**Charles University in Prague**

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# Inflammation and cancer in germ-free vs. conventionally reared animals

# Zánět a rakovina v bezmikrobních a konvenčně chovaných zvířatech

*Doctoral thesis*

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# ABSTRACT

Inflammation is considered as one of the main defence mechanisms of the immune system against threats that occur in the body. When present in its acute form, minimal or no detectable subsequent damage of original affected tissue exists. The more pathological form, chronic inflammation, is associated with permanent damage of the tissue and typically a hallmark of various diseases such as ulcerative colitis or colon carcinogenesis. These two pathologies are evolving in the unique colon microenvironment, where intensive interaction between the host cells and bacteria is present. The aim of our study was to investigate the immunological (ELISA, FACS, RT-PCR) and structural (histology, confocal microscopy) changes in the colon mucosa of Wistar-AVN rats induced by dextran sodium sulphate (DSS) to produce colon colitis and by azoxymethane (AOM) to produce colon carcinogenesis. Conventional (CV) and also germ-free (GF) reared animals were used to investigate the effects of the mucosal inflammation activated by the administered inducers as well as the role of colon microbiota - as promoters of a continuous immune activation - in the modulation of immunity and collagen scaffold remodelling. Our results showed that even in the early period after the induction, both inducers produced a smouldering inflammation, able to activate immunological and collagen structural changes in the colon mucosa. This can be indicated as a consequence of variation of what we can call “inflammatory threshold”, representing the limit of tolerance for not dangerous inflammation. Significant differences were found between CV and GF mucosa structure with higher complexity in the CV rats associated to a more activated immune environment. The cytokine production in CV rats was more balanced, regulated to achieve homeostatic levels present in GF rats. This project has achieved the main result attended, i.e., identification of a new marker (collagen scaffold modification) linked to the immunological environment in the colon mucosa, with predominance of IL-6, perspective to be used for very early detection of cancer risk and local immunological pathology.

# ABSTRAKT (CZ)

Zánět je považován za jeden z hlavních obranných mechanismů imunitního systému před hrozbami, které se v těle vyskytují. Pokud jsou přítomny v akutní formě, existuje minimální nebo žádné detekovatelné následné poškození původní postižené tkáně. Patologičtější forma, chronický zánět, je spojena s trvalým poškozením tkáně a je typickým znakem různých onemocnění, jako je ulcerózní kolitida nebo karcinogeneze tlustého střeva. Tyto dvě patologie se vyvíjejí v jedinečném mikroprostředí tlustého střeva, kde je přítomna intenzivní interakce mezi hostitelskými buňkami a bakteriemi. Cílem naší studie bylo vyšetřit imunologické (ELISA, FACS, RT-PCR) a strukturální (histologické, konfokální mikroskopie) změny v sliznici tlustého střeva potkanů Wistar-AVN vyvolané dextranem síranem sodným (DSS) za vzniku kolitidy tlustého střeva a azoxymethanem (AOM) za vzniku karcinogeneze tlustého střeva. Konvenční (CV) a také bezmikrobní (GF) zvířata byla použita k vyšetřování účinků zánětu sliznice vyvolaného podanými induktory a role mikrobioty tlustého střeva - jako promotéru kontinuální imunitní aktivace - při modulaci přestavba imunity a kolagenového lešení. Naše výsledky ukázaly, že i v časném období po indukci vyvolaly oba induktory „doutnající zánět“, schopný aktivovat imunologické a kolagenové strukturální změny ve sliznici tlustého střeva. Lze to označit jako důsledek variace toho, co můžeme nazvat „zánětlivým prahem“, což představuje hranici tolerance pro nebezpečný zánět. Byly nalezeny významné rozdíly mezi strukturou CV a GF sliznice, významnější u CV potkanů spojených s aktivnějším imunitním prostředím. Produkce cytokinů u CV potkanů byla vyváženější, regulovaná k dosažení homeostatických hladin přítomných u GF potkanů. Tento projekt dosáhl hlavního očekávaného výsledku, tj. identifikace nového markeru (modifikace kolagenového lešení) navázaného na imunologické prostředí ve sliznici tlustého střeva, s převahou IL-6, s perspektivním použitím pro velmi včasné odhalení rizika rakoviny a lokální imunologická patologie.

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# LIST OF ABBREVIATIONS

**AOM**-azoxymethane
**APC-**antigen-presenting cells
**APRIL**- a proliferation-inducing ligand
**CD**-cluster of differentiation
**CRC**-colorectal cancer
**CrD**-Crohn´s disease
**CTL**-cytotoxic T cell
**CTLA-4**- cytotoxic T-lymphocyte-associated protein 4
**CV**-conventional
**DAMP-**danger-associated molecular pattern
**DC**-dendritic cell
**DMH**-dimethyl hydrazine
**DSS**-dextran sodium sulphate
**ECM-** extracellular matrix
**ELISA**-enzyme-linked immunosorbent assay
**EP3-**erytropoetin 3
**FACS**-fluorescence-activated cell sorting
**FGF-**fibroblast growth factor
**FPXP3**- forkhead box P3
**GALT**-gut-associated lymphoid tissue
**GF**-germ-free
**GIT**-gastrointestinal tract
**HGF-**hepatocyte growth factor
**HIF-α**-hypoxia-inducible factor α
**HLA-**human lymphocyte antigen
**HSP-**heat shock protein
**IBD**-inflammatory bowel disease
**ICAM-** intercellular adhesion molecule
**IFN-γ**-interferon gamma
**IL**-interleukin
**ILC**-innate lymphoid cell
**KO-**knock out
**LFA-1-** leukocyte function-associated antigen
**LN-**lymp node
**MadCAM-1-** mucosal vascular addressin cell adhesion molecule 1
**MAP-** mitogen-activated protein
**MDSC-**myeloid-derived suppressor cell
**MHC**-major histocompatibility complex
**MLN-**mesenteric lymph node
**MMP-12-**matrix metalloproteinase 12
**MW-**molecular weight
**NET-**neutrophil extracellular net
**NK-**natural killer
**NKT**-natural killer T cell
**NLR**-NOD-like receptor
**NO-**nitric oxide
**PAMP-**pathogen-associated molecular pattern
**PD-ECGF-** platelet derived-endothelial cell growth factor

**PDGF-**platelet-derived growth factor
**PG**-prostaglandin
**PGD2**-prostaglandin D2
**PGE2**-prostaglandin E2
**PGF2α**-prostaglandin F2α
**PGI2-**prostaglandin I2
**PG-PS-** peptidoglycan-polysaccharide
**PhIP**-2-amino-1-methyl-6-phenylimidazol (4,5-b) pyridine
**ROS**-reactive oxygen species
**RT-PCR-**real-time polymerase chain reaction
**S1P-**sphingosine 1-phosphate
**SC-**subcutaneous
**SCID-**severe combined immunodeficiency
**SHG-**second harmonic generations
**SPARK-** separation of phases-based activity reporter of kinase
**TAM-**tumor-associated macrophage
**TGF-β1**-transforming growth factor β1
**Th**-T helper
**TLR-**Toll-like receptor
**TM**-tumor microenvironment
**TNBS-** trinitrobenzene sulfonic acid
**TNF-α**-tumor necrosis factor α
**Treg-**T regulatory
**TSLP-** thymic stromal lymphopoetin
**UC**-ulcerative colitis
**VCAM-** vascular cell adhesion molecule
**VEGF-**vascular endothelial growth factor
**VLA**- very late antigen

# INTRODUCTION

The immune system is composed by the complex of cells, circulating or present in tissues, organs (like spleen, lymph nodes and thymus) and operates through various types of molecules in a network able to intervene and to protect the integrity of the organism from external (e.g. microbes) or internal (e.g. transformed cells) treats. Its activity is classically divided in innate and adaptive immune responses. The first, the most ancient in the phylogenesis, is immediate and aspecific, the second, characteristic of vertebrates, is following the first and addressed to recognize and aggress specific targets, antigens (Bonilla and Oettgen 2010, Boehm and Swann 2014).

Inflammation is important part of the innate immune responses. Recognized since the ancient medicine observations of the reactions to wound or infections, it was clinically described by Celsus (25 B.C.-50 A.C.) in its acute characteristics (Rather 1971): redness (corresponding to vasodilation), local increase of temperature (due to increase of blood flow), swelling (due to vessel permeabilization and plasma accumulation in the perivascular spaces), pain (due to tension in the tissue and stimulation of the nervous terminations by inflammatory products), to which were later added the alteration of function (Virchow, XIX century) (Bagot and Arya 2008). Only more recently the immunological and molecular mechanisms producing this response were progressively enlightened and are still under study for their complexity. The general principle of inflammation includes recruitment of immune cells to the site of tissue damage to permit the limitation and elimination of the treat, the clearing of the damaged tissue and its repair (Medzhitov 2008). The inflammation can be differentiated to acute or chronic form with different pathological, immunological and molecular aspects. The acute form is more common and physiologically self-resolving, becoming pathologic only under special conditions even life-threatening such as septic shock, cytokine storm or allergic reactions (Angus and van der Poll 2013, Karbian, Abutbul et al. 2020). The chronic form is a not resolving inflammation, more dangerous and pathogenic, associated with the permanent tissue damage and remodelling, leading to malfunction of organs, and even cancer promoting factor recognized as a typical hallmark of evolving tumours (Shacter and Weitzman 2002, Hanahan and Weinberg 2011, Zhao, Liang et al. 2016). It is also the common pathological feature of inflammatory bowel diseases (IBDs), which are characterised by permanent inflammation of intestinal tissue inducing mucosal damage and tissue remodelling toward fibrosis and even cancer (Zhang and Li 2014, Latella, Di Gregorio et al. 2015, Axelrad, Lichtiger et al. 2016). The tissue remodelling

is an example how immunological changes in organs can be very often mirrored into physiological and morphological changes of tissues. In the case of chronic inflammation, very typical phenomenon is the progression toward fibrosis of tissues, caused by upregulated production of collagen fibres by activated fibroblasts. The increased collagen production can be found also in various tumours (e.g., pancreas, breast cancers) (Chandler, Liu et al. 2019, Thomas and Radhakrishnan 2019, Dey, Varelas et al. 2020).

In consideration of the effects that differently activated immunity can have also on the structural organization of a tissue, we decided to evaluate this relationship in experimental animals and to find the evidence of its importance both in tumoral and non–tumoral conditions.

The main goal of our project was to study on rodent animal models the aspects of inflammation in conditions mimicking IBDs or cancer in human patients and also to see the effect of the continuously activated immunity in the colon mucosa of conventional (CV) animals versus germ-free (GF) animals. This last model was taken as a direct evidence of the importance of chronic inflammation, at various degrees, on the tissue structure organization and modelling. We used dextran sodium sulphate (DSS) to produce the IBD model and azoxymethane (AOM) to induce carcinogenesis model. We evaluated microscopic and immunological changes in the colon mucosa as well as immunological changes in the spleen and mesenteric lymph nodes. The colon was chosen as an ideal model for immunity-structure relationship because one of the most important immunological organs, continuously activated and regulated by its own microbiota, and common place for development of colitis as well as of cancer. Since the possibility to follow the evolution of both illnesses in the chosen models, we particularly focused on the early period post-induction, preliminary to the establishment of the definitive illnesses. It is a not yet evaluated period despite its importance to clarify the conditions preparing evolution of the pathological environment.

