Thesis for the Master's degree in Molecular Biosciences

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Are Chemokines Stored for Regulated Secretion in Rodent Endothelial Cells?

60 study points

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Abbreviations

A	Absorbance
Ab	Antibody
AR	Antigen Retrieval
Ag	Antigen
CD	Crohn`s Disease
CLP	Cecal ligation and puncture
DSS	Dextran Sulphate Sodium
EC	Endothelial cell
GCP-2	Granulocyte Chemotactic protein
HEV	High endothelial venule
HRP	Horseradish Peroxidase
HUVEC	Human umbilical vein endothelial cell
IBD	Inflammatory Bowel Disease
IHC	Immunohistochemistry
IL	Interleukin
kDa	kilo Daltons
KLH	Keyhole-Limpet Hemocyanin
mAb	Monoclonal Antibody
MIP-2	Macrophage inflammatory protein 2
PBS	Phosphate-buffered saline
RT	Room temperature
TNF	Tumor necrosis factor
UC	Ulcerative Colitis
vWf	von Willebrand factor
WPB	Weibel-Palade bodies

Summary

Chemokines are small, secreted proteins (5-15 kDa) that mediate leukocyte transmigration and can be secreted from endothelial cells in a constitutive or regulated manner. Regulated secretion shortens the response time to inflammatory stimuli because the chemokine is produced in advance and not dependent on *de novo* synthesis. The existence of regulated chemokine secretion from endothelial cells has not yet been demonstrated in animal models. A homologue of the potent neutrophil-attracting chemokine IL-8 has not been found in rodents. However, other potent neutrophil chemoattractants such as CXCL1, CXCL2, and CXCL6 were therefore analyzed by immunohistochemistry in experimental acute peritonitis/sepsis and in DSS-induced colitis, with special emphasis on their possible sorting to Weibel-Palade bodies (WPB). The data presented imply that sorting to WPB for regulated chemokine secretion is not a prominent feature of rodent endothelial cells but also indicate that MIP-2 may be sorted to another granular compartment in resting mesenteric vessels, perhaps related to the type II compartment of regulated secretion recently described in human endothelial cell cultures

1. Introduction

The inside of blood vessels is lined with endothelial cells that constitute the interface between circulating blood and surrounding tissues. Here they provide a tightly controlled barrier that regulates recruitment of leukocytes to the underlying tissues, vascular tone and hemostasis. Depending on their location within the vasculature, endothelial cells have distinct gene expression profiles in healthy individuals [1], [2]. Moreover, their phenotype is dramatically altered when exposed to proinflammatory or immunoregulatory cytokines as well as other humoral mediators [3].

During normal bloodflow, red and white blood cells generally travel along the central axis, leaving a cell-poor layer of plasma in contact with endothelium. As vascular permeability increases in early inflammation, fluid exits the vascular lumen and blood flow slows down. As a result, the leukocytes settle out of the central column, marginating to the vessel periphery. The identity of emigrating leukocytes varies depending on the nature of the inciting stimulus, and also changes as the inflammatory site ages. Thus, in most forms of acute inflammation, neutrophils predominate for the first 6-24 hours and are followed by monocytes in the subsequent 24 to 48 hours. This pattern is best explained by the sequential expression of different adhesion molecules and chemotactic factors as detailed below [4]

1.1 The leukocyte adhesion cascade

The recruitment of leukocytes from the blood to surrounding tissues takes place in lymphoid organs and in inflammatory lesions in a multistep process. It is mainly initiated by selectins which enable tethering and rolling of leukocytes along the endothelial cell surface until chemokines or other chemoattractants trigger firm arrest and enable trans-endothelial migration [5]. P-selectin and E-selectin are expressed by inflamed endothelial cells and interacts with PSGL1 (P-selectin glycoprotein ligand 1) found on the leukocyte microvilli [6]. L-selectins is expressed by most leukocytes

and interacts with PNAd (peripheral lymph node addressin) in high endothelial venules (HEVs) as well as with less characterized glycosylated ligands on inflamed venules. The interaction of selectins with their ligands is characterized by exceptionally high on- and off-rates which determine the speed with which bonds are formed and broken respectively [6]. Selectin-mediated leukocyte rolling allows activating signals to be transmitted through selectins or selectin-ligands but a crucial event appears to be the activation of leukocytes via G protein-coupled receptors by chemokines or other chemoattractants presented on the endothelial cell surface. Upon such activation leukocyte arrest is rapidly triggered via integrins that change their conformation to an activated state and then bind to immunoglobulin superfamily members expressed by endothelial cells, such as ICAM1 and VCAM1 [7]. Resting vessels are thought to have neither selectins, their ligands nor other chemoattractants on their surface. Moreover, immunoglobulin superfamily members are expressed at low levels and the absence of chemoattractants does not allow substantial integrin activation. The resting endothelial cell phenotype will therefore not permit a strong flux of leukocytes across the vessel wall. During inflammation, on the other hand, endothelial cells are activated by bacterial products such as LPS (lipopolysaccharide) or inflammatory cytokines to enhance the expression of adhesion molecules and to induce synthesis of chemokines and lipid chemoattractants for presentation on their luminal surface.

Although resting endothelial cells display no early adhesion determinants on their surface, they have an excellent system to allow rapid activation and recruitment of neutrophils. Firstly, they sort P-selectin to Weibel-Palade bodies (described in more detail below) that can be rapidly translocated to the surface membrane upon exposure to mast cell-derived histamine, coagulation-induced thrombine or complement-derived C5a. Secondly, the WPB also contains the chemokine IL-8 and eotaxin-3, but so far such sorting has been observed only in human tissues and cell culture models [8], [9]. Of relevance to this thesis, endothelial cells can also by means of transcytosis from their abluminal surface to the lumen present chemokines that are made by perivascular cells [10] [11].

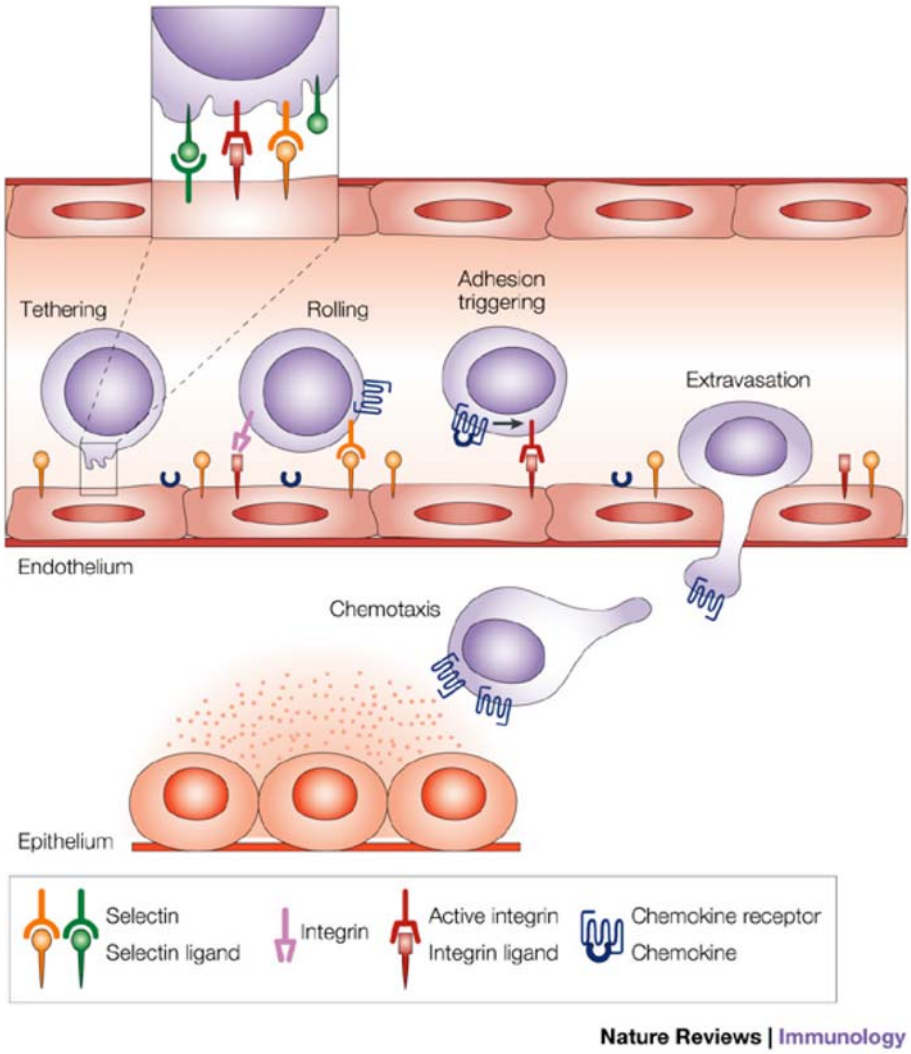

Figure 1 –The leukocyte adhesion cascade. Circulating leukocytes can tether to and roll on the endothelium, generally through transient selectin-carbohydrate ligand interactions and/or intergrin-interactions. Rolling is thought to allow leukocytes to sample the endothelium for chemoattractants signals such as chemokines, that in turn trigger firm adhesion by activating integrins on the leukocyte surface. Chemokines are also thought to direct the adherent leukocyte to migrate across the endothelium into the extravascular space and to further navigate leukocytes to the inflammatory focus. Figure copied from Kunkel et al, Nature Rewiews, 2003.

1.2 Chemokines

Chemokines are small proteins (5-15 kDa) that play a fundamental role in the development and homeostasis of the immune system as well as in immune responses [12]. Chemokines are also involved in angiogenesis and tumorigenesis [13]. During host responses against infections, the subtle interplay between chemokines and

adhesion molecules allows specific leukocyte subsets that express corresponding chemokine receptors to be selectively recruited to the site of inflammation.

Based on the position of the first two out of four conserved cysteine residues, chemokines can be classified into two major subgroups; CC chemokines and CXC chemokines. The nomenclature is defined by the structure-related acronyms followed by an L (for ligand) and the number of their gene. Earlier designations involved consecutively numbered genes that were designated as SCY (small inducible cytokines). In addition, most chemokines have names based on their initially observed functions and the discovering laboratory, in many cases also leading to different names in different species. For example, CXCL1 is known as Groα, KC or CINC in human, mouse and rat, respectively (see table 1) [14].

