which are separated from the underlying lamina propria by only a single layer of epithelial cells. The epithelial-cell layer is comprised of absorptive and secretory cells, goblet cells and Paneth cells. Goblet cells contribute to the formation of the protective mucus layer. Microfold cells (M cells) and dendritic cells (DCs) sample intestinal luminal contents.
The presence of either pathogenic bacteria or disruption of the epithelial-cell barrier results in activation and migration of DCs to the mesenteric lymph nodes, where they activate naive T cells, which then undergo differentiation under the influence of factors released by DCs and other stromal elements. A central challenge facing researchers of inflammatory bowel diseases is defining the mechanisms of crosstalk between components of the innate and adaptive immune systems in light of the multiple established genetic variants associated with Crohn's disease. SED, subepithelial dome; TGFβ, transforming growth factor-β; TH, T helper; TReg, T regulatory (From Cho JH; Nat Publ Group 2008).

The mucosa of patients with Crohn's disease is dominated by $CD4^+$ lymphocytes with a Th1 phenotype, characterized by the production of IFN-γ and IL-12 by lamina propria mononuclear cells (Parronchi *et al*., 1997). In addition, in Crohn's disease there is also a elevated production of IL-17 by Th17 cells and dual IFN-γ and IL-17-producing Th cells, and also IL-21 that regulates IL-17 production (Fujino *et al*., 2003; Monteleone *et al*., 2005).

More precisely, in patients with Crohn's disease, the activation of classic antigen-presenting cells, such as dendritic cells, or direct stimulation through pattern-recognition receptors promotes the differentiation of Th1.

In mice, it has been suggested that the stereotypical Th1 cytokines promote a self-sustaining cycle of activation with macrophages: Th1 cytokines activate macrophages, which in turn, produce IL-12, IL-18, and macrophage migration inhibitor factor which further stimulate Th1 (Podolsky, 2002). The macrophages produce also a potent mix of active inflammatory cytokines, including TNF-α, IL-1, and IL-6. These factors stimulate a variety of other type of cells such as vascular endothelial cells, which facilitate the recruitment of leukocytes to the mucosa from the vascular space, fibroblasts and epithelium. The recruitment of additional leukocytes from the vascular space to sites of disease activity is especially important in maintaining inflammation and depends on the expression of cytokines, chemokines and adhesion molecules in the local microvasculature.

The activation of intestinal immune system is linked to a variety of non nonspecific mediators of inflammation such as many other cytokines, chemokines, and growth factors as well as metabolites of arachidonic acid (prostaglandins and leukotrienes) and reactive nitrogen metabolites such as nitric oxide (NO).

These mediators enhance the inflammatory process and tissue destruction, which lead to the clinical manifestations of disease.

IV. Therapeutic approaches

Crohn's disease is a disease whose anatomical location is fairly stable but the evolution of disease may vary during its course.

A study on population-based cohorts showed that 13–20% of patients with Crohn's disease have a chronic active course of disease activity, 67–73% has a chronic intermittent course and only 10–13% remains in remission for several years. After 20 years old, most patients with Crohn's disease will require surgery (Loftus *et al*., 2002). The ideal therapeutic strategies for Crohn's disease patients should be able to induce remission and maintain long-term remission, reducing to a minimum the surgery. Furthermore, although surgery might be necessary to treat complications or to induce remission in patients with Crohn's disease, surgery is unable to cure the disease.

Among the different treatment, corticosteroids have been used in the treatment of Crohn's disease for many decades. These drugs are effective in inducing a remission of disease but are unable to secure a long-term remission and are associated to severe and irreversible side effects.

A better understanding of the immunologic mechanism associate with the disease led to the introduction a new concept of therapies, biologic therapies, aimed to correcting the imbalance of the intestinal immune system observed in disease.

Among the molecules that have been shown to be effective in treating the disease, stand the TNFα inhibitors. TNFα is a proinflammatory cytokine that induces cell proliferation and differentiation and promotes the inflammatory response. Three anti-TNFα molecules are currently used to treat IBD: infliximab, adalimumab and cerolizuman pegol. In particular, infliximab is a chimeric monoclonal IgG1 against TNFα that was introduced in 1997 and has

proved highly effective in patients with refractory luminal and fistulising Crohn's disease (Present *et al*., 1999; Sands *et al*., 2004). Infliximab treatment reduces hospitalizations and surgery and improves the quality of life in patients with Crohn's disease (Lichtenstein *et al*., 2005).

A series of promising new molecules for treatment of Crohn's disease were assessed in controlled clinical trial: anti–IL-12/IL-23 p40, anti-IFN-γ and anti-IL-6 antibodies. The patients with active Crohn's disease treated with anti-IL-12 display a decrease of production of proinflammatory cytokines such as IL-12, IFN-γ and TNFα (Mannon *et al*., 2004).

Reagents that blocked a receptor complex responsible of T cell activation, such as CD4 and CD3, are also, under development in treatment of Crohn's disease (Plevy *et al.,* 2007).

Recently, a large number of small molecules involved in specific immune pathway disease such as eicosanoides (thromboxane A2, leukotrienes and platelet activating factor (PAF)) and inhibitors of phosphodiesterase 4 (PDE4) are also studied for the treatment of IBD. Particularly, a new promising small molecule is the agonist of activator of peroxisome proliferator activated receptor γ (PPARγ) that is associated with an inhibition of signal transduction pathway linked to production of proinflammatory cytokines (van Deventer, 2002).

Furthermore, the alternative approaches therapeutic are being tested especially in patients with severe disease, such as stem cell transplantation (Rutgeerts *et al*., 2009).

Finally, the progress in the discovery of gene implicated in the pathogenesis of the disease and in understanding of their interaction, could lead in the future, to use of the specific treatments for different disease phenotypes.

V. Crohn’s Disease and Vascular Alterations

Several anatomic and pathological studies have shown, in both IBD patients and in animal models, an altered microvascular anatomy and function in inflamed gut. Then, vascular alterations may be one of the causes of inflammatory disease.

In 1989, Wakefield *et al.* (1989) have reported vascular lesions in both segments of inflamed and noninflamed intestine in Crohn's disease patients and a remodelled vascular network in inflamed segments, suggesting that Crohn's disease is mediated by multifocal gastrointestinal infarction and that vasculitis may be one of the first events leading to mucosal injury.

Recent studies have described an impaired vasodilatation and tissue remodelling in human submucosal arterioles isolated from areas of chronic inflammatory damage of intestine of inflammatory bowel disease patients. This reduced vasodilatory capacity, is due to a functional alteration in endothelium-dependent dilatation and is linked to a reduced endothelial generation of NO and high levels of oxidative stress in blood vessels (Hatoum *et al*., 2003).

Some authors have proposed that increased expression of arginase enzymes that convert L-Arg into urea and L-ornithine, and that are important for tissue homeostasis and lesion repair, play an important role in the mechanism involved in microvascular dysfunction present in IBD patients. Effectively, an increase of gene expression and activity of arginase enzymes that was found in inflamed IBD submucosal tissue could decrease microvascular endothelial access to L-Arg that is the fundamental substrate required for NO production (Horowitz *et al*., 2007).

Microvascular dysfunction is also linked to impaired tissue perfusion and oxygenation (Mori *et al*., 2005) that may lead to ramifications of microvascular dysfunction.

In contrast, unchanged endothelium-dependent and -independent relaxations in mesenteric arteries from Crohn's disease patients and a decrease of vascular tone are also reported. The reduction of contractile response, involve an excessive NO production through the overexpression of inducible NOS in vascular smooth muscle. These authors suggest that this hyporeactivity could improve the blood perfusion and could be a possible response of host organism against the injury (Lebuffe *et al*., 2000).

Tabernero *et al.* (2003) revealed, despite an increase of release of proinflammatory cytokines in mucosa of Crohn's disease patients, an unmodified vascular reactivity in small mesenteric arteries from patients with Crohn's disease. The regulation of vascular contraction involves vasoconstrictor metabolites of cyclooxygenase (COX) and, interestingly; there is a balance between vasoconstrictor products from COX-2 and vasodilator products that maintained the vascular reactivity unchanged.

All these data, suggest a possible implication of vascular alterations in pathogenesis of disease (Fig 4).

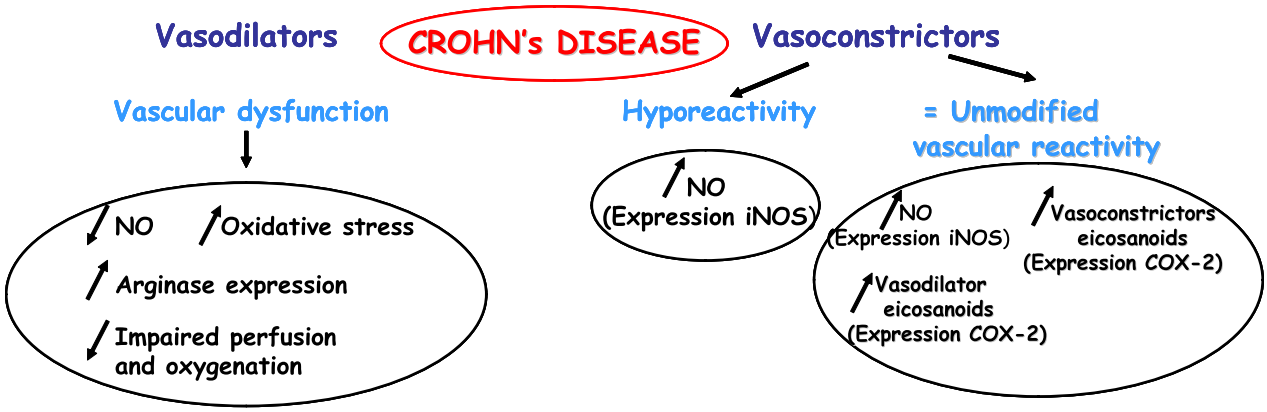

Fig 4: Vascular alterations in Crohn's Disease: vascular alterations include both vascular dysfunction and modification of vascular tone. These alterations involve NO pathway, oxidative stress, COX-2-derived metabolites and impaired perfusion and oxygenation.

Part II

Microparticles

I. General aspects

In the multicellular organisms, the communication between cells occurs through direct cell-cell contact or mediator production such as protein that binds to receptors on neighboring cells. Another way of intercellular communication which has recently generated an increasing interest is the release of membrane vesicles.

The consequences of this membrane transfer involved the induction, amplification or modulation of immune response and the acquisition of new functional properties in the recipient cells. Furthermore recent studies suggest a possible transfer of genetic material between cells achieved through the transfer of mRNA present in small vesicles.

In this context, microparticles (MPs) are vesicles shed from the blebbing plasma membrane that have been widely studied. Recent data show that MPs can be considered as biological vectors able to transfer information from one cell to another. In addition, MPs can mediate long-range signalling, acting on cells different from their cells of origin (Martínez *et al*., 2005).

The MPs can allow intracellular communication trough different pathways. They can interact directly with the ligands present on the surface of target cells transferring proteins, mRNA, miRNA, receptors and bioactive lipids (Hunter *et al*., 2008). MPs may also enable communication between cells without direct cell contact. MPs can bind to the membrane of target cells which can acquire new surface antigens and then new biologic properties and activities. Finally, MPs can be absorbed by fusion or by internalization.

MPs are present in the blood from healthy subjects and patients, they could than participate in the maintenance of homeostasis under physiological conditions or have a deleterious effect in pathological situations.

II. Definition and generation of MPs

The concept of MPs was introduced in 1967 by Wolf that describes inert "cellular dust" released from activated platelet in human plasma (Wolf, 1967). Based on several data, it is now well established that MPs are small vesicles heterogeneous in size (0.05-1 μm) and composition with procoagulant and proinflammatory properties. Although MPs can potentially be produced by all cell types, in general, MPs derive from circulating cells such as platelets (that represent the highest percentage of MPs), leukocytes, monocytes and erythrocytes, and cells that compose vessel wall such as endothelial cells, macrophages and smooth muscle cells; MPs can derived also from cancer cells (Martínez *et al*., 2005); (Mostefai *et al*., 2008).

The mechanism that leads to the formation of MPs is not completely clear but there are two well-known cellular processes that can stimulate the production of MPs: chemical and physical cell activation (by agonist or sheer stress) and apoptosis (growth factor deprivation or apoptotic inducers) (Benameur *et al*., 2009).

Following cell activation by different agonists, MPs formation is dependent on an increase of cytosolic calcium that is associated with calpain activation required for proteolysis of cytoskeletal proteins and then cytoskeleton disruption (Pasquet *et al*., 1996). The cytoskeleton disruption involved also a kinase activation and phosphatase inhibition caused by increase of cytosolic calcium level (Heemskerk *et al*., 1997; Kunzelmann-Marche *et al*., 2002).

MP formation is linked to a loss of membrane asymmetry. The membrane bilayer is formed by two leaflets each of which having a specific lipid composition. Aminophospholipids (phosphatidylserine (PS) and phosphatidylethanolamine) are specifically segregated in the inner leaflet, whereas phosphatidylcholine and sphingomyelin are present in the outer leaflet. During MP formation, there is an alteration of bilayer structure that is controlled by three major players: an aminophospholipid translocase which is an inward-directed pump that transport PS and phosphatidylethanolamine from the outer to inner leaflet of plasma membrane against the concentration gradient; an outward-directed pump, an ATP-dependent floppase which acts in conjunction with the translocase and a lipid scamblase, can rapidly move aminophospholipids between the membrane leaflets by a calcium-dependent mechanism and may lead to collapse of membrane asymmetry (Bevers *et al*., 1999; Hugel *et al*., 2005). A sustained rise of intracellular calcium concentration regulates positively scramblase and floppase activities and inhibiting the translocase. The main effect of redistribution of membrane lipids is the exposure of PS and a consequent release of MPs. The PS exposure is present in the surface of most but not all MPs and PS plays two important functions: promote the blood coagulation and is involved in signal recognition for clearance of senescent cells by the reticuloendothelial system (Hugel *et al*., 2005) (Fig 5).

MPs production can be also induced by physical stimulation such as changes in blood flow. Holme et al (Holme *et al*., 1997) have shown that in arteries with several stenosis, high shear stress activates platelets and triggers platelet MP formation. In another study, it has been found that platelet MP formation increases with the duration of shear stress and that activation of protein kinase C (PKC) promotes shear-dependent MP formation. Furthermore, both MPs and platelets showed the exposure of procoagulant activity on their surfaces suggesting that MPs formation could contribute to arterial thrombosis by providing and expanding a catalytic surface for the coagulation cascade (Miyazaki *et al*., 1996).

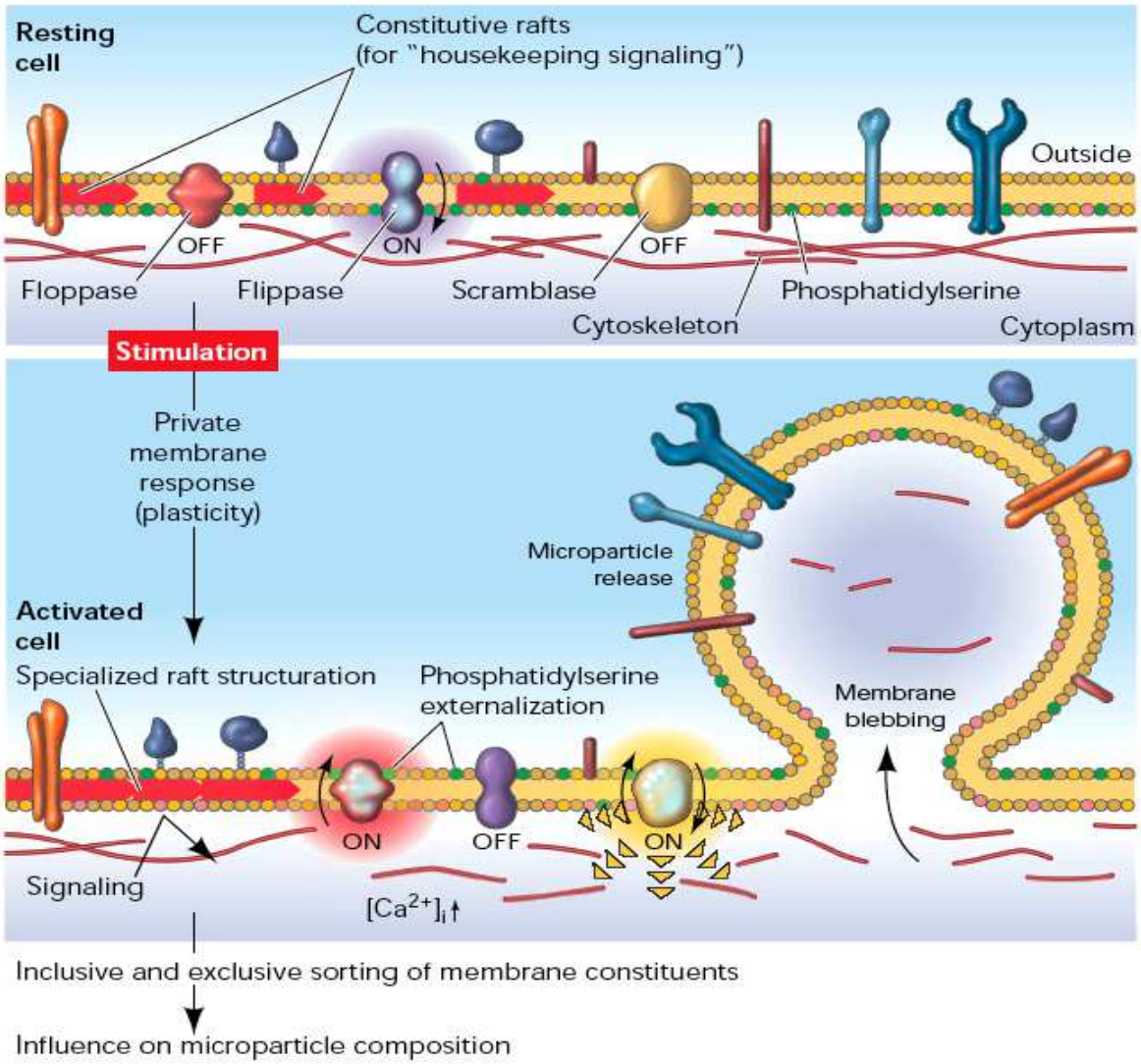

Fig 5: **The plasma membrane response to cell stimulation:** The plasma membrane is a well-structured entity characterized by a controlled transverse distribution of lipids and proteins between the two leaflets but also by a lateral organization in domains termed "rafts." Following stimulation, a general redistribution occurs, leading to raft structuration, phosphatidylserine externalization, and microparticle release. The private membrane response characterized by controlled inclusion or exclusion of specific constituents into rafts leads to the release of microparticles of a particular composition. (From Hugel *et al*., 2005).

The second main process that leads to production of MPs is apoptosis. Apoptosis is an important biological process for normal cellular homeostasis in multicellular organisms that can be induced by different extracellular stimuli such as: irradiations, proinflammatory cytokines, chemotherapeutic drugs, hormones or bacterial agents. The cell death by apoptosis

consists of an intracellular cell death program that involved changes of the cell morphology (condensation of the cytoplasm and segregation of chromatin from compact masses on the nuclear membrane inner leaflet), cytoskeleton disruption, cell shrinkage and membrane blebbing (Thompson, 1995). The membrane blebbing is followed by shedding of membrane vesicles that transport nuclear fragments, and release of apoptotic bodies or MPs which expose PS on their surface. The PS exposure characterizes apoptotic bodies and apoptotic cells allowing their recognition and phagocytosis by macrophages and mesenchymal cells.

MP formation by apoptosis involved a Rho-associated kinase (ROCK I) activity, due to caspase 3 activation. ROCK I promotes generation of actin-myosin force, couples actin-myosin filaments to the plasma membrane cell and than leads to disruption of membrane skeleton structure and consequently membrane blebbing and MP formation (Coleman *et al*., 2001).

Another study showed that thrombin plays a role in endothelial MP generation by activating RhoA/ROCK II via caspase-2 pathway in endothelial cells, despite the absence of cell death (Sapet *et al*., 2006).

III. Composition of MPs

The composition of MPs reflects the organization of plasma membrane of the origin cell at the precise moment of MP generation and this allows their characterization with respect to their cellular source. The membrane and cytoplasmic constituents of MPs influence their biological

activities and their phenotype. Several studies showed that the type of MPs can vary depending on a given stimulus which initiates their production. For example, endothelial cells release qualitatively and quantitatively distinct endothelial MPs (EMPs) during activation compared to apoptosis. In particular, EMPs issued the following inflammatory activation are characterized by their richness in E-selectin. On the contrary, MPs produced by apoptotic endothelium are particularly rich in CD31 (Jimenez *et al*., 2003).

Furthermore, recently a comparative proteomic analysis indicated that EMPs generated from human umbilical vein endothelial cells by stimulation with plasminogen activator inhibitor type 1 (PAI-1) or tumor necrosis factor-alpha (TNF-alpha) showed a distinct proteins compositions (Peterson *et al*., 2008).

Martinez et al (Martínez *et al*., 2006) have reported that MPs generated from activated (by phytohemagglutinin, PHA and phorbol-myristate-acetate, PMA) and apoptotic (actinomycin D, ActD) CEM T lymphocytes (cell line) or lymphocytes from diabetic patients expose on their surface the morphogen Sonic Hedgehog (Shh) (protein implicate in embryonic and adult development). In the presence of PHA alone, PMA alone and ActD alone, these cells generate MPs lacking Shh.

Another study has shown a different protein composition in MPs produced by human monocyte THP-1 depending on whether activated with lipopolysaccharide or a soluble P-selectin chimera (Bernimoulin *et al*., 2009).

The membrane of MPs is formed mainly of lipids and several proteins and results negatively charged because of presence of PS and phosphatidylethanolamine. Several reports have shown that the lipid environment could modify the activity of certain proteins carried by MPs. For example cholesterol enrichment of human monocytes/macrophages induces the generation of highly biologically active PS-positive and TF-positive MPs (Liu *et al*., 2007).

Furthermore, these vesicles express antigens derived from the original cell and carry different membrane, cytoplasmic and nuclear constituents (Fig 6). For example, platelet MPs (PMPs) incorporate plasma membrane glycoproteins (GP), such as GP IIb/IIIa (CD41) and GP Ib/IX complex and the alpha-granulate membrane protein (GMP-140) (Wiedmer *et al*., 1990). PMPs are also major carrier of platelet-activating factor (PAF) a potent phospholipid involved in the pathogenesis of inflammation (Iwamoto *et al*., 1997). In addition, they can expose markers of activated cells such as P-selectin (CD62P) (Diamant *et al*., 2004).

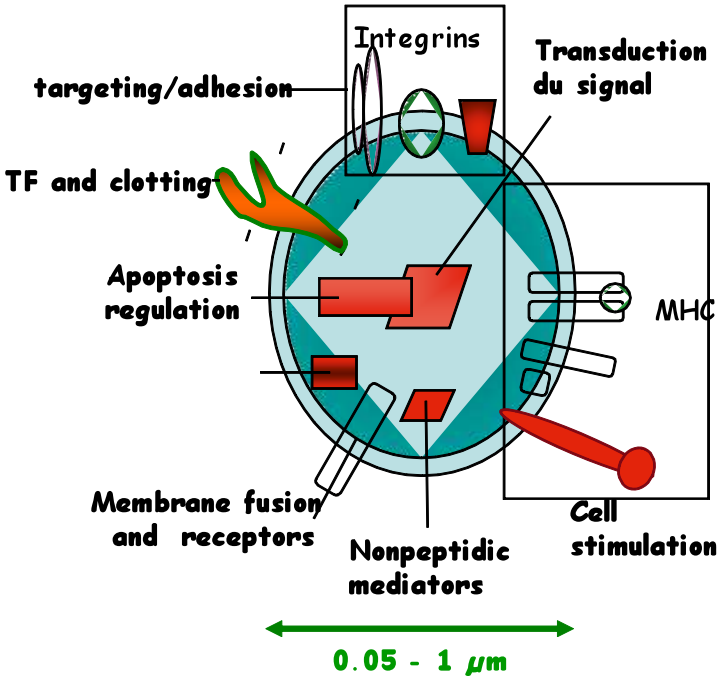

Fig. 6: Cellular MPs: a disseminated storage pool of bioactive effectors. MPs are shed from the plasma membrane of stimulated cells. They harbor membrane and carry cytoplasmic proteins as well as bioactive lipids implicated in a variety of fundamental processes. This representation does not intend to be exhaustive with respect to the different hijacked components. MHC, major histocompatibility complex. (From Hugel *et al*., 2005).

Smalley et al. (Smalley *et al*., 2007) have shown a comparative analysis of MPs isolated from plasma (plasma MPs) versus PMPs in which it was identified 21 proteins that were present in plasma MPs but were essentially absent in PMPs. These proteins include proteins associated with apoptosis (CD5-like antigen, galectin 3 binding protein), iron transport (transferrin, transferrin receptor, haptoglobin), immune response (complement components, immunoglobulin J and kappa chains), and the coagulation process (protein S, coagulation

factor VIII). Only two proteins were present in both types of MPs: von Willebrand Factor and albumin.

Functional and proteomic analysis of PMPs size fractions has shown that PMPs can be separated into different size classes that differ in their contents of plasma membrane receptors and adhesion molecules, chemokines, growth factors and protease inhibitors and functional effects on platelets and endothelial cells (Dean *et al*., 2009).

A proteomic approach has revealed that the protein content of EMPs consists mainly of metabolic enzymes, proteins involved in adhesion and fusion processes, cytoskeleton-associated proteins and nucleosome (Banfi *et al*., 2005). There are a limited number of endothelial-specific markers such as CD31, CD146 and E-selectin (CD62E).

MPs released by leukocytes, are normally present in low concentration in blood healthy subjects but their production can be enhanced by inflammatory cell stimulation *in vitro* or in the presence of various diseases (Sabatier *et al*., 2002). Leukocyte-derived MPs carried different complex such as CD4, CD3 or CD8 (Martin *et al*., 2004).

Regarding the overall content of proteins of MPs, another proteomic analysis has identified 390 proteins in MPs among which 34% were localized in the plasma membrane. Half of the detected proteins are intracellular proteins and only a few number of proteins derived from nucleus, mitochondria, Golgi apparatus and endoplasmic reticulum. (Miguet *et al*., 2006). Recent studies reveal the presence of MPs released by endothelial progenitor cell cultures (EPCs). The analysis of the composition of these MPs obtained by proteomic approach has identified 618 proteins among which there is a large concentration of platelet proteins such as glycoprotein IIb/IIIa (Prokopi *et al*., 2009).