We found that even in early period very significant associations between molecular changes in the local immunity of the colon and its morphology are already present, sustained by a “smouldering” inflammation. The prevalence of inflammatory molecules, even though in a downregulated immune environment, associated to clearly measurable alterations of the collagen scaffold in the colon mucosa suggests that immunity plays an important role in modulation of colon tissue structure. An increased production of pro-inflammatory cytokine IL-6 was found one month after the end of DSS treatment, accompanied by decreased

production of interleukin-1α (IL-1α), transforming growth factor β1 (TGF-β1) and, partially, interferon-γ (IFN-γ). The expression of *Il1a*, *Il1b*, *Ifng* and *Tgfb1* genes was decreased after both treatments. Colon tissue structure was significantly more irregular after DSS and AOM, with intensively increased collagen production and alteration of the mucosa symmetry. For the first time, we showed that changes in the colon tissue architecture can be observed even in the early periods after the treatment, i.e., initial evolution of the illnesses, which brings possibility to their use as a new diagnostic marker for recognizing IBD or colon cancer prone patients.

Our experiments further focused on the modulation of local immunity and colon tissue structure in healthy rats under either CV, with microbiome, or (GF), without microbiome, conditions. Very significant differences between CV and GF rats were observed at macroscopic, microscopic and molecular levels, confirming important role of the continuous challenge and regulated immune activation induced by the intestinal microbiota, locally and in the body. Production of cytokines was lower in GF rats than in CV ones, except of IL-6. The expression of all cytokine genes was lower in GF rats. Very interesting differences were found by confocal microscopy. The collagen structure of GF rat colon mucosa was more irregular with higher distances between centres of the glands and with lower integrated density of fibres. When evaluating immune cell distribution, all studied tissues (peripheral blood, spleen, mesenteric lymph nodes) showed higher population of natural killer (NK) cells in GF rats. All data indicated that the condition of continuously activated immunity induced by the commensal microflora in the colon mucosa was able to shape the structure of the mucosal scaffold, confirming its plasticity and adaptation to the immune stimulations.

Additionally, very preliminary experiments were also designed, trying to evaluate the immuno-biological and structural changes in the colon mucosa of CV and GF rats in the early period after the DSS-induced colitis.

The final preliminary project included the study of cytokine production and structural changes of collagen scaffold in the colon of CRC patients before the chemotherapy or radiotherapy treatment in the frame of a collaboration with Dr. Pavel Vodička and his team at the Institute of Experimental Medicine of the CAS, v.v.i. to associate a genetic approach to the CRC-promoting factors, in the view of a comparison between results in the human samples and in the experimental animal models.

# 1. LITERATURE OVERVIEW

## 1.1 Generalities of immunity

The general role of immunity is to defend the organism against microbes and other non-self agents to preserve the organism integrity and homeostasis. It is the complex of various cells and molecules involved in this defence constitutes the immune system. Its importance for body is evident in individuals with defective immune system who develop infections even from commonly non-pathogenic microorganisms, often with life-threatening conditions (De Silva, Gunawardena et al. 2007, Chinen and Shearer 2010). However, the immune reactions can also have adverse impacts on healthy tissues during processes such as inflammation or when abnormal activation of immunity is stimulating the development of autoimmune diseases or allergies (Ferguson 2010, Aksu, Donmez et al. 2012). During the tumour development, the immunity can also generate contrasting responses, depending on the phase of tumour evolution (Pawelec, Derhovanessian et al. 2010, Thorsson, Gibbs et al. 2018).

The immune responses are divided mainly to innate (native) and adaptive immunity. Both types of responses have some common and specific mechanisms by which they recognise and eliminate threats to prevent the damage of the organism.

The cells of the immune system can be generally divided to either resident or circulating, which can be attracted to a damaged tissue by chemotactic gradient when danger signals are arising. Resident immune cells (macrophages, dendritic cells, mast cells) recognise the dangerous agents (e.g., microbes, transformed cells), kill and ingest them and present antigens to other immune cells of adaptive immunity, the B and T lymphocytes. These cells circulate in the body through lymphoid organs to peripheral tissues and, once primed, they produce antibodies or directly kill infected cells. Finally, effector cells (neutrophils, monocytes, T lymphocytes) are present at the site of infection and actively try to eliminate infectious agents, infected or transformed cells. The reciprocal communication between these groups of cells is necessary for successful immune response. The overview of innate and adaptive immune cells is show in **Fig. 1**.

Macrophages are long-living cells present in all connective tissues and organs of the body, able to present antigens to adaptive immune cells. To become activated, macrophages need to receive more activating signals from Toll-like receptors (TLRs), NOD-like receptors (NLRs), mannose, scavenger and complement receptors. Two distinct pathways of

macrophage activation exist: 1) classical through TLRs and by IFN-γ activates M1 macrophages that are involved in the inflammation and destroying microbes; 2) alternative in the absence of TLRs but with interleukin-4 (IL-4) and IL-13 contribution, produce M2 macrophages which play repair functions and inhibit inflammation (Sica and Mantovani 2012). These M2 are also common in the tumour microenvironment and enhance tumour growth by supressing other immune cells.

Another group of (APCs) are the dendritic cells (DCs), which very effectively present antigens to T lymphocytes and produce cytokines for their activation and differentiation from naïve to effector T cells. The presentation of the antigen linked to the major histocompatibility complex (MHC) molecules to T lymphocytes is in the peripheral lymph nodes. Very specific functions play DCs in the immunity of colon mucosa, where in the colon lumen they recognise the antigens originating from commensal bacteria, normally developing intensive immunological tolerance, which turns to immune response in the case of infection by pathological bacteria (Esterhazy, Loschko et al. 2016). The all described activities show the integration between innate and adaptive immune system.

Mast cells are evolving in the bone marrow and are localised in the skin and mucosal epithelia. When activated, they release many potent inflammatory mediators that defend mainly against parasite infections, or cause symptoms of allergic diseases.

Other very important cells of innate immunity are granulocytes (neutrophils, eosinophils, basophils), which play different functions in the body. Neutrophils are the most abundant cells of innate immunity, circulating in the blood cells and involved mainly in the acute inflammatory reactions. The major function of neutrophils is to phagocytose microbes and products of necrotic cells. Moreover, neutrophils produce antimicrobial substances that kill extracellular microbes. Basophils constitute less than 1% of blood leukocytes and they are normally not present in tissues. They are recruited to the inflammatory sites, express IgE receptors, bind IgE on infected cells and play similar functions as mast cells, mainly in allergic reactions. Eosinophils express cytoplasmic granules containing enzymes that are harmful to the cell walls of parasites, these cells circulate in the blood, from which they can migrate to the site of infection. Some eosinophils are also present in peripheral tissues, especially in mucosal linings of the respiratory, gastrointestinal (GIT), and genitourinary tracts, and their numbers can increase by recruitment from the blood in the setting of inflammation.

NK cells are specific components of innate immunity (approx. 10 %), they recognise infected or damaged cells and respond directly killing these cells. Additionally, they produce

IFN-γ as major activator of macrophages (Dungan, McGuinness et al. 2014). Many cytoplasmic granules are present inside of NK cells, they lack antigen or T cell receptors but express activating and inhibitory receptors on their surface. The resulting response of NK cells to host cells that lack class MHC I is determined by a balance between signals from these two types of receptors.

The innate immunity uses also soluble proteins defensis, cathelicidins, pentraxins and complement system to kill the infected cells. The complement system involves around 60 soluble plasma proteins that work together to opsonize microbes, to promote the recruitment of phagocytes to the site of infection, and in some cases, to directly kill the microbes. Complement proteins are activated through three distinguished pathways: classical, alternative and lectin pathway. Recognition of microbes by any of these pathways lead to formation of protease complexes killing the infected cells.

Very special group of innate immune cells represent NK T lymphocytes (NKT) and γδ T lymphocytes that are sharing the characteristics of innate and adaptive immune cells. In general, they kill the infected cells by innate immune mechanisms but express on their surface molecules that are typical for the adaptive immunity. Because NKT cells can recognise lipids on the surface of microbes, they are mainly involved in defence against mycobacteria (Pfeffer, Schoel et al. 1991). γδ T cells recognize many different types of antigens, including some proteins and lipids, phosphorylated molecules and amines and they are involved mainly in the response to antigens present on epithelial tissues.

The last group of immune cells, exclusively involved in the adaptive immunity are the lymphocytes. More information about these cells is discussed in Chapter 1.1.2.

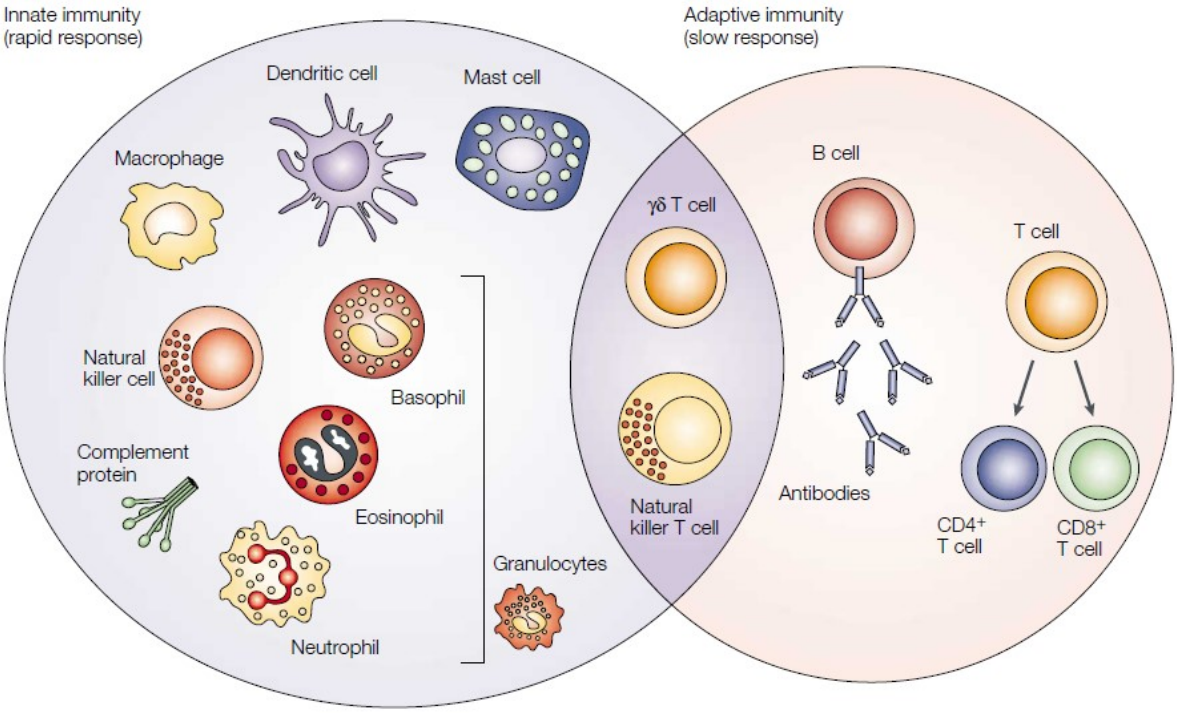


Figure 1: **Schematic overview of immune cells involved in innate and adaptive immunity.** The cells of innate immunity include granulocytes, macrophages, dendritic cells, mast cells and NK cells, completed by soluble proteins such as complement. NK T cells and γδ T cells are on the edge between innate and adaptive immune responses. These cells share the characteristics of both innate and adaptive immune cells. Adaptive immunity is exploited exclusively by lymphocytes, divided in antibody-producing B cells and T cells that can directly kill transformed or infected cells (CD8+) or play supportive functions for B and CD8+ T cells (CD4+ T helper cells). Adapted from (Dranoff 2004).