Table 1 - Chemokine nomenclature in human, mouse and rat

Chemokine Nomenclature	**Human**	**Mouse**	**Rat**
CXCL1	Gro-α	KC	CINC
CXCL2	Gro-β	MIP-2	MIP-2
CXCL6	GCP-2	GCP-2/LIX	LIX
CXCL8	IL-8	Has not been described	Has not been described

The CXC chemokine family can be further divided based on the presence or absence of the amino acid sequence Glu-Leu-Arg (ELR) immediately preceding the two N-terminal cysteine residues. ELR^+ CXC chemokines are predominantly activators of neutrophilic granulocytes and the ELR motif is indispensable for this chemotactic activity. Moreover, the ELR motif also appears to discriminate angiogenic (ELR^+) from angiostatic (ELR^-) CXC chemokines [15]. Like cytokines, chemokines are proteins produced by leukocytes and tissue resident cells, either constitutively or after activation. They act locally in a paracrine or autocrine fashion. The chemokines are usually much smaller than cytokines and act via heptahelical G-protein coupled

receptors, a feature common to several types of leukocyte attractants [16]. Through interactions with specific G-protein-coupled seven-transmembrane receptors, chemokines induce intracellular signalling leading to integrin activation during leukocyte adhesion (see above), oxidative burst, release of lysosomal enzymes and chemotaxis.

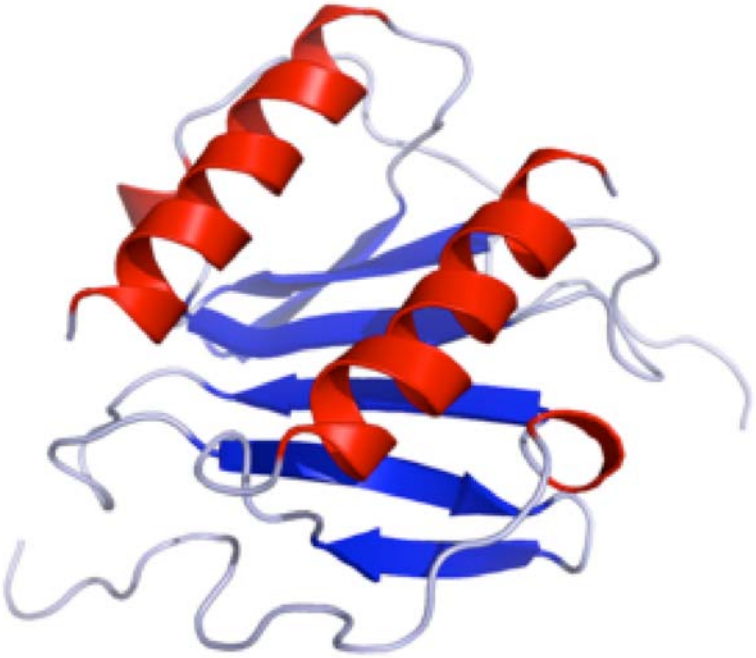

Figure 2 – Protein structure of an ELR+ chemokine,. CXCL8/IL-8 is shown as a dimer. One monomer contains three β-sheets and one alpha helix. The quarternary structure differs between different classes of chemokines.

1.2.1 Chemokine receptors

The receptors are defined by the chemokines structure-related acronyms such as CXC, CC, XC and CX3C, followed by R and a number [14]. Six CXC chemokine receptors, and 10 CC chemokine receptors, as well as receptors for lymphotactin (XCR1) and fractalkine (CX3CR1), have been identified to date. Most receptors recognize more than one chemokine and several chemokines bind to more than one

receptor [16]. Chemokines can synergize with each other or with other inflammatory mediators to enhance leukocyte infiltration in an early phase.

1.2.2 ELR+ chemokines in mice and men

Seven different ELR+ chemokines that all attracts neutrophils have been identified in man (Figure 3). Of these, IL-8/CXCL8 and GCP-2/CXCL6 are by far the most efficient and are found in large amounts in acute inflammatory lesions. A particular feature of IL-8 is that it signals predominantly through the chemokine receptor CXCR1 whilst the effects of most other ELR+ chemokines are mediated mainly via CXCR2 or via CXCR2 alone. Of special interest to this study is that a homologue of IL-8 is missing in rodents. Other potent neutrophil chemoattractants exist in the mouse, such as KC/CXCL1, MIP-2/CXCL2, GCP-2/CXCL6, Thymus chemokine-1/CXCL7 and CXCL15/lungkine. Moreover, a rodent homologue of the main IL-8 receptor, CXCR1, has recently been cloned and characterized [17, 18] and on the grounds of GCP-2 being the only other chemokine apart from IL-8 to signal efficiently via CXCR1 [18], it was of additional interest to assess whether GCP-2 like IL-8 can be sorted to Weibel Palade bodies for regulated secretion.

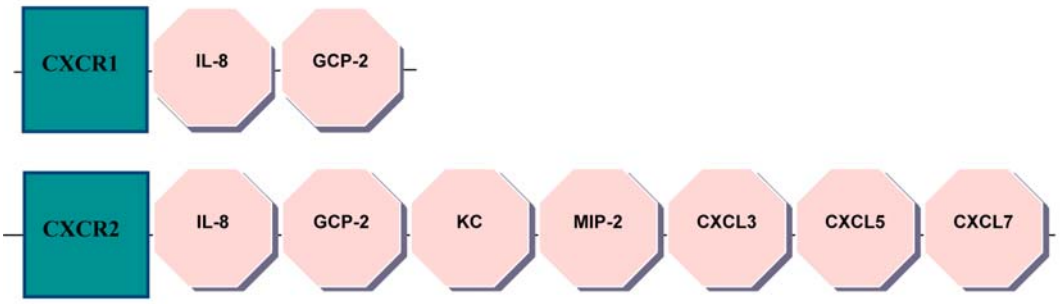

Figure 3 - The chemokine receptors CXCR1 and CXCR2 with the corresponding chemokine ligands. Modified from Zlotnik et al, Immunity, 2000.

1.2.3 Chemokine clusters

Chemokines differ in their biological activity, regulation of expression, receptor-binding specificities and the chromosomal location of the genes that encode them. In addition to the classification of chemokines based on structure (e.g. CC vs CXC chemokines), they also fall into two major groups based on their expression patterns and functions: those that are expressed in inflammation and therefore called inflammatory chemokines, and those that are constitutively expressed in discrete locations of the immune system, called homeostatic chemokines. Moreover, the genomic organization of chemokines enables us to divide chemokines into two alternative groups: those whose genes are located in large clusters at particular chromosomal locations and the non-cluster or mini-cluster chemokines whose genes are located separately in unique chromosomal locations. There are four major clusters of chemokine genes, two which are among the CXC chemokines plus numerous non-clustered or mini-cluster genes in both the human and the mouse genomes. The explanation for this dispersion is the force of evolution. Once duplication occurs, the two copies evolve independently and develop unique and specialized functions. The members of a given gene cluster usually bind to multiple receptors and vice versa. Furthermore, cluster chemokines often do not correspond well between species, for example between human and mouse [19] [20]. An explanation for the large number of ligands for these receptors may be that, during inflammation, multiple chemokines are needed to induce a robust leukocyte response [21]. Moreover, differential expression of these chemokines among different tissues may finally orchestrate the recruitment of leukocytes to the tissues and by this enable a customization of the inflammatory responses. Most cluster chemokines belong to the inflammatory category of chemokines [20, 21].