IV. MPs and disease

The role of MPs has been studied in various diseases states and their number, cellular source and composition are altered compared to healthy controls. It is well established that MPs play an important role in inflammation, coagulation and vascular dysfunction but the mechanisms involved are not yet fully clear.

High levels of circulating MPs have been shown in diseases linked with an increased risk of thromboembolic events such as diabetes, myocardial infarction, preeclampsia, rheumatoid arthritis, hypertension, cancer-associated thrombosis and metabolic syndrome (Sabatier et al. 2002; Mallat *et al*., 2000; Meziani *et al*., 2006; Preston *et al*., 2003; Knijff-Dutmer *et al*., 2002; Agouni *et al*., 2008) but the phenotype of circulating MPs may change in different pathological states.

Differences on the phenotype of MPs have been reported by Sabatier *et al*. (Sabatier *et al*., 2002) in diabetic patients. Indeed, although there is an elevated level of circulating MPs in type I and type II diabetic patients, the cellular origin and the procoagulant activity of MPs differ according to type of diabetes: in type I diabetic patients, there is an elevated level of EMPs, PMPs and procoagulant activity, instead in type II diabetic patients only the level of annexin-V positive (procoagulant) is increased. The large increase of EMPs in type I diabetic patients suggests that they could be markers in endothelial damage observed in microvascular complications associated to this disease.

Diabetes, particularly type II diabetes, is associated with another disease linked to cardiovascular risk and inflammation, the metabolic syndrome (MS), in which was observed, in the same way, an increase of the rate of circulating MPs. Interestingly, in addition to the increase of MPs from endothelial cells, erythrocytes and platelets in MS patients, there is also

an increased level of procoagulant (annexin V^+) MPs that could participate to fibrinolysis impairment and thrombogenesis involved in this disease (Agouni *et al*., 2008).

An elevated level of MPs with procoagulant potential has been also detected in patients with acute coronary syndrome compared with patients with stable angina and noncoronary heart disease. The cellular origin of these MPs is endothelial and this suggest an important role for endothelial injury in inducing the procoagulant potential and that MPs may contribute to the generation and perpetuation of intracoronary thrombi (Mallat *et al*., 2000).

The inflammatory process and an enhanced cardiovascular morbidity and mortality are aspects that have also been observed in the rheumatoid arthritis. In the pathology of this disease, has been suggested the involvement of PMPs. Indeed, level of PMPs is increased and is correlated to activity of disease (Knijff-Dutmer *et al*., 2002).

High concentration of circulating MPs is often associated with high blood pressure. In particular, in patients with several uncontrolled hypertension, the pressure-induced endothelial and platelet activation is linked with a release of EMPs and PMPs (Preston *et al*., 2003). Therefore, MPs could be markers of organ injury resulting from the combined effects of EMPs and PMPs on coagulation, leukocytes, and endothelium.

Two others pathologies associated with systemic vascular inflammation and risk of thrombosis are preeclampsia and sepsis.

Women affected by preeclampsia showed various disorders characterized by a generalized endothelium dysfunction that lead to hypertension and proteinuria. Van Wijk and co-workers (Vanwijk *et al*., 2002) have shown an unaltered total number of MPs in preeclamptic patients and normal pregnancy despite en increase level of T-lymphocytes and granulocytes MPs in preeclamptic patients compared to women with normal pregnancy. However, Meziani *et al*., (Meziani *et al*., 2006) have found in preeclamptic patients, an increased level of MPs and

phenotypic characterization of their cellular origin showed increased leukocyte-and platelet-derived MPs in the bloodstream of these patients. Furthermore, MPs from preeclamptic women are also involved in vascular alteration and proinflammatory process. Then, preeclamptic MPs could act as vectors of vascular inflammation in this disease.

Sepsis is an acute and systemic immune response mainly to bacterial infection. The production of procoagulant MPs has been shown in patients with meningococcal sepsis. These MPs originating from platelets and granulocytes could contribute to development of disseminated intravascular coagulation that affects these patients (Nieuwland *et al*., 2000). Conversely, Soriano et al. have demonstrated a positive correlation between high circulating levels of MPs and survival in septic patients (Soriano *et al*., 2005) and this protective role has been confirmed in a recent study. In this work, Mostefai *et al*. (Mostefai *et al*., 2008) have suggest a possible protective role in vascular function of MPs which are present in high amounts in septic patients compared with nonseptic subjects. Particularly, there is an increase of 1.7- and 3-fold of the circulating level of PMPs and EMPs, respectively, in septic patients compared with nonseptic subjects. These MPs are capable to counter the hyporeactivity associated with sepsis, suggesting that, during sepsis, MPs may have a protective role rather than a deleterious role. Indeed, the septic MPs enhanced the contraction of mouse aorta in response to serotonin and this effect was associated with increased thromboxane A2 production, and was sensitive to a selective thromboxane A2 antagonist.

The inflammation is the key pathogenic component of atherosclerosis and is orchestrated by the interaction by inflammatory cells such as smooth muscle. In particular, it has been showed that smooth muscle cells could be able to release tissue factor-bearing MPs in the atherosclerotic plaque that could contribute to thrombus formation during atherosclerotic plaque disruption (Schecter *et al*., 2000).

Finally, recent data show a possible involvement of MPs on endothelial dysfunction in patients with obstructive sleep apnea (OSA), a disease characterized by recurrent episodes of partial or complete obstruction of the upper airways during sleep, leading to repeated falls in oxygen saturation. Indeed, MPs seem to participate to endothelial dysfunction involved in vascular complications of OSA. Although the level of circulating MPs is unchanged between the two groups considered (desaturators and nondesaturators patients), there is higher levels of granulocytes and activated leukocyte ($CD62L^{+}$)-derived MPs in desaturators compared to nondesaturators. In addition, MPs from desaturator patients increased expression of endothelial adhesion molecules including E-selectin, "intercellular adhesion molecule-1" (ICAM-1) and integrin-α5, and COX-2 (Priou *et al*., 2010).

All these data suggest that, in several pathological states, MPs could be used as biomarkers to identify a disease or to highlight the disease-related complications.

V. Microparticles and inflammation

Several reports suggest the involvement of MPs in the inflammatory process. Indeed, MPs can induce the production of potent pro-inflammatory mediators by cells and the up-regulation of several pro-inflammatory enzymes. Furthermore, the production of platelet, endothelial and leukocyte MPs can be increased during inflammatory conditions (Daniel *et al*., 2006).

MPs are rich in aminophospholipids and are a preferential substrate for phospholipase A2 that is involved in production of lysophosphatidic acid which can triggers platelet aggregation and inflammatory process (Fourcade *et al*., 1995). The first study about the effect of the PMPs on endothelial cells has shown that they facilitate the transcellular transport of arachidonic acid (AA) which could lead to an increase expression of COX-2 and ICAM-1. Both COX-2 and ICAM-1 regulate the vascular and platelet functional interaction. Thus, the MP-induced modulation of COX-2 expression in human monocytoid cell line induces the translocation of protein kinase C from the cytosol to the membrane and triggers activation of different kinase (Barry *et al*., 1999; Barry *et al*., 1997). Furthermore, the unmetabolized AA present in PMPs is involved in platelet aggregation and interactions of platelets and monocytes with endothelial cells (Barry *et al*., 1998). This suggests a mechanism whereby MPs modulate cell function by the transcellular delivery of bioactive substances.

Also, it has been shown that MPs from platelet and leukocyte origins participate in the production of several endothelial (IL-1β, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1)) and monocytes (IL-1β, TNF-α, IL-8) cytokines that could facilitate the interaction between leukocytes and endothelium (Mesri and Altieri, 1999). In this respect, Mesri and Altieri showed that after incubation of human umbilical vein endothelial cells (HUVECs) with leukocyte-derived MPs, there is an increased production of IL-6, IL-8 and membrane adhesion molecules implicated in interaction between endothelial cells and leukocytes. In

addition, an up-regulation of IL-6 mRNA was observed suggesting the ability of MPs from leukocytes-to modify gene expression (Mesri and Altieri, 1998).

In turn, cytokines also can be involved in the production of MPs. For example, in patients with arteriosclerosis, it has been shown that increased levels of IL-6 are associated with enhanced expression of P-selectin and generation of PMPs in condition of high shear stress (Nomura *et al*., 2000). MPs from activated platelets can also mediate in vitro leukocyte-leukocyte interactions via binding of P-selectin to its ligand PSGL-1 on leukocytes. These attachments can then lead to increased accumulation of leukocytes on a P-selectin surface, for example, activated endothelium at sites of vascular injury (Forlow *et al*., 2000).

Furthermore, MPs derived from platelets could be able to facilitate the recruitment of various immune cells such as monocytes, T and B lymphocytes and NK cells that play a major role in inflammatory process (Ogura *et al*., 2001a).

A possible role of MPs as pro-inflammatory vectors in disease, has been suggest in preclamptic patients, since, circulating MPs are able to induce an up-regulation of iNOS and COX-2 and activate the NF-κB pathway, suggesting that MPs could be a pro-inflammatory vectors (Meziani *et al*., 2006).

All these data suggest that MPs may therefore contribute to the increased risk of thrombosis in systemic inflammatory diseases where increased numbers of MPs have been identified, or in localised inflammatory environments, such as atherosclerotic lesions where activated monocytes, endothelial cells and platelets are co-localised.

On the contrary, other authors suggested potentially anti-inflammatory role of MPs. Indeed, it has been reported that MPs released by neutrophils do not possess proinflammatory activity on human macrophages since they increase the release of transforming growth factor beta1 (TGF β1) and then down-modulate cellular activation in macrophages (Gasser *et al*., 2004).

VI. Microparticles and vascular function

The endothelium is a primary target of cardiovascular risk factors and endothelial dysfunction is the primary event leading to the failure of vasoactive, anticoagulant, and anti-inflammatory effects of healthy endothelium.

The endothelial response can be immediate following the release of several factors, or delayed involving the modulation of genes expression responsible of structural and functional regulation of the vascular wall. In this context, MP-associated effects may represent an adaptive phenomenon or contribute to the aggravation of disease. For this reason, several groups have studied the effects of MPs on vascular function and it was established that MPs are able to affect endothelial (Fig 7) and smooth muscle cells.

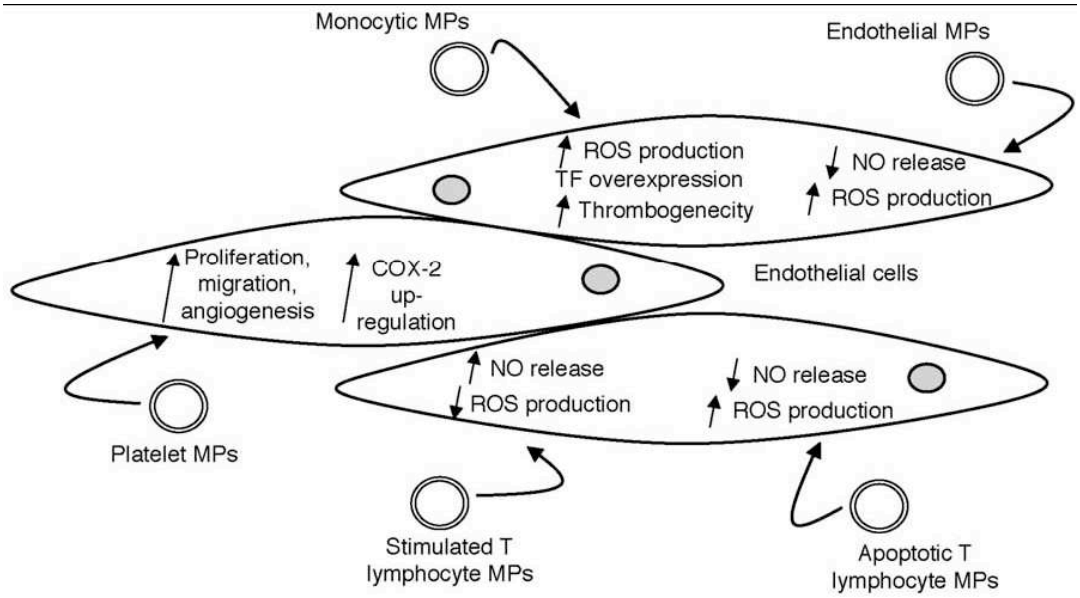

Fig 7. Microparticle (MP) effects on endothelial cells. Depending on the cell origin and the stimulation used for their generation, MPs have different effects on endothelial cell function. MPs can have beneficial or deleterious effects by acting on the nitric oxide pathway, pro-inflammatory enzymes, reactive oxidative species production, and angiogenesis. COX-2, inducible cyclo-oxygenase; NO, nitric oxide; ROS, reactive oxygen species; TF, tissue factor (From Benameur et al., 2009).

Concerning the role of MPs in the regulation of vascular function, it has been observed in several studies, that circulating MPs are able to reduce NO production via the reduced activity of endothelial NO-synthase (eNOS) and/or by decreasing its bioavailability. For example, MPs isolated from patients with myocardial infarction, diabetes or preeclampsia induce endothelial dysfunction by impairing endothelial NO transduction pathway (Boulanger *et al*., 2001; Martin *et al*., 2004; Vanwijk *et al*., 2002).

A potentially deleterious effect of MPs on the cardiovascular system and, notably, on the endothelial cells were observed in other disease such as MS, OSA and end-stage renal failure. Indeed, MPs from patents with MS are able to induce *in vitro*, the reduction of NO and

superoxide anion productions in endothelial cells, resulting in protein tyrosine nitration; in vivo, the same MPs impair the endothelium-dependent relaxation by decreasing eNOS activity (Agouni *et al*., 2008).

A direct correlation between a specific phenotype of MPs and endothelial dysfunction has been shown in patients with OSA. Indeed, in these patients, there is an increased level of MPs from granulocytes and activated leukocytes ($CD62L^+$) and levels of $CD62L^+$ MPs correlate with endothelial dysfunction that may initiate atherogenic processes in patients with OSA (Priou *et al*., 2010).

In patients with end-stage renal failure, an increased level of MPs is associated *in vitro* with impaired endothelium-dependent relaxations and cyclic guanosine monophosphate generation. In addition, endothelial dysfunction correlated with endothelial-derived MPs that induced a decrease of endothelial nitric oxide release (Amabile *et al*., 2005).

Furthermore, using a model of rat pulmonary arterial hypertension has been showed that circulating MPs from hypoxic rats reduce NO bioavailability by decreasing eNOS activity and by enhancing oxidative stress in pulmonary endothelial cells (Tual-Chalot *et al*., 2010).

MPs issued by smooth muscle cells may also have effects on vascular system. Indeed, MPs from apoptotic smooth muscle cells cause endothelial dysfunction *via* diminished NO production. In addition, pre-treatment of endothelial cells with two blockers of β3-integrins, abciximab or eptifibatide, restored NO production, suggesting that β3-integrins are implicated in the effects of smooth muscle MPs (Essayagh *et al*., 2005).

On the other hand, MPs can interact with smooth muscle cells, induce vascular inflammation and modify the vessel contractility. Indeed, in preeclamptic patients, MPs cause *ex vivo* vascular hyporeactivity in human omental arteries and mouse aortas by increasing NO

production via iNOS and COX-2-derived vasoconstrictor metabolites. Interestingly, MPs from leukocytes could be probably responsible for the COX-2 vascular component while MPs of platelet origin would be involved mainly in the production of NO (Meziani *et al*., 2006). These data suggest that MPs from platelets may serve as a protective mechanism against hypertension during preeclampsia.

Vascular hyporeactivity is induced also by T lymphocyte-derived MPs by increasing of NO and prostacyclin (PGI_2) production resulting from upregulation of iNOS and COX-2 expression. The mechanism involves Fas-Fas ligand interactions between lymphocyte MPs and target smooth muscle cells that lead to NF-κB activation which in turn upregulates iNOS and COX-2 expression (Tesse *et al*., 2005). These effects induced by MPs suggest a potential role of these MPs as vectors of transcellular exchange of message in promoting vascular dysfunction during inflammatory diseases.

Recent data showed that vascular hyporeactivity induced by MPs can be prevented by *in vitro* treatment or after administration of rosiglitazone. Indeed, rosiglitazone is a selective agonist of peroxisome proliferator activated receptor γ (PPARγ) able to prevent MP-induced vascular hyporeactivity trough the regulation of proinflammatory agents such as proinflammatory cytokine, NO and NF-κB (Tesse *et al*., 2008).

Another important contribution of MPs to the vascular system function consists in their ability to promote angiogenesis. These effects could be mediate by lipid components of PMPs such as sphingosine 1-phosphatase, that promote the proliferation and survival, migration, and tube formation in HUVECs culture (Kim *et al*., 2004). Furthermore, endothelial cells release MPs carrying metalloproteinases that could be responsible for mechanisms for regulating focalized proteolytic activity vital to invasive and morphogenic events during angiogenesis (Taraboletti *et al*., 2002).

Different studies show that MPs do not only have deleterious effects on the vascular system but a possible beneficial role has been suggested. Indeed MPs are able to carry molecules involved in proliferation and differentiation of cells. Recently, Benameur et al. (Benameur *et al*., 2010 b) have found that differentiation and angiogenesis of endothelial progenitor cells can be regulated by PPARα carried by MPs. These effects involve the Akt and NF-κB pathway activation.

MPs issued by apoptotic/stimulated human T lymphocytes harbour Sonic hedgehog (Shh), a morphogen that play a role of inter-cellular signal responsible for various cellular fate decisions and involved in induction of megakaryocyte differentiation (Martínez *et al*., 2006). Furthermore, Shh carried by the same MPs induces NO release from endothelial cells, triggers changes in the expression and phosphorylation of enzymes related to the NO pathway, and decreases production of reactive oxygen species. *In vivo* these MPs improve endothelial function in mice aorta by increasing NO release, and it reversed endothelial dysfunction after ischemia/reperfusion (Agouni *et al*., 2007).

Finally, a recent study, using a model of mice whit hind limb ischemia, showed that MPs bearing morphogen Shh (MP^{Shh+}) may contribute to reparative neovascularization after ischemic injury by regulating NO pathway and genes involved in angiogenesis *(*Benameur *et al*., 2010a).

All these data suggest an involvement of MPs in the mechanisms responsible for cardiovascular complications of different diseases and that MPs, behaving as biological vectors may represent a new therapeutic approach in conditions of several endothelial damage.

VII. Microparticles and Crohn's Disease

In the literature, there are few studies concerning the involvement of circulating MPs in Crohn's disease and particularly the possible role of MPs in the vascular alterations observed in Crohn's disease patients. Chamouard *et al.* (2005) have shown an increased levels of MPs isolated form peripheral circulation of patients with Crohn's disease compared with healthy subjects but this increase was not correlated with the level of Crohn's disease activity. The cell origin of MPs is mainly from platelets and low levels of leukocyte-derived MPs are also found. Furthermore, the treatment with infliximab reduces significantly the level of circulating MPs from Crohn's disease patients. In another study, the presence of elevated level of PMPs in blood of patients with IBD has been reported. In particular, there is a relationship between the circulating levels of PMPs and both active phase of the inflammatory disease and activation of platelet aggregation evaluated by P-selectin expression. It is also demonstrated that the level of MPs from platelets and P-selectin were significantly reduced after remission of disease (Andoh *et al*., 2005).

These data showed an involvement in Crohn's disease of MPs derived from cells involved in inflammation and therefore the release of MPs could be linked to the type of inflammatory response underlying disease.

The aim of study

The high level of MPs and the presence of vascular alterations induced by MPs have been demonstrated in different diseases characterized by an inflammatory component (Meziani *et al*., 2006; Mostefai *et al*., 2008; Agouni *et al*., 2008; Priou *et al*., 2010). These data suggest that on the one hand, MPs may represent differential markers of disease; they may act as biological vectors of inflammation and vascular remodelling and therefore could be considered as new drug targets to combat these diseases.

At present, the participation and the role of MPs in Crohn's disease have not been widely studied. There is no study that has established a correlation between the rate of circulating MPs, vascular dysfunction and inflammation.

For this reason, we are interested in the potential role and effects of MPs isolated from plasma of Crohn's disease patients and in the evaluation of their possible contribution in the pathophysiology of the disease.

We wanted to test the hypothesis that MPs could be markers of inflammatory and thrombotic complications of disease and that circulating MPs can be vectors responsible of vascular dysfunction and tissular alteration such as the colon observed in Crohn's disease patients.

In this study, we investigated the possible relationship between the levels and cellular origin of MPs and disease activity.

- For this, we have quantified the MPs from inactive and active Crohn's disease patients and healthy subjects and assessed their cellular origins. Particular interest is given to the characterization of MPs according to their procoagulant and proinflammatory properties.

- Next, using an animal model, we evaluated the consequences of *in vivo* treatment of inactive and active Crohn's disease MPs and healthy subjects MPs on endothelial function and vascular reactivity in order to test their pathophysiolgical relevance.

In particular, the quantification of MPs allows us to use, in mice, the same concentration of circulating MPs found in Crohn's disease patients and healthy subjects to analyze their effects on different tissues of animal.

- Furthermore, we evaluated the mechanisms that underlie the vascular and tissue dysfunction focusing analysis on pathways involved in the production of NO, COX-derived metabolites and oxidative stress.

- In a second part, we consider the potential effects of MPs on a target organ of disease, the colon, with particular attention to the involvement of NO pathways, oxidative stress and inflammatory cytokines.

Manuscript I

Microparticles are relevant markers of Crohn's disease activity and cause endothelial and vascular dysfunctions

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Background & Aims: Microparticles (MPs) are small vesicles released from the membrane surface during cell activation and apoptosis, and have been implicated in coagulation, inflammation and vascular dysfunction in a number of diseases including Crohn's Disease (CD). We aimed to characterize circulating MPs from patients with CD and to evaluate their effects of *in vivo* treatment on *ex vivo* endothelial function and vascular reactivity. **Methods:** Circulating MPs and their cellular origins were examined by flow cytometry of blood samples from healthy subjects (HS) and inactive or active CD patients. MPs from patients or HS were injected into mice intravenously. Then, endothelial function and vascular reactivity were assessed with respect to cyclo-oxygenase pathway, oxidative and nitrosative stress. **Results:** Circulating levels of MPs did not differ between HS and inactive CD patients except the increased levels of MPs from leukocytes in the later. In contrast, active CD patients compared to HS displayed increased circulating levels of MPs, especially those from platelets, pro-coagulant, endothelial cells, erythrocytes, leukocytes, activated leukocytes and activated platelets. A significant correlation was found between total levels of MPs, those from platelets and endothelial cells, CD activity indexes and C reactive protein levels. In addition, pro-coagulant MP levels and those from erythrocytes were positively correlated with C reactive protein. Injection of MPs from CD, but not from HS, into mice impaired endothelium-dependent relaxation in aorta and flow-induced dilation in small mesenteric arteries. Unexpectedly, MPs from inactive CD patients were more effective in inducing endothelial dysfunction compared those from active CD patients. Injection of MPs from both inactive and active CD patients, but not those from HS, into mice induced vascular hypo-reactivity in response to serotonin in aorta. Interestingly, hypo-reactivity was reversed by a nitric oxide (NO)-synthase inhibitor, and was associated with increased production of NO and decreased superoxide anion content in aorta. The selective cyclo-oxygenase-2 inhibitor reduced serotonin-induced contraction in vessels from vehicle and HS MPs but did not affect response

to the same agonist in aorta from CD MP-treated mice. These data provide evidence that MPs from CD patients are relevant prognostic markers of the diseases activity and inflammation. Moreover, MPs can be not only considered as triggers of endothelial dysfunction or injury, but also as effectors able to amplify pre-existing vascular dysfunction, including vascular hypo-reactivity and inflammation.

Introduction

Crohn's disease (CD) is a relapsing inflammatory disorder of the digestive tract the cause of which remains largely unknown. Three main interacting actors contribute to the pathogenesis: genetic susceptibility factors; environmental factors; and priming by the enteric flora.[1,2] As a result, inappropriate intestinal inflammatory and immune response occur leading to immune-mediated tissue injury and the subsequent biological, histological and clinical symptoms and signs of the disease. Beside this current pathophysiological hypothesis, several data indicate that CD is also associated to intestinal microvascular dysfunction.[3] Early pathological studies have shown occlusion of the vessels supplying the mesenteric margin that might cause ischemia in CD inflamed terminal ileum.[4] Moreover, mesenteric vascular damage appears before inflammatory infiltration of the intestinal *lamina propria*.[5] Peripheral arterial mesenteric thrombosis and multifocal mesenteric ischemic infarction in distal small arteries at the mesenteric border, next to the mucosal lesions have also been reported in CD.[6,7]

Among the direct consequences of these vascular alterations, the risk of thrombosis, endothelial dysfunction and modifications of vascular tone are well described.[8,9] In human submucosal arterioles isolated from areas of chronic inflammatory damage of intestine from intestinal bowel disease (IBD) patients, endothelial dysfunction is associated with reduced nitric oxide (NO) and increased oxidative stress.[10] However, other authors described an unchanged endothelium-dependent and independent relaxation in the mesenteric arteries from CD patients but a decrease of vascular tone. The later could improve the blood perfusion as a possible response of host organism against the injury.[11] In contrast to these results, we found no modification in vascular reactivity of small mesenteric arteries from patients with CD despite an increase of release of proinflammatory cytokines in mucosa of CD patients. Indeed, a balance between vasoconstrictor products from cyclo-oxygenase-2 (COX-2) and unidentified vasodilator products maintains the vascular reactivity unchanged.[12]

Besides, functional alterations in inflamed gut can result from over-productions of different inflammatory mediators including not only NO and eicosanoids but also endotoxin and cytokines.[13] Other factors that may trigger these effects can be microparticles (MPs) which are small membrane vesicles shed from cells in response to activation and apoptosis.[14-16] Present in blood of healthy subjects (HS), MPs have been studied in various disease states, like diabetes, metabolic syndrome, sepsis, obstructive sleep apnea or pre-eclampsia, in which their number, cellular source or composition are altered.[17-21] Recent data showed that MPs might play a major role in interactions with circulating cells or the components of the vessel wall. MPs have been implicated in coagulation, inflammation and vascular function.[22, 28]

With regard to CD, increased circulating levels of pro-coagulant MPs that are reduced upon treatment with infliximab in active CD patients with no correlation to the clinical Van Hees disease activity index has been reported.[23] On the other hand, elevated MPs derived from platelets correlate with disease activity indexes and activation of platelet aggregation evaluated by P-selectin expression. The level of platelet MPs decreased after remission of disease.[24]

To the best of our knowledge, the correlation between total circulating MPs and the relative contribution of the cellular origins with biological and clinical characteristics of CD patients has never been assessed in details. Moreover, the role of these MPs in the regulation of endothelial dysfunction and vascular reactivity in CD patients is not known.