### 1.1.1 Innate immunity

Innate immunity is ancient and less elaborated system that immediately recognises and eliminates pathogens or foreign agents. The first line of defence against microbes are tissue barriers, such as skin or mucous membranes. In case microbes succeed to break these barriers and enter the body, innate immune responses are activated, cells called phagocytes and NK cells are recognising microbes and try to eliminate them. Another part of innate responses includes production of antimicrobial peptides and several plasma proteins such as complement system molecules, which recognise and react against the microbes. The innate and adaptive immunity are not separated but connected each other, the innate immune responses to danger signals stimulate adaptive immune responses. Conversely, adaptive immune responses are enhancing the protective mechanisms of innate immunity, making them more capable of effectively eliminating the source of danger. The innate system has no

capacities to recognise specific antigens, instead its cells and molecules recognise general molecular patterns using restricted defence mechanisms against them.

### 1.1.2 Adaptive immunity

Adaptive immunity is considered as more evolved, sophisticated system able to specifically recognise and target unlimited number of various antigens, typically present in vertebrates only. In comparison with the innate immunity, the reaction of adaptive immunity to threats takes more time to develop, needing the presentation of antigens by specialized cells. Antigens become available during the activity of innate immunity response as illustrated in **Fig. 2**, showing the immune response against the viral infection. Adaptive immunity is specific and effectively activated against concrete targets. Effector cells of adaptive immunity are T and B lymphocytes, which after priming by a specific antigen expand, creating two different subpopulations, one immediately active and the other creating a colony of memory cells ready to be reactivated in the case of repeating contact with the threat. The B lymphocytes are producing the antibodies (humoral immunity), while T lymphocytes (cell-mediated immunity) directly contact and kill the target (cytotoxicity).

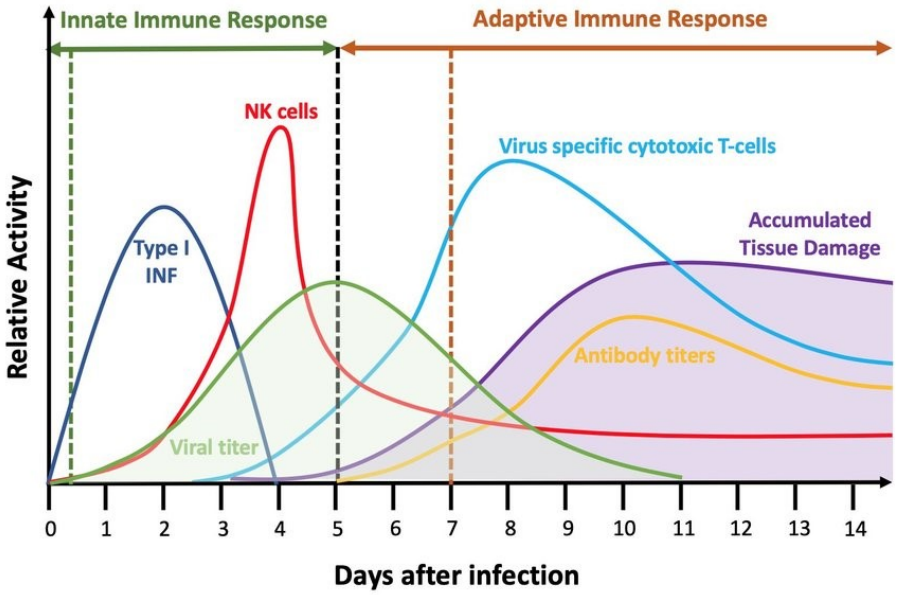


Figure 2: **Development of innate and adaptive response to viral infection.** The immune response to viral infection firstly activating the innate immunity with the involvement of type I interferons and significant proliferation of NK cells. Later, the adaptive immunity is activated, cytotoxic T cells are proliferating, and B cells are producing specific antibodies against virus. The titre of virus is progressively decreasing, as well the damaged tissue is repaired. This figure illustrates the timing of immune response to infection and the cooperation between innate and adaptive immunity in general. Adapted from (Sego, Aponte-Serrano et al. 2020).

While B lymphocytes recognise antigens by the surface immunoglobulin receptors, T lymphocytes bind to antigens presented by MHC (major histocompatibility complex) receptors on the surface of APCs by the T cell receptor complex (**Fig. 3**). However, B lymphocytes are also able to process antigens and present them through MHC II molecules to CD4+ T helper lymphocytes (Th). Cytotoxic T lymphocytes (CTLs) exert the cytotoxic activity by creating the immunological synapses with the targeted cells through which perforin and granzyme B molecules are released and killing the targeted cells. Th cells are on the other hand necessary for sustaining of the proper function of cytotoxic T and B lymphocytes by specific cytokine release (IL-2, IL-4, IL-5, IL-17) and can be divided into six subgroups, including Th1, Th2, Th9, Th17, Th22 and T follicular helper cells according to either specific type of response (Th1, Th2) or a specific cytokine production, e. g. Th17 releasing IL-17 (Cosmi, Maggi et al. 2014). Th1 are involved in pro-inflammatory responses to agents, mediated mainly by IFN-γ and activating CD8+ T lymphocytes. Th2 are activated as a downregulating and tissue-remodelling function in the inflamed tissue and are sustaining B lymphocytes and the humoral immune responses (antibodies). Th17 cells are involved in the host defences against extracellular pathogens and T follicular helper cells are localised in the germinal centres of secondary lymphoid organs, where they mediate the selection and survival of B cells capable of producing high-affinity antibodies (Crotty 2019). A specific subtype of CD4+ cells are the regulatory T lymphocytes (Tregs) that have regulatory functions in the immune responses. Tregs are able to inhibit T cell proliferation and cytokine production and play also critical roles in preventing autoimmunity. This particular type of CD4+ cells can exert either protective functions or impeding the immune reaction against tumours. They are attracted to tumour microenvironment by chemokine gradient, producing variety of molecules involved in suppression mechanisms, including CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), IL-2, IL-10, IL-35 and TGF-β1. The infiltration of Treg into tumour microenvironment is associated with poor survival in various types of cancer (Sakaguchi, Yamaguchi et al. 2008, Ohue and Nishikawa 2019).

Lymphocytes exit from a lymph node via the cortical sinuses, which lead into the medullary sinus and then the efferent lymphatic vessel. This process is driven by lipid molecule sphingosine 1-phosphate (S1P), which has chemotactic activity and signalling properties similar to those of chemokines. The subsequent migration of effector lymphocytes to peripheral tissues requires the activation of changes in the expression of surface molecules. For example, T lymphocytes decrease the expression of L-selectin and CCR7 on one hand but increase the expression of integrins and E-selectin and P-selectin ligands on

the other hand. These molecules mediate cell binding to endothelium at peripheral inflammatory sites. Effector lymphocytes also express receptors for chemokines that are produced in infected peripheral tissues, e. g. CXCR3 and CCR5 in Th1 cells, Th2 cells express CCR3, CCR4, and CCR8 in Th2 cells (Kim and Broxmeyer 1999).

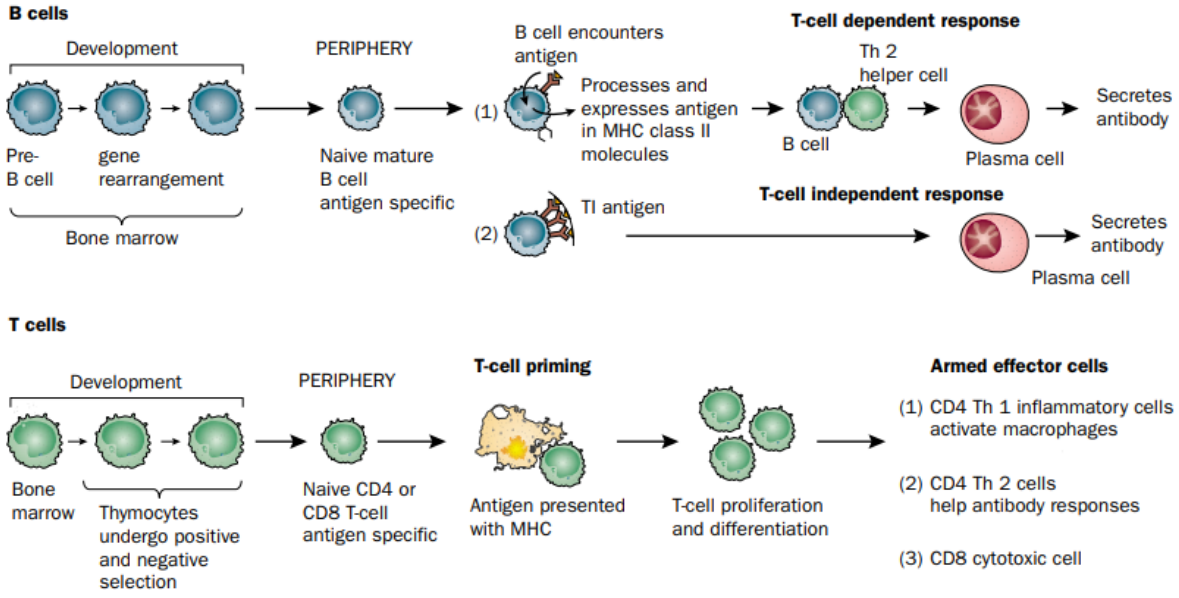


Figure 3: **The roles of B and T lymphocytes in adaptive immunity.** Adapted from (Parkin and Cohen 2001)

Cutaneous and mucosal structures (e. g. gastrointestinal and respiratory tracts) are other very specific sites of immune cell localisation (lymphocytes, APCs) and immune recognition of agents. The immune cells are present either diffusely beneath the epithelia or organised in more complex structures like tonsils in the respiratory tract and Peyer´s patches in the GIT or in intramucosal follicles in the colon (**Fig. 4**). Cutaneous and mucosal structures can be considered as the biggest immune organ in the body because of around 25 % of lymphocytes are concentrated here.