Table 2 The chemokine superfamily

Human	Other names	Chromosome	Function	Cluster	Mouse	Other names	Chromosome	Function	Cluster	Receptor
CXC family										
CXCL1	Groα	4q13.3	I	GRO	CXCL1	Gro/KC	5qE2	I	GRO	CXCR2, CXCR1
CXCL2	Groβ	4q13.3	I	GRO	CXCL2	MIP-2	5qE2	I	GRO	CXCR2
CXCL3	Groγ	4q13.3	I	GRO	Gm1960	Dcip1	5qE2	I	GRO	CXCR2
CXCL4	PF4	4q13.3	U	GRO	CXCL4	PF4	5qE2	U	GRO	CXCR3B^
CXCL4VI		4q13.3	U	GRO						
CXCL5	ENA-78	4q13.3	I	GRO	CXCL5	LIX*	5qE2	I	GRO	CXCR2
CXCL6	GCP-2	4q13.3	I	GRO		LIX*				
CXCL7	NAP-2	4q13.3	I	GRO	CXCL7	Ppbp	5qE2	I	GRO	Unknown
CXCL8	IL-8	4q13.3	I	GRO	Unknown					CXCR1, CXCR2
CXCL9	MIG	4q21.1	I	IP10	CXCL9	MIG	5qE3	I	IP-10	CXCR3, CXCR3B
CXCL10	IP-10	4q21.1	I	IP10	CXCL10	IP-10	5qE3	I	IP-10	CXCR3, CXCR3B
CXCL11	I-TAC	4q21.1	I	IP10	CXCL11	I-TAC	5qE3	I	IP-10	CXCR3, CXCR3B, CXCR7
CXCL12	SDF-1α/β	10q11.21	H		CXCL12	SDF-1α/β	6qF1	H		CXCR4, K61CXCR7
CXCL13	BLC, BCA-1	4q21.1	H	IP10	CXCL13	BLC, BCA-1	5qE3	H	IP-10	CXCR5
CXCL14	BRAK, Bolekine	5q31.1	I		CXCL14	BRAK	13qB2	I		Unknown
Unknown					CXCL15	Lungkine, Weche	5qE2	U		Unknown
CXCL16	DMC	17q13.2	I		CXCL16	CXCL16	11qB4	I		CXCR6
CXCL17		19q13.2	U		CXCL17	DMC	7qA3	U		Unknown
CC family										
CCL1	I-309	17q11.2	I	MCP	CCL1	TCA-3	11qB5	I	MCP	CCR8
CCL2	MCP-1	17q11.2	I	MCP	CCL2	JE	11qB5	I	MCO	CCR2
CCL3	MIP-1α, LD78α	17q11.2	I	MIP	CCL3	MIP-1α	11qB5	I	MIP	CCR1, CCR5
CCL3L1	LC78β	17q12	I	MIP						
CCL3L3	LC78β	17q13	I	MIP						
CCL4	MIP-1β	17q14	I	MIP	CCL4	MIP-1β	11qB5	I	MIP	CCR5
CCL4L1	AT744.2	17q15	I	MIP						
CCL4L2		17q16	I	MIP						
CCL5	RANTES	17q17	I	MIP	CCL5	RANTES	11qB5	I		CCR1, CCR3, CCR5
CCL7	MCP-3	17q11.2	I	MCP	CCL7	MARC	11qB5		MCP	CCR1, CCR2, CCR3
CCL8	MCO-2	17q11.2	I	MCP	CCL8, CCl	MCP-2 MCP-5	11qB5	I	MCP	CCR1, CCR2, CCR3, CCR5
CCL11	Eotaxin	17q11.2	I	MCP	CCL11	Eotaxin	11qB5	I	MCP	CCR3
CCL13	MCP-4	17q11.2	I	MCP	Unknown				MCP	CCR1, CCR2, CCR3
CCL14	HCC-1	17q12	H	MIP	Unknown	MMRP2, CCF18				CCR1
CCL15	HCC-2	17q12	H	MIP	CCL19	MIP-1γ	11qB5	H	MIP	CCR1, CCR3
CCL16	HCC-4 LEC	17q12	H	MIP	Pseudogene		11qB5		MIP	CCR1, CCR2, CCR5, HRH4
CCL17	TARC	16q13	D	MIP	CCL17	TARC	8qC5	D		CCR4
CCL18	PARC	17q12	H		Pseudogene					Unknown
CCL19	MIP3β, ELC	9p13.3	H		CCL19	MIP3β	4qB1	H		CCR7
CCL20	MIP3α, LARC	2q36.3	D		CCL20	MIP3α, LARC	1qC5	D		CCR6
CCL21	SLC	9p13.3	D		CCL21a, CCL21b, CCL21c	SLC	4qB1	D		CCR7
CCL22	MDC	16q13	D		CCL22	ABCD-1	8qC5	D		CCR4
CCL23	MPIF-1	17q12	I	MIP	CCL6	C10	11qB5	I	MIP	CCR1, FPRL-1
CCL24	Eotaxin-2	7q11.23	I		CCL24	Eotaxin 2	5qG1	I		CCR3
CCL25	TECK	19p13.3	H		CCL25	TECK	8qA1.2	H		CCR9
CCL26	Eotaxin 3	7q11.23	I		CCL26l	Eotaxin 3-like	5qG1	I		CCR3
CCL27	CTACK, ILC	9p13.3	H		CCL27a, b	CTACK, ILC	4qB1	H		CCR10
CCL28	MEC	5p12	U		CCL28	MEC	13	U		CCR10, CCR3
Other classes										
XCL1	Lymphotactin	1q24.2	D		XC11	Lymphotactin	1qH2	D		XCR1
XCL2	SCM-1β	1q24.2	D							
CX3Cl1	Fractalkine	16q13	I		CX3C11	Fractalkine	8qC5	I		XC3CR1

Functions are as follows: I, inflammatory, H, Homeostatic, D, dual (homeostatic and inflammatory), U, unknown. The list of alternative names are not comprehensive. Chromosomal location data are derived from the Ensebl (39) or Mouse Genome Informatics (40) databases. GRO, GRO region of the CXC major gene cluster, IP10, IP10 region of the CXC major gene cluster. MIP, MIP region of the CC major gene cluster. Adapted from Zlotnik, Genome Biology 2006; 7:243. *CXCL5 and CXCL6 appear to have originated from the common ancestor LIX by gene duplication, see discussion.

1.3 The secretory pathway - regulated and constitutive secretion

Eukaryotic cells are highly compartmentalised into distinct membrane-bound organelles. The organelles of the secretory pathway are involved in sorting of proteins to a variety of intracellular membrane compartments and to the cell surface.

The fusion of secretory vesicles with the plasma membrane occurs in all cells as constitutive exocytosis, and is required for the insertion of new cell membrane. Moreover, many extracellular molecules like plasma proteins and antibodies are secreted by the constitutive secretory pathway [22]. On the other hand, regulated secretion is a process in which the organelles with their cargo reside in the cytoplasm until the cell receives a second stimulus inducing fusion of the organelle with the plasma membrane and release of its cargo [23]. Chemokines can be secreted both in a constitutive and in a regulated manner [8].

1.3.1 The Weibel-Palade Body

The best characterized intracellular storage pools in endothelial cells are Weibel-Palade Bodies (WPB). They are rod-shaped, elongated structures that appear like “chocolate sprinkles” in the cytoplasm [24], [25]. The major constituent of WPB is the multimeric protein von Willebrand factor (VWF), which is required for correct hemostasis at sites of vascular injury through its role in platelet adhesion. Several lines of evidence support the idea that VWF is the driving force behind the biogenesis of these organelles. Expression of VWF in nonendothelial cells results in the formation of VWF-containing, rod-shaped organelles that closely resemble WPB. Over the past few years it has been appreciated that multiple components are stored together with VWF in WPB. The growing list of components that are present within this subcellular organelle suggests a role for regulated release of WPB in inflammation, hemostasis and angiogenesis [24], [25]. Several studies have provided evidence for the existence of different subsets of WPB that apart from VWF do not

contain the same set of additional constituents. A clear example is the neutrophil-attracting chemokine IL-8. This chemokine is stored in WPB only after induction of its synthesis by inflammatory mediators such as IL-1β, thus providing a rapidly releasable pool of IL-8, independent of *de novo* synthesis [8]. Up to this date there are 12 proteins in addition to VWF that have been identified as constituents of WPB [26].

1.3.2 Other compartments for regulated secretion in endothelial cells

More recently, another granular compartment in vascular endothelial cells containing the chemokines GROα/CXCL1 and MCP-1/CCL2 was discovered in vascular endothelial cells. Based on the absence of other proteins known to reside in similar granules as well as differential responsiveness to cAMP and PKC mimetics it was designated, the type II compartment of regulated secretion in endothelial cells [27]. In this manner, prestored chemokines can originate from different compartments for rapid release and presentation upon demand, allowing rapid recruitment of leukocytes in acute inflammation [8].

2. Methodological background

2.1 Immunohistochemistry

Immunochemistry started in 1941, when it was realized that antibodies could be labelled with fluorescent molecules and at the same time preserve their binding activity. Accordingly, immunohistochemistry is an antibody-based technique that allows the scientist to study epitope distribution in tissues. Such analyses can be performed using fluorochrome- or enzyme-conjugated antibodies. The latter will generate a coloured precipitate from a chromogenic substrate. While an advanced fluorescence-microscope is needed to observe fluorochromes, a light microscope is sufficient when using enzyme conjugated antibodies. The validity and quality of immunohistochemistry depends on the antibody quality, how the tissue is fixed and processed, the unmasking of epitopes and the sensitivity of the detection system [28]. Like other antibody-based techniques, immunohistochemistry requires careful selection and titration of antibodies and the use of appropriate controls. Complete and regularly updated websites of commercially available antibodies, reagents and kits are for example The Linscotts Directory (http://www.linscottsdirectory.com) and the Biocompare website (http://www.biocompare.com/index.asp)

2.1.1 Antibodies

Primary antibodies that give unwanted background sometimes make it necessary to block the tissue sections with serum from the species of the secondary antibody before applying the primary antibody. If non-specific background persist, further dilution of the primary antibody combined with an increase in the concentration of secondary reagents is often effective. If there is cross-reactivity between the secondary antibody and antigens present in the target cells or tissue, this is often

caused by cross-reaction with immunoglobulins in the target tissues, and such binding can be reduced by pre-absorbing the antibody with serum from the target species.

2.1.2 Controls

Proper controls must always be included with each staining procedure. Either preimmune serum or isotype- and concentration-matched Ig should be used as a negative control to ensure that the staining observed is not due to background, and a positive control slide should always be included to verify the activity of the secondary reagents. One slide that omits a primary antibody is also useful to indicate background associated with the secondary antibody. Given that antibodies raised to peptide fragments are often linked to keyhole limpet hemocyanine (KLH) as a hapten, an optimized control for such reagents would be an antibody purified from immunoserum by means of KLH alone.

2.1.3 Fixation

The fixation of tissues is required to stop diffusion of soluble components, block unwanted enzyme activity, stop degradation of tissue structures and allow the preparation to withstand the different steps of the staining procedure [29]. Acetone is a gentle fixative used mainly after cryopreservation, and causes minimal antigenic denaturation, sufficient membrane permeabilization for antibodies to enter the cells and in many cases an acceptable morphology. Formalin- or paraformaldehyde-fixed tissue retains excellent morphology, but the antigen often suffers extensive denaturation that may diminish or completely prohibit epitope recognition by the primary antibody. This problem can be solved via two approaches; either by using antibodies that have been raised to specifically recognize formalin-fixed antigens and/or using a protocol designed to expose antigens in fixed tissues, such as the method of antigen retrieval (see below) [30].

2.1.4 Antigen Retrieval (AR)

Formalin forms intra-molecular crosslinks with amino groups of proteins and can therefore influence the accessibility of the antigen for an antibody. Heat treatment is the most commonly used method used for the antigen retrieval in immunohistochemical studies of formalin-fixed and paraffin-embedded tissues. Heat treatment cleaves the formalin-induced intra- and intermolecular crosslinks of proteins [31]. In addition to the temperature, pH and buffer composition are important parameters that contribute to proper AR. Therefore, several different buffers at specific pH should be tested to find the most optimal AR procedure for your antigen of interest. After AR, the antibody might need to be re-titrated properly, since more epitopes becomes available after the AR treatment. The potential disadvantages using AR are detrimental effects on tissue morphology and increases the general background staining.