Thus, the aim of this work was to characterize circulating MPs from patients with CD according to their cellular origins and their correlation with biological and clinical status. Then, MPs were injected *i.v.* into mice to test their pathophysiological relevance on both endothelial function and vascular reactivity in conductance and small mesenteric arteries with respect to NO and superoxide anion (O_2^-) productions and to the involvement of COX pathway.

Methods

Patients

Thirty four patients (24 women, 10 men) with CD confirmed according to the Lennard-Jones criteria[25] were recruited at the Hepatogastroenterology Department of Caen University Hospital. Exclusion criteria were: current or recent (1 month before) intestinal infection or infectious complication of CD (i.e. intra-abdominal or perineal abcess), extra-digestive infection, or pregnancy. Their median age was 26 years (extremes: 19-60). Clinical characteristics of patients were summarized in Table 1. In addition to clinical characteristics and clinical activity (determined by the Harvey-Bradshaw index[26]), biological parameters were also determined at the time of blood collection for MP isolation in CD patients: total leukocytes, polymorphonuclear leukocytes (PMN), lymphocytes and platelets counts, hemoglobin, C-reactive protein (mg/L) and albumin (g/L) concentrations.

The control group consisted of 28 HS (Controls: 13 women, 15 men) aged 25.5 years old that did not differ with those of CD patients (22-56; $P = 0.15$ compared to CD patients). HS had no history of significant extra-digestive disease and, except oral contraception in women, do not take any medication.

In accordance with the ethical guidelines of the Declaration of Helsinki and the ethical French guidelines, the local Ethics Committee (Comité de Protection des Personnes (CPP) Nord-Ouest III, Caen, France) approved the study. All subjects provided written informed consent during an introductory session prior to the start of the study.

Blood collection and cell-derived circulating MP isolation

Blood samples from CD patients and healthy volunteers (20 mL) were collected by sterile venous puncture in trisodium citrate glass tubes (BD Vacutainer) at a final volume ratio of 9:1. Blood collection was in all cases performed in the morning in overnight fasting patients and controls. The isolation procedure of circulating MP began less than 30 minutes after the

blood collection. Samples were centrifuged for 20 minutes at 270 *g*, and plasma was then harvested and centrifuged 20 minutes at 1,500 *g* to obtain platelet-free plasma (PFP). Two hundred µL of PFP were frozen and stored at –80°C until subsequent use. As previously described, remaining PFP was subjected to three series of centrifugations at 21,000 *g* for 45 minutes in order to eliminate plasma and to pellet MPs for *in vivo* studies and supernatant was replaced by 0.9 % saline salt solution.[18,19] Finally, MP pellets were recuperated in 150 µL of 0.9 % saline salt solution and stored at 4°C until subsequent use. Washing medium for the last supernatant was used as control.

Characterization of MP phenotype

MP subpopulations were discriminated in PFP according the expression of membrane-specific antigens by flow cytometer.[18,19] MPs derived from platelets, erythrocytes, leukocytes, endothelial cells and granulocytes were carried out using anti-CD41, anti-CD235a, anti-CD45, anti-CD146 and anti-CD66b antibodies, respectively. Anti-CD62P and anti-CD62L antibodies were used to identify P-selectin$^+$ and L-selectin$^+$ MPs, respectively. Irrelevant human IgG was used as an isotype-matched negative control for each sample. Annexin V (Beckman Coulter, Villepinte, France)-binding was used to numerate phosphatidylserine-expressing MPs (2 µL of annexin V/5 µL PFP). To determine concentration of MPs, 10 µL of PFP were incubated with either 5 µL of specific antibody (Beckman Coulter). After 45 minutes of incubation, samples were diluted in 300 µL of 0.9 % saline salt solution or annexin-V labelling buffer, respectively. Then, an equal volume of sample and Flowcount beads were added in order to calculate MP concentration and samples were analyzed in a flow cytometer 500 MPL System (Beckman Coulter). Regions corresponding MPs were identified in forward light scatter and side-angle light scatter intensity dot plot representation set at logarithmic gain, depending on their diameter (0.1-1 µm). Sample analysis was stopped after the count of 10,000 events.

Vascular Reactivity

All animal studies were performed using approved institutional protocols. MPs were injected into the tail vein in male Swiss mice (6-8 week old) at the circulating level of MPs detected in the blood of CD patients (CDMPs) and HS (HSMPs) or with saline solution (vehicle). Due to the findings from our preliminary studies in previous projects investigating MP effects on vascular function that the 24 hours duration was the best time to observe changes at the functional level for vascular function [21, 27, 28], we injected the mice with MPs or vehicle control for 24 hours prior to sacrifice. After 24 hours mice were sacrificed, aortic rings and small mesenteric arteries were isolated and segment (2 mm-long) were mounted on a wire myoghraph and arteriograph for measurement of vascular reactivity. The functionality of the endothelium was assessed by cumulative application of acetylcholine (1 nmol/L to 10 µmol/L, Sigma-Aldrich, St. Quentin, Fallavier, France) in aortas precontracted with U46619 (0.1 µmol/L, Sigma-Aldrich).

Mouse small mesenteric arteries (SMA) (100 to 130 µm in diameter) were mounted in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, VT) to study the physiological endothelial dilatation in response to shear stress. Diameter changes were measured by increasing flow rate (0 to 92 µl/min) under a constant intraluminal pressure of 75 mmHg. The integrity of the endothelium was studied by using of acetylcholine (10 µmol/L) in the arteries precontracted with U46619 (1 µmol/L). The different components of the dilation were determined by using the following inhibitors: the NO-synthase inhibitor N^G-nitro-L-arginine (L-NA, 100 µmol/L; Sigma Aldrich), the non-selective COX inhibitor indometachin (10 µmol/L; Sigma Aldrich). The NO-dependent component of dilation was calculated as the difference between the dilation in the absence of inhibitors and the dilation in the presence of L-NA. The dilation dependent to COX products was obtained as the difference between the dilation in the presence of L-NA alone and the dilation in the presence of L-NA plus

indomethacin. The EDHF component was assessed in the presence of L-NA plus indomethacin.

In another set of experiments, the vascular reactivity was evaluated by cumulative application of serotonin (5-HT, 3 nmol/L to 10µmol/L; Sigma Aldrich) to vessels with functional endothelium in the absence or presence of the given inhibitor: L-NA (100 µmol/L), indometachin (10 µmol/L), the selective COX-1 inhibitor 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole (SC-560 10, µmol/L) and the selective COX-2 inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398, 10 µmol/L; Sigma Aldrich). All inhibitors were used at maximal active concentrations at which they inhibit the release of either NO from all isoforms of NO-synthases, metabolites from COX-2 isoform or metabolites from COX in blood vessels, as reported in many of our previous studies.[19, 28] Higher concentrations of L-NA, NS-398 or indomethacin did not induce further inhibition.

NO Spin Trapping and Electronic Paramagnetic Resonance (EPR) Studies

The detection of NO production was performed using the technique with Fe^{2+} diethyldithiocarbamate (DETC, Sigma Aldrich) as spin trap. Isolated aortas from mice injected respectively with CDMPs, HSMPs and salt solution were incubated for 45 minutes in Krebs-Hepes buffer (BSA (20.5 g/L), $CaCl_2$ (3 mmol/L) and L-arginine (0.8 mmol/L)) and after treated with 250 µL of colloid $Fe(DETC)_2$ and incubated at 37°C for 45 minutes. The organs were immediately frozen in plastic tubes. NO measurements were performed on a tabletop x-band spectrometer miniscope (MS200; Magnettech, Berlin, Germany). Values are expressed as amplitude of signal per weight of dried tissue.

Superoxide Anion (O_2^-) Determination by EPR

The aortas isolated from mice injected respectively with CDMPs, HSMPs and salt solution were dissected and allowed to equilibrate in deferoxamine-chelated Krebs-Hepes solution containing 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH; Noxygen, Mainz, Germany) (500 µmol/L), deferoxamine (25 µmol/L), and DETC (5 µmol/L) under constant temperature (37°C) for 45 minutes. The reaction was stopped by putting samples on ice. The organs were frozen in plastic tubes and analyzed in a Dewar flask by EPR spectroscopy. Values are expressed as amplitude of signal per weight of dried tissue.

Data Analysis

Data are represented as mean ± SEM, *n* represents the number of experiences. Statistical analyses were performed by a one-way analysis of variance and Mann- Whitney *U*-test for data analysis between groups or two-way analysis of variance for repeated measures and subsequent Bonferroni post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Patient Characteristics

Clinical characteristic of CD patients are summarized on Table 1. Twenty one patients had active CD with a median Harvey-Bradshaw index of 9 (extremes: 5-19), among which 13 were considered as having active non severe disease (Harvey-Bradshaw index between 5 and 10) and 8 patients presenting severe disease (Harvey-Bradshaw index > 12). Thirteen CD patients had inactive disease (median Harvey-Bradshaw index: 1; extremes: 0-3).

Disease duration in severely active CD patients was of 49 months (extremes: 0.5-180) and in inactive patients of 108 months (extremes: 17-304) ($P = 0.13$, Mann-Whitney). Among active patients, 17 had “inflammatory” type of CD (81%), 4 “stenotic” disease compared to 9 with “inflammatory” type (69%), 4 “stenotic” disease in inactive patients ($P = 0.49$). In active CD

patients, 5 (24%) had only ileal disease, 8 (38%) had only colonic disease, and 8 (38%) had ileocolonic disease. In inactive patients, 4 (31%) had only ileal disease, 4 (31%) had only colonic disease, and 5 (38%) had ileocolonic disease. Thus, disease extension was not statistically different between active and inactive patients.

Considering biological parameters, CRP concentration was significantly higher in active CD patients ($P < 0.0001$), whereas albumin concentration was significantly lower ($P = 0.0099$) compared to inactive CD patients. Total leukocytes ($P = 0.74$), PMN ($P = 0.81$), and lymphocytes ($P = 0.86$) were not different between active and inactive CD patients. Platelet count was significantly increased in active CD patients ($P = 0.014$) whereas hemoglobin was decreased ($P = 0.039$).

Finally, Harvey-Bradshaw index was positively correlated to CRP concentration (r' = .70, $P < 0.0001$) and negatively to albumin concentration (r' = - 0.52, $P = 0.0042$). C-reactive protein and albumin concentrations were negatively correlated to each others (r' = - 0.67, $P = 0.0002$).

Circulating MPs and Their Cellular Origin

The total number of circulating MPs was significantly increased in active CD patients compared with HS and inactive CD patients (Figure 1A). Phenotypical characterization of cellular origin of MPs between HS and inactive CD patients did not show significant different between the two groups including pro-coagulant (annexing V^+) MPs, and those from platelets ($CD41^+$), endothelial ($CD146^+$), macrophages ($CD11b^+$), granulocytes ($CD66b^+$), erythrocytes ($CD235a^+$), activated leukocytes ($CD62L^+$) and activated platelets ($CD62P^+$) except that of leukocytes ($CD45^+$) MPs (Figure 1B-1F). Interestingly compared to HS, active CD patients displayed increases of pro-coagulant MPs and those from platelets, endothelial, leukocytes, erythrocytes, activated leukocytes and activated platelets (Figure1C-1J). MPs from macrophages and granulocytes were not significantly different between HS and active CD

patients (Figure 1E and 1G). Finally, active CD patients showed increased total circulating MPs, platelet- and endothelial-derived MPs when compared to inactive CD patients (Figure 1A, 1B and 1D).

Relation between MP levels and clinical characteristics

Total MPs levels were not correlated to disease duration (r' = 0.104, *P* = 0.55).

Correlation analysis for disease activity showed that total circulating MPs (r' = 0.384, *P* = 0.0274; Spearman rank test) and those from platelets (r' = 0.35, *P* = 0.045) and endothelial (r' = 0.49, P = 0.0054) were positively correlated to the Harvey-Bradshaw index.

Among biological data, total circulating (r' = 0.56, *P* = 0.0014) MPs, pro-coagulant (r' = 0.44, *P* = 0.011) MPs, platelet- (r' = 0.51, *P* = 0.0032), endothelial- (r' = 0.67, *P* = 0.0001) and erythrocyte MPs (r' = 0.36, *P* = 0.039) correlated with CRP concentration. Only a trend of correlation was observed for activated leukocyte MPs (r' = 0.33, *P* = 0.0563). There was no correlation between total MPs with albumin concentration, hemoglobin level, leukocytes, PMN or lymphocytes counts, although there was a trend with platelet count (r' = 0.34, *P* = 0.058). Endothelial-derived MPs were negatively correlated to albumin concentration (r' = -0.37, *P* = 0.05). There was no correlation between the levels of macrophage-, leukocyte-, granulocyte- and activated platelet-MPs with other measured biological characteristics of CD patients.

Neither total MPs nor their cellular origins were significantly different considering disease extension despite a trend to a decrease in ileal compared to colonic or ileocolonic disease for $CD62P^+$ MP levels (135.77 ± 30.23 *versus* 330.96 ± 68.34 *versus* 300.92 ± 57.32, *P* = 0.0723).

The type of disease did no influence MPs levels. Patients with perineal disease showed lower levels of $CD66b^+$ MPs (49.63 ± 10.52 *versus* 145.40 ± 23.35, *P* = 0.0207) and a trend to lower $CD235a^+$ MPs (266.56 ± 95.76 *versus* 543.73 ± 266.56, *P* = 0.074).

Patients with associated extra-intestinal manifestations exhibited lower levels of $CD66b^+$ MPs (55.78 ± 8.50 *versus* 144.09 ± 23.37, $P = 0.0117$).

MPs from CD induce endothelial dysfunction in the aorta and the small mesenteric arteries

The endothelium-dependent relaxation to acetylcholine was significantly impaired in aorta isolated from mice injected with CDMPs compared to aorta isolated from mice injected with salt solution and mice injected with HS MPs (E*max*: 69.3±2.1%, 65.2±2% and 45.1±1.02% for CTL, HSMPS and CDMPs, respectively P<0.001) (Figure 2A). Injection of either inactive or active CDMPs reduced acetylcholine-induced aortic endothelium-dependent relaxation when compared to those taken from HSMP- treated mice. Unexpectedly, inactive CDMPs were more effective than active CDMPs in impairing endothelial function (E*max* inactive CDMPs 41.2±1.28; E*max* active CDMPs 49.8±1.03; P<0.01) (Figure 2B, 2C and 2D).

In SMA, MPs from HS significantly enhanced flow-induced dilation when compared to control (Figure 3A). MPs from CD patients impaired flow-induced dilation when compared both to controls (P<0.01) and HSMPs (P<0.001) (Figure 3A). Compared to control, inactive CD MPs (P<0.001) but not active CDMPs reduced flow-induced dilation (Figure 3B-3C). As for the aorta, inactive CDMPs (P<0.001) were more effective in eliciting endothelial dysfunction compared to active CDMPs in SMA (Figure 3D). The reduced efficiency of active CDMPs to decrease flow-induced dilation resulted in the decrease of NO-component (Figure 3E) compensated by the increase of EDHF-component (Figure 3G) without affecting prostacyclin-component (Figure 3F) of the response. The increase flow-induced dilation by HSMPs was due to increase prostacyclin- and EDHF-component of the response.

CDMPs induce ex vivo vascular hypo-reactivity in mouse aorta

5-HT produced a concentration-dependent increase in tension in aortic rings from all groups of mice; however, vascular reactivity to the agonist was markedly decreased in mice treated with CDMPs compared to those treated either with vehicle or HSMPs (Figure 4A). Both

inactive and active CDMPs reduced vascular reactivity to 5-HT to the same extent when compared to vehicle or HSMPs (Figure 4B-4D). Since no difference of hyporeactivity was observed between inactive and active CD patients, the mechanisms involved for this process was only investigated in vessels from mice treated with active CD patients.

Involvement of NO and reduced ROS in active CD MP-induced vascular hypo-reactivity

To investigate the role of NO, the effect of the NO-synthase inhibitor, L-NA, was studied on the response to 5-HT treatment. Interestingly, we found that inhibition of the NO pathway did not affect response to 5-HT in vessels from HSMPs (Figure 5A) but completely prevented the vascular hypo-reactivity induced by active CDMPs (Fig. 5B), suggesting that NO may be involved in the mechanism of this vascular hypo-reactivity. Direct *in situ* measurements of the NO production were performed by EPR spectroscopy using $Fe(DETC)_2$ as a spin trap. Aortas from vehicle, HSMP- and CDMP-treated mice, exhibited an EPR feature of signals derived from $NO\text{-}Fe(DETC)_2$. The $NO\text{-}Fe(DETC)_2$ EPR signal was greater in aortas from active CDMP-treated mice compared to vehicle- and HSMP-treated mice (Figure 5C).

Unexpectedly, vascular hyporeactivity to 5-HT by active CDMPs was not associated with increased ROS production. Indeed, the measurement of O_2^- production by EPR showed that HSMPs and CDMPs active rather reduced O_2^- production compared to control (Figure 5D).

Involvement of COX metabolites in active CDMPs-induced vascular hypo-reactivity

To investigate the role of COX metabolites in 5-HT-induced vaso-reactivity, the effects of a non-selective inhibitor of COX (indomethacin) and a selective inhibitor of either COX-1 (SC-560) or COX-2 (NS398) were examined.

In the presence either of indomethacin or SC-560, contractile response to 5-HT was reduced in aorta from all groups of mice such as control, HS MPs and active CDMPs (Figure 6A-6F). Thus, vascular hypo-reactivity to 5-HT was still present in aortas from mice treated with CDMPs compared to HSMPs or vehicle. These results highlight that vasoconstrictor

metabolite(s) sensitive to indomethacin and SC-560 participate in 5-HT-induced contraction. When COX-2 was specifically silenced using NS398, the response to 5-HT was impaired in vessels from vehicle and HSMP-injected mice (Figure 6G-6H), suggesting the contribution of COX-2-derived vasoconstrictor metabolites. By contrast, blockade of COX-2 did not modify the response induced by active CDMPs (Figure 6I). These results suggest that active CDMPs treatment leads to the release of both vasodilator and vasoconstrictor metabolites and the former blunted the effect of the latter.

Discussion

The present study reports an increased level of circulating MPs during the active but not inactive state of CD compared to HS. Detailed analysis of cellular origin of MPs showed that only MPs from leukocytes were increased during the inactive CD patients whereas active CD patients displayed increased circulating levels of MPs from platelets, pro-coagulant, endothelial cells, erythrocytes, leukocytes, activated leukocytes and activated platelets. Most importantly, a significant correlation was found between total levels of MPs, those from platelets and endothelial cells, CD activity indexes and CRP levels. In addition, pro-coagulant MP levels and those from erythrocytes were positively correlated with CRP. Finally, only endothelial MPs were negatively correlated with albumin concentration.

Evidence of the pathophysiological relevance of MPs was provided *ex vivo.* On the one hand, CDMPs impaired endothelium-dependent relaxation in response to acetylcholine in aorta and the endothelial response to flow in SMA, MPs from inactive CD patients being more deleterious than those from active CD patients. On the other hand, CDMPs promoted vascular hypo-reactivity by inducing the production of NO, decreased O_2^- contents and altered the release of COX metabolites especially those from COX-2. Altogether, we demonstrate that MPs from CD patients are relevant prognostic markers of the diseases activity and inflammation. They also showed that MPs trigger endothelial dysfunction and vascular hypo-reactivity.

Since circulating levels of MPs are increased in several pathologies, they have been considered as biomarkers of several diseases including cardiovascular and inflammation. With regard to CD, only two studies investigate their possible role as biomarkers of the disease. The first reports that increased circulating MPs could be linked to the type of inflammatory response underlying CD although the amounts of MPs do not correlate with disease activity. It is important to note that only pro-coagulant MPs positive to annexin V have been explored

with the technique used. The cellular origin of these procoagulant MPs is mainly from platelets with low amounts from leukocytes. The second measures only MPs from platelets and demonstrates that they are elevated in active but not inactive CD and correlate with diseases activities and soluble P-selectin, suggesting that platelet MPs may be useful markers to evaluate activated state of platelets in this disease. Both procoagulant and platelet MPs probably participate to fibrinolysis impairment and thrombinogenesis responsible for the increased risk of thrombotic and bleeding disorders. Thus, the association between increased risk of thromboembolic events with altered conditions of coagulation and fibrinolysis and IBD[8] may involve platelet MPs.

The correlation between total circulating MPs and the relative contribution of the cellular origins with biological and clinical characteristics of CD patients has been assessed in details and we report important information demonstrating MPs as strong biomarkers of the diseases with good correlation with the 3 main criteria for CD including disease activity, inflammation and albumin concentration. Total levels of MPs were elevated in the active but not inactive CD patients compared to HS suggesting that the number of the circulating MPs participate in the active form of the disease and probably play a role in the deleterious processes during this phase. MPs from leukocyte origin were enhanced both in inactive and active CD patients. Although, the degree of inflammation may vary between active and inactive state of the disease, leukocytes MPs are probably involved in this process inasmuch they participate in the production of several pro-inflammatory monocytes cytokines such as IL-1β, TNF-α and IL-8 that could facilitate the interaction between leukocytes and endothelium.[29] Interestingly, active CD patients displayed increased several other populations of MPs including those from platelets, pro-coagulant, endothelial cells, erythrocytes, leukocytes, activated leukocytes and activated platelets. The burst release of the combination of these types of MPs probably participates in the inappropriate intestinal inflammatory and immune response during this

state of the disease. Thus, the present study reveals the contribution of different types MPs in addition to pro-coagulant and platelet MPs in the increased of thrombotic and bleeding disorders described in IBD patients including CD. These over type of MPs may act in a synergistic pathway to promote increased inflammatory process leading to the active state of the disease.

One of the most important findings of the present study is that the determination of 3 types of MPs can be reliable biomarkers the disease including total, platelet- and endothelial-derived MPs. Indeed, we report a great correlation between total MPs and those from platelet and endothelial origins, disease activity and CRP. Moreover, endothelial-derived MPs negatively correlated with albumin concentration. The two types of MPs probably participate in the severity of the disease, platelet MPs as discussed and, most importantly endothelial MPs. With respect to increased platelet MPs, our data are in accordance with those reported in the literature using a new tool in the evaluation of thrombotic and bleeding disorders, the endogenous thrombin potential (ETP), the increase of which is related to the activity of this disease.[30] Regarding the involvement of endothelial MPs, an increased level of this type of MPs has been observed in several pro-inflammatory and pro-thrombotic pathological states such as pulmonary hypertension, venous thromboembolism and acute coronary syndrome.[31-33] Furthermore, endothelial MPs play a role in mechanism of coagulation, inflammation and angiogenesis.[34] Thus, the combination of these populations of MPs may participate in the inflammatory process inasmuch they can induce neutrophils activation, monocyte adhesion, endothelial activation and recruitment of different inflammatory cells.[14-16]. All of these effects contribute to mesenteric thrombosis and multifocal mesenteric ischemic infarction in distal small arteries of the ileum and/or colon. They may also induce endothelial dysfunction that is the primary event leading to the failure of vasoactive, anti-coagulant and anti-inflammatory effects of healthy endothelium. Indeed, *in vivo* treatment of mice with MPs from both inactive

and active CD patients reduced both the ability of acetylcholine to promote endothelium-dependent relaxation in aorta, and also flow-induced vasodilatation in SMA. Thus, these data strongly suggest that circulating MPs from CD patients are able to induce *ex vivo* endothelial dysfunction and demonstrate one of their pathophysiological relevance in this disease. These data are in accordance with our former works with MPs isolated from patients with preeclampsia, metabolic syndrome and lately obstructive sleep apnea.[18,20,34] Unexpectedly, MPs from inactive CD patients were more deleterious than those from active CD patients. Thus, firstly endothelial dysfunction still occurs during the inactive state. Moreover, one can advanced the hypothesis of a potential attempt by the organism in the active phase of disease to partially offset these deleterious effects these MPs towards the endothelium. Further studies are needed to sort out the underlying mechanism. Indeed in SMA, the reduced NO component of flow-induced vasodilatation was compensated by an increased EDHF-component of the response in vessels taken from mice treated with MPs from active CD patients.

Finally, we provide further evidence that MPs from both inactive and active CD patients are able to promote vascular hypo-reactivity in response to vaso-constrictor agonists in mouse aorta. These findings are in line with our previous studies on other disease such as diabetes, preeclampsia and recently metabolic syndrome.[21,28,35,36] Indeed in these pathologies, MPs induce vascular hypo-reactivity by a mechanism that involve NO and COX pathways through enhanced expression of inducible NOS and COX-2 with subsequent increased NO and prostacyclin production respectively. Altogether, these results suggest that CDMPs are able to act on smooth muscle and induce the release of vasodilatory factors, at least NO without modification of oxidative stress in this case, or alternatively, CDMPs evoke alteration of the balance between vasoconstrictor or relaxant factors (COX-2 metabolites) in smooth muscle cells. The reduced response to 5-HT induced by CDMPs is independent of activity of disease.

We have reported in SMA isolated from inflamed colon of CD patients a marked inducible NOS and COX-2 expression and a balance between vasoconstrictor products from COX-2 and unknown vasodilator products that maintained vascular reactivity in a physiological range.[12] In the present study, we highlight the fact that MPs from CD patients play a significant role in vascular dysfunction including reduced response to vasoconstrictor agents in this disease.

In conclusion, the present study demonstrates that MPs from CD patients can be relevant prognostic markers of the diseases activity and inflammation. Moreover, MPs can be not only considered as triggers of endothelial dysfunction or injury, but also as effectors able to amplify pre-existing vascular dysfunction, including vascular hypo-reactivity.

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Table 1. Clinical characteristics of Crohn's disease patients.