The other cells and mechanism of immune reactions to threats will be discussed in the next chapters. Cytokines represent a special category of proteins mediating the immune reactions in the body, functions of immune cells and communication between them. Cytokines are divided into several groups: type 1 cytokines such as interleukins (IL), type 2 cytokines such as interferons (IFN), tumor necrosis factor superfamily including tumor necrosis factor α (TNF-α), interleukin-1 family including IL-1α, IL-1β, IL-18, IL-33 and

other cytokines such as TGF-β and chemokines (Kelso 1998, Kelso 2000, Cameron and Kelvin 2003). The production and monitoring of levels of cytokines can be measured by various methods, enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (RT-PCR), fluorescence-activated cell sorting (FACS) and can be used both in the clinical and experimental practice to monitor the physiological and pathological conditions. In our studies, we evaluated the variation of pro-inflammatory or regulatory cytokines in induced colitis and colorectal carcinogenesis in experimental animals as well as in the comparison between GF and CV (with microbiota) animals.

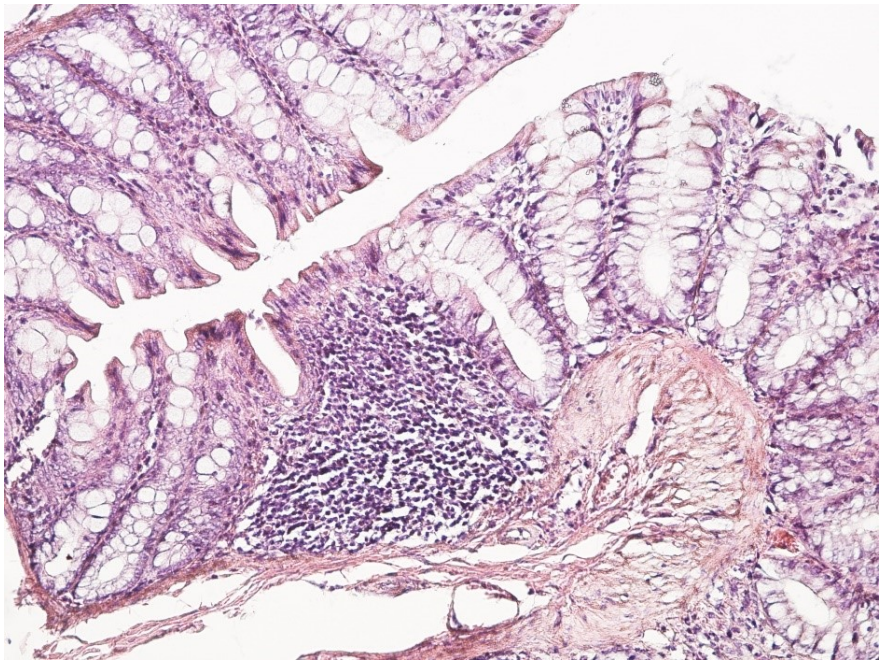

Figure 4: **The representative histological visualisation of lymphoid follicle localised in the rat colon mucosa.** Method: Haematoxylin-Eosin staining, original magnification: 20x

## 1.2 Colon as a model for immunological study of inflammation and tumour development

### 1.2.1 Anatomy of the colon

Colon represents a unique structure suitable for studying the immune processes in the body. IBD and cancer can be easily induced in the colon of experimental animals and resemble the ulcerative colitis and CRC in human. Since these two important pathologies arise in the same organ and share inflammation as one of the important factors that characterise their development, colon is an ideal system for evaluating in experimental animals and in the same organ the importance of inflammation and its effects on the involved structures. Thanking to availability of GF animals, it is also possible to evaluate the role of microbiota in the modulation of local immunity and structure of colon under different conditions.

The colon is the last part of GIT, conventionally divided to caecum, ascending or right colon, transversal part, descending or left colon, sigmoid and rectum. From the small bowel, colon receives digested food, from which it absorbs water and electrolytes to form faeces. The colon is intensively vascularized, and it is the place of sophisticated immune system that maintains the homeostasis with microbiota resident there. The colon wall is made up of four layers of specialised tissue, stratified from the lumen outward following this sequence: mucosa, submucosa, muscular layer and serosa (peritoneum) (**Fig. 5**). Immune processes are present mainly in the mucosa part, which can be divided to other three layers: 1) the epithelium is the innermost layer where most digestive, absorptive and secretory processes occur; 2) the lamina propria, a layer of connective tissue within the mucosa; 3) the muscularis mucosae, a thin layer of smooth muscle. The submucosa consists of a dense layer of connective tissue with the blood vessels, lymphatics, and nerves branching into the mucosa and muscular layer. The muscular layer also known as the muscularis propria consists of two layers of muscle, important for the peristalsis. The last layer of colon wall, serosa, consists of several layers of connective tissue, covered by a simple squamous epithelium called the mesothelium, which reduces frictional forces during digestive movements. Muscular and vessel structures are controlled by parasympathetic nervous system (vagus nerve). According to some studied, vagus nerve activity is also associated with the modulation of the inflammation (Borovikova, Ivanova et al. 2000). (http://www.histology.leeds.ac.uk/digestive/GI_layers.php)

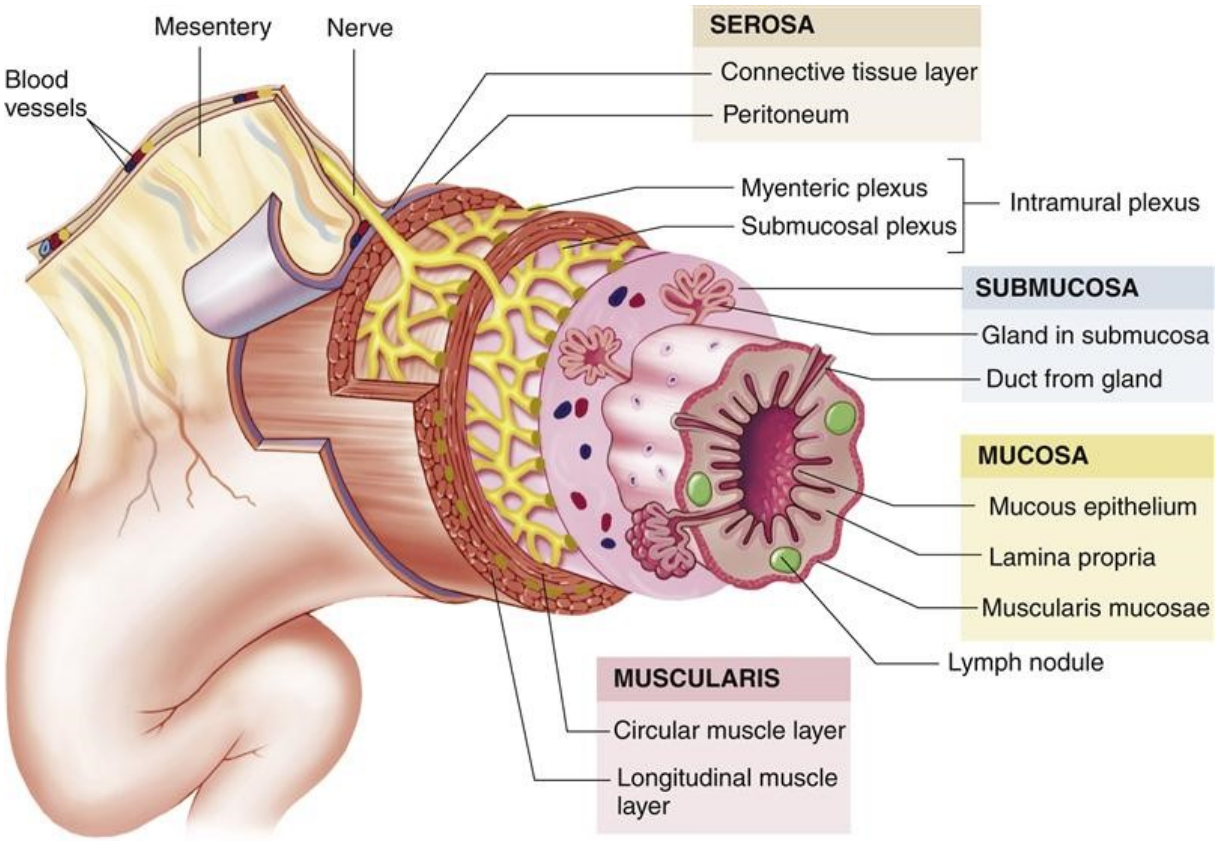


Figure 5: **The anatomy of colon wall.**

Adapted from https://basicmedicalkey.com/gastrointestinal-function/

### 1.2.2 Mucosa as an immunological organ

The GIT itself has an enormous surface and can be considered as the biggest immune system in the body. The continuous presence of bacteria that in human counts about 10-times higher than the body cells and about 30-times higher number of genes than in human body, forms the universe that can influence its own host, including the immune system. The bacterial and food antigens permanently activate the innate and adaptive immune responses in the mucosa that, on the other hand, need to be regulated to avoid severe damage of own GIT tissues. The study of GIT-associated immune processes brings an opportunity to understand the complexity of immunity. The basic mechanism that protects the body against infections from intestinal lumen include: 1) presence of thick layer of mucus in the intestinal lumen; 2) the production of antimicrobial peptides by epithelial cells; 3) the massive

production of IgA antibodies by plasma cells resident in the lamina propria layer below the epithelial cells. The production of IgA counts for about 20 % of all antibodies produced in the body (Gill, Wlodarska et al. 2011).

The innate immunity of the GIT involves even the cells of intestinal wall. The goblet cells producing the mucus, the M cells capturing antigens and Paneth cells producing antimicrobial peptides intensively cooperate with other cells of innate immunity localised beneath the mucosal epithelium (Kurashima and Kiyono 2017). The macrophages and dendritic cells evolved specific functions in the intestinal tract. They are able to recognise the antigens, but the expression of inflammatory genes is not activated, and these cells produce high amounts of IL-10 and TGF-β1 as immunosuppressive cytokines (Ueda, Kayama et al. 2010, Worthington, Czajkowska et al. 2011, Farache, Zigmond et al. 2013). Also other types of immune cells are present in the intestinal mucosa like innate lymphoid cells (ILCs), NKT cells and γδT cells (Ciccia, Guggino et al. 2015, Shiromizu and Jancic 2018, Brailey, Lebrusant-Fernandez et al. 2020). Among them, ILC3s respond to IL-1β and IL-22 by increased production of IL-17 (Ciccia, Guggino et al. 2015). The special type of innate immune cells in the intestines represent mucosal-associated invariant T cells, localised mainly in the liver thus protecting the body against bacteria and fungi that escaped from the intestines to the blood stream (Jeffery, van Wilgenburg et al. 2016).