2.1.5 Animal models

It is important to have animal models that closely simulate human disease. The model of cecal ligation and puncture (CLP) in rodents has been used to investigate the clinical conditions of sepsis and septic shock. Aspects of sepsis research using the CLP-model include, among others, immune function, mediator release and cytokine expression patterns [32], and CLP represents a good model for acute systemic inflammation. The driving agents of inflammation in the CLP model are bacterial products that leak into the peritoneal cavity and are recognized by the host immune system, leading to the release of proinflammatory cytokines

Dextran Sulphate Sodium (DSS) -induced colitis is another widely used model in rodents. It simulates human inflammatory bowel disease (IBD), and is a useful model of chronic inflammation [33]. It is popular because it is reproducible and easy to induce: it involves cheap, easily maintained, and widely available animal species: it has a predicable time course of inflammation and it resembles the clinical course, therapeutic response and inflammatory mediator profile of human IBD [33]. The

most severe disease in this model takes place in the distal colon, where a mixed cell inflammatory infiltrate comprising lymphocytes, macrophages and granulocytes develops. High levels of ELR^+ chemokines have been reported in both clinical sepsis and IBD as well as in rodent CLP and DSS model systems. The DSS model has proved particularly useful for testing epithelial repair agents, and has also been used for testing inhibitors of TNFα, IL-18 and IL-1 [34-36]. Considering the availability of these models, we chose to use these model systems to study the expression of ELR^+ chemokines.

Bacterial products such as LPS from gram negative bacteria are powerful activators of Toll-like receptors (TLR) that induce the release of inflammatory cytokines via activation of nuclear factor (NF)-κB. Intestinal epithelial cells are the first line of defense against invasion of intestinal bacteria. LPS also stimulates the release of IL-8 from these cells. The release of these inflammatory cytokines and NF-κB activation in macrophages and intestinal epithelial cells plays a key role in the pathology of inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn`s disease (CD) [37] They are characterized by chronic inflammation and a constant influx of leukocytes out of the blood stream into the mucosa, mediated by pro-inflammatory cytokines and chemokines [38].

3. Aims

- Establish whether formalin-fixed, paraffin-embedded tissue is suitable for detection of vessel-associated chemokines
- Establish protocols for detection of rodent ELR+ chemokines in tissue sections
- Assess the vascular expression of such chemokines in healthy and inflamed tissue
- Establish whether such chemokines are sorted to Weibel-Palade bodies of rodents for regulated secretion in vivo.

4. Materials and Methods

4.1 Materials

Table 3 - Primary antibodies

1 2	Mouse anti-human IL-8	LeukoSite Inc, Cambridge MA	1 µg/ml
3	Rabbit anti-human VWF	Dako, Glostrup Denmark	1:2000
4	Rabbit anti-mouse KC	Peprotech EC LTD, London	1 µg/ml
5	Rabbit anti-mouse MIP-2	Peprotech EC LTD, London	1 µg/ml
6	Rabbit anti-human GCP-2	Peprotech EC LTD, London	5 µg/ml
7	Rabbit anti-mouse LIX	Peprotech EC LTD, London	1 µg/ml
	TRITC-conjugated, goat anti-human VWF	Unknown	1:100
8	Rabbit anti-hemocyanin	Sigma, Missouri USA	Matched to the test antibody

Table 4 - Secondary and tertiary reagents

1	Biotinylated horse anti-mouse IgG	Vector Laboratories, Burlingame USA	1:200
2	Streptavidin Cy2	Amersham Biosciences, GE Healthcare, Oslo Norway	1:1000
3	Cy3-conjugated donkey anti-rabbit IgG	Jackson ImmunoResearch Laboratories, West Grove USA	1:500
4	HRP-conjugated donkey anti-mouse IgG	Jackson ImmunoResearch Laboratories, West Grove USA	1:300
5	HRP-conjugated goat anti-rabbit IgG	Invitrogen Molecular Probes	1:100

Table 5 - Other reagents

1	TSA kit	Invitrogen Molecular Probes
2	H_2O_2	Ventana Medical Systems, France

4.2 Methods

4.2.1 Human tissue

Sections of biopsy material taken from human non-inflamed colon were retrieved from the Diagnostic Biobank at the Division of Pathology according to a protocol approved by the regional Committee for Research Ethics (S-05152).

4.2.2 DSS-model of colitis

Intestinal samples from DSS-fed mice were kindly provided by Anders Sandvik, Institute. of Pathology, UiO). To induce colitis these animals were given DSS, (MP Biomedicals, Inc. Solon, Ohio, USA) dissolved in the drinking water (1.5% w/v) for 7 days according to a protocol approved by the national ethics committee for animal experiments. Control animals were given regular drinking water. Following a recovery phase of 4 days animals were anesthetized by subcutaneous injection of Hypnorm® and Midazolam (50-75µL/10g body weight) prior to cardiac puncture. Postmortem mice were soaked in 70% ethanol and fixed to a dissection board. The abdomen was opened and the colon excised, before flushing with cold PBS and partitioning into proximal, middle, and distal segments prior to fixation. All tissue samples were kept on ice and fixed in 4 % formalin for 24 h at 4°C before transfer to PBS with 0.1% formalin for storage at 4°C or immediate tissue processing.

4.2.3 CLP model of peritonitis and sepsis

Intestinal samples from adult Wistar male rats with peritonitis caused by cecal ligation and puncture (CLP), were kindly provided by J.E. Wang and Y.Y. Wang. CLP was performed by a method described elsewhere (Irshad Chaudry, 1979), using isoflurane anesthesia and buprenorphin (Temgesic, Schering Plough International, Kenilworth, NJ, US) to provide analgesia. The rats were fasted for 16 hours before anaesthesia, with water allowed *ad libitum*. The abdomen was opened and the colon exposed. The cecum was ligated just below the ileocecal valve to maintain bowel continuity and punctured twice with an 18 Gauge needle before closing the abdominal wall in two layers. Sham animals were subjected to surgery, but not CLP. Animals were given 3ml/100g body weight physiological saline subcutaneously immediately after and 12 hours after surgery to compensate for loss of fluid. Animals were sacrificed after 24 hours and an array of tissues excised and transferred to fixative as described for the mouse samples.

4.2.4 Tissue processing and histology

Biopsies were embedded in paraffin using an automated tissue processor (Leica TP1050, Leica Instruments GmbH, Nussloch, Germany) and cut in 4 μm sections that were arranged onto polysine-coated microscope slides, incubated at 60°C for 30 minutes and cooled. To remove paraffin from the tissue surface, tissue sections were incubated in xylene, rehydrated in a serial grade of absolute, 95%, and 70% ethanol (15 seconds each) and finally washed in PBS (2 x 2 minutes).

Haematoxylin and eosin staining (H&E) is the most commonly used tissue staining for animal histology and routine pathology. The basic dye, haematoxylin, gives a purplish blue stain to basophilic structures, such as nuclei, ribosomes and rough endoplasmic reticulum due to their content of DNA and RNA, respectively. By cont5rast, eosin/azophloxin is an acidic dye that stains eosinophilic structures, such as most cytoplasmic proteins, pinkish red. A particular feature of the H&E staining protocol at the Division of Pathology is the addition of saffron to also stain

connective tissue orange [39]. The staining is performed as follows; Deparaffinised tissue sections are dipped in ddH_2O before incubation in haematoxylin (10 minutes), hexamine (2 minutes), azophloxin (3 minutes) and in saffron (3 minutes). Between each step, the slide is cleansed under running tap water. The tissue is then dehydrated in absolute alcohol (2 x 2 minutes) and mounted in Eukitt.

4.2.5 Immunostaining and Antigen Retrieval

Antigenic epitopes masked by formalin-fixation were retrieved by incubation in pre-heated Tris-buffer (pH 9.0) for 20 minutes on a water bath at 100°C, followed by cooling to room temperature in the same buffer. To determine the localization of the different chemokines, different methods of immunolabelling were used, each time validating the specificity of the labelling by means of concentration-matched irrelevant antibodies. Immunohistochemistry can be performed as a direct method, in which the primary antibody is conjugated, and the staining is performed in one operation, or as an indirect method, in which the primary antibody is un-conjugated and the secondary antibody is conjugated. If the secondary antibody is polyclonal, several secondary antibodies can bind each primary antibody. This makes the indirect method more sensitive than the direct method. The use of biotin-avidin interactions is another popular approach based on the extremely high binding affinity between molecules, and the ability of one biotin molecule to bind four streptavidin molecules. Multiparameter immunohistochemistry allows the simultaneous detection of different epitopes by carefully selecting primary and secondary reagents that do not cross-react and by labelling each epitope with fluorochrome-conjugates of one specific colour.

In one series of experiments, human IL-8 was detected by mixing mouse anti-human IL-8 together with rabbit anti-human VWF in in PBS containing 1.25% BSA and incubating sections over night at 4°C. The sections were washed in PBS (2 x 3 minutes) before applying a mixture of biotinylated horse anti-mouse and Cy3-conjugated donkey anti-mouse antibody and incubating for 1.5 hour at RT. The washing procedure was repeated and tertiary Cy2 conjugated streptavidin was applied and incubated for another 1.5 hour. Finally, slides were washed as described above,

counterstained with Hoechst nuclear dye, and washed once in ddH_2O before mounting with polyvinyl alcohol (PVA)

Another series of experiments employed tyramide signal amplification (TSA™), an enzyme-mediated detection method that utilizes the catalytic activity of horseradish peroxidase (HRP) to generate high-density labelling of a target. TSA-labelling combines three elementary processes:

1. Binding of a primary antibody to the target followed by secondary detection of the probe with an HRP-labelled antibody or a streptavidin conjugate.
2. Activation of multiple copies of a dye- or hapten-labelled tyramide derivative by HRP
3. Covalent coupling of the resulting highly reactive, short-lived tyramide radicals to nucleophilic residues in the vicinity of the HRP-target interaction site, resulting in minimal diffusion-related loss of signal localization.