Clinical characteristics (total Crohn's disease patients population; n = 34)	
Gender (women/men)	24/10
Age (median, extremes)	26 (19 – 60)
Disease duration (months) (median, extremes)	54 (0.5 – 304)
Disease activity; Harvey-Bradshaw index (HB) median(extremes)	Inactive: n = 13; HB: 1 (0 – 3) Non-severe: n = 13; HB: 8 (5 – 10) Severe: n = 8; HB: 14 (13 – 19)
Extension	Only ileal: n = 9 Only colonic: n = 12 ilocolonic n = 13
Type of Crohn's disease	Inflammatory: n = 26 Stenotic: n = 8 Perforating: n = 0
Perineal disease (yes/no)	8/26
Extraintestinal manifestations	No: n = 23 Yes: n = 11 *Rheumatologic (n = 8)* *Skin lesions (n = 4)* *Eye lesions (n = 1)* *Chronic sclerosing cholangitis (n = 0)*
Treatment at inclusion	Infliximab: n = 12 Adalimimab: n = 5 Azathioprine: n = 10 Methotrexate: n = 3 Oral prednisone: n = 14

Figure Legends

Figure 1. Circulating MP levels in patients with inactive CD and active CD compared to healty subjects (HS). Total circulating MPs (**A**), platelet-derived ($CD41^+$) MPs (**B**), procoagulant (annexin V^+) MPs (**C**), endothelial-derived ($CD146^+$) MPs (**D**), macrophage-derived ($CD11b^+$) MPs (**E**), leukocyte-derived ($CD45^+$) MPs (**F**), granulocyte-derived ($CD66b^+$) MPs (**G**), erythrocyte-derived ($CD235a^+$) MPs (**H**), L-selectin+ ($CD62L^+$) MPs (**I**), P-selectin$^+$ ($CD62P^+$) MPs (**J**). MPs from healthy subjects (HS n=25), MPs from inactive CD patients (inactive CD n=13) and MPs from active CD patients (active CD n=21). Results are expressed as events/µl of plasma and given as mean ± SEM. **P<0.05,* ****P<0.001* versus HS; †*P<0.05,* ††*P<0.01* active CD versus inactive CD.

Figure 2. MPs from CD patients impair endothelium-dependent relaxation in mouse aorta. Acetylcholine (Ach) -induced relaxation in aortic rings isolated from mice injected *in vivo* with salt solution (controls (CTL)), MPs from healthy subjects (HSMPs) and MPs from CD patients (CDMPs) (**A)** subsequently separated in inactive CD patients (inactive CDMPs) (**B**, **D**) and active CD patients (active CDMPs) (**C, D**). Results are expressed as a percentage of U46619-induced precontraction and given as mean ± SEM (n=*5-11*). ****P<0.001* versus CTL; †††*P<0.001* versus HSMPs; **$$$***P<0.001* inactive CDMPs versus active CDMPs.

Figure 3. MPs from CD patients impair flow-induced dilation in small mesenteric arteries. Flow-induced dilatation obtained in small mesenteric arteries isolated from mice injected *in vivo* with salt solution (controls (CTL)), MPs from healthy subjects (HSMPs) and MPs from CD patients (CDMPs) (A) separated in inactive CD patients (inactive CDMPs (B, D) and active CD patients (active CDMPs) (**C, D**). Dilation expressed in µm in response to flow expressed in µL/min: dilation dependent to nitric oxide (NO) (**E**), to cyclooxygenase products

prostaglandin (PGI_2) (**F**)**,** and to endothelium-derived hyperpolarizing factor (EDHF) (**G**). Results are given as mean ± SEM (n= 5–10). ****P<0.001* versus CTL; †††*P<0.001* versus HSMPs; **$$$** *P<0.001* inactive CDMPs versus active CDMPs.

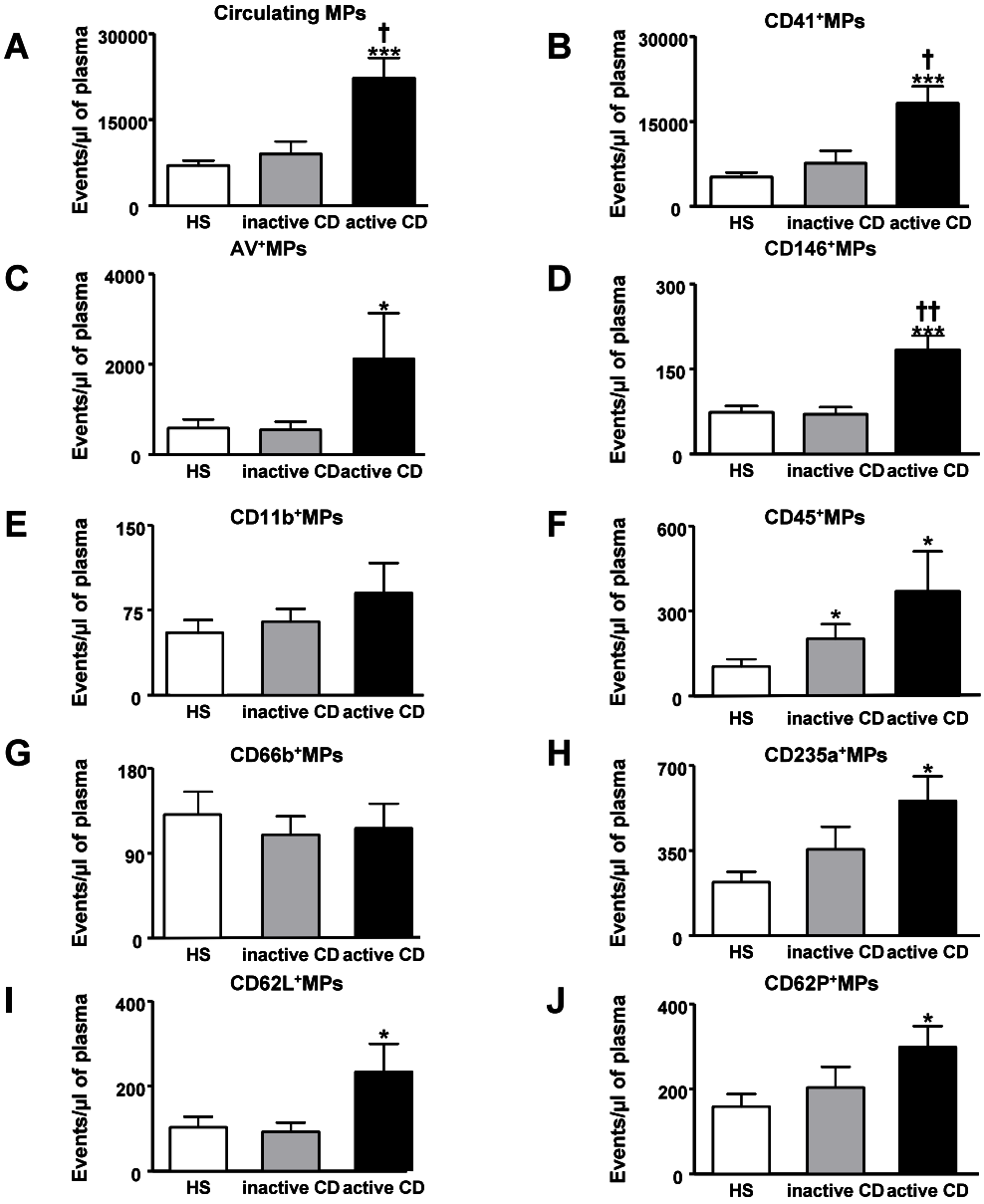
Figure 4. MPs from CD patients decrease 5-HT contraction in mouse aorta. Concentration–effect curves in response to increasing concentration of 5-HT in aortic rings isolated from mice injected *in vivo* with salt solution (controls (CTL)), MPs from healthy subjects (HSMPs) and MPs from CD patients (CDMPs) (**A**) subsequently separated in inactive CD patients (inactive CDMPs) (B, D) and active CD patients (active CDMPs) (**C, D**). Contraction are expressed in mN/mm. Results are given as mean ± SEM (n=6-13). ***P<0.01,* ****P<0.001* versus CTL; ††*P<0.001,* †††*P<0.001* versus HSMPs.

Figure 5. MPs from active CD patients induce nitric oxide overproduction in mouse aorta. Concentration–effect curves of 5-HT in the presence and in the absence of L-NA in aortic rings isolated from mice injected *in vivo* with salt solution (controls (CTL)) and MPs from healthy subjects (HSMPs) (**A**) and from mice injected *in vivo* by MPs from active CD patients (active CDMPs) (**B**). Contraction are expressed in mN/mm. Results are given as mean ± SEM (n=7-8). Nitric oxide production (C) assessed by the amplitude of NO-Fe(DETC)2 complex signal and superoxide anion production (D) assessed by the amplitude of O_2^--CMH complex signal in unit/weight in mouse aorta from mice injected *in vivo* with salt solution (CTL), HSMPs and active CDMPs. Results are given as mean ± SEM (n=5-7). ***P<0.01;* ****P<0.001* versus CTL.

Figure 6. Involvement of COX-1 and COX-2 products in vascular reactivity of mouse aorta. Concentration–effect curves of 5-HT in the presence and in the absence of indomethacin (**A**,

B, **C**), SC-560 (**D**, **E**, **F**) and NS-398 (**G**, **H**, **I**) in aortic rings isolated from mice injected *in vivo* with salt solution (controls (CTL)) (A, D, G), MPs from healthy subjects (HSMPs) (B, E, H) and MPs from active CD patients (active CDMPs) (C, F, I). Contraction are expressed in mN/mm. Results are given as mean ± SEM (n=5-7). *$P<0.05$, ***$P<0.001$ versus control aortic rings.

Figure 1


A
Circulating MPs
Events/µl of plasma
30000
15000
0
HS
inactive CD
active CD
†
***
B
CD41+MPs
Events/µl of plasma
30000
15000
0
HS
inactive CD
active CD
†
***
C
AV+MPs
Events/µl of plasma
4000
2000
0
HS
inactive CD
active CD
*
D
CD146+MPs
Events/µl of plasma
300
150
0
HS
inactive CD
active CD
††
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E
CD11b+MPs
Events/µl of plasma
150
75
0
HS
inactive CD
active CD
F
CD45+MPs
Events/µl of plasma
600
300
0
HS
inactive CD
active CD
*
*
G
CD66b+MPs
Events/µl of plasma
180
90
0
HS
inactive CD
active CD
H
CD235a+MPs
Events/µl of plasma
700
350
0
HS
inactive CD
active CD
*
I
CD62L+MPs
Events/µl of plasma
400
200
0
HS
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CD62P+MPs
Events/µl of plasma
400
200
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HS
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Figure 2

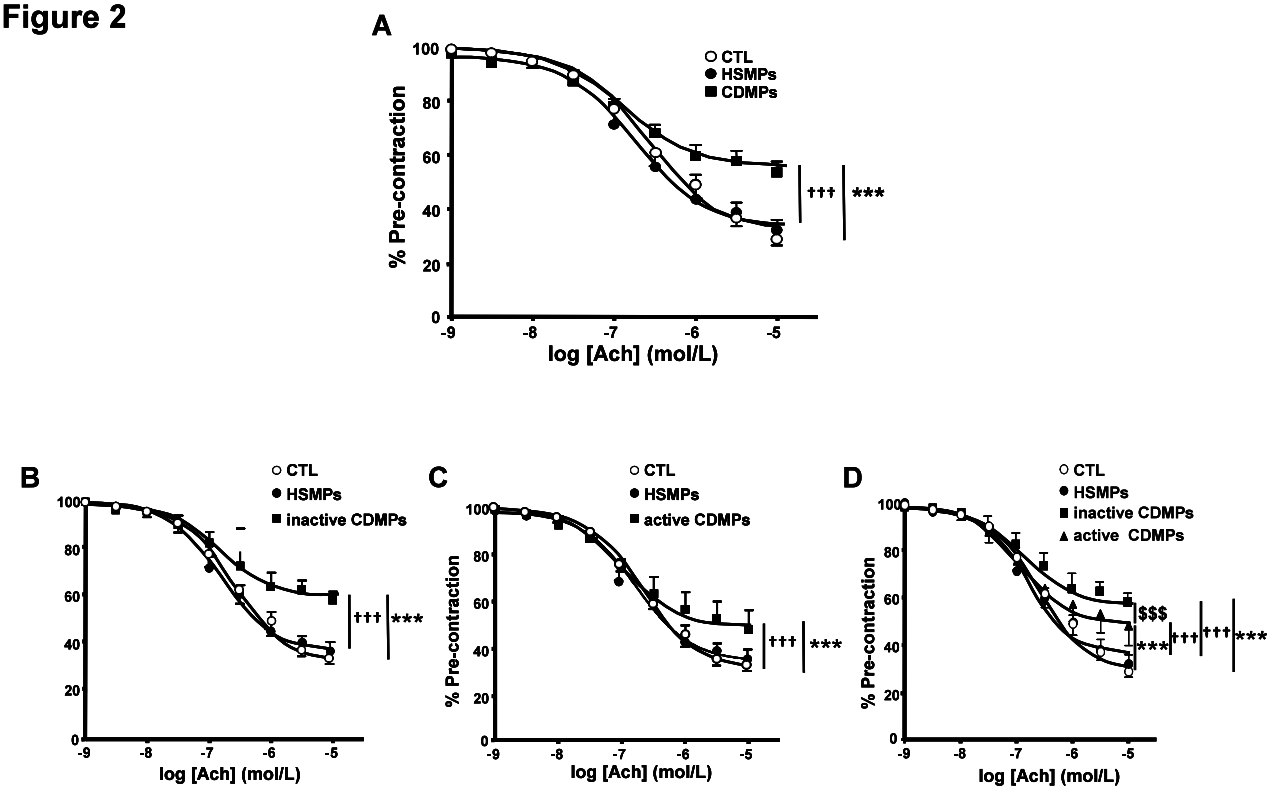
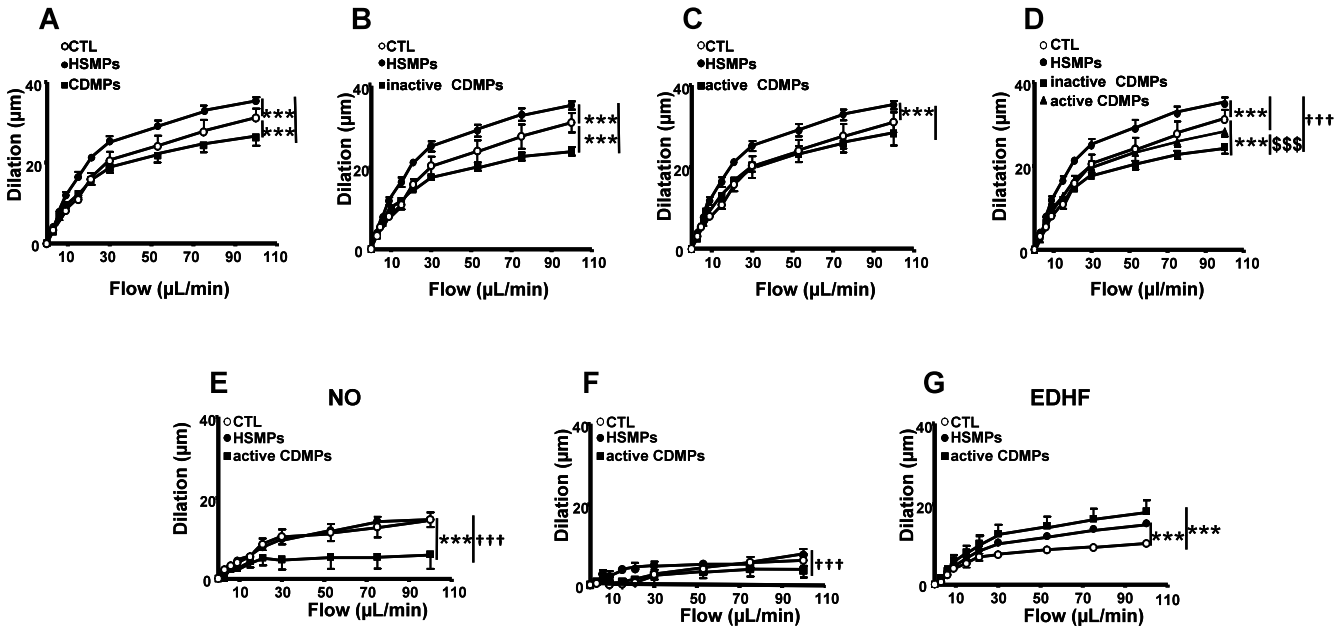

A
CTL
HSMPs
CDMPs
% Pre-contraction
log [Ach] (mol/L)
†††
***
B
CTL
HSMPs
inactive CDMPs
log [Ach] (mol/L)
†††
***
C
CTL
HSMPs
active CDMPs
% Pre-contraction
log [Ach] (mol/L)
†††
***
D
CTL
HSMPs
inactive CDMPs
active CDMPs
% Pre-contraction
log [Ach] (mol/L)
$$$
***
†††
†††
***

Figure 3


A
CTL
HSMPs
CDMPs
Dilation (µm)
Flow (µL/min)
***
***
B
CTL
HSMPs
inactive CDMPs
Dilation (µm)
Flow (µL/min)
***
***
C
CTL
HSMPs
active CDMPs
Dilatation (µm)
Flow (µL/min)
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D
CTL
HSMPs
inactive CDMPs
active CDMPs
Dilatation (µm)
Flow (µl/min)
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***$$$
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E
NO
CTL
HSMPs
active CDMPs
Dilation (µm)
Flow (µL/min)
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F
CTL
HSMPs
active CDMPs
Dilation (µm)
Flow (µL/min)
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G
EDHF
CTL
HSMPs
active CDMPs
Dilation (µm)
Flow (µL/min)
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***

Figure 4

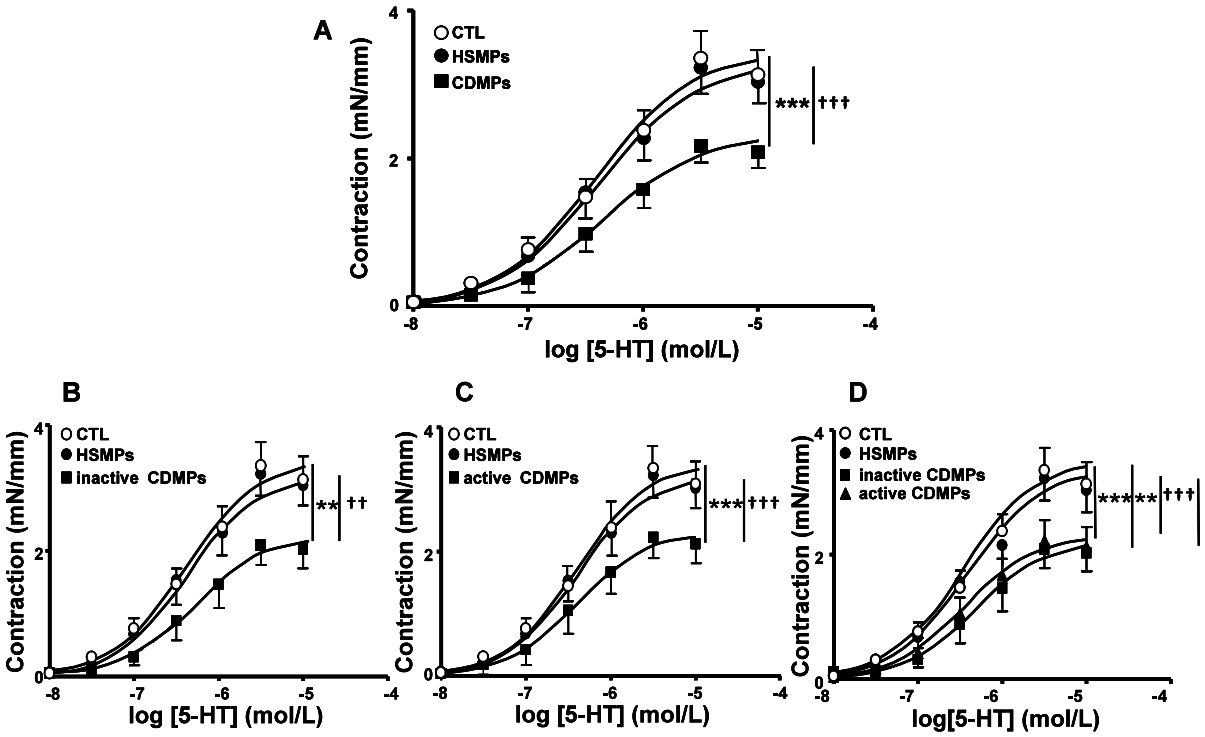

A
CTL
HSMPs
CDMPs
***†††
Contraction (mN/mm)
log [5-HT] (mol/L)
B
CTL
HSMPs
inactive CDMPs
**††
Contraction (mN/mm)
log [5-HT] (mol/L)
C
CTL
HSMPs
active CDMPs
***†††
Contraction (mN/mm)
log [5-HT] (mol/L)
D
CTL
HSMPs
inactive CDMPs
active CDMPs
***†††
Contraction (mN/mm)
log[5-HT] (mol/L)

Figure 5

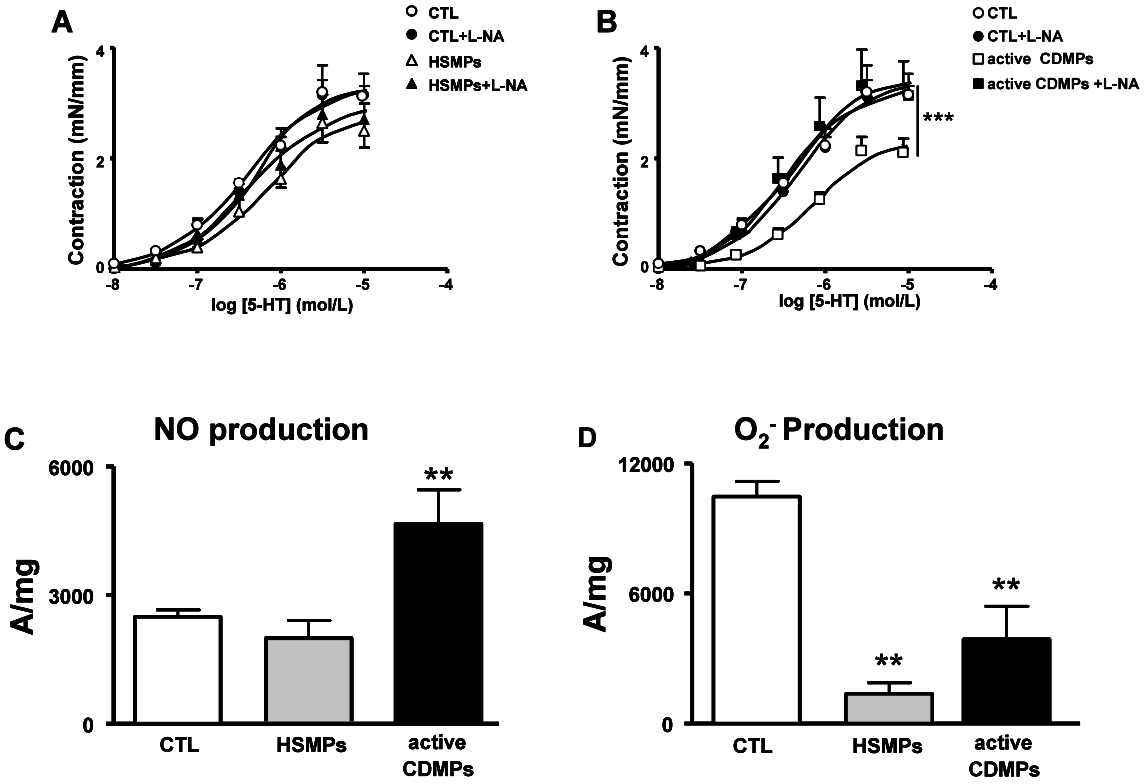
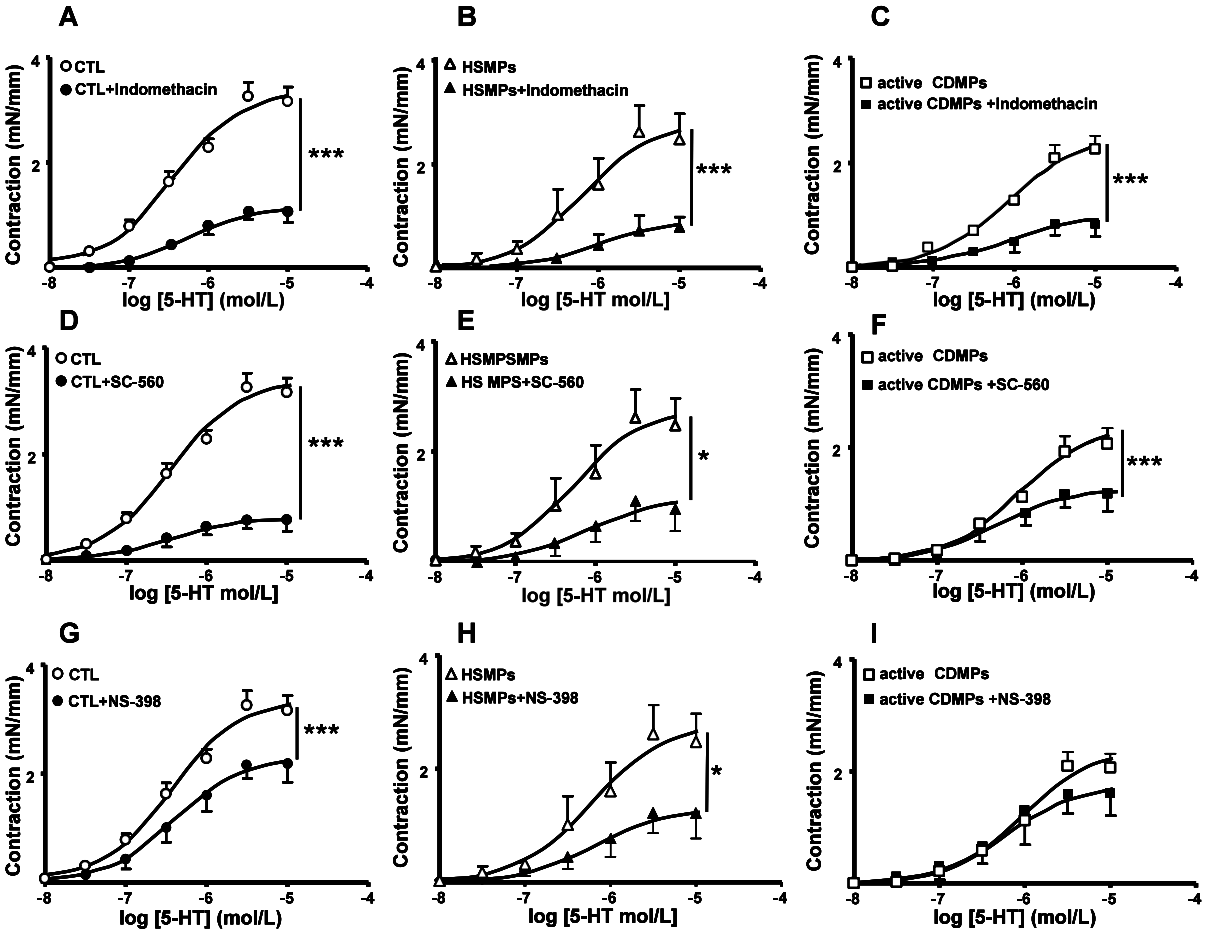

A
CTL
CTL+L-NA
HSMPs
HSMPs+L-NA
Contraction (mN/mm)
log [5-HT] (mol/L)
B
CTL
CTL+L-NA
active CDMPs
active CDMPs +L-NA
***
C
NO production
A/mg
**
CTL
HSMPs
active CDMPs
D
O2- Production
A/mg
**
**
CTL
HSMPs
active CDMPs

Figure 6


A
CTL
CTL+Indomethacin
***
Contraction (mN/mm)
log [5-HT] (mol/L)
B
HSMPs
HSMPs+Indomethacin
***
log [5-HT mol/L]
C
active CDMPs
active CDMPs +Indomethacin
***
log [5-HT] (mol/L)
D
CTL
CTL+SC-560
***
log [5-HT mol/L]
E
HSMPSMPs
HS MPS+SC-560
*
log [5-HT mol/L]
F
active CDMPs
active CDMPs +SC-560
***
log [5-HT] (mol/L)
G
CTL
CTL+NS-398
***
log [5-HT] (mol/L)
H
HSMPs
HSMPs+NS-398
*
log [5-HT mol/L]
I
active CDMPs
active CDMPs +NS-398
log [5-HT] (mol/L)

Manuscript II

Circulation microparticles from Crohn's disease patients exert differential effects on nitric oxide and superoxide anion productions, and inflammatory markers

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Abstract

Crohn's disease (CD) is one of the major forms of inflammatory bowel disease (IBD). The specific pathways that lead to mucosa damage in colon are still not completely understood. Proinflammatory mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and various cytokines are involved in inflammatory cascade which plays a critical role in the pathogenesis of CD. Microparticles are small vesicles released from the membrane surface during cell activation by agonists, physical or chemical stress and apoptosis with procoagulant and proinflammatory properties. Elevated number of microparticles occurs in various disease states in which microparticles are able to modulate nitric oxide (NO) production and oxidative stress. We evaluated possible effects of microparticles from CD patients on oxidative and nitrosative stresses and inflammatory markers. Microparticles obtained from blood samples from healthy subjects and inactive and active CD patients were injected *in vivo* in mice for 24 hours. Then, NO and superoxide anion (O_2^-) productions were assessed in different isolated tissues of mice: colon, liver, heart, lungs and kidneys. The expression of mRNA was investigated in isolated colon from mice, for 12 transcripts related to inflammation by RT–PCR. Inactive CD microparticles induce an overproduction of NO in colon from mice, conversely active CD microparticles evoke an increase level of O_2^- production in the same tissue. Furthermore, an increase of production of O_2^- is observed in isolated liver from mice treated with active CD microparticles. Finally, in colon from mice treated whit active CD microparticles, an increased expression of IL-23R mRNA is detected.