The adaptive immunity of the gut-associated lymphoid tissue (GALT) is specific and differs from the adaptive immune mechanisms in other organs. The immune responses are based on one hand on humoral immunity through IgA production (Macpherson and Uhr 2004). On the other hand, on cell-mediated responses based on Th2 cells (McCoy, Ignacio et al. 2018). All immune responses against bacteria and food antigens are strictly regulated by IL-10-producing Treg cells, which are more abundant in GALT than in other immunological organs (Sakaguchi, Sakaguchi et al. 1995, Mottet, Uhlig et al. 2003). The adaptive immune system in GALT is based on the lymphocytes and APCs spread beneath the mucosal epithelial layer. These immune cells also accumulate in Peyer´s patches that, especially localised in the distal ileum. The homing of T and IgA-producing B lymphocytes is based on expression of specific surface receptors ($\alpha_4\beta_7$ integrin) and mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) on the endothelial cells (Erle, Briskin et al. 1994). The intestinal epithelial cells produce inactive TGF-β1 and DCs release $\alpha_v\beta_8$ integrin that activate TGF-β1 (Travis, Reizis et al. 2007, Fenton, Kelly et al. 2017). This cytokine together with a proliferation-inducing ligand (APRIL) and thymic stromal lymphopoetin (TSLP) enhance the isotype switching in B lymphocytes. The immune

processes in the Peyer´s patches are similar to those in LNs except of the formation of germinal centres and the delivery of antigens to B lymphocytes is direct (Craig and Cebra 1971, Reboldi and Cyster 2016). A special type of the intestinal epithelial cells called M cells enhance the uptake of the antigens from the intestinal lumen (Corr, Gahan et al. 2008). The various substances from the lumen are transported to APCs beneath the epithelial layer by phagocytosis or clathrin-coated vesicles (transcytosis) (Rios, Wood et al. 2016).

The GALT adaptive immunity is based on the same principles as that in other tissues except of one difference in T helper cells. Within the T cells, Th17 lymphocytes result more abundant especially in the small bowel, in relation to colonization of special type of filamentous bacteria in the postnatal period (Atarashi, Tanoue et al. 2010). By their cytokine production they contribute to the antimicrobial immunity in the gut stimulating β-defensin release by the epithelial cells (Liang, Tan et al. 2006). Th2 cells are able to stimulate the mucus production and increase the peristalsis by IL-4 and IL-13 release in the case of parasitosis (Svetic, Madden et al. 1993). These are two examples of adaptive immunity cells that indirectly activate non-immunological defence mechanisms, underlining the complexity of immune-biological network of the bowel mucosa. Th1 cells are not very abundant in the gut but expand in pathologies such as IBD, where the control of inflammation is deregulated (Niessner and Volk 1995).

The immunity in the gut must be very precisely regulated to avoid the development of damaging inflammation. This regulation is based on the presence of abundant Treg cells that produce immunosuppressive cytokines IL-10 and TGF-β1. More studies showed that deficient production of these cytokines is associated with severe inflammation in the bowel (Ebert, Panja et al. 2009, Chen, Berin et al. 2021). Ebert found that about one-third of IBD patients may have a relative deficiency of TGF-beta, IL-2 or IL-10 due to an increase in neutralizing antibodies in their sera. Chen recently published that epicutaneous T cell inoculation-based immunotherapy in IBD patients can induce Treg cell enrichment, resulting the decreased colonic inflammatory cytokine production and histological colitis due to IL-10 and TGF-β1 released from Tregs.

Another specific factor that very significantly influence the immunity in the gut is microbiota itself. More recent studies showed that practically each individual has unique composition of the bowel microbiota which depends on many factors, type of delivery, diet, travels and changes caused by diseases or therapies (Tojo, Suarez et al. 2014, Bibbo, Ianiro et al. 2016). Also aging is considered to be associated with the changes in the bowel microbiota (An, Wilms et al. 2018, Bana and Cabreiro 2019). Some studies showed that

common pathologies as asthma or allergies may be induced by dysbiosis of commensal bacteria in the gut (Russell, Gold et al. 2012, Aitoro, Paparo et al. 2017, Hong, Kim et al. 2017, Huang, Wang et al. 2018). The full understanding of the bacterial functions in the balance of bowel microbiome may help to address patient-tailored treatments for controlling diseases by recovering the bowel homeostasis. The schematic overview of bowel-associated immunity is showed in **Fig. 6**.

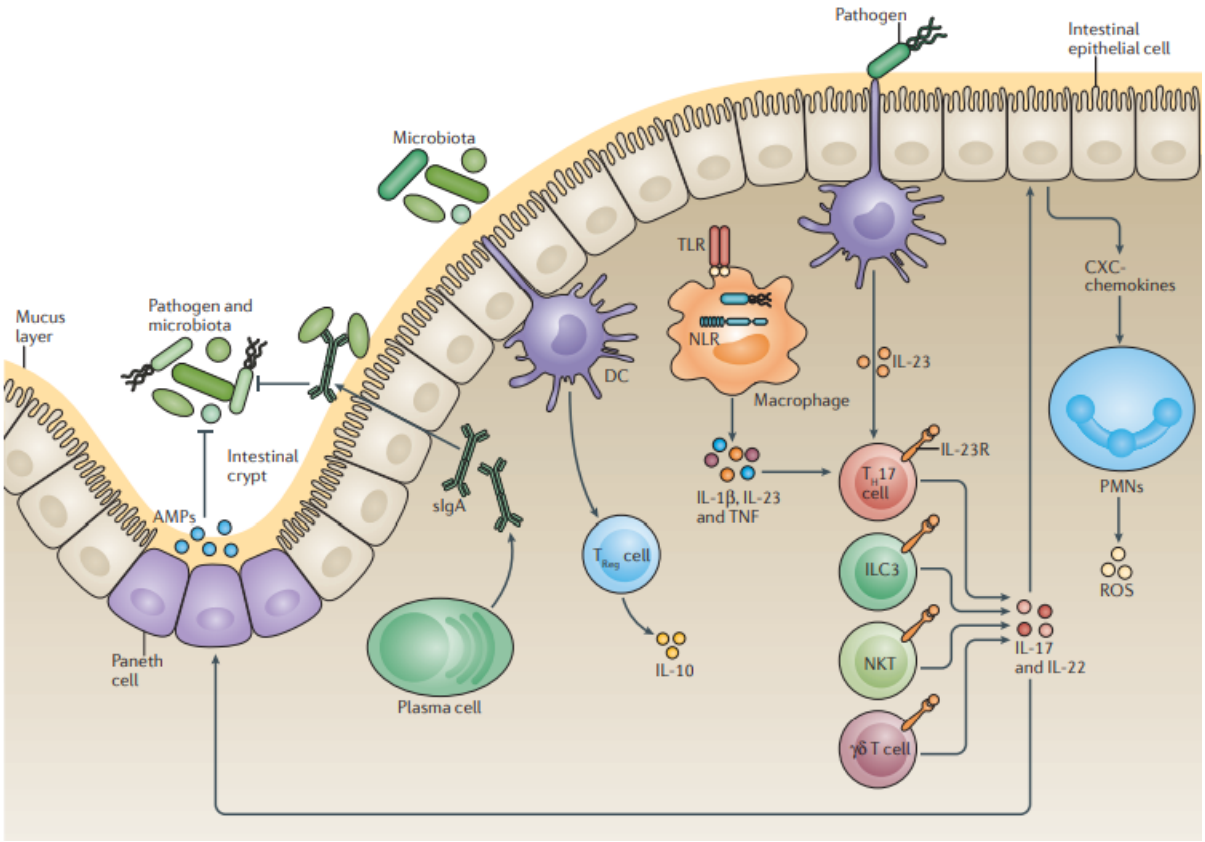


Figure 6: **The schematic diagram of mucosal immunity to commensal and pathogenic bacteria.** This scheme shows the main processes of mucosal immunity against the bacteria present in this compartment. DCs sample intestinal microorganisms, then induce a tolerogenic response by activating Treg cells to secrete IL-10. In case of infection by pathological bacteria, resident macrophages and DCs are activated and secrete IL-23, which stimulates several subsets of T cells (Th17, γδ T cells, NK cells, NKT cells and group 3 ILCs to secrete IL-17 and IL-22. These cells then promote amplification of the host response by stimulating the intestinal epithelium to secrete CXC-chemokines that attract neutrophils. IL-17 and IL-22 induce also the production of antimicrobial peptides (AMPs) which modulate the microbial composition of the intestinal lumen. Plasma cells control the microbiota and pathogens via secretory IgA (sIgA).
IL-23R, IL-23 receptor; NLR, NOD-like receptor; PMNs, polymorphonuclear cells; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF, tumour necrosis factor Adapted from (Perez-Lopez, Behnsen et al. 2016).

## 1.3 Inflammation

Inflammation is the initial reaction of living tissue to infection, wound or other injuries as well as to tumour cells. It is a multistep process including various immune cells and protein or non-protein mediators which aim is to eliminate the source of injury, clear out the necrotic cells and restore the homeostasis of the damaged tissue. In general, the inflammatory reaction has three stages: initiation, progression and resolution (Medzhitov 2010).

1) Initiation - chemokines such as histamine or thrombin are released from injured cells. These mediators induce the expression of specific adhesion molecules on the leukocytes circulating in the blood stream and on the surface of endothelial cells, further driving the evolution of inflammatory response (Lorant, Patel et al. 1991).
2) Progression – neutrophils, the first immune cells recruited to the inflammatory site through chemokine gradient, firmly bind to adhesion molecules on the endothelial cells, squeeze through loosened junctions between endothelial cells by the process of diapedesis, migrate to the inflamed tissue and destroy the non-self agents by phagocytosis. This phase of inflammatory response includes also other immune cells – circulating monocytes and eosinophils and resident tissue mast cells (Sullivan 1979, Wintroub and Soter 1981). Monocytes migrate to the site of tissue injury by chemotactic factors and differentiate into macrophages, which are the main source of growth factors and cytokines regulating the epithelial, endothelial and immune cells (Geissmann, Manz et al. 2010). The inflammatory process progresses by activation of resident macrophages which release pro-inflammatory cytokines IL-1β and TNF-α. These stimulate macrophages itself to release other chemokines, which attract more neutrophils to inflamed tissue (Cavaillon 1994). To maintain the chemokine gradient towards the inflamed tissue, chemokines bind to extracellular matrix proteins such as heparan sulphate that serves as scaffold (Schenauer, Yu et al. 2007). Lymphocytes are normally involved only in chronic inflammatory responses.
3) Resolution - the last phase of inflammatory response. The complement proteins are eliminated by various inhibitors, proteases released from neutrophils and

macrophages are degraded by protease inhibitors. The resolution phase as final part of inflammatory response is regulated mainly by TGF-β1, stimulating fibroblast activation and production of extracellular matrix components such as collagen (Ignotz and Massague 1986). The resolution of inflammation is very important to protect the tissue against permanent damage which occurs in the case of chronic inflammation which will be discussed in chapter 1.3.3.