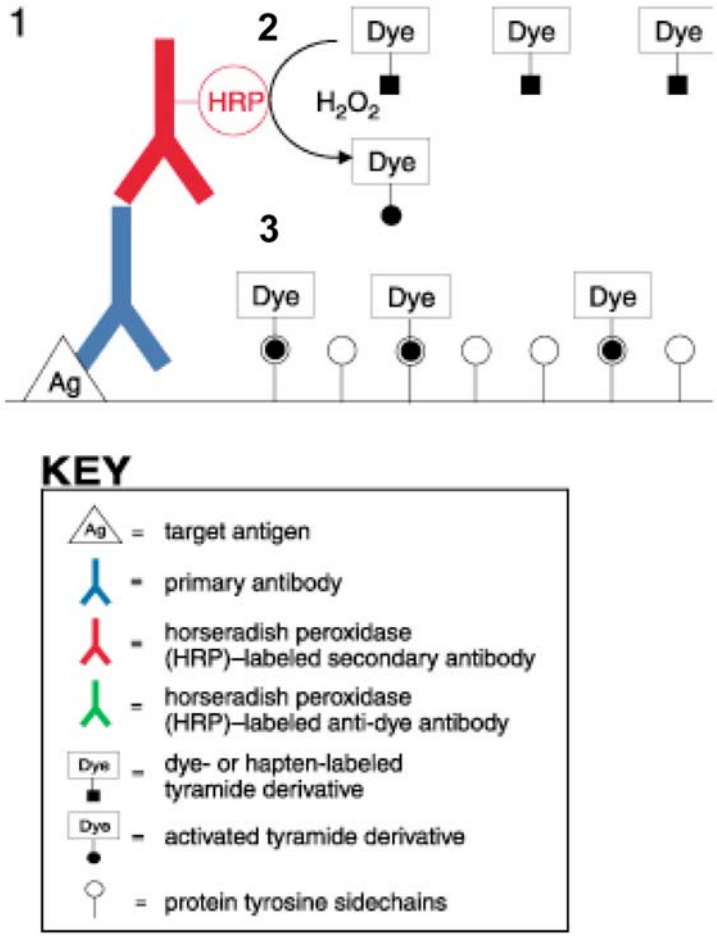

Figure 4 - The principle of Tyramide Signal Amplification

When using the Tyramide Signal Amplification Kit, endogenous peroxidase activity was quenched with H_2O_2 for 10 minutes at RT. The specimens were then blocked with 1% blocking buffer for 60 minutes. Primary antibody was diluted together with TRITC-conjugated goat anti-human VWF in 1% blockingbuffer and incubated over night at 4°C. The secondary HRP conjugate (Jackson Immunoresearch Laboratories, West Grove PA or Invitrogen Molecular Probes) was applied and incubated for 2 hours at RT. After the antibody incubation the slides were washed 2 x 3 minutes with PBS, before incubation 10 minutes at RT in tyramide solution prepared according to the protocol from the manufacturer (Invitrogen Molecular Probes). The sections were protected from light at all times. Finally, slides were washed as described above, counterstained with Hoechst nuclear solution, and washed once in ddH_2O before mounting in PVA.

4.2.6 Microscopy

To study the localization of chemokines and to compare it to that of WPB as demonstrated by staining for VWF, the immunostained tissues were examined with a Nikon E-800 fluorescence microscope or a Zeiss Axioplan Imaging LSM 5 Pascal confocal laser scanning microscope.

5. Results

5.1.1 IL-8 can be detected in formalin-fixed samples of human intestine

Colocalization of IL-8 and VWF has previously been demonstrated in cryopreserved sections of human intestine [8]. Due to the excellent morphology provided by formalin-fixation, the question was raised whether these findings could be reproduced after such fixation, given the risk that the antigen retrieval might cause soluble chemokines to detach from the tissue sections. As demonstrated in figure 5, IL-8 was indeed detectable by immunofluorescence in formalin-fixed tissue, and colocalization with VWF in WPB could be observed. Moreover, this protocol also allowed us to observe IL-8 in a pattern compatible with its binding to the surface membrane of endothelial cells (data not shown). We therefore concluded that formalin-fixation of tissues would facilitate the detection of chemokines under conditions of superior morphology.

5.1.2 Robust detection of ELR$^+$ CXC chemokines in rodent vessels requires non-biotin amplification of the signal

Initial efforts to detect chemokines in rodent tissue by modifying the biotin/streptavidin protocol used to detect human IL-8, revealed that rodent tissue contained high amounts of endogenous biotin rendering them less suitable for this method of signal amplification. A commercially available kit for signal enhancement based on the tyramide signal amplification protocol was therefore tested and established, revealing strong induction of chemokines as described below. To investigate the endothelial cell expression of CXCL1, CXCL2 and CXCL6 in rodents, suitable models of inflammation were needed. It was desirable to find models in which the global expression of these chemokines had been previously described, and in which they are presumed to play an important role in the pathogenesis. The DSS colitis model and the CLP model appeared to fulfil both of

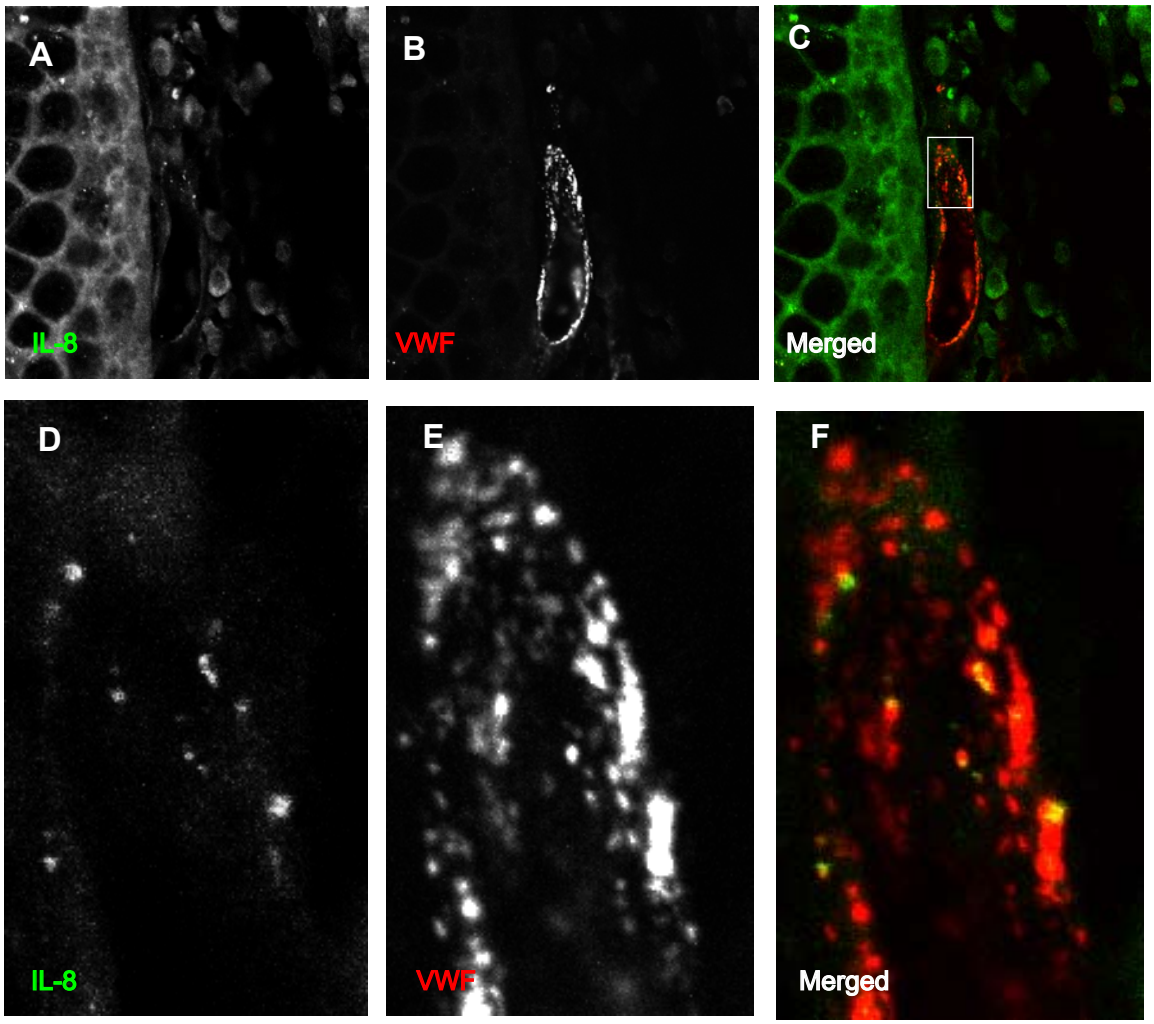

Figure 5 - CXCL8/IL-8 colocalizes with VWF in endothelial cells of healthy human intestine. Immunostainings of intestinal vessel for IL-8 in green (A) and VWF in red (B). The merged image of the green and red channel demonstrates colocalization of IL-8 and VWF (C). (D-F) Detail of vessel at higher magnification

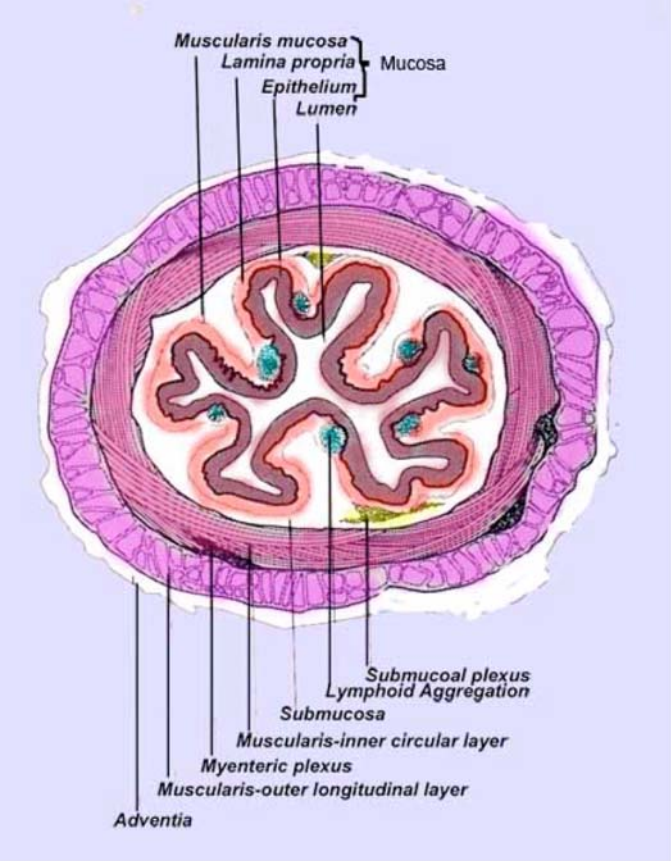

Figure 6 – Microanatomy of the intestine. Consists of four distinct functional layers; mucosa, submucosa, muscularis propria and adventitia. The mucosa is made up of three components; the epithelium, the supporting lamina propria and the muscularis mucosae, a thin smooth muscle layer. The submucosa is a thin layer of loose collagenous tissue which supports the mucosa and contains the larger blood vessels, lymphatics and nerves. The muscularis propria is the next layer which consists of smooth muscle, usually arranged as an inner circular layer and an outer longitudinal layer. Adventitia is the outer layer of loose supporting tissue and conducts the major vessels and nerves

these criteria, and the availability of relevantly preserved tissues in our lab provided the additional benefit of avoiding unnecessary animal experiments. The first series of experiments were focused on the CLP model that initially mimics acute peritonitis as puncture of the cecum will release a massive efflux of bacteria into the peritoneal cavity. Moreover, this situation rapidly develops into fulminant sepsis. Despite the greatly deteriorated condition of these animals after 24 h we observed quite subtle histopathological changes in the intestinal samples including moderate numbers of inflammatory leukocytes in vessels (fig 7). To complement these acute findings, samples of DSS colitis that had been harvested after a period of 7 days of exposure to