These data provide evidence of involvement of microparticles in inflammatory process observed in this pathology and suggest that microparticles could be considered as new markers of CD.

Introduction

Crohn's disease (CD) is, with ulcerative colitis, one of the major forms of inflammatory bowel disease (IBD) that affects ≈ 0.1% of Western population (Logan *et al*, 2010). This chronic inflammatory disorder is due to a transmural inflammation that extends through all layers of the bowel wall and can affect the entire gastrointestinal tract, with greater involvement of colon and terminal ileum.

The pathogenesis of CD is complex and consists of the interaction of several factors mainly genetic susceptibility factors, enteric microflora and immune-mediated tissue injury (Xavier *et al*, 2007). Particularly, the specific pathways that lead to mucosa damage are still not completely understood. The inflammatory cascade, which plays a critical role in the pathogenesis of CD, begins by infiltration of inflammatory cells into the mucosa and release of proinflammatory mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and various cytokines (Grisham, 1994; Kruidenier *et al*, 2002; Rezaie *et al*, 2007; Cross *et al*, 2003; Reimund *et al*, 1996; Radford-Smith *et al*, 1996). Moreover, the activated immune cells, notably neutrophils, during inflammatory process release ROS molecules that are highly reactive and can compromise cell integrity and function (Harris *et al*. 1992; Weiss, 1989). In this respect, epithelial cell injury linked to ROS production has been observed in mucosa of IBD patients (Kruidenier *et al*, 2003). Furthermore, using monolayers of human colonic cells in culture, Banan *et al*. (2000; 2001) showed that ROS and RNS can oxidize actin and tubulin and that the ensuing cytoskeletal disruption causes barrier dysfunction.

Thus, in CD patients, it has been suggested an imbalance between ROS production and the antioxidant defense that would cause an increase of oxidative stress, lipid peroxidation and inflammation (Wendland *et al*, 2001; D'Odorico *et al*, 2001). Indeed, ROS have been shown to contribute to the expression of genes encoding inflammatory cytokines through activation

of the NF-κB, whereas, antioxidant molecules inhibit its production in CD patients (Reimund *et al*, 1998). In this context, Maor *et al*. (2008) demonstrated a significant correlation in active CD patients between elevated levels of inflammation molecules such as C-reactive protein (CRP) and TNF-α and an oxidative index, malondialdehyde (MDA).

Concerning the involvement of RNS in inflammatory process of disease, several studies showed an involvement of nitric oxide (NO) in the active phase of IBD (Ramchilewitz *et al*, 1995; Kimura *et al*, 1998; Keshavarzian, 2003) with an up-regulation of inducible NO synthase (iNOS) enzyme (Cross *et al*, 2003). In particular, an increase of NO levels has been detected in colon of CD patients directly linked to NOS activity and the clinical and endoscopic indices of disease activity (Rachmilewittz *et al*, 1998).

Microparticles (MPs) are small vesicles (0.05-1 μm) released from the membrane surface during cell activation by agonists, physical or chemical stress and apoptosis with procoagulant and proinflammatory properties (Martinez *et al*, 2005). MPs are present in blood from healthy subjects but their numbers become elevated in disease states where can exert both beneficial and deleterious effects. Several studies conducted by our team and others have provided that MPs play a role in various pathophysiological situations and notably they are able to modulate NO production and oxidative stress (Sabatier *et al*, 2002; Meziani *et al*, 2006; Mostefai *et al*, 2008; Agouni *et al*, 2008; Priou *et al*, 2010; Tual-Chalot *et al*, 2010; Huang *et al*, 2010; Helal *et al*, 2010).

Recently, we reported (Leonetti, Reimund & Andriantsitohaina (In preparation)) that CD patients displayed elevated levels of MPs (CDMPs) especially procoagulant-, endothelial-, erythrocyte-, leukocyte- and activated leukocyte-derived MPs and this increase is correlated whit the activity index of disease. Furthermore, we provided evidence *in vivo* of the pathophysiological relevance of CDMPs. Indeed, CDMPs impaired endothelium-dependent relaxation both in aorta and in small mesenteric arteries and induced hyporeactivity in mice

aorta. These results suggest potential deleterious effects of MPs and their involvement in vascular alterations observed in CD disease patients.

Then, the aim of this study was to obtain additional information on involvement of MPs in CD disease. Using a mice model, we have assessed whether CDMPs can affect NO and superoxide anion (O_2^-) productions as well as inflammatory markers in different tissues, notably, the colon one the main target tissue involved in this disease.

Materials and Methods

Patients

Sixteen patients (10 women, 6 men) with CD confirmed according to the Lennard-Jones criteria (Lennard-Jones, 1971) who where seen at the Hepatogastroenterology Department of Caen University Hospital were considered for the study. Exclusion criteria were: current or recent (1 month before) intestinal infection or infectious complication of CD (i.e. intra-abdominal or perineal abcess), extra-digestive infection, or pregnancy. Their median age was 32 years (extremes: 19-59). Clinical characteristics of patients were summarized in Table 1. In addition to clinical characteristics and clinical activity (determined by the Harvey-Bradshaw index) (Harvey *et al*, 1980), biological parameters were also determined at the time of blood collection for MP isolation in CD patients: total leukocytes, polymorphonuclear leukocytes (PMN), lymphocyte and platelet counts, hemoglobin, C-reactive protein (mg/L) and albumin (g/L) concentrations.

The control group consisted of 12 healthy subjects (Controls: 8 women, 4 men) and their median age was 24 years old (extremes: 21-34). No healthy control subject had a personal or familial, medical or surgical history of intestinal disease. Healthy controls had no history of significant extra-digestive disease and, except oral contraception in women, do not take any medication.

In accordance with the ethical guidelines of the Declaration of Helsinki and the ethical French guidelines, the local Ethics Committee (Comité de Protection des Personnes (CPP) Nord-Ouest III, Caen, France) approved the study. All CD patients and healthy controls gave their written informed consent.

MPs isolation

Blood samples from CD patients and healthy volunteers (20 mL) were collected by sterile venous puncture in trisodium citrate glass tubes (BD Vacutainer) at a final volume ratio of 9:1. Blood collection was in all cases performed in the morning in overnight fasting patients and controls. The isolation procedure of circulating MP began less than 30 min after the blood was obtained as the medical department was located in the same building than the laboratory. Tubes were first centrifuged at 170 *g* during 10 min at room temperature to recover platelet-rich plasma. Platelet-rich plasma was further centrifuged at room temperature for 20 min at 1500 *g* in order to obtain plasma containing MPs (PCMP), which then was distributed in 2 mL tubes (1 mL PCMP/tube) and centrifuged a first time at 21000 *g* during 90 min at 4°C. Centrifugation supernatant was harvested and conserved at 4°C, and MP were pelleted, pooled, and recovered in 1 mL of 0.9% sterile NaCl. These samples were centrifuged again twice at 21000 *g* for 45 min for washing, and finally, MP were resuspended in 150 µL of 0.9% sterile NaCl and conserved at 4°C until analysis and experimental use.

For each subject, MP concentration in plasma was determined by flow cytometry. Briefly**,** an equal volume of sample and Flowcount beads (Beckman Coulter, Villepinte, France) were added and samples were analyzed in a flow cytometer 500 MPL System (Beckman Coulter), (Agouni *et al*, 2008).

Animal treatment

All animal studies were performed using approved institutional protocols. MPs were injected into the tail vein in male Swiss mice (4-8 week old) at the circulating level of MPs detected in the blood of Crohn's disease patients (CDMPs) and healthy subjects (HSMPs) or with saline solution (vehicle). After 24 h mice were sacrificed and colon, liver, heart, lungs and kidneys were isolated.

NO Spin Trapping and Electronic Paramagnetic Resonance (EPR) Studies

The detection of NO production was performed using the technique with Fe^{2+} diethyldithiocarbamate (DETC; Sigma- Aldrich, St. Quentin, Fallavier, France) as spin trap. Animals were sacrificed 24 h after administration of MPs or vehicle and colon, liver, heart, lungs and kidneys were isolated and incubated for 45 min in Krebs-Hepes buffer (BSA (20.5g/L), $CaCl_2$ (3mM) and L-arginine (0.8 mM)) and after treated with 250 µL of colloid $Fe(DETC)_2$ and incubated at 37°C for 45 m. The organs were immediately frozen in plastic tubes. NO measurements were performed on a tabletop x-band spectrometer miniscope (MS200; Magnettech, Berlin, Germany). Values are expressed as amplitude of signal per weight of dried tissue.

Superoxide Anion (O_2^-) Determination by EPR

The colon, liver, heart, lungs and kidneys isolated, after 24 h, from mice injected respectively with vehicle, HSMPs and inactive and active CDMPs, were dissected and allowed to equilibrate in deferoxamine-chelated Krebs-Hepes solution containing 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH; Noxygen, Mainz, Germany) (500 µmol/L), deferoxamine (25 µmol/L), and DETC (5 µmol/L) under constant temperature (37°C) for 45 m. The reaction was stopped by putting samples on ice. The organs were frozen

in plastic tubes and analyzed in a Dewar flask by EPR spectroscopy. Values are expressed as amplitude of signal per weight of dried tissue.

Western Blotting

The colon isolated from mice injected respectively with vehicle, HSMPs and active CDMPs were dissected and homogenized for protein extraction. Proteins (80µg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blots were probed with anti-eNOS, anti-iNOS (BD Bioscences, San Jose, CA), anti-COX-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-COX-2 (Calbiochem, London, UK).

Quantitative real-time reverse transcription–polymerase chain reaction analysis

The colon isolated after 24 h from mice injected respectively with vehicle, HSMPs and active CDMPs were used to investigate the expression of messenger RNA (mRNA) for 12 transcripts related to inflammation by real-time reverse transcription–polymerase chain reaction (RT–PCR).

RT– PCR analyses were carried out by Service Commun de Cytométrie et d'Analyses Nucléotidiques from Angers University, using a Chromo 4TM (Bio-Rad, Hercules, CA) and SYBR Green detection. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Quantifications were realized according to the ΔCt method and the relative gene expression levels were normalized using the geometric mean of three housekeeping genes as described previously (Vandesompele *et al*., 2002).

Data Analysis

Data are represented as mean ± SEM, *n* represents the number of experiences. Statistical analyses were performed by Mann- Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

Results

MPs from inactive and active CD exerted differential effects on NO and O_2^- production in colon from mice

Colon isolated from mice treated respectively with vehicle, HSMPs and CDMPs exhibited an EPR feature of signals derived from NO-$Fe(DETC)_2$. As shown in Figure 1A, NO production was not significantly different between three groups of mice. After separation of patients depending on the inactive or active phase of disease, we observed that injection of inactive CDMPs induced a significantly increase on NO release in colon from mice compared to colon from control mice (Figure 1C). In contrast, active CDMPs did not affect NO production in colon from mice. In the aim to evaluate molecular pathways governing the NO production, we analyzed, by Western blotting, expression of eNOS and iNOS in colon from control mice and from HSMPs- and active CDMPs-treated mice. MPs from either HS or active CD patients did not modify both the expression of eNOS and iNOS in colon from mice (Figure 2A-2B).

EPR measurement of O_2^- production showed that CDMPs but not HSMPs significantly increased O_2^- production in colon from mice compared to those injected with vehicle (Figure 1B). In addition, only active CDMPs were able to induce significantly increase of O_2^- production in isolated colon from mice compared to vehicle (Figure 1D).

MPs from active CD patients did not affect COX-1 and COX-2 expressions in colon from mice

In order to evaluate a possible involvement of COX metabolites in the effects induced by MPs in colon from mice, we evaluated expression of COX-1 and COX-2 in colon from three groups of mice (control, injected with HSMPs and injected with CDMPs). Both HSMPs and active CDMPs did not modify the expression of COX-1 and COX-2 (Figure 2C-2D).

Effects of active CDMPs on NO and O_2^- productions in isolated tissues from mice

EPR measurement of NO production in isolated liver, heart, and kidneys did not differ among the three groups (control mice and mice injected with HSMPs or CDMPs). Only in isolated lungs, we showed a significantly increase on NO production in mice injected with HSMPs compared with CDMPs-treated mice (Table 1).

The analysis of O_2^- production did not display difference in heart, lungs and kidneys. Conversely, in liver CDMPs increase significantly O_2^- production compared to control mice and mice injected with HSMPs (Table 2).

MPs from active CD patients increased expression level of mRNA of IL-23R in colon from mice

The possible regulation of expression by MPs of inflammatory markers was further evaluated. The analysis by real-time quantitative RT-PCR showed that the expression of specific transcripts for the interleukin-23 receptor (IL-23R) were enhanced in colon isolated from mice injected with active CD patients compared to colon injected with MPs from HS (Table 3).

Discussion

We have previously shown that CDMPs induce vascular alterations such as endothelial dysfunction and hyporeactivity in mouse aorta by a subtle alteration of the balance between NO, reactive oxygen species and metabolites from COX-2. In the present study, we show that MPs issued from plasma from CD patients affect also the main target tissue involved in CD, the colon. Interestingly, whereas injection of inactive CDMPs induced an overproduction of NO without changes in O_2^- production in colon from mice, active CDMPs did not affect NO pathway but enhanced O_2^- production in colon and liver. In addition, active CDMPs did not modify both COX-1 and COX-2 expressions. Finally, in colon from mice treated with active CDMPs, an increase in IL-23R mRNA expression was observed. These data suggest that CDMPs are main actors in the induction of oxidative and nitrosative stresses, depending on the phase of the disease, as well as in the inflammatory process associated with this pathology.

In the present study, we report that inactive, but not active, CDMPs enhance NO production. Although we have not evaluated the effects of these MPs on NOS expression, it has been observed that colonic epithelial cells represent a major source of NO production and, NOS activity (particularly iNOS) has been reported to be increased in the mucosa of IBD patients. In addition, this production is regulated by T cell through the modulation of proinflammatory cytokines (Kolios *et al*, 2004).

Interestingly, active CDMPs do not affect NO release but induce an increase of O_2^- production. This result suggests a possible role of MPs, at least during the active phase of CD, in oxidative stress observed in CD (Kruidenier *et al*, 2003; Tüzün *et al*, 2002). According whit these data, Maor et al. (2008) found that patients with active CD showed an enhanced oxidative stress level which decreased when the patients improve in terms of CD status and become clinically stable.

Under oxidative stress conditions, the production of ROS exceeds the available antioxidant system. Several evidences suggested that in CD there is en imbalance between ROS production and antioxidant defense system leading to increased oxidative stress, lipid peroxidation and inflammation (Geerling *et al,* 1998; Kruidenier *et al*, 2003). In this respect, it has been reported that, in serum from active CD patients, an enhanced lipid peroxidation as well as an increase susceptibility of the serum to oxidation are accompanied with a low level of antioxidant β-carotene and a high activity of glutathione peroxidase (Maor *et al*., 2008). Regarding the enzyme systems that can potentially produce ROS species, they include NADPH oxidase, xanthine oxidase and enzymes of the mitochondrial respiratory chain. An up-regulation of NADPH oxidase expression has been observed in CD intestinal macrophages (Hausmann *et al*, 2001). In addition, an increased expression of NOX-1 mRNA, a ROS-producing NADPH oxidase, has been shown in lymphocytes in lesions of CD patients (Szanto *et al*., 2005). Furthermore, the ability of MPs to modulate the expression of NADPH oxidase subunit has been found in other pathologies such as sepsis and metabolic syndrome (Agouni *et al*., 2008; Mostefai *et al*., 2008). These data suggest a possible mechanism by which CDMPs could cause an increase of oxidative stress.

Recently, some authors have found a mitochondrial dysfunction related to oxidative damage that occurs in CD and that could suggest a mitochondrial origin of ROS. Indeed, in active CD patients, a significant inhibition of mitochondrial membrane potential (ΔΨm) has been observed. Conversely, inactive CD patients showed a complete recovery to the normal value of ΔΨm, showing that mitochondrial dysfunction is related to disease activity (Beltrán *et al*, 2010).

Another hypothesis that could explain the increase in the production of O_2^- observed in colon from mice treated with active CDMPs could be linked to a lower activity of the antioxidant systems such as the superoxide dismutase (SOD). SOD is a primary defense against ROS

which is activated in cells when excessive O_2^- production occurs. There are three SOD isoforms: cytoplasmic copper/zinc (Cu/Zn)-SOD, mitochondrial manganese (Mn)-SOD, and an extracellular SOD. In support of this hypothesis, some studies reported lower levels of Cu/Zn-SOD protein and activity in IBD peripheral blood granulocytes and in inflamed mucosa from IBD active patients (Verspaget *et al*., 1988; Mulder *et al*., 1991). Furthermore, a more recent study showed an increase in Mn-SOD protein levels in non-inflamed and inflamed IBD mucosa, but this increase is not correlated to activity of enzyme. Instead, the Cu/Zn-SOD and EC-SOD content decreased with inflammation (Kruidenier *et al*., 2003). These findings might indicate a decreased endogenous intestinal protection against ROS in IBD which could contribute to the pathogenesis of the disease.

In the present study, we found also that active CDMPs evoke an increase production of O_2^- in liver from mice. In light of this, one could assume that CDMPs may be involved in extra-intestinal manifestation observed in CD patients. Indeed, liver is one of the organs most involved in the complication of this pathology since hepatobiliary manifestations constitute some of the most common extraintestinal manifestations of IBD (Navaneethan *et al*., 2010).

Finally, expression of specific transcripts for the IL-23R mRNA is increased in colon from mice treated with active CDMPs. The gene encoding IL-23R is the one the strongest gene associated to CD (Duerr *et al*., 2006). According with the present study, there is evidence that IL-23R signalling pathway plays a key role in mediating organ inflammation in mouse model of IBD (Ma *et al.* 2010; Durrant *et al*., 2010). The IL-23R mRNA is expressed by various cells involved in inflammatory cascade such as natural killer (NK) cells, NKT cells, $CD4^+$ and $CD8^+$ T cells. It could be argued that these cells release MPs which would act as vector of inflammatory messages.

Of particular interest is the ability of IL-23 to induce IL-17 expression by T helper (Th)-17 cells. The signalling pathway of IL23R, following engagement of IL-23, involves a series of

STAT molecules, and in particular STAT3 and STAT4 play an important role in the differentiation of Th-17 cells and Th-1 cells (Watford *et al*, 2004; Cho, 2008). Th-17 cells represent a novel subset of $CD4^+$ T cells that are protective against extracellular microbes, but are responsible for autoimmune disorders in mice (Annunziato *et al*, 2007). In intestinal lamina propia of CD patients, it has been shown an increased expression of IL-17 (Fujino *et al*., 2003). Therefore, an interesting aspect would be to evaluate a possible implication of CDMPs in this signalling pathway, in light of the fact that MPs can allow intracellular communication by different ways and can transfer different molecules including mRNA.

In conclusion, we show that CDMPs play a role in inflammatory process and increased oxidative stress observed in CD. Furthermore, CDMPs could also evoke extraintestinal effects related to the pathology. The increased expression of IL-23R induced by CDMPs in colon of mice highlights a possible role of CDMPs in specific signalling pathways that involve inflammatory cytokines directly involved in this pathology. All these data confirm the deleterious effects played by MPs in CD and that MPs can be considered as markers of inflammation.

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Figure Legends

Figure1. Inactive CDMPs increase NO production and active CDMPs increase O_2^- production in colon of mice. Quantification of the amplitude of NO-Fe(DETC)$_2$ (A, C) and O_2^- CMH (B,D) complex signals in colon of mice injected *in vivo* by salt solution [controls (CTL)], MPs

from healthy subjects and MPs from CD patients (CDMPs). CDMPs are separated into inactive CDMPs and active CDMPs (C, D). Values are expressed as unit/weight of dried colon (n=5-10) *$P<0.05$, * * $P<0.01$ versus CTL.

Figure 2. Active CDMPs do no affect eNOS, iNOS, COX-1 and COX-2 expressions. Western blots reveal expression of eNOS (A), iNOS (B), COX-1 (C), COX-2 (D) in colon of mice treated *in vivo* with salt solution [controls (CTL)], MPs from healthy subjects (HSMPs) and MPs from active CD patients (active CDMPs) (n=5-8).

Table 1. Clinical characteristics of CD patients (n=16).

Clinical characteristics of Crohn's disease patients	
Number	**16**
Age (median, extremes)	**32 (19-59)**
Sex ratio (male:female)	**6:10**
Disease duration (months) (median, extremes)	**91 (9-304)**
Disease activity; Harvey-Bradshaw index (HB) (median, extremes)	**Inactive: n=7 HB:1 (0-3)** **Non-severe: n=6 HB:8 (6-10)** **Severe: n=3 HB:14 (13-16)**
C-reactive protein (CRP) (mg/L)	**23.52±11.33**
Albumin (g/L)	**34.97±1.67**
Total leucocytes (number/mm^{3}of blood)	**9165.33±893.19**
Polymorphonuclear leucocytes (PMN) (number/mm^{3}of blood)	**5745.13±779.88**
Lymphocytes (number/mm^{3}of blood)	**2065.53±211.85**
Platelets (number/mm^{3}of blood)	**312437.50±29886.30**
Hemoglobin (g/dL)	**12.90±0.41**
Extension	**Only ileal: n =5** **Only colonic: n =2** **Ileocolonic n = 9**
Type of Crohn's disease	**Inflammatory: n = 11** **Stenotic: n = 5** **Perfotaring n= 0**
Perineal disease (yes/no)	**4/12**
Extraintestinal manifestations (yes/no)	**7/9**
Treatments	**Infliximab: n =4** **Adalimumab: n =3** **Azathioprine: n =3** **Methotrexate: n = 1** **Oral prednisone: n =8** **NO treatment n=1**

Table 2. Effects of active CDMPs on NO production in liver, heart, lungs and kidneys isolated from mice. Nitric oxide (NO) production assessed by the amplitude of NO-$Fe(DETC)_2$ complex signal in unit/weight in tissues from mice injected *in vivo* with vehicle [controls (CTL)], MPs from healthy subjects (HSMPs) and MPs from active CD patients (active CDMPs) (n=5-8). †† *P<0.01* HSMPs versus active CDMPs.