### 1.3.1 Acute inflammation

Acute inflammation is a quickly established process involving the innate immunity responding to the damage of the tissues produced by biological or non-biological agents (chemical, physical). Main players of inflammatory reactions are on one hand neutrophils and macrophages, on the other hand pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. These cytokines can modify the local microenvironment (e. g. inducing the adhesion molecules and chemotactic signals recruiting granulocytes to damaged tissues) and also to induce fever, one of the basic mechanisms killing microbes, through their action on hypothalamus (Dinarello, Cannon et al. 1986, Dinarello, Cannon et al. 1991). Acute inflammation includes initiation, progression and resolution. In general, inflammatory response include inducers, sensors, mediators and effectors. The inducers can be divided to exogenous or to endogenous originating from the components of own organism. Tissue healing as the final phase of acute inflammation includes these processes: degradation of inducers, clearing of mediators and immune cells, replacement of injured cells and restoring the normal function of injured tissue (Germolec, Shipkowski et al. 2018, Varela, Mogildea et al. 2018).

### 1.3.2 Cells, mediators and mechanisms of activation and remission of acute inflammation

The activation, evolution and remission of acute inflammation is a multi-step process including the interplay of many cell types and molecules (**Fig. 7**). First, microbes or damaged tissues stimulate through pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) the resident immune cells like macrophages and dendritic cells that release pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and chemokines (CCL2, CXCL8), eliciting the expression of adhesion molecules intercellular

adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) on the surface of endothelial cells to permit the adhesion, rolling and translocation of other immune cells (mainly neutrophils) to the site of inflammation and injury (Osborn, Hession et al. 1989, Medzhitov 2010, Chigaev and Sklar 2012). The expression of integrins leukocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4) on rolling cells is further sustained by chemotactic cytokines such as CXCL8 and CCL2 that are released in the subendothelial space (Gerlza, Nagele et al. 2021). The release of pro-inflammatory cytokines and other factors of inflammation like kinins, leukotrienes, prostaglandins or serotonin, produced by immune cells or by the involved tissue itself, induce the vasodilatation and leakage from vessels with the possibility of extravasation of plasma proteins into interstitial spaces of the tissue.

The following phase of acute inflammation is characterised by the activation of phagocytes. These cells ingest agents as well as debris of the injured tissue. Once the phagocytosis process is activated by the receptor-ligand interaction, the element to be phagocytosed is internalized inside vesicle called phagosome (Gray and Botelho 2017, Lancaster, Ho et al. 2019). Then the fusion of phagosome with the lysosome (phagolysosome) produces the elimination of the agent through lytic enzymes and toxic molecules present in the lysosome. IFN-γ and signals from TLRs, activates the enzyme phagocyte oxidase, producing the release of reactive oxygen species (ROS), killing the microbes (Nathan, Murray et al. 1983).

Macrophages play additional role in the inflammatory processes by producing cytokines IL-1β and TNF-α, which function as attractants of leukocytes and other proteins (Miossec, Yu et al. 1984, Jinquan, Frydenberg et al. 1995). After the elimination of microbes, the healing process of the infected tissue is induced with the contribution of macrophages which release growth factors for fibroblasts (FGF) and endothelial cells (VEGF). This function is becoming permanent in the case of chronic inflammation, resulting in excessive fibrosis and angiogenesis in the tissue (Barrientos, Stojadinovic et al. 2008). Moreover, released IL-1β and IL-6 also induce production of pentraxins, C reactive protein, serum amyloid P and fibrinogen from hepatocytes, which have protective roles in the tissue repair (Steel and Whitehead 1991, Alles, Bottazzi et al. 1994, Fujita, Ito et al. 2012, Ishikawa, Kobayashi et al. 2015).

As mentioned, cytokines are the modulators of the immune system, some of them essential in the inflammation processes. Among many cytokines, IL-1α, IL-1β, IL-6, TNF-α and IFN-γ can be listed as examples of pro-inflammatory and IL-10, TGF-β1 as one of the

most important anti-inflammatory cytokines. IL-1α and IL-1β are considered as very important pro-inflammatory cytokines with pleiotropic functions (Apte and Voronov 2002). These cytokines are classic endogenous pyrogens, proteins inducing fever by activating the hypothalamus-pituitary-adrenal axis (Yazdi and Ghoreschi 2016). IL-1α is associated with the necrosis, where it functions as an alarm molecule (alarmin) and thus plays a critical role early in inflammation (Rider, Carmi et al. 2013). IL-1α prolongs the lifespan and stimulates the effector function of neutrophils and macrophages and modulates the differentiation and effector function of innate and adaptive immune cells (Smith, Bowman et al. 1986, Mantovani, Dinarello et al. 2019). Interestingly, IL-1α and IL-1β are encoded by different genes but bind to the same IL-1R1 receptor. The main difference between these two cytokines is that the precursor of IL-1α is constitutively present in the cytosol and/or nucleus of epithelial layers of the entire GIT, lung, liver, kidney, endothelial cells and astrocytes, while IL-1β is a secreted cytokine with higher pyrogenic effect (Bersudsky, Luski et al. 2014). IL-1β is upstream, pro-inflammatory cytokine that is generated primarily by myeloid cells, it initiates and propagates inflammation, mainly by inducing a local cytokine network and by enhancing inflammatory cell infiltration. IL-1β is also a major mediator in the tumor microenvironment, which acts together with VEGF in mounting and maintaining tumor-mediated angiogenesis (Voronov, Carmi et al. 2014). Macrophage respond to PAMPs by production of two important cytokines, IL-6 and IFN-γ. IL-6 is a pro-inflammatory cytokine secreted by macrophages in response to PAMPs. IL-6 is responsible for stimulating acute phase protein synthesis, as well as the production of neutrophils in the bone marrow. It supports the growth of B cells and is antagonistic to regulatory T cells (Rose-John 2018). Macrophages after the contact with PAMPs produce IFN-γ that by autocrine stimulation induces macrophages to produce direct antimicrobial and antitumor activities, upregulation of antigen processing and presentation. IFN-γ also mediates leukocyte attraction, growth, maturation, and differentiation of many other cell types. Additionally, it is enhancing NK cell activity and regulating B cell functions such as immunoglobulin production and class switching (Schroder, Hertzog et al. 2004). TGF-β1 is regulating the proliferation and differentiation of all immune cells by direct binding to them. TGF-β1 is produced by many different types of immune cells, including macrophages, DCs, NK cells, B cells, and T cells. For example, in B cells, TGF-β1 regulates the expression of immunoglobulins or surface MHC II receptors (Tamayo, Alvarez et al. 2018). Also, the proliferation of naïve CD4+ cells and expansion and cytotoxic activity of CD8+ T cells is negatively regulated by TGF-β1 (Park, Shultz et al. 2005, Becker, Fantini et al. 2006). TGF-β1 plays a dual role in the cancer

evolution, on one hand it is a suppressor of cell division, on the other hand it can activate the fibroblasts in tumour microenvironment, enhancing the tumour growth.

The resolution is a period in which all pro-inflammatory factors are downregulated to return to physiological levels and reparation is activated to establish the complete homeostasis in the tissue. There are various mechanisms that cease inflammatory reaction. First, pro-inflammatory cytokines have short half-life in the body thus they are continuously produced in high amounts until the inflammatory signals persist. However, to stop the complete inflammation, other mechanisms are activated: apoptosis of pro-inflammatory cells, downregulation of leukotrienes, production of TNF receptors and IL-1 receptor antagonists, production of IL-10 and TGF-β1, release of anti-inflammatory proteins such as lipoxins and resolvins, desensitization of chemokine receptors (IL-16-induced desensitization of CCR5 and CXCR4), degradation of chemokines by matrix metalloproteinases (Rahangdale, Morgan et al. 2006, Ortega-Gomez, Perretti et al. 2013, Taams 2018).

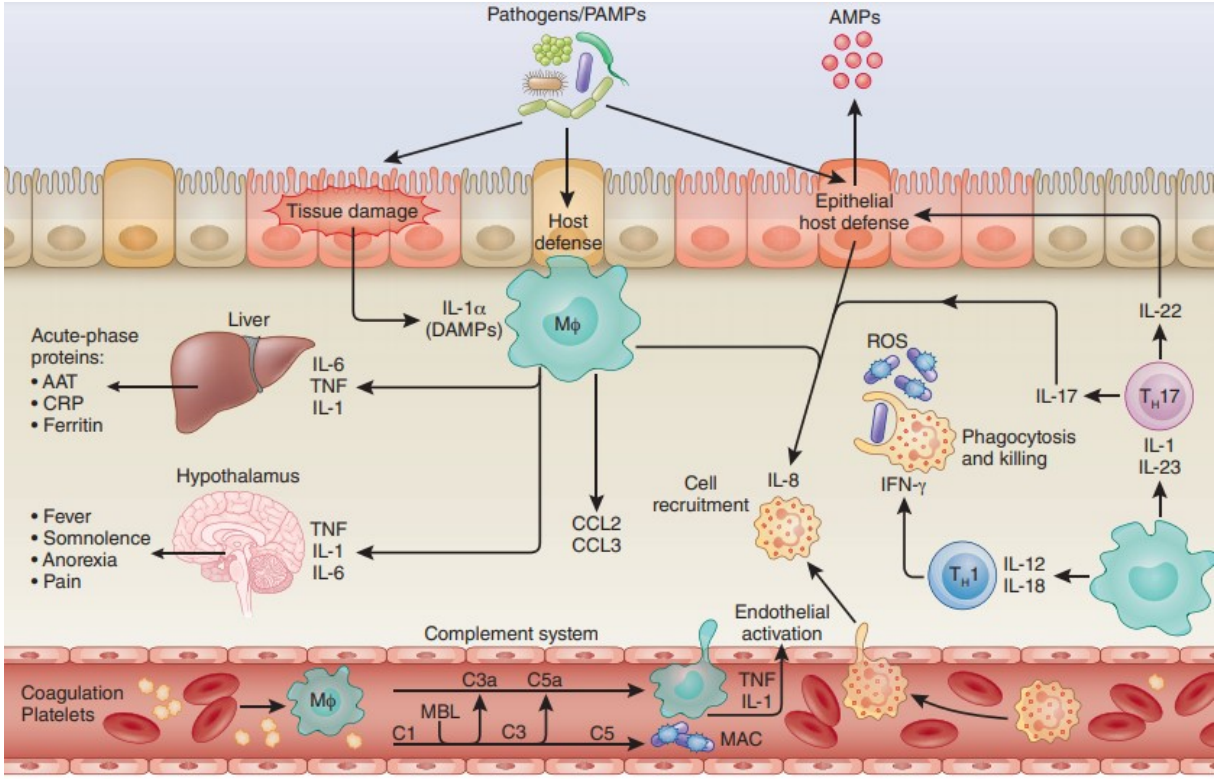


Figure 7: **The immunological mechanisms leading to inflammation during the host defence against invading pathogens.** AAT, a1-antitrypsin; PAMPs, pathogen-associated molecular patterns; AMPs, antimicrobial peptides; DAMPs, danger-associated molecular patterns; MAC, membrane attack complex; ROS, reactive oxygen species; CRP, C-reactive protein; MBL, mannose-binding lectin; Mf, macrophage or monocyte; CCL, C-C motif chemokine ligand. Adapted from (Netea, Balkwill et al. 2017).