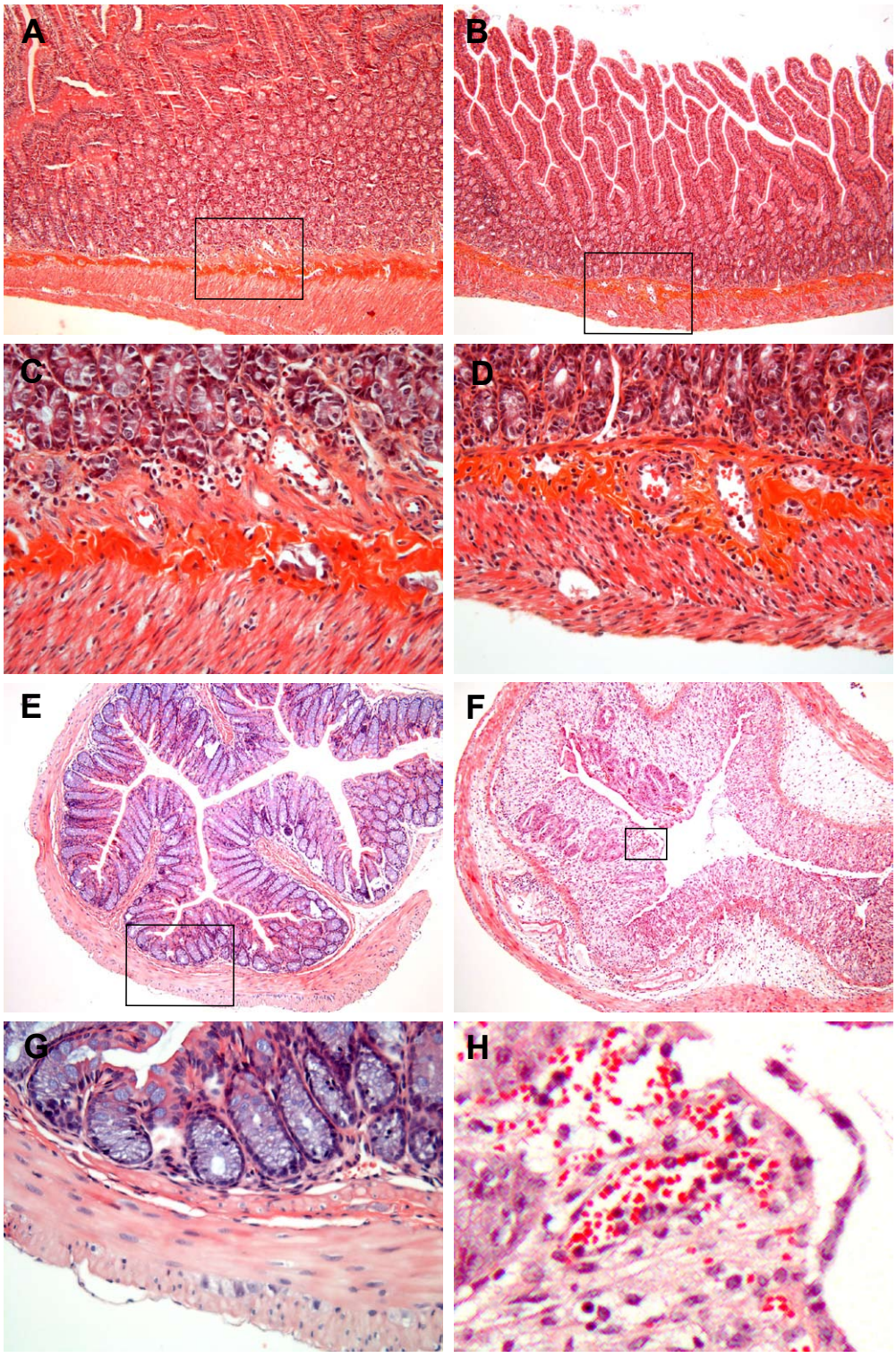

Figure 7 - Light microscopy of formalin-fixed, H&E-stained sections of rat (A-D) and mouse (E-H) intestine used for subsequent immunostainings. The images are obtained with x10 (A, B, E and F) and x40 (C, D, and G) lenses. H is a closeup image of F. Normal tissue architecture is demonstrated in control animals (A, C, E and G). In septic rats that have undergone caecal ligation and puncture (B, D), there is an increase in intraluminal leukocytes (arrow D), but no marked increase in cells in the lamina propria. In mice with DSS-induced ulcerous colitis (F, H) there is a marked increase in cells in the lamina propria, loss of normal crypt architecture (arrow F) and some epithelial sloughing.

DSS followed by 4 days recovery, were also included, revealing a mixed scenario of edema, inflammation and tissue regeneration and showing a moderate to massive influx of neutrophillic and eosinophillic granulocytes as well as mononuclear cells (Fig 7).

5.1.3 CXCL1 is induced in vessels of inflamed intestine:

Expression of CXCL1/KC in healthy rat intestine was assessed by means of a double staining protocol designed to allow simultaneous detection of chemokine (green) and VWF (red). No immunoreactivity was seen in intestinal vessels, but a weak, non-granular signal was observed in mesenteric vessels (fig 10) in endothelial cells. By contrast, inflamed tissue from CLP rats revealed high levels of expression of CXCL1 (Fig. 8B) above background levels (fig 8C) in both vessels and other cell types. In the former, CXCL1 was expressed in a diffuse pattern compatible with a surface membrane location, however, areas of enhanced perinuclear signal intensity indicated high levels of chemokine in Golgi stacks, reminiscent of the strong chemokine signal seen in Golgi stacks in vitro [9]. On the other hand, we observed no granular signal that colocalized with VWF and therefore concluded that CXCL1 is not sorted to Weibel-Palade bodies. It is also worth mentioning that not all vessels stained positive for chemokines, and in fact it appeared that arterioles in fact to have lower levels of chemokine expression than venules. The protocol of the present study did not allow identification of other CXCL1-positive cells observed but it is likely that dendritic cells and macrophages are among such candidates based on published *in vivo* data [40].

The expression of CXCL1/KC was also investigated in healthy and inflamed intestine of DSS-treated mice. While control tissues revealed no immunoreactivity on endothelial cells, inflamed samples showed some vascular chemokine expression, but fewer vessels with membranous staining than seen in the rat samples, see figure 8. Again, CXCL1 was neither seen in a granular pattern nor seen to colocalize with VWF.