	NO (A/mg)		
Tissues	**CTL**	**HSMPs**	**Active CDMPs**
Liver	100.43 ± 20.41	74.31±13.11	78.65±11.96
Heart	268.34 ± 34.65	334.82 ± 67.80	283.51 ±55.60
Lung	271.24 ± 35.82	387.52 ± 33.50 ††	187±18.89
Kidney	180.27 ± 36.50	149.40 ± 15.02	128.11 ± 20.18

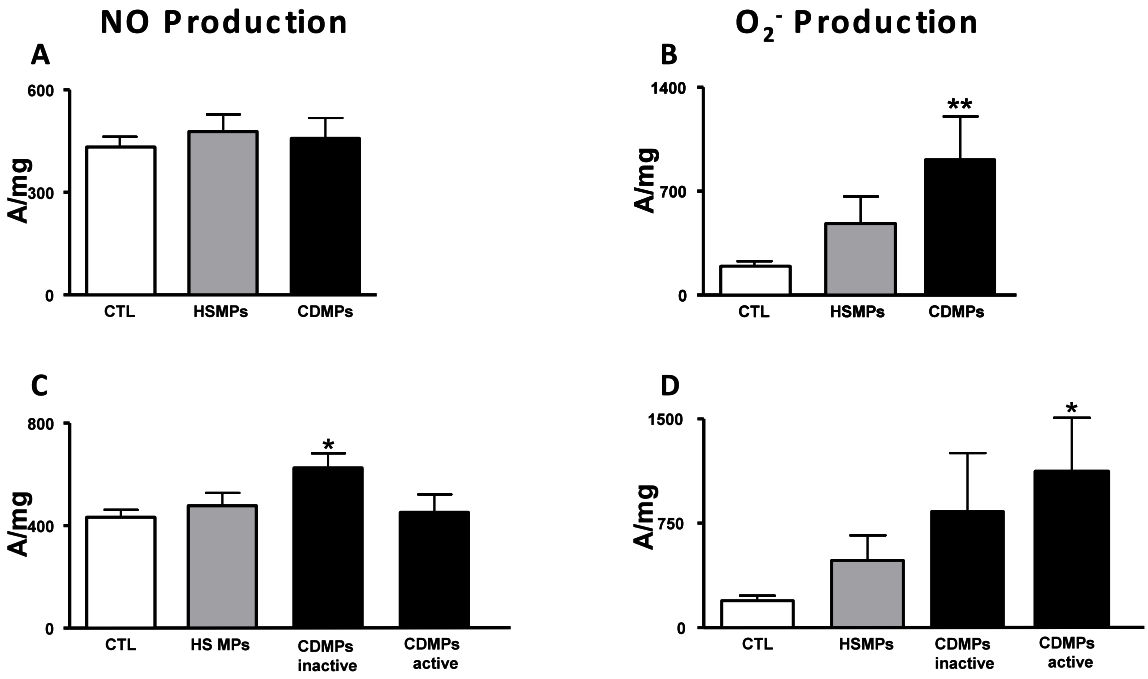
Table 3. Effects of active CDMPs on O_2^- production in liver, heart, lungs and kidneys isolated from mice. Superoxide anion production assessed by the amplitude of O_2^{-}-CMH complex signal in unit/weight in tissues from mice injected *in vivo* with vehicle [controls (CTL)], MPs from healthy subjects (HSMPs) and MPs from active CD patients (active CDMPs).(n= 5-7). ** *P<0.01* versus CTL, † *P<0.05* versus HSMPs.

	O_2^- (A/mg)		
Tissues	**CTL**	**HSMPs**	**Active CDMPs**
Liver	52.23 ± 8.57	66.09±20.38	214.36±37.46**†
Heart	229.65 ± 58.03	472.50±81.08	290.11±101.44
Lung	540.48± 155.96	431.47± 65.37	500.18 ± 251.05
Kidney	57.77 ± 28.08	99.93 ± 44.55	87.01 ± 33.39

Table 4. Effects of active CDMPs on different mRNA expression in isolated colon of mice. CTL= mice injected *in vivo* with vehicle, HSMPs= mice injected *in vivo* with MPs from healthy subjects, active CDMPs= mice injected *in vivo* with MPs from active CD patients, ND=No detected. (n=3-11) † *P<0.05* versus HSMPs.

	mRNA normalized quantity		
	CTL	**HSMPs**	**Active CDMPs**
IL-6	ND	ND	ND
TNF-α	269±128.0	179±69.9	346.5±95.12
MCP-1	947.24±413.20	494.17±165.96	490.75±191.01
IL-1α	431.14±122.94	271.5±101.83	322±101.83
TGF-β1	6950.8±1997.68	5247.33±1153.17	8697.12±2571.29
TGF-β3	1394.33±399.49	1032.42±407.02	702.25±229.78
IL-12p40	ND	ND	ND
IL-22	72.66±3.28	70.75±18.58	64±15.30
IL-23R	208.36±72.85	103.37±30.99	519.66±35.16†
IL-23 p19	154.66±69.66	300.2±215.28	159.75±25.92
IL-17F	97.25±43.63	41±11.23	24.33±6.64
INF-γ	815±351.16	329.33±103.68	265±65.01

Figure.1


NO Production
A
600
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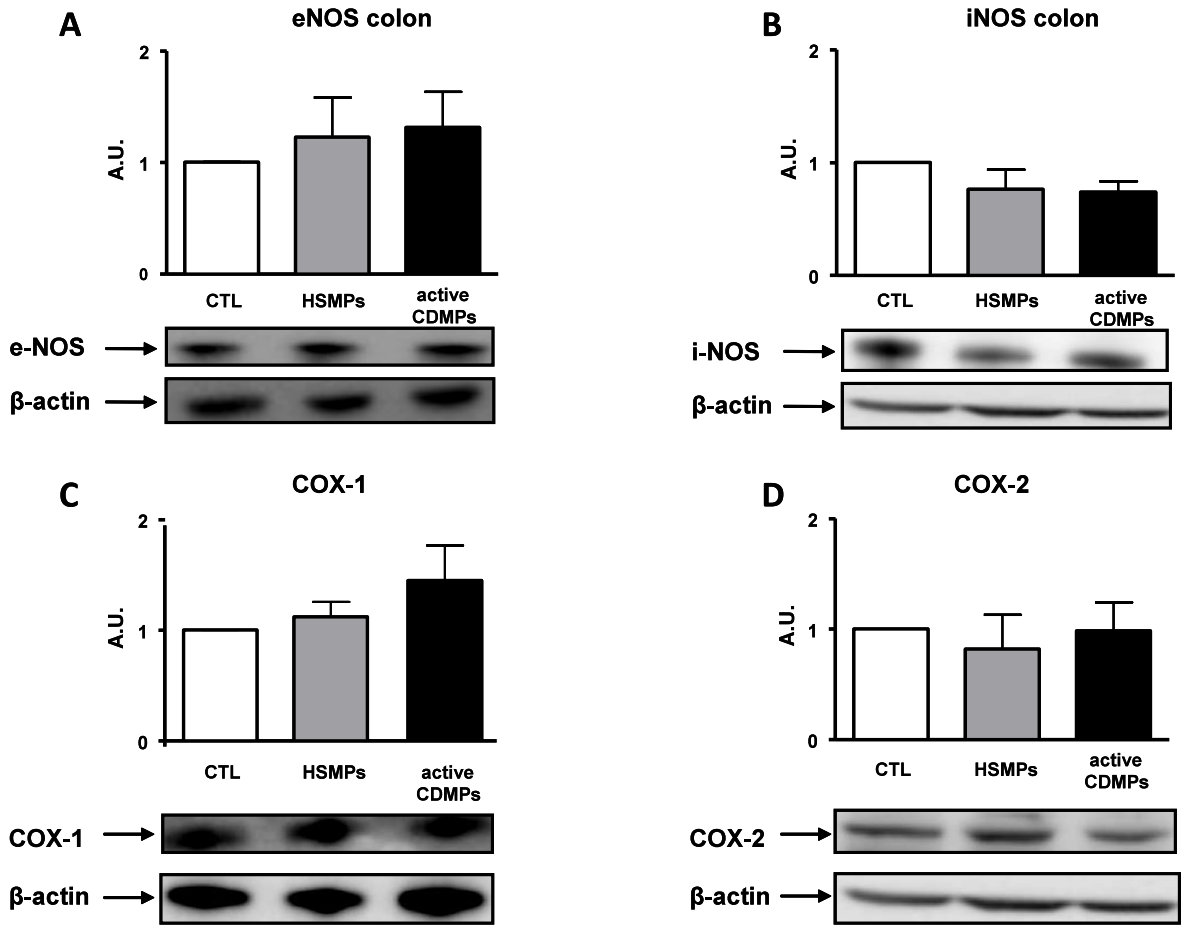
HSMPs
CDMPs inactive
CDMPs active

Figure. 2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Review

Microvesicles: intercellular vectors of biological messages

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Introduction

Cells communicate with other cells not only through direct cell-cell contact or mediator production, but also through secretion of microvesicles (MVs). MVs, small vesicles released from a wide variety of cells, can be considered truly as micromessengers. The term MVs includes microparticles (MPs) and exosomes (1). While exosomes are released into the extracellular compartment by exocytosis, MPs are shed from the blebbing plasma membrane, and their composition and effects on target cells differ. This lack in homogeneity in the MV definition is clearly underlined by some works which claim that broad definition impedes the understanding of MV actions (2). Nevertheless, both MPs and exosomes released from circulating cells, cells from the vascular wall or tumor cells constitute a mechanism of intercellular communication. This review focuses the mechanisms of MV formation and their effects on target cells depending on their production under physiological or pathophysiological conditions.

DEFINITION, FORMATION AND CONTENT OF MVs

Microparticles (MPs)

The concept of MPs was born in the 70's and described to MPs as inert dust ("platelets dust" exactly by Wolf (3) who noted the presence of small platelet-derived fragments in human plasma). Then, the evolution of knowledge and technology leads the scientific community for a better understanding and signification of the possible role played by MPs as proposed by Bastida et al. (4).

Nowadays, MPs are known as small vesicles heterogeneous in size (0.05-1 μm, a characteristic which is often used to distinguish MPs from exosomes (< 0.1 μm) and platelets (> 1 μm), respectively) and composition, with pro-coagulant and pro-inflammatory properties. Although all cell types can theoretically release MPs, the determination of the their origins have allowed to establish that MPs can be released from the plasma membrane of circulating

cells (such as platelets, erythrocytes, T and B cells, and monocytes), cells from the vascular wall (endothelial and smooth muscle cells) and tumor cells (5-7). This central position of MPs suggests a general role in cellular regulations.

Even if the mechanisms governing MP formation are complex and not completely elucidated, cell activation or apoptosis are the two main cellular processes which lead to their formation (Figure 1). The phenotype and the quantity of MPs released vary depending on the way they are produced, either activation or apoptosis, as nicely reported by the group of Jimenez regarding endothelial cells (8).

During cell activation by agonists or different stresses, MP formation is dependent on the patterns on intracellular calcium increase with respect to the kinetic and/or the amplitude of the signal. Influx of extracellular calcium is necessary to induce the formation of MPs upon agonist activation and is associated with calpain activation required for cytoskeleton disruption (9). Although the relationship between calpain activation and microvesiculation has been confirmed (10), the participation of calpain in MP liberation remains controversial. Indeed, Wiedmer et al, (11) have shown that formation of platelet-derived MPs is dependent of calcium influx, but do not require metabolic energy or calpain-mediated proteolysis of cytoskeletal proteins, suggesting the existence of alternative mechanisms for microvesiculation independently of calpain.

In addition, blebbing and microvesiculation are often, but not always, preceded by the loss of membrane asymmetry resulting from local perturbation of the bilayer structure and, the appearance of aminophospholipids localized in the inner leaflet to the outer leaflet, like phosphatidylserine (12). The regulation of aminophospholipid asymmetry is ruled by three distinct activities which promote their bidirectional redistribution across the bilayer (for review see: 13). The first complex is an aminophospholipid translocase which is able to transport phosphatidylserine and phosphatidylethanolamine from the outer to inner leaflet of

plasma membrane against the concentration gradient. The differential transbilayer orientation of aminophospholipids is ruled by an outward ATP-dependent floppase which act in unison with the translocase. The third complex, the lipid scramblase, can rapidly move aminophospholipids between the membrane leaflets by a calcium-dependent mechanism and may lead to collapse of membrane asymmetry. An elevated intracellular calcium concentration regulates positively scramblase and blocks cooperation between translocase and floppase. This mechanism leads to phosphatidylserine exposure in the outer leaflet and is followed by MP formation. Indeed, phosphatidylserine exposure is expressed on most but not all MPs, and has functional consequence including the stimulation of coagulation process by binding the positively charged domain of coagulation proteins.

In addition, protein kinases have been implicated in the regulation of MP formation and phosphatidylserine exposure. Cytosolic calcium increase may activate kinases and inhibit phosphatases, which are responsible for cytoskeleton disruption (14-16). Recently, it has been reported that protein kinase A (PKA) plays key roles in the regulation of platelet MP formation and phosphatidylserine exposure, and PKA-mediated MP shedding is dependent on calpain activation (17).

Moreover, other calcium-insensitive mechanisms can be involved in MP formation. Cauwenberghs et al (18) found that integrin αIIbβ3 pathway is responsible for MP generation by stored platelets, in the absence of agonist, by destabilization of actin, independently of calcium and calpain, but they cannot explain whether both pathways (calcium-sensitive and -insensitive) operate alongside each other or synergize.

The other main process of MP formation is apoptosis. Apoptosis is a regulated process of fundamental significance in the maintenance of homeostasis, and is accompanied by changes of the cell morphology and notably cytoskeleton disruption, cell shrinkage and dynamic membrane blebbing (19). Whereas MP blebbing occurs mainly during the early phases of

apoptosis, apoptotic bodies are produced in the late stages of death process (20). Apoptotic bodies are larger than MPs and represent the compacted or condensed remnants of the shrinking apoptotic cells (21).

During apoptosis, caspase 3-mediated cleavage leads to Rho-independent activation of ROCK I, which promotes generation of actin-myosin force, cell contractility and consequently membrane blebbing and formation of MPs, without affecting phosphatidylserine externalization (22, 23).

Finally, another mechanism exists at the interface between cell activation and apoptosis for MP formation and release. A study based on thrombin-induced endothelial MP releases shows that thrombin, a multifunctional enzyme, generates MPs by activating RhoA/ROCK II via caspase-2 pathway in endothelial cells, despite the absence of cell death (24). The same group, based on thrombin-induced endothelial MP release model, found that TRAIL and its receptor TRAIL-R2, involved in the signaling pathway of apoptotic cell death (25), mediated endothelial MP release by initiating the recruitment of adaptor proteins and the activation of NF-κB. They also found a new function of TRAIL as a mediator between coagulation and inflammation in response to thrombin (26). These different data clearly underline the complexity and possible connection in pathway that lead to the formation of MPs.

A better understanding of the mechanisms governing cell activation- and apoptosis-induced MP release would contribute to determine emergent therapeutic approaches in order to decrease MP production under pathological conditions. However, the main limitation concerning studies related to MP formation is that they are performed in *vitro* and very often on cell lines with pharmacological inhibitors. It remains to be elucidated whether the same pathways are involved for the *in vivo* production of MPs.

An important parameter that determines the biological effects of MPs is their protein and lipid compositions, which may vary depending on the cell they originate from and the type of

stimulus involved in their formation. MPs contain membrane, cytoplasmic and nuclear constituents of their precursor cells, which allow characterizing them using antibodies directed against specific epitopes. Indeed, proteins from endothelial MPs are mainly metabolic enzymes, proteins involved in adhesion and fusion processes, and cytoskeleton-associated proteins (27), whereas proteins from platelet MPs are surface glycoproteins or chemokines (28, 29).

Concerning proteins, global composition of MP proteins can be related to stimulus at their origin. For instance, MPs generated *in vitro* from activated (by phytohemaggglutin (PHA) and phorbol-myristate-acetate (PMA)) and apoptotic (Actinomycin D (ActD)) CEM T lymphocytes or lymphocytes from diabetic patients expose on their surface the morphogen Sonic Hedgehog (morphogen implicate in embryonic and adult development). However, treatment of these cells with PHA alone, PMA alone and ActD alone generates MPs lacking in Sonic Hedgehog (30). Moreover, the comparison of protein composition of MPs obtained from the CEM T-cell line either in mitogenic (PHA) and apoptotic conditions shows that MPs generated from apoptotic stimulation are more rich in proteins than those from MPs activated with mitogenic stimulus (31), and showing that MP membranes, not only express surface proteins, but also engulf some cytoplasm and organelle proteins during membrane blebbing. Also, activation of human monocyte THP-1 with lipopolysaccharide or a soluble P-selectin chimera evoke generation of MPs with similar size, organization of cytoskeleton and procoagulant activity, but different protein composition (32).

Regarding lipid composition, MP bilayer consists mainly of phospholipids and results negatively charged because of presence of phosphatidylserine and phosphatidylethanolamine. In particular, phosphatidylserine exposure in the exoplasmic leaflet of membrane and vesiculation are dependent on lipid raft organization, since cholesterol depletion inhibits both events (16). Dean et al. (33) have recently reported that the active component of middle size

class of platelet MPs may be a lipid but it nature remains to be determined. Furthermore, several reports have shown that the lipid environment could modify the activity of certain proteins carried by MPs. For instance, cholesterol enrichment of human monocytes induces the generation of highly procoagulant active MPs (34). Taken together, one can advanced the hypothesis that MPs from patients with metabolic pathologies, for instance, may have different lipid composition, which may account for different functional effects. Future lipidomic studies should be addressed to evaluate the role of lipid environment of MP effects.

Exosomes

Exosomes are small natural membrane vesicles released by a wide variety of cell types into the extracellular compartment by exocytosis. Exosomes were described initially during the secretion of MVs of endocytic origin by reticulocytes (35). When compared to MPs, exosomes are smaller (usually less than 0.1μm), more homogeneous in size and produced in the endocytic lysosomal system (Figure 2). Indeed, exosomes are formed within endosomes, by invagination of the limiting membranes, resulting in the formation of multivesicular bodies. Then, multivesicular bodies can fuse with the plasma membrane and release exosomes into the extracellular environment (36). Secretion of exosomes can be spontaneous or induced depending on the cell type. Reticulocytes (35), T cells (37), mastocytes (38) and resting B cells secrete detectable levels of exosomes only following the activation of a cell surface receptor. In contrast, dendritic cells (39), macrophages (40) or epithelial cells (41) constitutively secrete exosomes in vitro, as do most tumor cells. However, the molecular pathway involved in the exocytic fusion to release exosomes is still under investigation. Since secretory multivesicular bodies have characteristic similar to those of secretory lysosomes (42), it was hypothesized that exosomes use the same way to be secreted. The release of exosomes in the extracellular space requires the transport of the formed multivesicular bodies toward the cell periphery and its fusion with the plasma membrane. Exosome release is a

complicate process involving multiple protein complexes (36), such as an endosomal sorting complex required for transport (ESCRT) which recognizes mono-ubiquitinated transmembrane protein (43, 44), a passive mechanism (45, 46), a Rab (small GTPase) mechanism (47), and an alternative pathway requiring ceramide (48). Also, calcium is fundamental in regulating exocytosis, early endosome fusion, and exosomes-mediated release (49). Indeed, it has been shown, in a hematopoietic cell line (K562 cells), that exosome release is an event dependent of calcium from both extracellular source and intracellular stores, and suggests that a signal transduction mechanism is involved in the activation of exosomes-carrying cells to release these small vesicles at the proper site (50). Finally, very little is known about the molecular mechanisms responsible for the sorting of exosomes and further works are clearly needed to highlight the machinery involved, an essential step to further understand their functions and significance.

The identification of MVs as exosomes is based on morphological and biochemical criteria. Exosomes were obtained after high speed centrifugation (51) and to confirm the presence and purity, western blot analysis were provided with antibody directed against exosomal markers like tetraspanins, heat shock protein (HSP)70, HSP90 or EF-1α. Also, electron microscopy or flow cytometry could be used for detection of exosomes, even if some works claim that exosomes would be too small to be detected by flow cytometry. Once again, the protocol described for isolation and detection of exosomes needs to be refined.

Exosomes do not contain any proteins from nuclear, mitochondrial, endoplasmic-reticulum or Golgi-apparatus. As a consequence of their endosomal origin, proteomic evaluation shown that nearly all exosomes derived from cell lines or body fluids, independently of the cell type from which they originate, share common structures and contain proteins involved in membrane transport and fusion, in multivesicular body biogenesis, in processes requiring HSPs, integrins and tetraspanins. Tetraspanins can be a central component for exosomes

binding and integration into distinct target cells (52). In addition, exosomes display a discrete set of proteins involved in antigen presentation, such as MHC-I and MHC-II (53), characteristic of cells from which they are released. Exosomes contain also selectively enriched mRNA and miRNA, allowing genetic exchange between cells (54, 55). It has been suggested that since MVs "hijacks" the cytoplasm, capturing cytoplasm components and miRNA, prior to release from the cell, the packaging of the miRNAs in the cell may be random (56).

INTERACTION BETWEEN MVs and TARGET CELLS

Communication between cells is based on protein signaling cascades that require direct cell-cell apposition or receptor engagement by secreted molecules. In this part, we summarized the different pathways required to MV-induced cell stimulation.

Microparticles

MPs may interact with target cells through surface-expressed ligands, transferring surface receptors, and delivering proteins, mRNA, miRNA, and bioactive lipids. Moreover, they may serve as vehicles to transfer infectious particles (Trojan horse mechanisms) and to deliver intact organelles. Up to now, four mechanisms (ligand/receptor interaction, transfer, fusion and internalization) of information transfer used by MPs have been reported (Figure 1).

Several studies demonstrate that MPs can directly stimulate receptors expressed in target cells by the interaction with ligands present on their surface or by the mediators released by MPs. Thus, platelet MPs expressing P-selectin enhance leukocyte aggregation and accumulation by binding to P-selectin glycoprotein ligand-1 (57). Likewise, MPs harboring Sonic Hedgehog promote megakaryocytic differentiation, nitric oxide (NO) production from endothelial cells and angiogenesis by activating Sonic Hedgehog cascade, since all the effects were reversed after silencing of the Shh receptor (30, 58, 59). Moreover, MPs released from T cells can

interact with smooth muscle cells through the Fas/Fas Ligand (FasL) pathway evoking NF-κB activation (60).

Another way used by MPs indicating a selective transfer of different components by close contact which can affect functions of the target cells. Several studies point out that MPs can transfer receptor on the surface of target cells. Indeed, the release and intercellular trafficking of $CD81^+$ MPs regulate the expression of CD81 surface receptors in lymphocytes (61). Rozmyslowicz et al (62) provide evidence that MPs may play an important role in spreading HIV infection by transferring the CXCR4 co-receptor to $CD4^+/CXCR4^-$ cells. Besides, arachidonic acid transported by platelet MPs can be transferred to endothelial cells and lead to an increase in cyclooxygenase-2 (COX-2) and ICAM-1 expression (63).

Finally, MPs can be also absorbed by fusion or by internalization (or engulfing). Fusion of MPs with their target cells leads to non selective transfer of MP components, and affect property of cells (64). Indeed, MP released from endothelial progenitor cells are internalized in endothelial cells by interaction with α4 and β1 integrins. Besides, these MPs are shuttling mRNA that is able to activate angiogenesis in endothelial cells (65).

This enumeration does not exclude the possibility that MPs might be involved in different pathways and the different possibility of interaction raises a question: could one single MP interacts with many cells, or once single MP interacts with one recipient cell, this MP is then eliminated. Moreover, it is important to note that the technology used for the study of MP metabolism and MP clearance is not accurate at present and thus their half-life in vivo is not known.

All these data indicate that the precise characterization of cell origin and of the biological message carried and transferred by MPs is essential to understand whether they possess pathogenic or beneficial properties.

Exosomes

The presence of exosomes in urine (66), circulating blood (67) or cerebrospinal fluid (68) and many studies on their fate and functions suggests that they may play a role in intercellular communication in physiological and pathological processes. However, the exosomes-cell interaction mode and the intracellular trafficking pathways of exosomes in their target cells remain unclear. Once released, exosomes interact like MPs with their target cells by direct binding, adhesion, fusion or internalization (69-71) (Figure 2). Exosomes can bind to cells through receptor-ligand interactions, similar to cell-cell communication mediating. Dendritic cell-derived exosomes contain MFG-E8 which potentially can bind integrins expressed by dendritic cells or macrophages (53). Alternatively, exosomes could putatively attach or fuse with the target cell membrane, delivering exosomal surface proteins and cytoplasm to the recipient cell. Finally, exosomes may also be internalized by target cells by endocytosis or phagocytosis. Exosomes are internalized efficiently by phagocytes, by actin- and phosphatidylinositol 3-kinase mechanism-dependent mechanisms, without implication of caveolae, macropynocytosis and clathrin-coated vesicles (72). Exosomes can also be internalized by immature dendritic cells for presentation to CD4 T cells (71).

The understanding of intercellular transfer of exosomes might allow developing strategies to interfere with the release of pathogenic components by exosomes. Recently, it has been proposed a novel mechanism of genetic exchange between cells referred as “exosomal shuttle RNA” by a transfer of mRNAS and miRNAs mediated by exosomes (54), opening a possibility to use exosomes for gene therapy. Also, exosomes secreted by dendritic cells could modulate immune responses directly by exposing MHC and T cell costimulatory molecules and indirectly by carrying internal components to surrounding cells (73, 74).

MVs: BIOMARKERS AND EFFECTORS IN DISEASES

Although basal levels of MVs are found in fluids of healthy donors, elevated levels have been detected in many pathological conditions. Based on the literature, we describe the effects mediated by MPs on the cardiovascular system, and exosomes-induced dialogue between immune system and tumor cells. This choice is made by taking in consideration that the main MP effects target the cardiovascular system, and on the other hand, exosomes display important immunomodulatory functions and may be considered as potential tools against tumor development.

MPs and cardiovascular diseases

Circulating MPs isolated from blood have been considered as biomarkers of vascular injury and inflammation in several cardiovascular pathologies such as acute myocardial infarction, preeclampsia, atherothrombosis, diabetes, hypertension and metabolic syndrome. Indeed, in these diseases, elevated levels of MPs have been detected and frequently correlated with the severity of the pathology. Only since recently, MPs are also considered as effectors being able to vehicle biological messages on the target cells. Interestingly, this transfer of information between cells is independent of the number of MPs but rather on the different composition and/origin between MPs (75, 76). Here, we develop some studies dedicated to determine the biological effects mediated by MPs in the cardiovascular system.

Owing to the procoagulant ability of tissular factor and phosphatidylserine that they carry, elevated levels of MPs have been described to participate in the development and maintenance of prothrombotic status in atherosclerosis (77), pre-eclampsia (78) and hematological diseases (79) as well as in cancer-associated thrombosis (80).