### 1.3.3 Chronic inflammation

Typical features of chronic inflammation include: 1) persistent presence of resistant microbes, viruses or other antigens in the site of infection, autoimmune disease or permanent injury of tissue; 2) significant involvement of adaptive immunity in the inflammation (lymphocytes), presence of fibroblasts, platelets and macrophages; 3) permanent release of various cytokines, growth factors, ROS and hydrolytic enzymes; 4) tissue damage, angiogenesis, fibrosis and necrosis. It is important to mention that Th2-mediated adaptive immunity is a very significant factor that contribute to prolonged action of chronic inflammation. The composition of immune cells in this type of inflamed tissue is different with higher invasion of lymphocytes and subsequently fibroblasts that are responsible for fibrosis of the original tissue with subsequent permanent loss of function of original tissue. Diabetes, cancer, cardiovascular diseases, allergies and chronic obstructive pulmonary disease (COPD) are examples of diseases associated with the chronic inflammation (Barnes 2016, Kim, Kim et al. 2019, Luc, Schramm-Luc et al. 2019). More detailed mechanisms of chronic inflammation are described on concrete examples of diseases in the following chapters.

### 1.3.4 Complications of chronic inflammation

The process of inflammation which lasts for longer period, negatively affecting the function of the original tissue, is classified as chronic inflammation. Tissue injury, inflammation and healing processes are acting simultaneously in a loop sustaining the inflammatory cell infiltration, tissue damage and its remodelling (**Fig. 8**). The combination of prostaglandins (PGs) and cytokines which stimulate immune, mesenchymal and epithelial cells for amplified reaction to damage or infection, is considered as the main cause of permanent inflammation (Yao and Narumiya 2019). The most potent PGs include thromboxanes, $PGD_2$, $PGE_2$, $PGF_{2\alpha}$ and $PGI_2$, which are metabolic cyclooxygenase products of fatty acids such as arachidonic acid (Ricciotti and FitzGerald 2011). The signalling cascades by which these ligands act include G protein-coupled receptors specific for each type of PG. Some studies proved that PGs are the main factors that mediate conversion of acute inflammation to chronic form (Aoki and Narumiya 2012). Mainly macrophages, lymphocytes and plasma cells infiltrate damaged tissue and release angiogenesis- or fibrosis-promoting molecules. The suppression of balancing negative feedback mechanisms is

present with additional epigenetic regulatory mechanisms. They are represented, for instance, by demethylation and permissive histone modifications of *Foxp3* and *Ctla4* genes in Treg cells. (Ohkura, Kitagawa et al. 2013).

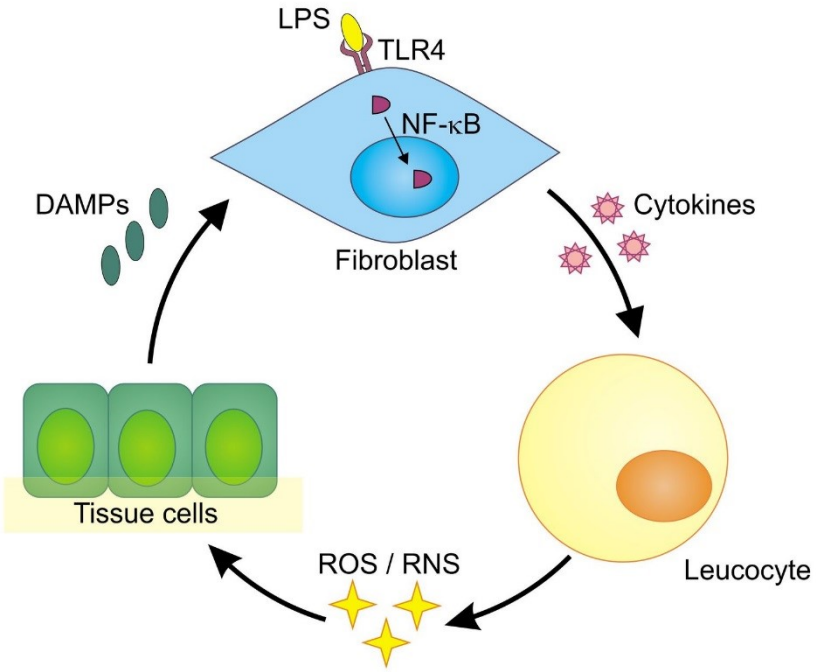


Figure 8: **The chronic inflammatory cycle as response to bacterial infection.** This scheme represents an example of bacterial infection which can induce the chronic inflammatory loop. Infected cells release PAMPs (e. g. LPS) that recognise TLRs (TLR4) on fibroblasts, responding by NF-κB translocation into the nucleus and release of pro-inflammatory cytokines. These recruit to the site of infection leukocytes, releasing reactive oxygen species (ROS) and reactive nitrogen species (RNS), contributing to tissue damage. Adapted from Neumann, J., Scherhag, A., Otten, S., Shen, F., Leifke, A., Birk, U., . . . Cremer, C. (2016). Chronic Inflammation Under the Microscope. Microscopy Today, 24(6), 38-45. doi:10.1017/S1551929516000894.

Understanding the principles of chronic inflammation is very important because of its association with many serious diseases. Chronic inflammation pathologically impacts the tissue physiology due to repeated destruction and repair of affected tissues, leading to remodelling processes, with possible evolution towards fibrosis, angiogenesis. These can also affect the tissue cells with the phenomenon of metaplasia, a pre-carcinogenic condition.

Enhanced angiogenesis is a part of chronic inflammation, associated with many autoimmune diseases such as psoriasis, rheumatoid arthritis, inflammatory bowel disease, diabetes and cancer but also obesity and osteoarthritis (Carmeliet 2005). Angiogenesis is classified as growth and remodelling process by which vascular system forms branching

network with mature vasculature as happens also in the wound repair. It is a multistep process starting with the release of proteolytic enzymes that degrade blood vessels basement membrane, endothelial cells change shape, invade stroma and by action of various pro-angiogenic factors proliferate and form tubular structures. These factors include endothelial growth factor (VEGF), FGF, angiopoietin, platelet-derived growth factor (PDGF) and platelet derived-endothelial cell growth factor (PD-ECGF). The chronically inflamed tissue and the progressive fibrotic stimulation imply the hypoxic environment inducing the production of pro-angiogenic molecules to compensate the condition. Immune cells participate through production of various angiogenic factors, proteases and NO, necessary for degradation of extracellular matrix (ECM), proliferation and migration of endothelial cells. These enhance chronic inflammation providing cytokines IL-1β, TNF-α and IFN-γ, oxygen and nutrients, and enabling inflammatory cells chemotaxis (**Fig. 9**). This positive loop accelerates invasion of immune cells to injured tissue with subsequent prolonged duration of inflammation (Costa, Incio et al. 2007).

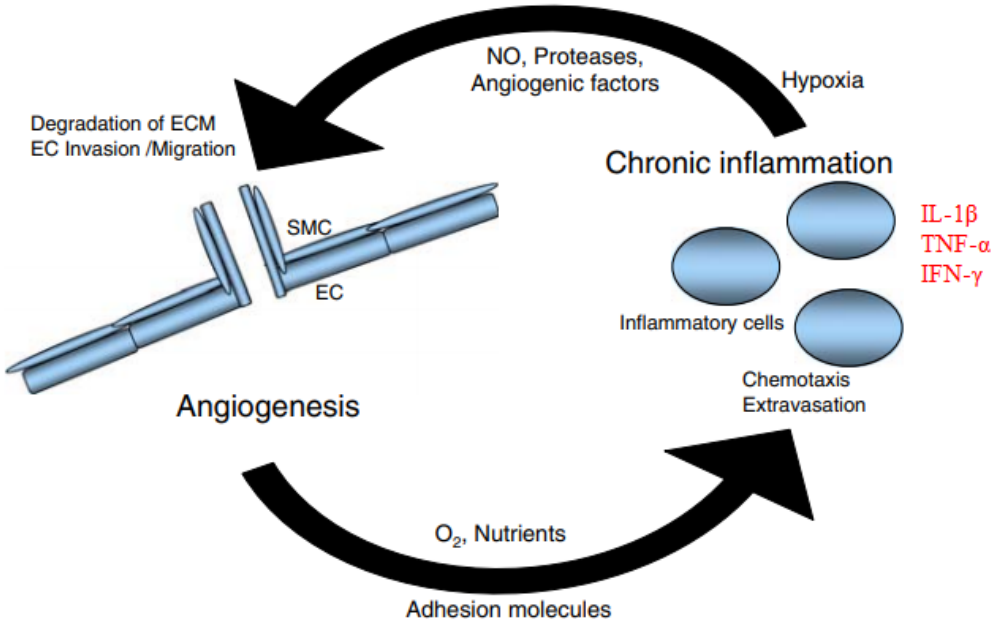


Figure 9: **Schematic diagram illustrating the cross-talk between chronic inflammation and angiogenesis.** Inflammatory cells involved in chronic inflammation release pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ), growth factors and proteases that degrade the ECM. By this, endothelial cells provide the oxygen, nutrients and adhesion molecules for inflammatory cells, enabling their chemotaxis to the site of inflammation. The inflammatory loop is repeating, sustaining the inflammation in chronic state.

As an example, the signalling through $PGE_2$-EP3 axis is stimulating the expression of chemokines in endothelial cells, sustaining the enhanced angiogenesis process (Amano, Hayashi et al. 2003). The studies on CRC showed that $PGE_2$-induced expression of CXCL12 recruits to tumour microenvironment CXCR4+S100A4+ fibroblasts from bone marrow, which produce collagen necessary for tumour stroma formation and lymphangiogenesis (Katoh, Hosono et al. 2010). The production of ECM proteins is a normal process as part of healing tissues during or after inflammation. However, tissue fibrosis in chronic inflammation is associated with the disruption of normal tissue architecture and functions due to permanent production of ECM proteins, e. g. collagen (Kilic, Sonar et al. 2011).

## 1.4 Examples of chronic inflammatory diseases: Crohn´s disease and ulcerative colitis

The bowel is an environment continuously challenged by the direct contact with the commensal microflora and food or other ingested antigens. To maintain the homeostatic condition in the mucosa, the activated response is highly regulated to avoid the damage to mucosa. By this way, this very controlled response can be even defined as "physiological inflammation" (Tlaskalova-Hogenova, Tuckova et al. 2005). This regulation doesn't abrogate the ability to respond to pathogenic bacteria.

IBD acronym defines heterogenous group of disorders, characterised by chronic inflammation of small and large bowel. The causes of these diseases are yet to be fully clarified but very probably they develop due to defects in immune responses to commensal bacteria, in combination with genetic and environmental factors (Jostins, Ripke et al. 2012, Wawrzyniak and Scharl 2018). Even the therapies are not yet fully assessed and are mainly based on anti-inflammatory drugs are immune suppressants (Mao and Hu 2016, Nadpara, Reichenbach et al. 2020).