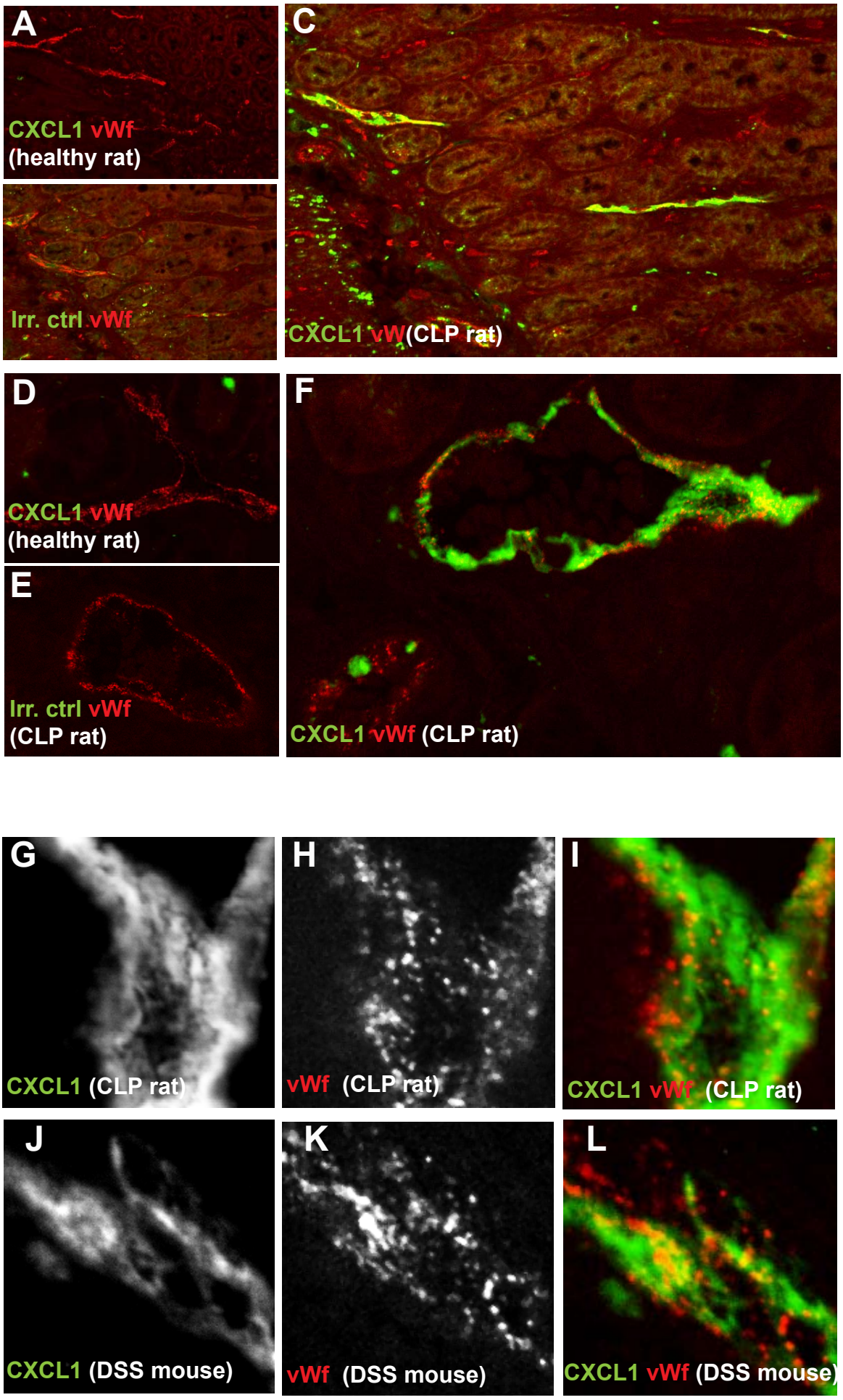

Figure 8 - Expression of CXCL1/KC in healthy and inflamed intestine. Vessel in the lamina propria costained for CXCL1 (green) and VWF (red). Panel A-C shows overview, panel D-F shows details of one single vessel and panel G-I shows higher magnification of vessel in CLP rat. Panel J-L shows detail of vessel in DSS mouse.

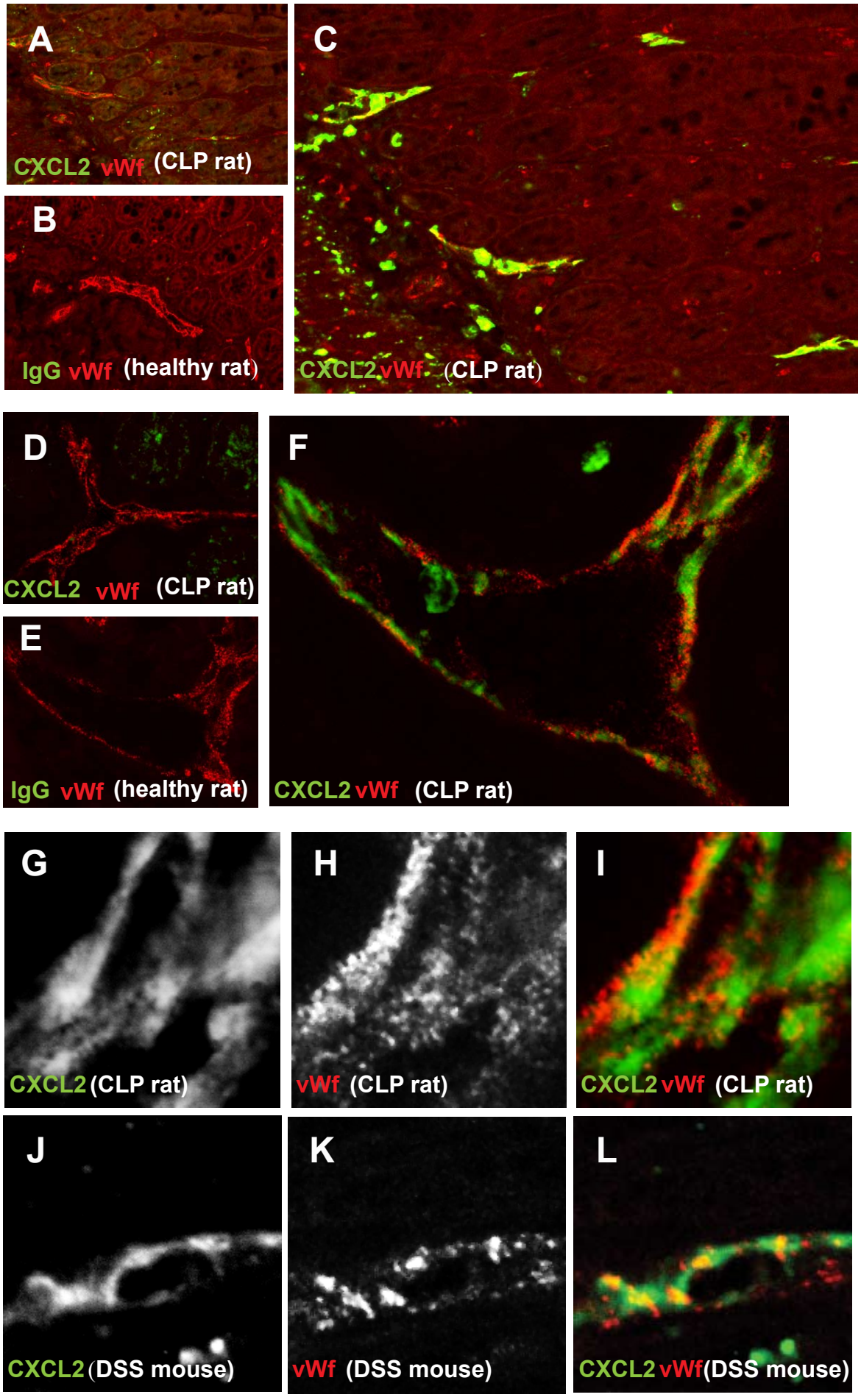

Figure 9 - Expression of CXCL2/MIP-2 in healthy and inflamed intestine. Vessel in the lamina propria costained for CXCL2 (green) and VEF (red). Panel A-C shows overview, panel D-F shows details of one single vessel and panel G-I shows higher magnification of vessel in CLP rat. Panel J-L shows detail of vessel in DSS mouse.

5.1.4 Immunostaining for CXCL6

The distribution of CXCL2/MIP-2 in healthy rat intestine revealed an interesting difference to that of CXCL1. While no immunoreactivity was found in vessels of the lamina propria, (Fig 9A+B), larger venules in the mesentery revealed a granular signal for CXCL2 that was quite convincingly associated with endothelial cells but did not colocalize with VWF (Fig 10). By contrast, the distribution of vascular CXCL2 expression in inflammation was quite similar to that of CXCL1 (fig 9 C, F-I) and colocalization with VWF was not observed. In the mouse, control intestinal tissue revealed no immunoreactivity, while inflamed tissue revealed expression of CXCL2 on fewer vessels than in the CLP rat (Fig 9J-L).

5.1.5 CXCL6 is not found on endothelial cells in rodents

Staining with an antibody to human CXCL6 in healthy intestine was mainly restricted to red blood cells in rat and mouse intestine. The staining pattern obtained with the antibody to human CXCL6 was so strong that it was hard to ascertain what subset of tissue cells might also be stained. However, no colocalization of CXCL6 with VWF was observed (fig 11). Moreover, there was no difference in the distribution of CXCL6 when comparing healthy and inflamed intestine, data not shown.

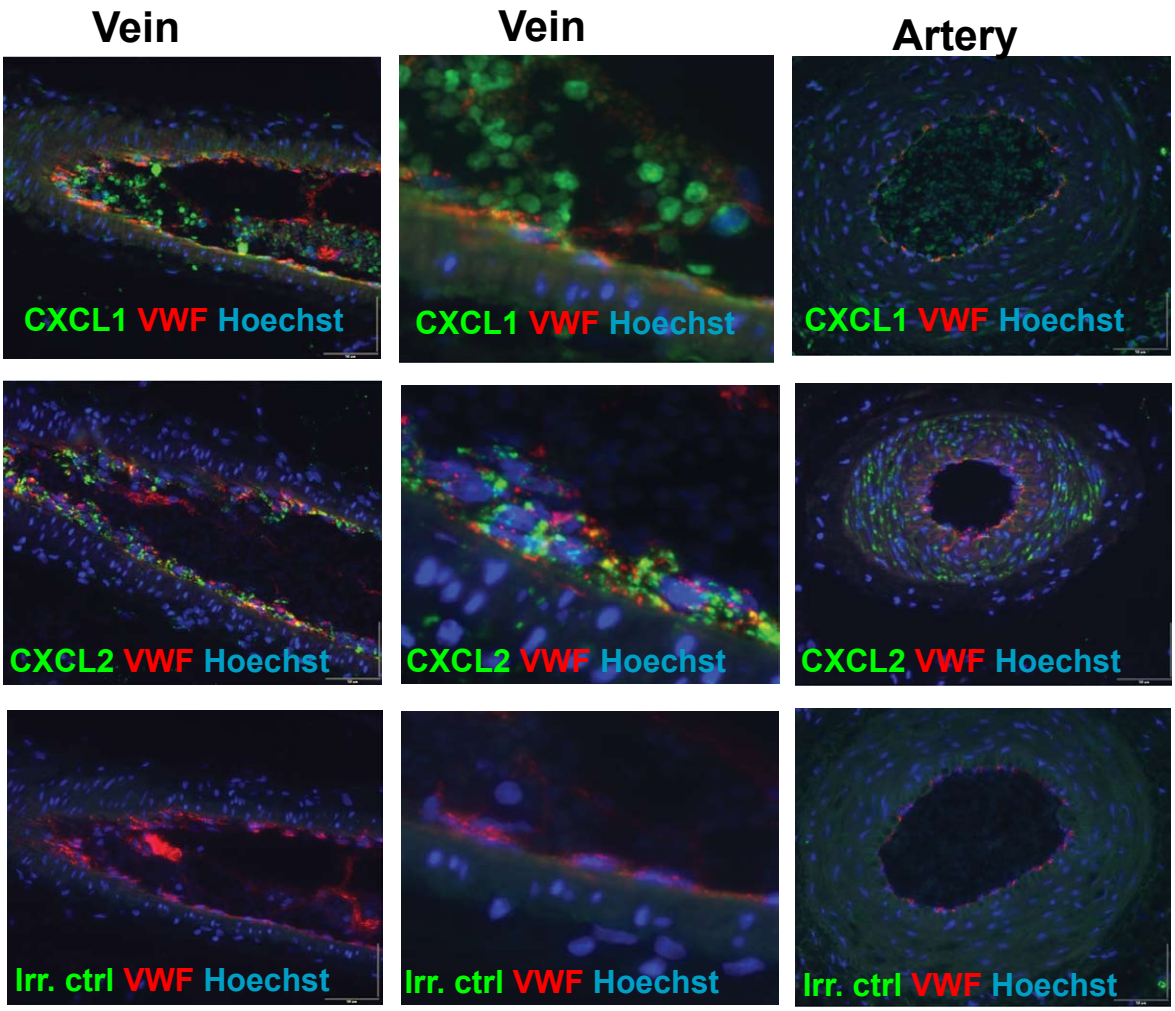

Figure 10 – Expression of CXCL1 and CXCL2 in mesenteric vessels of the rat. Three colour immunostainings showing chemokine (green), VWF (red) and Hoechst nuclear staining (blue). Panel A-C shows expression of CXCL1 in venule (A and B) and artery (C). Panel D-F shows expression of CXCL2 in venule (D and E) and artery (F). Panel G-I shows negative control staining with anti-KLH antibody (green) and VWF (red).