Concerning the direct effects of MPs on the vascular function, it has been proposed by several groups that circulating MPs affect NO production by decreasing its production via the reduced activity of endothelial NO-synthase and/or by decreasing its bioavailability. Thus, MPs from

patients with myocardial infarction, diabetes or pre-eclampsia induce endothelial dysfunction by impairing endothelial NO transduction pathway (81-83). In patients with metabolic syndrome, it has been shown that circulating number of total MPs, but also those from platelets, erythrocytes and endothelial cells, are enhanced and evoke endothelial dysfunction by decreasing endothelial NO-synthase activity and NO production, by increasing protein nitration on endothelial cells (84) and by enhancing plasmatic oxidative stress markers (85). Interestingly, patients with obstructive sleep apnea (OSA), a highly prevalent disease characterized by recurrent episodes of partial or complete obstruction of the upper airways during sleep, leading to repeated falls in oxygen saturation, display the same levels of circulating MPs than health subjects, but levels of MPs from granulocytes and activated leukocytes ($CD62L^+$) were higher in OSA patients. In addition, levels of $CD62L^+$ MPs correlate with endothelial dysfunction that may initiate atherogenic processes in patients with OSA (75). Using a model of rat pulmonary arterial hypertension, we have shown that circulating MPs from hypoxic rats reduce NO bioavailability by decreasing endothelial NO-synthase activity and by enhancing oxidative stress in pulmonary endothelial cells (76). Altogether these data underscore the deleterious effects of circulating MPs on the cardiovascular system, and in particular, on the endothelial cells.

Furthermore, circulating MPs can interact with smooth muscle cells, induce vascular inflammation and modify the vessel contractility. Indeed, during preeclampsia it has been reported that circulating MPs evoke vascular hyporeactivity to contractile agonists by increasing NO production via inducible NO-synthase and COX-2-derived vasoconstrictor metabolites (86). In addition, the separation of the MPs depending on their origin shows that whereas MPs from leukocyte origin induce both the release of NO and COX-2 vasoconstrictor products, those from platelet origin are able to stimulate the release of NO only, suggesting that MPs from platelets may serve as a protective mechanism against hypertension during

preeclampsia. These results suggest an involvement of MPs in the mechanisms responsible for cardiovascular complications of different diseases and can be considered as markers of cardiovascular dysfunction and participate in the amplification of pre-existing dysfunction.

Finally, one key feature of the effects of MPs in the cardiovascular system is their ability to modulate the angiogenic program of both endothelial mature and progenitor cells. Taraboletti et al. (87) reported that endothelial MPs bearing metalloproteinases promote formation of tubule-like structure in human endothelial cells suggesting that MPs may regulate focalized proteolytic activity vital to angiogenic steps involving migration of endothelial cells. Other proteins, such as PPARα carried by MPs seems to be essential for their ability to pro-angiogenic reprogramming on endothelial progenitor cells through the Akt and NF-κB pathway activation (88). Recently, it has been described that isolated MPs from vitreous fluid from patients with diabetic retinopathy stimulated endothelial cell proliferation and increased new vessel formation in mice, indicating that MPs could contribute to disease progression through their ability to promote angiogenesis (89). Also, MPs contribute to the development of atherosclerosis because they are able to amplify the initial endothelial dysfunction and accelerate the progression of atherosclerotic lesions by promoting intraplaque neovascularization and increasing their thrombogenicity (90). Altogether, these data highlight the potential effects of circulating MPs on promote new vessel generation and may constitute a new therapeutic target against the progression of these diseases.

Despite all these studies, an enhanced level of circulating MPs is not always accompanied by a deleterious effect of them; indeed, a fraction may deliver protective biological messages preserving endothelial function and/or vascular integrity. Thus, circulating MPs from septic patients have been shown be protective, rather that deleterious, by recovering contraction in vessels treated with LPS through an increased thromboxane A2 production (91), confirming a previous study showing a positive correlation between high circulating levels of MPs and

survival in septic patients (92). In addition, MPs from septic patients treated with recombinant activated protein C carry this protein and may be related to the anti-apoptotic and barrier protective effects mediated by activated protein C on endothelial cells (93).

Exosomes: a link between immune responses and cancer

Activation of immune cells occurs during a large number of pathological situations and leads to exosome generation. Depending on their origin, exosomes can serve as both immunostimulatory and immunoregulatory entities (94). As described above, T and B cells as well as dendritic cells release exosomes. Thus, *in vitro* studies have shown that exosomes released from B cells can directly activate $CD4^+$ T cells (69). Also, dendritic cell-derived exosomes initiate specific cytotoxic T lymphocyte response *in vivo* and suppress growth of established murine tumors in a T cell-dependent manner (39). In the same way, tumor-derived exosomes transfer tumor antigens to dendritic cells, which induce potent $CD8^+$ T cell-dependent antitumor effects on mouse tumors (95). Since then, numerous studies have reported the ability of exosomes to act as potential relevant elements for immunointerventions, by amplifying the generation of donor-reactive T cells following transplantation (96) or by suppressing antigen-specific responses through a Fas/FasL-dependent mechanism in the treatment of autoimmune diseases (97).

Recent studies show that quantification and identification of several proteins carried by exosomes have allowed considering them as potential tools as prognostic, diagnostic, or detection markers in cancers. For instance, claudin-4 can be shed from ovarian cancer cells and detected in exosomes from plasma from ovarian cancer patients (98). Logozzi et al. (99) found that the levels of plasma exosomes expressing CD63 and caveolin-1 in melanoma patients were significantly increased when compared to healthy donors. Also, CYP17A1, a target for total androgen blockade in advanced prostate cancer patients, is expressed in plasma exosomes for these patients (100), while the presence of two known prostate cancer

biomarkers, PCA-3 and TMPRSS2:ERG, have been found in exosomes isolated from urine of patients (101), suggesting that exosomes may be used as a novel approach in detection and prostate tumor surveillance. Also, exosomes from glioblastoma tumor cells containing mRNA, miRNA and angiogenic proteins, such as VEGF, can be detected in serum from glioblastoma patients, suggesting that on one hand, exosomes are capable to promote angiogenesis favoring cancer growth and on the other hand, they can give information on the diagnosis of disease and the choice of appropriated therapy (102). Cancer specific miRNA isolated from circulating tumor-derived exosomes from patients with ovarian cancer constitutes a new biomarker of this pathology (103).

Most interestingly, exosomes can represent a nexus between the immune system and tumor environment. Revoltini and colleagues isolated exosomes from blood from patients with colorectal cancer. These MVs expressed typical proteins of exosomes (CD63) but also FasL and TRAIL which mediated their pro-apoptotic effects on $CD8^+$ T cells (104). This mechanism of immunosuppression has potential implications as a prognostic factor and could be targeted for the development of new antitumor therapies in colorectal cancer patients. Also, exosomes present in blood and ascites from patients with ovarian carcinoma promote differentiation and proliferation of Treg as well as up-regulation of Treg-mediated suppression that may potentially contribute to tumor escape (105). Furthermore, these exosomes injected to tumor-bearing mice resulted in augmented tumor growth (106).

Altogether, exosomes act as vectors for the transfer of immunological information at distance and can be considered as novel biomarkers and effectors.

MODULATION AND THERAPEUTIC ACTION OF MVs

It is becoming clear that MPs and exosomes can be considered as potent biological vectors capable of transmitting information by interacting with target cells and are able to induce both

beneficial and deleterious responses through their ability to modulate gene expression. Consequently, the comprehension of the role of MVs in diseases is not only important for deciphering the pathophysiological mechanisms associated with these pathologies but also suggests a promising therapeutic use of MVs (Figure 3).

Concerning MPs, we have proposed to use MPs as therapeutic tools based on the production of *in vitro* engineered MPs (107). Indeed, engineered MPs can be generated in order to modify their molecular composition and consequently, their cardiovascular properties. In this way, we have engineered MPs, from activated/apoptotic T cell line, bearing Shh and they evoke NO production on endothelial cells, restore endothelium-dependent relaxation after ischemia-reperfusion injury, and favor *in vitro* angiogenesis and new vessel formation in an ischemic hindlimb model (58, 59, 108). These findings suggest that MPs^{Shh+} may represent a potent tool in stimulating neovascularization in disease states associated with impaired angiogenesis. More audacious, transfection of MPs with "new" proteins or mRNA and their subsequent delivery to target cells may represent a new opportunity to transfer a "desired" biological message into target cells and modify their phenotype, for instance recovering the function of mutated/failed protein.

Regarding exosomes, very recent studies report their cardioprotective effects. Exosomes released from human mesenchymal stem cells reduced infarct size in a mouse model of myocardial ischemia/reperfusion injury (2, 109). Taking in consideration the complexity of exosome composition, proteomic and/or metabolomic analysis should allow identifying the molecular mechanisms implicated in these effects.

Another example of the use of exosomes as therapeutic tools is to consider them as anti-tumor vaccines. Since exosomes released from dendritic cells induce anti-tumor immune responses (39), exosomes may be considered as potential cell-free cancer vaccine. Thus, several clinical trials develop exosomes as vaccines to allow cancer patients to prolong survival after

stabilization of the disease through chemotherapy and radiation therapies and could be combined with other therapeutic tools to enhance the immune response (110, 111).

In summary, MVs constitute a promising therapeutic tool taking in consideration the easy/feasibility of high amount production, the lack of toxicity and the preclinical and clinical data obtained in different pathologies.

Figure legends

Figure 1. Schematic representation of mechanisms of microparticle (MP) formation and interaction with target cells. During cell activation, an increase in intracellular calcium induces externalization of phosphatidylserine through activation of scramblase and blockade of cooperation between translocase and floppase. Also, calcium increase is associated with calpain activation required for cytoskeleton disruption and MP formation. Under apoptotic induction, cleavage of caspases induces ROCK activation, leading to cytoskeleton alteration and blebbing. Once MPs are released, they can interact with target cells through four mechanisms: ligand/receptor interaction (1), protein transfer (2), membrane fusion (3) or internalization (4). p-MLCK, phospho-myosin light chain kinase; ROCK, Rho kinase; TRAIL, TNF-related apoptosis-inducing ligand.

Figure 2. Schematic representation of mechanisms of exosome formation and interaction with target cells. Exosomes are formed within endosomes, by invagination of the limiting membranes, resulting in the formation of multivesicular bodies. Then, multivesicular bodies can fuse with the plasma membrane and release exosomes into the extracellular environment. Exosome release involves an endosomal sorting complex required for transport (ESCRT) complexe, a Rab-dependent (small GTPase) mechanism, a passive mechanism, and an alternative pathway requiring ceramide. Also, calcium can regulate exocytosis. Once exosomes are released, they can interact with target cells through four mechanisms: internalization (1), membrane fusion (2), protein transfer (3) or ligand/receptor interaction (4).

Figure 3. Modulation and therapeutic action of microvesicles. (A) Engineered microparticles (MP) generated from isolated cells can over-express several "normal" proteins that can be transferred to endothelial cells and consequently, rescue the function of a mutated protein. (B) Dendritic cells treated with peptides generate exosomes able to activate T cell activation leading to tumor regression.

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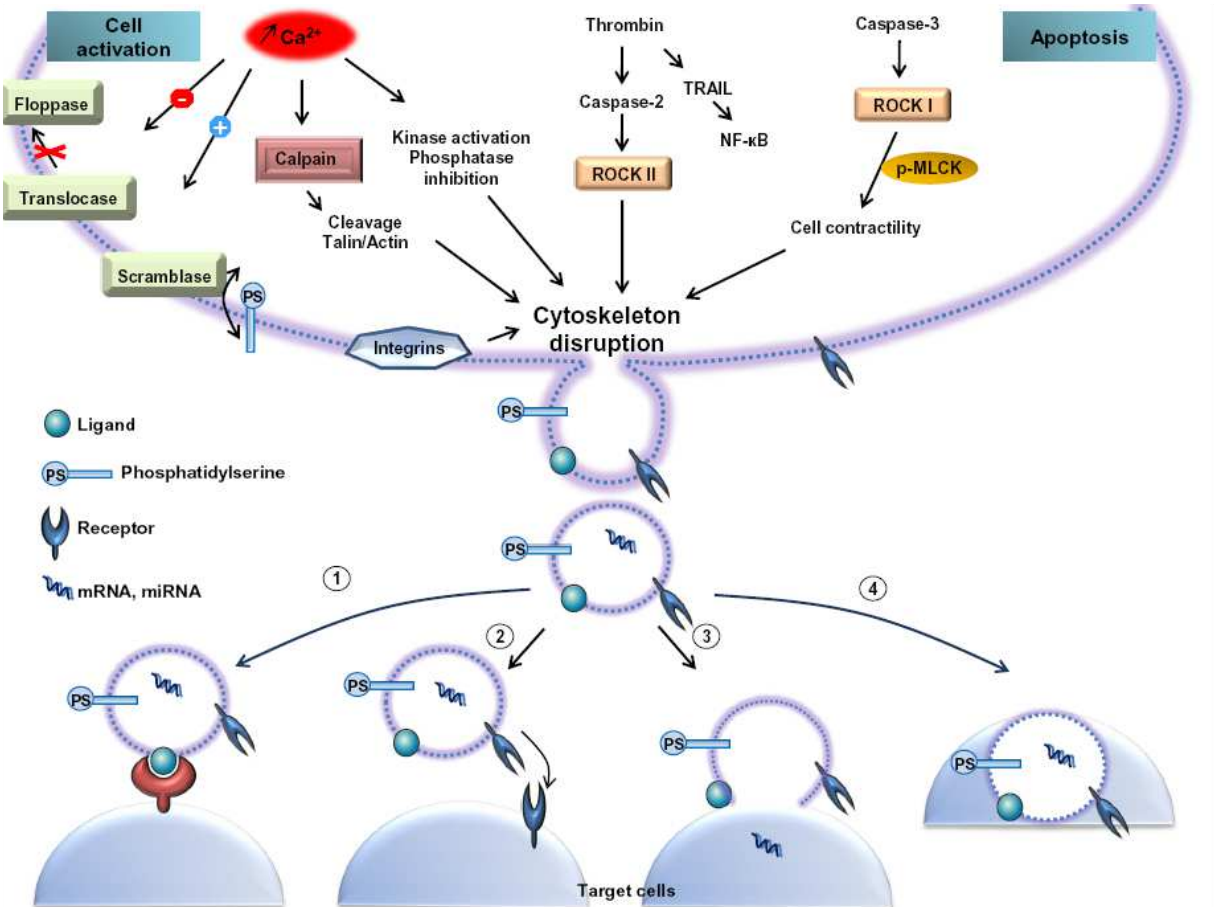
Figure 1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Floppase
Caspase-2
TRAIL
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NF-κB
ROCK II
p-MLCK
Cleavage
Talin/Actin
Cell contractility
Scramblase
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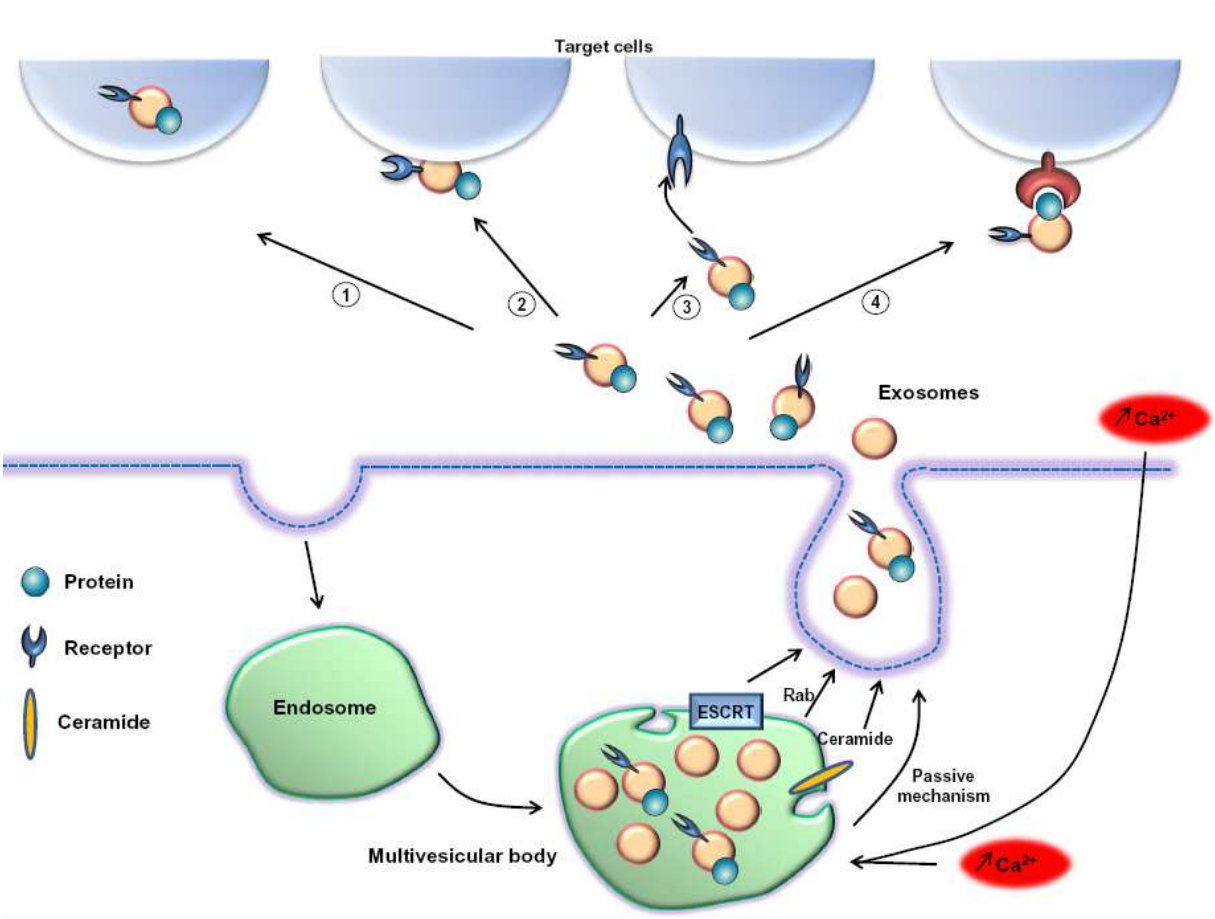
Target cells

Figure 2


Target cells
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Exosomes
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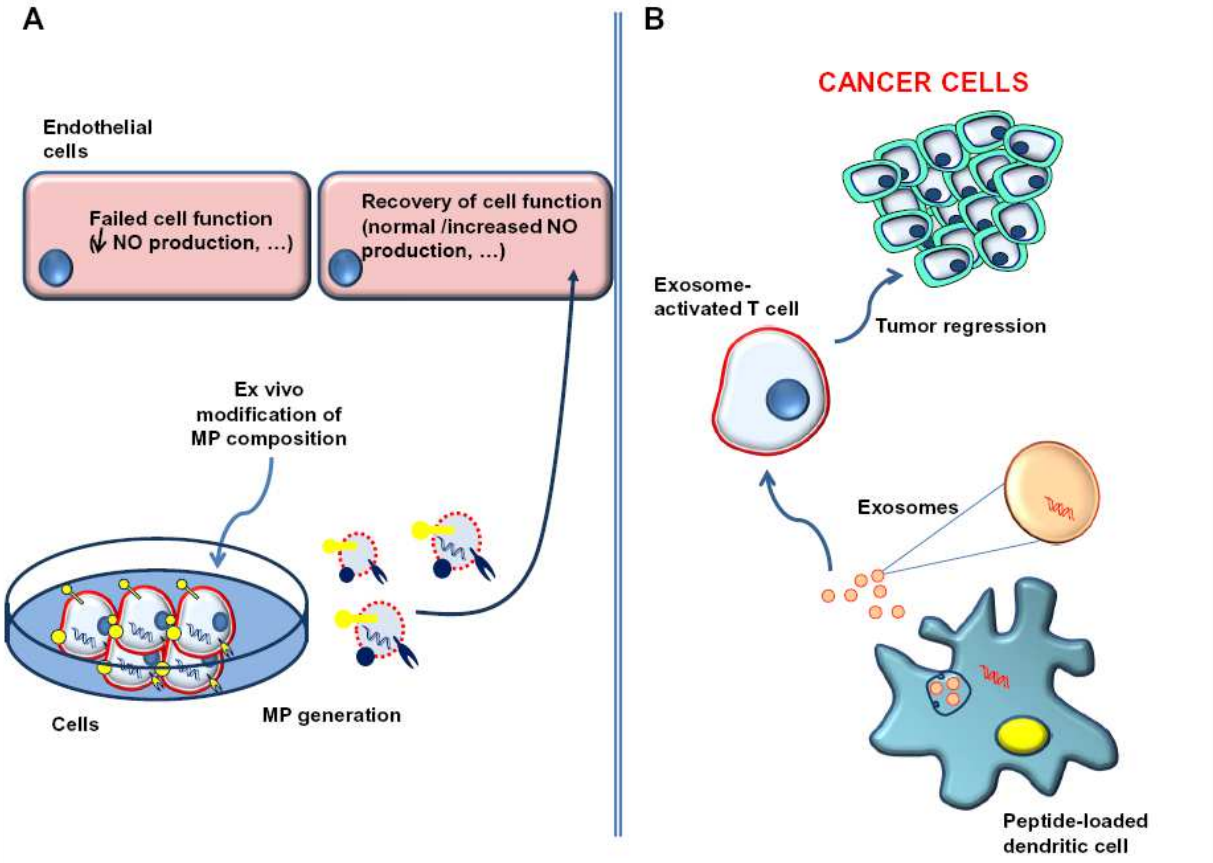
Ceramide
Endosome
ESCRT
Rab
Ceramide
Passive mechanism
Multivesicular body
↗Ca2+

Figure 3


A
Endothelial cells
Failed cell function (↓ NO production, ...)
Recovery of cell function (normal /increased NO production, ...)
Ex vivo modification of MP composition
Cells
MP generation
B
CANCER CELLS
Exosome-activated T cell
Tumor regression
Exosomes
Peptide-loaded dendritic cell

Discussion

Considered as inert cellular debris, MPs appear today as vectors of biological messages that may actively participate in the pathophysiology and development of diseases, notably cardiovascular diseases. The comprehension of the role of MPs in cardiovascular function may allow a better understanding of the pathophysiology of diseases and may provide a new therapeutic approach in their treatment.

Among diseases with cardiovascular complications, Crohn's disease incidence has significantly increased in the last years, linked especially to an improvement in living standard and currently, this disease represents a major burden on public health-care resources. The pathogenesis of Crohn's disease is not yet fully understood and involves the interaction of several factors. One of the factors that could play a role in this pathology is represented by MPs.

In this context, there is a little information in literature on the possible implication of MPs in the pathophysiology of Crohn's disease and in particular in vascular alterations observed in Crohn's disease patients.

In the present study, we show an increased level of circulating MPs from patients with Crohn's disease compared to MPs from healthy subjects and this increase is dependent of level of Crohn's disease activity. Indeed, the rate of MPs from active Crohn's disease patients appears to be increased since the MPs from inactive Crohn's disease patients.

Interestingly, the total levels of MPs were positively correlated in the one hand, to the Harvey-Bradshaw index, parameter that is used to assess disease activity and in the other hand to a marker of inflammation as C-reactive protein (CRP). About the cellular origin of MPs, both levels of PMPs and EMPs were positively correlated to Harvey-Bradshaw index and CRP. Finally, a positive correlation with CRP was also found with procoagulant MPs and

erythrocyte-derived MPs. These findings suggest a possible involvement of MPs in the pathogenesis of disease and that increased level of MPs could be reflect the inflammatory state observed in Crohn's disease patients. Indeed, inflammation that is a processes involved in this pathology can be orchestrated by the interaction between circulating cells, such as lymphocytes, platelets, vascular cells, endothelial cells, and smooth muscle cells, which under activation or apoptosis release circulating MPs.

Another important aspect is the increase of procoagulant-, erythrocyte-, leukocytes, activated leukocyte- and activated platelet- derived MPs, only in active Crohn's disease patients compared to MPs from healthy subjects. Furthermore, MPs from platelet and endothelial cells appear increased in active Crohn's disease patients, not only compared to healthy subjects but also to inactive Crohn's disease patients. The latter showed just an increase of leukocytes derived MPs compared to healthy subjects. The level of macrophages- and granulocytes-derived MPs were not significantly different between the three groups (healthy subjects, inactive and active Crohn's disease patients) considered although patients with perineal disease and patients with associated extra-intestinal manifestations exhibited lower levels of granulocytes-derived MPs.

In agreement with the results found in this study, Andoh *et al*. (Andoh *et al*., 2005), have shown an elevated level of PMPs in blood from patients with IBD, and this increase is associated, both in ulcerative colitis and Crohn's disease, with the level of disease activity and with platelet activation, evaluated by P-selectin expression. These data suggest that PMPs levels represent a direct marker for the detection of platelet activation in IBD patients.

Several studies have demonstrated the presence of abnormalities in platelet number, size and function in IBD (Danese *et al*., 2004). In particular, a lower mean corpuscular volume of platelets has been considered as potential marker of IBD activity (Kapsoritakis *et al*., 2001).

These platelet dysfunctions could be related to a risk of thromboembolism that is a frequent extraintestinal complication in IBD (Quera and Shanahan, 2004). Indeed, histopathological studies have shown the presence of mucosal capillary thrombi in biopsy specimens from patients with Crohn's disease as a result of increased platelet activation and aggregation (Wakefield *et al*., 1989). Moreover, activated platelets are capable to produce different proinflammatory products such as IL-1β and IL-7 and express the CD40 ligands which are able to bind to CD40 present on the surface of immune cells as T and B cells, macrophages, monocytes, and also endothelial and mesenchymal cells (Weyrich and Zimmerman, 2004). Danese *et al*. (Danese *et al*., 2003) showed that CD40-expressing platelets produce elevated levels of chemokines when they are in contact with microvascular endothelial cells. Furthermore, PMPs upregulate expression of CD11b on leukocytes and increase their phagocytic activities (Nomura, 2001) and stimulate COX-2 expression in vascular endothelial cells (Barry *et al*., 1999). All these factors contribute to amplify the immune and inflammatory response.