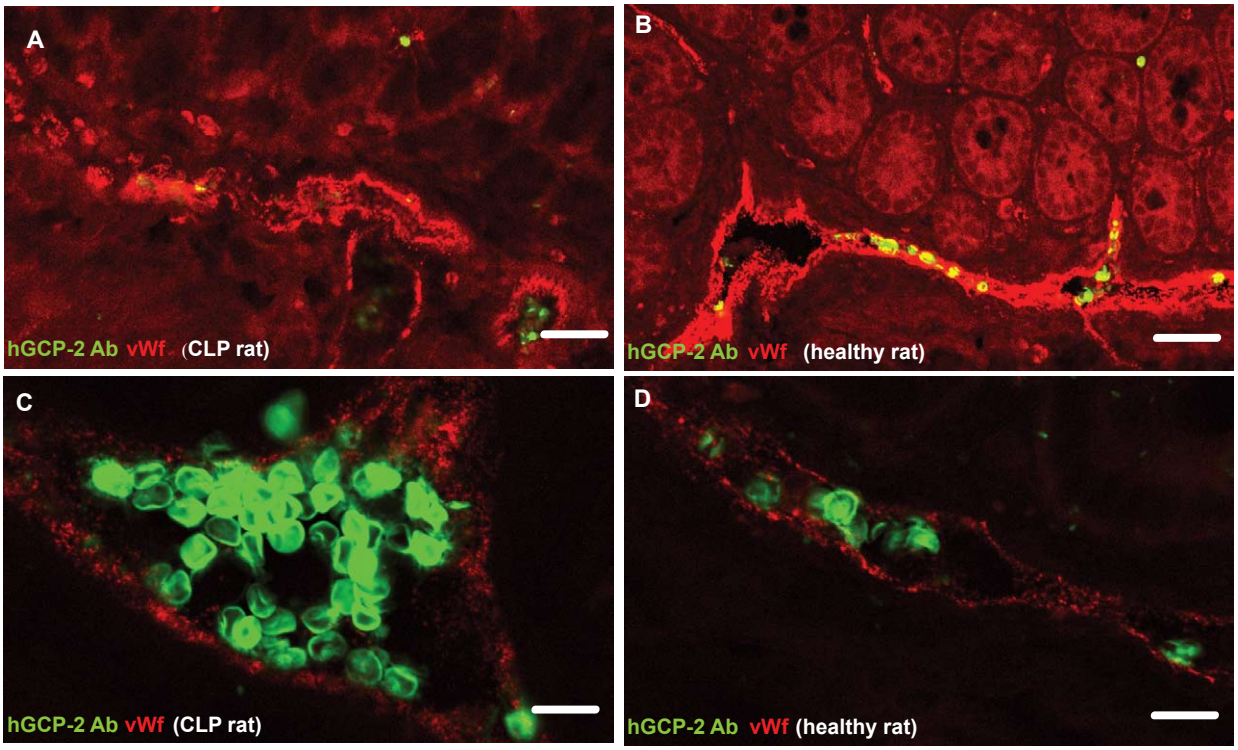

Figure 11 – Immunostaining for CXCL6/GCP-2 in CLP intestine. Two colour immunostainings with anti human GCP-2 (green) and anti-VWF (red), showing positive red blood cells in healthy (A-C) and inflamed (B and D) intestine. Scale bars in A and B = 100 µm, scalebars in C and D = 10 µm

6. Discussion

The capacity of endothelial cells to prestore chemokines in the same compartments as P-selectin has profound implications for the early vascular responsiveness in acute inflammation as in this manner, leukocytes can be rapidly recruited without the delay of *de novo* protein synthesis. In principle, regulated secretion of chemokines has been conceptually and mechanistically demonstrated *in vitro* and the immunohistochemical analysis of human tissue has brought evidence of its relevance *in vivo* [8]. Nevertheless, functional studies of the rapid release of chemokines from the vasculature *in vivo* has yet to be performed. The present study was therefore undertaken to examine whether known ELR^+ chemokines were subject to such sorting in rodents. Such knowledge is the key to design future *in vivo* studies that could assess the relative importance of rapid chemokine release in clinical settings such as ischemia/reperfusion injury and acute allergic reactions.

The relevance of animal models for understanding human chemokines depends in part on the extent to which their structures and functions have been conserved in evolution. Evolutionary relationships among mouse and human ELR^+ chemokines are not straight forward [41]. In mice, only five neutrophil chemoattracting ELR^+ chemokines have been identified; CXCL1/KC, CXCL2/MIP-2, GCP-2/LIX, CXCL7/thymus chemokine-1, and CXCL15/lungkine [42]. Based on available literature KC, MIP-2 and LIX were chosen as the most likely candidates for playing the role in rodent inflammation that IL-8 plays in man [43]

The data obtained in this study indicate that KC and MIP-2 are not sorted in detectable amounts to Weibel-Palade bodies that represent the best characterized compartment of regulated secretion in vascular endothelial cells. The antibody to GCP-2 used in this study was raised against the human homologue and produced an interesting staining pattern that did not fit well with that seen in preliminary experiments using an antibody to the murine homologue GCP-2/LIX (see below). It is therefore possible that we have not detected true murine GCP-2/LIX and a possible

sorting of GCP-2/LIX to WPB has therefore not been formally excluded. It is also possible that either of these chemokines are instead subject to regulated secretion from the murine counterpart of a non-WPB compartment recently characterized in human endothelial cells and shown to contain GRO-α, the human homologue of KC [9]. Unfortunately, these granules have not been sufficiently characterized to allow their specific recognition in tissue sections and the existence of chemokine secretion from such granules can hence not be evaluated in rodents. Nevertheless, it will be interesting to explore whether the granular pattern of MIP-2 observed in mesenteric venules corresponds to the human compartment containing GROα and whether it is responsive to secretagogues like histamine.

Although decisive data regarding regulated chemokine secretion could not be obtained in the course of this study, it nevertheless reveals substantial expression of KC and MIP-2 on blood vessels in two settings of inflammation. The demonstration of KC on vascular endothelial cells has not been demonstrated before, but is well in line with *in vitro* studies [44] and also *in situ* hybridization data of inflammatory lesions [45]. On the other hand, MIP-2 has to the best of our knowledge not been demonstrated on vessels *in vivo*, although *in vitro* data point to their ability to synthesize it. The data therefore needs confirmation in future studies, either by using independent antibodies taking possible differences between mouse and rat into account, or by using *in situ* hybridization for detection of mRNA transcripts.

The relationship between human GCP-2 and murine LIX deserves further discussion: In man, IL-8 and GCP-2 signal through the same chemokine receptors [46, 47] and GCP-2 is nearly as potent as IL-8 in recruiting neutrophils [48]. On these grounds and because murine LIX was a more potent stimulator of mouse neutrophils than KC and MIP-2 [49], it has been assumed that murine LIX might be the strongest candidate to compensate for the lack of IL-8 in rodents. Moreover, murine LIX was considered the homologue of human GCP-2 because they shares more identical amino acid residues than do murine LIX and human ENA-78 [49]. However, Smith *et al*. demonstrated that human GCP-2 and ENA-78 have a very high nucleotide similarity also in non-coding regions, suggesting that they are the result of an evolutionary recent gene

duplication event and that they developed after the phylogenetic branching of rodents and primates from a gene that in rodents later evolved into LIX [41]. The immunostainings with the antibody to human GCP-2 yielded a strong signal from red blood cells. The putative presence of chemokines on red blood cells is no surprise given the known broad chemokine binding affinity of the Duffy antigen receptor of chemokines (DARC) on these cells [50], but as no differences were seen between species or between illness and health. The question was raised if the anti-human GCP-2 reagent is suitable for detecting rodent GCP-2/LIX. To clarify that, experiments with a recently acquired anti-murine LIX reagent was performed after this study was ended. These stainings showed no similarity to the stainings with anti-human GCP-2 reagent and no conclusion can hence be drawn on the basis of this study about the role of LIX in murine inflammation.

The fact that some chemokine genes exist in one species but not others applies in particular to the inflammatory/cluster chemokines. Genes can also be similar in different species but they may not be exact structural or functional equivalents. This may also explain that KC in the mouse might not be exactly the same as GRO-α in human, and that MIP-2 in mice might differ from GRO-β in human. While the discrimination between KC and MIP-2 in murine tissue has been the focus of much attention, it has been difficult to distinguish between the expression pattern of GRO-α and GRO-β in human tissue, because specific antibodies to human GRO-β have not been available.

7. Conclusion

In conclusion, the fact that IL-8 does not yet have a discovered homologue in the mouse may implicate certain limitations when using the mouse models to understand human inflammatory diseases. However, for most studies this does not exclude the mouse as a valid model for human disease. An example of a situation where rodent models appear to be of limited value is the assessment of the clinical relevance of regulated chemokine secretion, since no convincing sorting to WPB was observed in this study. It is nevertheless possible that regulated chemokine secretion in rodents originates from the type II granules described in man and their analysis *in vivo* first requires a better phenotypic characterization. Moreover, animal models using different species could also have been used in this study, and deserves further focus in future work.

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