Another important complication associated to Crohn's disease is represented by risk of thromboembolic events such as an alteration of fibrinolysis. In this respect, Koutroubakis *et al* (Koutroubakis *et al.*, 2008), have shown an increase of level of plasminogen activator inhibitor-1 (PAI-1) in plasma of IBD patients, including patients with Crohn's disease, and a decrease of plasma level of thrombin-activable fibrinolysis inhibitor (TAFI) that suggest an imbalance of fibrinolysis system. Moreover, altered conditions of coagulation and fibrinolysis are highlighted in a large number of IBD patients compared to healthy subjects (Shen *et al*., 2009).

Then, the increase of procoagulant MPs observed in patients with active Crohn's disease compared with healthy subjects could be linked to bleeding disorders which have been widely described in IBD patients and notably in Crohn's disease (Hudson *et al*., 1992)

In particular, Saibeni *et al* (2010) have shown an increase of endogenous thrombin potential (ETP) in IBD patients, that is a parameter of the thrombin generation curve and represents a new tool in the evaluation of thrombotic and bleeding disorders. The increase of ETP is related to the activity of disease.

The cellular origin of procoagulant MPs can be varied and include platelets, endothelial cells, leukocytes and erythrocytes (Tesse *et al*., 2007; Hugel *et al*., 2005). MPs reveal their procoagulant properties by exposure of negatively charged phospholipids, which provides binding sites for activated coagulation factors Va, VIIIa, IXa and Xa. Alternatively, MPs may express tissue factor (TF) that is the initial activator of the blood coagulation pathways. The presence of TF has been showed in MPs from different type of cells such as monocytes, leukocytes, activated fibroblast and endothelial cells.

The involvement of endothelial MPs has already been shown, in other diseases associated with vascular inflammation and thromboembolic risk such as pulmonary hypertension, venous thromboembolism and acute coronary syndrome (Diehl *et al*., 2010; Chirinos *et al*., 2005); (Mallat *et al*., 2000). Indeed, MPs derived from endothelial cells play a role in mechanism of coagulation, inflammation and angiogenesis (Leroyer *et al*., 2010), in particular they are capable to promote coagulation and induce neutrophils activation and monocyte adhesion (Martínez *et al*., 2005).

Contrary to the results found in our study, Chamouard *et al.* (2005) revealed an elevated level of procoagulant MPs in patients with Crohn's disease but it is not related to the activity of the disease. The origin of these MPs is mainly from platelets and the presence of EMPs in peripheral circulation of Crohn's disease patients has not been found. The difference of data compared to those obtained in the present study may be due to different method used to quantify MPs. Indeed, Chamouard *et al.* immobilize only procoagulant MPs expressing PS by

using annexin V (Chamouard *et al*., 2005) that represent only a small fraction of total MPs required for the interpretation of the data. Conversely, in our study, all MPs are considered (expressing or not PS).

In this study we also observed, an increased level of leukocyte-MPs both in inactive and active Crohn's disease patients. Furthermore, in active Crohn's disease patients, there is also an increase of activated leukocyte-derived MPs. A possible involvement of these type of MPs in the inflammatory process is suggested by the participation of leukocyte-MPs in the production of several proinflammatory monocytes cytokines such as IL-1β, TNF-α and IL-8 that could facilitate the interaction between leukocytes and endothelium (Mesri and Altieri, 1999).

The presence of a lower level of MPs in inactive Crohn's disease patients compared to active Crohn's disease patients may be related to the type of therapy to which these patients underwent. For example, infliximab, besides its known TNF-alpha neutralizing property, downregulates the production of IFN-γ by Th1 cells in involved mucosa of Crohn's disease patients and alter TNF-α-specific augmentation of mucosal Th1 function (Agnholt and Kaltoft, 2001; Targan, 2000). Therefore, one could assume that also Th1 cells may be involved in the production of MPs. In support of this hypothesis, Chamouard *et al.* (2005) showed a significant reduction of amounts of circulating MPs in patients with Crohn's disease after treatment with infliximab.

In particular, in this study, we observed, only in active Crohn's disease patients, an increase of activated leukocyte-derived MPs. Evidence for involvement of $CD62L^+$ T cell in inflammatory process has been suggested using an animal model. Indeed transfer of $CD4^+CD62L^+$ T cells from wild-type mice resulted in clinical signs of severe colitis. Then,

according whit our data these types of cells could be directly linked to MPs production (Weigmann *et al*., 2004).

All these data suggest that MPs, mediating or resulting from transcellular exchange of biological information or phenotypes can be considered as potent disseminated effectors in this pathology.

MPs and vascular function: endothelial and vascular dysfunctions.

In this work, using an animal model, we demonstrate that MPs isolated from Crohn's disease patients are able to induce endothelial dysfunction both in conductance vessels, such as aorta, in response to acetylcholine, and in resistance vessels, such as mesenteric arteries in response to shear stress.

In aorta of mice, MPs from Crohn's disease patients impair endothelium-dependent relaxation and this effect is exacerbated by a loss of sensitivity to NO of smooth muscle. In particular, MPs from inactive Crohn's disease patients induce an endothelial dysfunction in mice aorta that is more important than this evoked by MPs from active Crohn's disease patients.

Similarly in mesenteric arteries, we observed a reduction of flow-induced vasodilatation in mice injected with MPs from Crohn's disease patients. This effect persists in the presence of MPs from inactive Crohn's disease patients but disappears in the presence of MPs from active Crohn's disease patients. This result could be explained by a decrease of NO-dependent component that was compensated by an increase of EDHF-dependent component of flow-induced vasodilatation.

This data suggest a potential deleterious effect of MPs in Crohn's disease and thus that they play a role in the alterations of endothelial function observed in Crohn's disease patients (Hatoum *et al*., 2003; Horowitz *et al*., 2007; Mori *et al*., 2005). The direct effect of MPs from

active Crohn's disease patients on endothelial function appear to be more attenuated when compared with MPs from inactive Crohn's disease and this suggests a potential attempt by the organism in the active phase of disease to partially offset these deleterious effects.

Furthermore, we have also observed that MPs from Crohn's disease patients induce vascular hyporeactivity in mice aorta and this reduced response to vasoconstrictor agonists is independent of activity of disease. The vascular hyporeactivity induced by Crohn's disease MPs is accompanied with increase of NO production. Interestingly, the inhibition of NOS by L-NA completely prevents the hyporeactivity in aorta of mice injected with active Crohn's disease MPs suggesting that NO play a major role in effects induced by MPs. These observations are supported by an increased production of NO in aorta of active Crohn's disease MPs-treated mice compared whit mice injected whit saline solution (control mice). These data differ from those observed in small mesenteric arteries taken from Crohn's disease patient in which both endothelial function and reactivity to vasoconstrictor agonists are preserved (Tabernero *et al*., 2003). In the one hand, the different effects observed on small mesenteric arteries, could be linked to the fact that in manuscript of Tabernero *et al*., have been considered only active Crohn's disease patient and therefore have not been evaluated possible differences associated to disease activity. In the other hand, these data might suggest that MPs drive only a part of the process seen in human vessels.

About the involvement of NO in the hyporeactivity, is established that NO is a factor constantly produced by endothelium that plays numerous features including relaxation of smooth muscle cells, inhibition of platelet aggregation, reduction of endothelial permeability and it can have effects on vascular tone. NO is also directly related to anti-atherosclerotic property of endothelium since it inhibits the formation of fibrous plaques. In normal conditions, NO stimulates the proliferation of endothelial cells and inhibits the proliferation of smooth muscle cells by blocking mitosis (Förstermann, 2010). In the presence of reduced

release of NO by endothelium, there is an alteration of vascular relaxation and an increase of platelet aggregation and proliferation of smooth muscle cells. The decrease in the level of NO from endothelial origin can be offset by the overproduction of NO by iNOS which may lead to hyporeactivity. Then, a similar mechanism could occur in our study. In support to this hypothesis, an overexpression of iNOS was also observed in mesenteric arteries from Crohn's disease patients who had a decrease of vascular tone or an unmodified vascular reactivity (Lebuffe *et al*., 2000; Tabernero *et al*., 2003).

In these study, we also found that the contraction in response to 5-HT involved the participation of COX-1 vasoconstrictor metabolites independently of treatment (salt solution, healthy subject MPs or Crohn's disease MPs). Thus, COX-1 metabolites did not affect the capacity of Crohn's disease MPs to promote vascular hyporeactivity. Interestingly, the blockade of COX-2 using a specific inhibitor resulted in a reduction in response to 5-HT in aorta from control mice and in those from mice injected with healthy subjects MPs. The abolition of ability of specific inhibitor of COX-2 to reduced vascular contraction in response to 5-HT, in vessels from Crohn's disease MPs-treated mice suggests that this enzyme is not involved in this process. Alternatively, Crohn's disease MPs activate COX-2 leading to release of COX-2 relaxant factors that are, probably, counterbalanced by a release of vasoconstrictor metabolites.

In light of these results, we provide further evidence that MPs from Crohn's disease patients are also able to act directly on smooth muscle cells and promote vascular hyporeactivity in response to 5-HT in mice aorta.

The ability of MPs to induce vascular hyporeactivity has already been showed in other disease such as diabetes and preeclampsia (Tesse *et al*., 2005; Meziani *et al*., 2006;Tesse *et al*., 2007) In this pathologies the mechanism that lead to hyporeactivity involve an enhance expression

of iNOS and COX-2 whit subsequent increased NO and prostacyclin production, respectively. In addition, recent study demonstrated that also in metabolic syndrome patients, MPs promote vascular hypo-reactivity and this effect resulted in an up-regulation of iNOS without changes in COX-1 and COX-2 expression, enhanced expression of the NADPH-oxidase subunits and involvement of Fas/FasL pathway (Agouni *et al*., 2010).

Furthermore, in small mesenteric arteries isolated from inflamed colon of Crohn's disease patients has been showed a marked COX-2 expression and a balance between vasoconstrictor products from COX-2 and unknown vasodilator products that maintained vascular reactivity in a physiological range (Tabernero *et al*., 2003).

Also, ROS could be involved in the contractile response to 5-HT in mice aorta. In this respect, although the SOD mimetic, MnTMPyP, reduced the contraction to 5-HT in an identical manner from three groups, we observed that the level of O_2^- measured was diminished by MPs from Crohn's disease patients, in mice aorta.

It is possible that O_2^- binds rapidly to NO to form $ONOO^-$ that is a strong oxidant involved in vascular damage through various mechanisms such as promotion of atherosclerosis and stimulation of apoptosis of endothelial cells (Martinez and Andriantsitohaina, 2009; Dickhout *et al*., 2005). Furthermore, it cannot be excluded that the affinity of $O_2^-$is greater in acting with NO compared to the spin trap used. Thus, $ONOO^-$ could be involved in the vascular alteration observed in aorta of mice

In summary (Fig 8), Crohn's disease MPs are able to induce endothelial dysfunction and vascular hyporeactivity in mice aorta by a subtle alteration of the balance between the production of NO, metabolites from COX-2 and ROS. All the results collected in the first part of our study allow us to deduce that MPs can play a major role in the pathophysiology of Crohn's disease. Furthermore, their capacity in reducing endothelial vasodilatation suggest

that they can be considered as marker of endothelial dysfunction or injury and that could act as effectors able to amplify existing vascular alteration like hyporeactivity and inflammation.

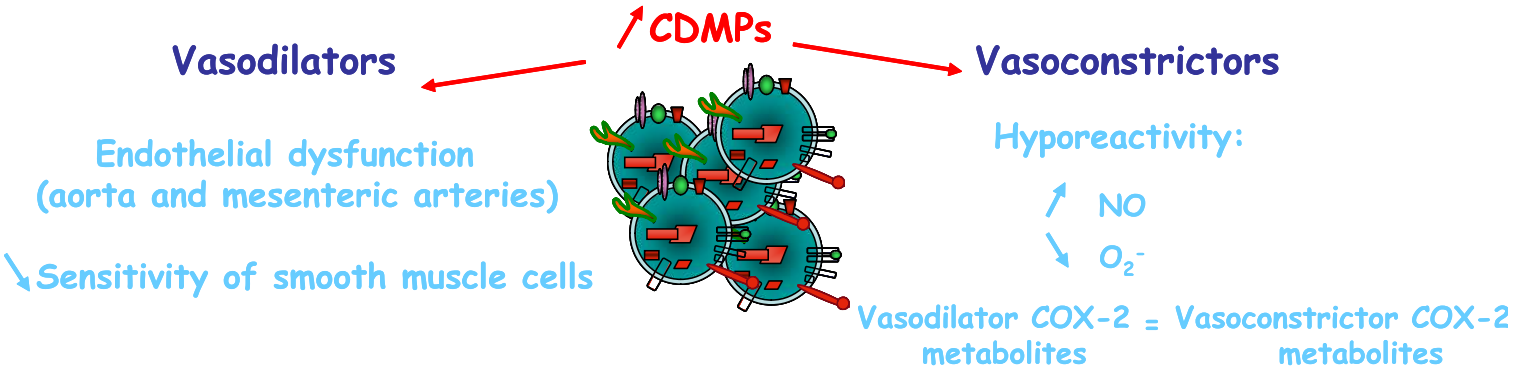


Fig 8: Schematic representation of *in vivo* effects induced by Crohn's disease MPs (CDMPs) on endothelium and smooth muscle cells in aorta and mesenteric arteries of mice.

In the second part of our study, we pursued the evaluation of potential effects of MPs from inactive and active Crohn's disease patients on different tissues and notably on the specific target tissue of this disease such as the colon. We are particularly interested in the role played by these MPs in NO and O_2^- production and in regulation and expression of inflammatory markers.

The MPs from Crohn's disease patients considered in their totality, do not modify the NO production but increase O_2^- production in colon of mice compared to control mice. Interestingly, inactive but not active Crohn's disease MPs induce an overproduction of NO in colon from mice injected with MPs compared to colon of control mice. Conversely, active Crohn's disease MPs evoke an increase level of O_2^- production in the same tissue, that is not observed in colon from mice injected with inactive Crohn's disease MPs. According with these results, in mice colon, active Crohn's disease MPs do not affect the expression of both eNOS and iNOS.

In order to evaluate a possible involvement of COX metabolites in the effects evoked by MPs, we evaluated the expression of COX-1 and COX-2 proteins. Either MPs from healthy subjects or from active Crohn's disease patients do not modify the expression of these enzymes.

Furthermore, we investigate the possible effects on NO and O_2^- production induced by MPs from active Crohn's disease patients in others tissue isolated from mice, notably liver, heart, lung and kidney. Concerning the NO production, we observed that MPs from healthy subjects induce an increase of NO production in isolated lung of mice compared to lung of active Crohn's disease MP-treated mice. Interestingly, active Crohn's disease MPs evoke an overproduction of O_2^- in mice liver compared of the same tissue isolated from healthy subjects MP-treated mice.

Finally, we are interested in a possible involvement of active Crohn's disease MPs in the regulation and expression of inflammatory markers. Interestingly, the results obtained by real-time quantitative RT-PCR, showed that, among the mRNA analyzed, in colon of mice treated whit active Crohn's disease MPs there is an increased expression of IL-23R mRNA, compared whit mice injected whit MPs from healthy subjects.

All these data provide additional information regarding the involvement of MPs in Crohn's disease. In light of these results, we revealed that Crohn's disease MPs are able to modulate NO production and oxidative stress in isolated tissues of mice and notably in a specific target tissue of disease, the colon. Furthermore we showed that Crohn's disease MPs could be directly involved in inflammatory cascade that is to origin of pathogenesis of disease by stimulation of production of proinflammatory mediators such as ROS, reactive species of nitrogen (RNS) and proinflammatory cytokines.

In particular, in colon of mice, inactive Crohn's disease MPs enhance NO production. An excess on NO release is considerably implicated in inflammatory process. Although we have not evaluated the effects of these MPs on NOS expression (eNOS and iNOS), it has been observed that colonic epithelial cells represent a major source of NO production and, NOS activity (particularly iNOS) has been reported to be increased in the mucosa of IBD patients. In addition, this production is regulated by T cells through the modulation of proinflammatory cytokines (Kolios *et al*., 2004). Taken together, these cells could be involved in Crohn's disease MP production that could participate in cell communication during intestinal inflammation.

Interestingly, active Crohn's disease MPs do not affect NO release but induce increase of O_2^- production. This result suggests a possible role of MPs in increased oxidative stress observed in Crohn's disease (Kruidenier *et al*., 2003a; Tuzun *et al*., 2002).

According whit these data, Maor et al. (2008) found that patients with active Crohn's disease showed an enhanced oxidative stress level which decreases when the patients improve in terms of Crohn's disease status and become clinically stable.

Under oxidative stress conditions, the production of ROS exceeds the available antioxidant system. Several evidence suggested that in Crohn's disease there is en imbalance between ROS production and antioxidant defense system leading to increased oxidative stress, lipid peroxidation and inflammation (Geerling *et al*., 1998; Kruidenier *et al*., 2003b). In this respect, a recent study showed that in serum of active Crohn's disease patients an enhanced lipid peroxidation as well as an increased susceptibility of the serum to oxidation are accompanied whit a low level of antioxidant β-carotene and a high activity of glutathione peroxidase (GSH-Px) that is a free radical scavenging mechanism (Maor *et al*., 2008). There are several enzyme systems that can potentially produce ROS species. These include NADPH oxidase, xanthine oxidase and enzyme of the mitochondrial respiratory chain. An up-regulation of NADPH oxidase expression has been observed in Crohn's disease intestinal macrophages (Hausmann *et al*., 2001). In addition, an increased expression of NOX-1 mRNA, a ROS-producing NADPH oxidase, has been shown in lymphocytes in lesions of Crohn's disease patients (Szanto *et al*., 2005). Furthermore, the ability of MPs to modulate the expression of NADPH oxidase subunit has been found in other pathologies such as sepsis and metabolic syndrome (Agouni *et al*., 2008; Mostefai *et al*., 2008). These data suggest a possible mechanism by which Crohn's disease MPs could cause an increase of oxidative stress.

Recently, some authors have found a mitochondrial dysfunction related to oxidative damage that occurs in Crohn's disease and that could suggest a mitochondrial origin of ROS. These authors have evaluated the mitochondrial membrane potential ($\Delta\Psi m$) as parameter of alteration in respiratory chain process of mitochondria. A significantly inhibition of $\Delta\Psi m$ has

been observed in active Crohn's disease patients and is maintained in patients who have a significant improvement of disease. Conversely, inactive patients showed a complete recovery to the normal value of ΔΨm, showing that mitochondrial dysfunction is related to disease activity (Beltrán *et al*., 2010).

Another hypothesis that could explain the increased production of O_2^- observed in colon from active Crohn's disease MP-treated mice could be linked to a lower activity of the anti-oxidant system such as superoxide dismutase (SOD).

SOD activity is a primary defence against ROS which is activated in cells when excessive O_2^- production occurs. Indeed, SOD induces a dismutation of O_2^- which is immediately metabolized to produce H_2O_2 ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$).

There are three SOD isoforms in humans; cytoplasmic copper/zinc (Cu/Zn)-SOD, mitochondrial manganese (Mn)-SOD, and an extracellular (EC)-SOD. In support of this hypothesis, some studies reported lower levels of Cu/Zn-SOD protein and activity in IBD peripheral blood granulocytes and in inflamed mucosa from IBD active patients (Verspaget *et al*., 1988; Mulder *et al*., 1991). Furthermore, a more recent study showed an increase in Mn-SOD protein levels in non-inflamed and inflamed IBD mucosa, but this increase is not correlated to activity of enzyme. Instead, the Cu/Zn-SOD and EC-SOD content decreased with inflammation (Kruidenier *et al*., 2003b). These findings might indicate a decreased endogenous intestinal protection against ROS in IBD which could contribute to the pathogenesis of the disease.

In the present study, we found also that active Crohn's disease MPs evoke an increase production of O_2^- in liver from treated mice. In light of this, one could assume that Crohn's disease MPs may be involved in extra-intestinal manifestation observed in Crohn's disease patients. Indeed, liver is one of the organs most involved in the complication of this pathology

since hepatobiliary manifestations constitute some of the most common extraintestinal manifestations of IBD (Navaneethan and Shen, 2010).

Finally, another remarkable result is the increase expression of specific transcripts for the IL-23R mRNA in colon of mice treated whit active Crohn's disease MPs.

The gene encoding IL-23R is the one the strongest gene associated to Crohn's disease (Duerr *et al*., 2006). According with data found in the present study, there is evidence that IL-23R signalling pathway plays a key role in mediating organ inflammation in mouse model of IBD (Ma *et al*., 2010; Durrant and Metzger, 2010).

The IL-23R mRNA is expressed by various cells involved in inflammatory cascade such as natural killer (NK) cells, $CD4^+$ T cells and $CD8^+$ T cells. It could be argued that these cells release MPs which would act as vector of inflammatory messages. Of particular interest is the ability of IL-23 to induce IL-17 expression by T helper (Th)-17 cells. The signalling pathway of IL23R, following engagement of IL-23, involves a series of STAT molecules and in particular STAT3 and STAT4 play an important role in the differentiation of Th-17 cells and Th-1 cells (Watford *et al*., 2004; Cho, 2008). Th-17 cells represent a novel subset of $CD4^+$ T cells that are protective against extracellular microbes, but are responsible for autoimmune disorders in mice, whereas the propriety of Th-17 in human is not still understood (Annunziato *et al*., 2007).

In intestinal lamina propria of Crohn's disease patients, it has been revealed an increase expression of IL-17 (Fujino *et al*., 2003). Therefore an interesting aspect would be to evaluate a possible implication of Crohn's disease MPs in this signalling pathway, in light of the fact that MPs can allow intracellular communication by different ways and can transfer different molecules including mRNA.

In conclusion (Fig 9), we have assessed that Crohn's disease MPs play a role in inflammatory process and increased oxidative stress observed in Crohn's disease. Furthermore, Crohn's disease MPs play a role in the extraintestinal symptoms related to the pathology. The increased expression of IL-23R induced by Crohn's disease MPs in colon of mice highlights a possible role of Crohn's disease MPs in regulation of expression of inflammatory markers of disease. All these data confirm the deleterious effects played by MPs in Crohn's disease and that MPs can be considered as marker of inflammation.

Thus, a better understanding of the mechanisms that underlie the effect observed in this study we allow us to clarify the signalling pathways using by MPs and probably considered Crohn's disease MPs as pharmacological target of disease.

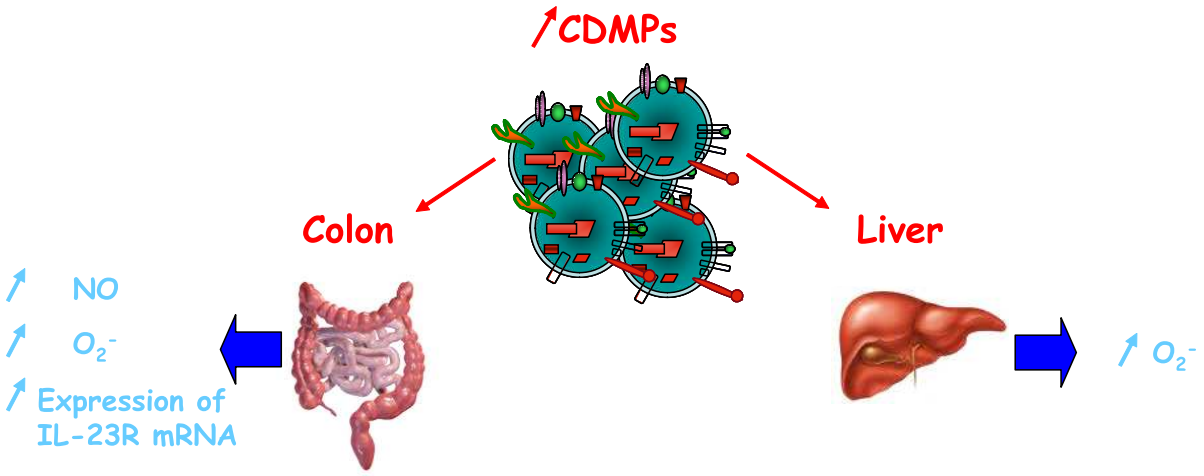


Fig 9: Schematic representation of *in vivo* effects evoked by Crohn's disease MPs (CDMPs) on colon and liver of mice.

General conclusion and Perspectives

In the first part of this thesis, we provided significant new information for understanding the pathophysiology of Crohn's disease. We observed increased levels of circulating MPs in Crohn's disease that is linked to activity of disease. Furthermore, we revealed the involvement of these MPs in the induction of vascular dysfunction both in conductance vessels and in resistance vessels affecting endothelium and vascular smooth muscle. The effects of Crohn's disease MPs at the level of smooth muscle cells result in a hyporeactivity that involves a subtle interaction between NO production, COX-2 metabolites and ROS. These results suggest a role of Crohn's disease MPs as marker of endothelial dysfunction or injury and highlight a promising possibility to consider Crohn's disease MPs as possible pharmacological targets of disease. Then, an interesting aspect would be to found a procedure that can reduce the number of MPs in order to improve the deleterious effects and in general the impact of Crohn's disease MPs in the evolution of disease.

In this context, increasing interest is caused by the ability of MPs to express microRNAs (miRNAs) that are a class of 21-23 nucleotide long non-coding RNA molecules, involved in the regulation of gene expression in critical biological processes. A role for aberrant miRNA expression in gastrointestinal disorders has been postulated. Particularly, a recent study has demonstrated a relationship between an up-regulation of miR-29a in microparticles isolated from the blood of IBD patients and increase intestinal membrane permeability of these patients (Zhou *et al*., 2010). These data suggest that a new diagnostic strategies could be based on the analysis of molecular profiling, notably miRNA arrays, of Crohn's disease MPs.

In the second part of this thesis we have gathered more information about the deleterious effects of Crohn's disease MPs. We demonstrate that Crohn's disease MPs not only are involved in inducing vascular alteration but they might participate directly in mediating organ inflammation of disease. Indeed, Crohn's disease MPs play a role in oxidative stress in different tissues and notably in a tissue target, the colon. Furthermore, an increased level of

Crohn's disease MPs could be linked, in tissue target, to specific signalling pathways that concerned inflammatory cytokine directly involved in this pathology, such as IL-23. These preliminary results lay the groundwork for a more detailed study of the mechanisms involved in these effects.

Finally, interesting perspectives of this work would be to extend the same type of study performed until now to the second most important form of IBD, ulcerative colitis.

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