Two main types of IBD are recognised: 1) Crohn´s disease (CrD) affecting whole GI tract but most frequently localised in the terminal ileum; 2) ulcerative colitis (UC) associated with the large bowel, mainly in its terminal part. Both diseases share typical symptoms: pain in the abdomen, bloody diarrhoea, fibrosis of the original tissue, vomiting and weight loss as a result of malnutrition, (Liu and Stappenbeck 2016, Borren, van der Woude et al. 2019). The pathologic mechanisms associated with IBDs can be summarized in four main parts: 1) Defect of immune system to avoid the invasion of commensal bacteria through the intestinal wall (e. g. the increased production of defensins suggests possible invasion of commensal

bacteria to subepithelial space) (Zaidi, Huynh et al. 2020); 2) Abnormal Th1 and Th17 cell activation (e. g. various animal models and studies on IBD patients showed high infiltration of Th17 cells in affected tissues) (Nielsen, Kirman et al. 2003); 3) Defective function of T regulatory cells - this defect can be considered as main cause of IBD which was observed in different animal strains in which depletion of Treg or knock-out of *Il10* gene caused development of IBD (Kuhn, Lohler et al. 1993, Zhang, Zheng et al. 2020); 4) Polymorphisms of genes associated with the macroautophagy - this defect causes self-destruction of intracellular organelles, causing the reduction of Paneth cells in the intestines which are responsible for secretion of defensins and other antimicrobial enzymes (Matsuzawa-Ishimoto, Shono et al. 2017).

Typical IBD associated with the chronic inflammation in the colon is the ulcerative colitis (UC) as well as the periodicity of disease progression and remission. Mainly the dysbalance of gut microbiota (dysbiosis) is considered as factor that has impact on UC development (van Nood, Vrieze et al. 2013). UC has relatively high prevalence in the developed countries (>0.3%) and rapidly increasing incidence in newly industrialised countries (Ng, Shi et al. 2017). UC is clinically heterogeneous because only 30 % of patients have extensive or aggressive colitis (Solberg, Lygren et al. 2009). Among the genetic factors, the mutations in *IL23-R, IL-12, JAK2, CARD9, TNFSF18,* and *IL-10* genes are considered to be associated with UC development (Beaudoin, Goyette et al. 2013). Sixteen human leukocyte antigen allelic associations were described for UC, including human lymphocyte antigen (HLA) DRB1*01*03 (Goyette, Boucher et al. 2015). Due to rapid increase of UC incidence in recently industrialized countries, the environmental factors are important. New urban lifestyle, exposure to pollution, change in diet, excessive use of antibiotics, better hygiene and fewer infections are all considered as general contributory factors (Kaplan and Ng 2017). This was proved in Integrative Human Microbiome Project including 132 IBD patients and healthy individuals, which showed that IBD gut microbiome as significantly less diverse but more variable over time (Lloyd-Price, Arze et al. 2019, Clooney, Eckenberger et al. 2021). It is still unclear if the microbiome dysbalance is the cause or consequence of the UC. However, some recent clinical studies showed that faecal microbial transplantation from healthy donors can successfully treat UC (Paramsothy, Kamm et al. 2017, Costello, Hughes et al. 2019). The disruption of epithelial barrier is also one of the pathological factors involved in UC. This is through either altered or impaired secretion of antimicrobial peptides or mucus) or caused by physical defects, for example the disruption of epithelial tight junctions (Turner 2009). The immunity itself is significantly contributing

to UC pathology too. The innate immune reactions are involved with abundant infiltration of neutrophils into the lamina propria (Park, Abdi et al. 2016). The inflammatory environment of UC supports the neutrophil survival, which escalates the tissue damage. Neutrophils are extensively proliferating but also dying by necrosis, forming the neutrophil extracellular traps (NETs) with high concentration of calprotectin (Dinallo, Marafini et al. 2019). This NETs can then function as traps for IL-1 family cytokines, IL-6 and TNF-α that support the inflammatory circuit in UC (Friedrich, Pohin et al. 2019). Among the adaptive immune responses, UC is associated with a Th2 response with high IL-4, IL-5, and IL-13 (Bouma and Strober 2003). Significant IL17 mRNA upregulation in CD4+ cells was also found in UC, induced by IL-23 (Kobayashi, Okamoto et al. 2008). This observation was further enlarged by Nalleweg et al., who found that also IL-9 mRNA expression was significantly increased in inflamed samples from patients with UC (Nalleweg, Chiriac et al. 2015). IL-6 and TGF-β1 are important factors involved in tissue remodelling (Becker, Fantini et al. 2004). TGF-β enhances enterocyte migration and regulates extracellular matrix turnover, while IL-6 is a key mediator in the fibrotic process, stimulating collagen synthesis (Camporeale and Poli 2012, Biancheri, Giuffrida et al. 2014, Zeng, Hu et al. 2019). Increment of collagen deposition leading to fibrosis is another pathological factor associated with IBD as Lawrance and Maxwell showed (Lawrance, Maxwell et al. 2001). These authors concluded that TGF-β1 and insulin-like growth factor-1 (IGF-1) are involved in intestinal ECM remodelling in IBD, and their enhanced expression depends on the presence and location of inflammatory infiltrates. The immunological markers and structural changes of collagen in IBD can be studied also in colitis-induced animal models, which is one of the aims of this thesis (**Fig. 10**). The overview of immune processes involved in IBD pathology is schematically showed in **Fig. 11**.

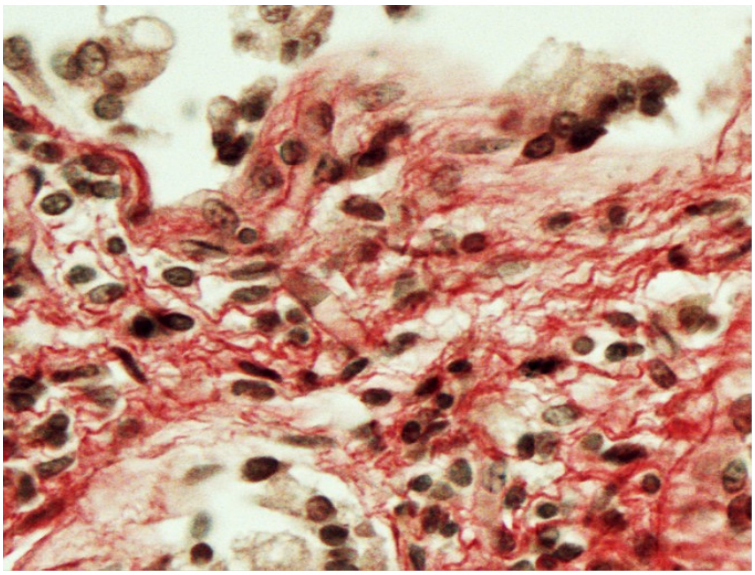

Figure 10: **Mucosa with increased collagen fibres in rat with chronic colitis induced by DSS.** Collagen fibres are evidenced by Picrosirius red staining, 40x original magnification.

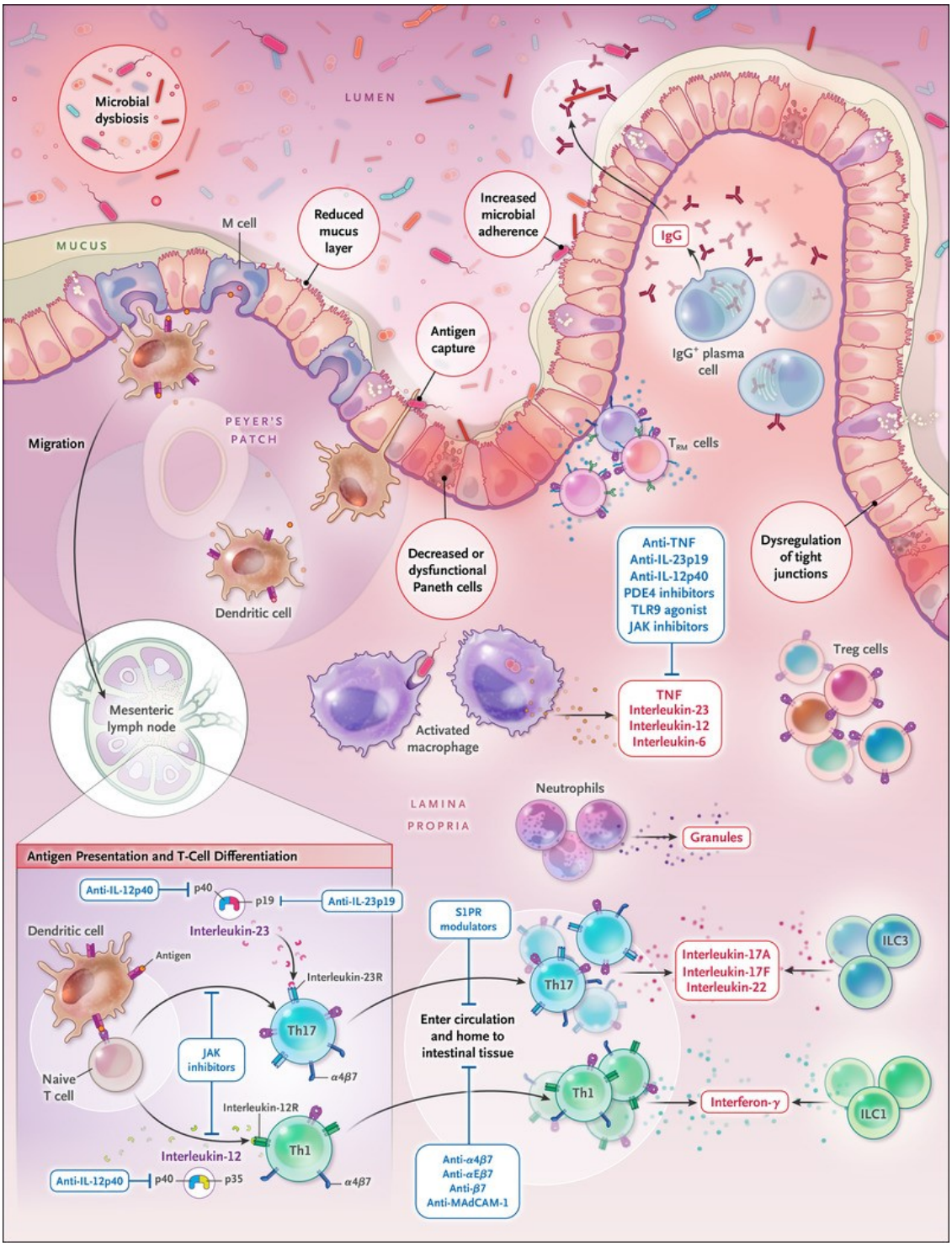


Figure 11: **Pathophysiology of inflammatory bowel disease.** Disruption of tight junctions and the mucus film covering the epithelial layer causes increased permeability of the intestinal epithelium, resulting in increased uptake of luminal antigens. Macrophages and dendritic cells change their functional status from tolerogenic to an activated phenotype, resulting in increased production of proinflammatory cytokines (TNF-α, IL-6, IL-12, IL-23). Another group of innate immune cells are neutrophils, releasing antibacterial peptides into the lamina propria. APCs migrating to local mesenteric lymph nodes stimulate the differentiation of naïve T cells to two subtypes involved in IBDs – Th1 cells producing pro-inflammatory IFN-γ and Th17 cells producing IL-17 and IL-22, pro-inflammatory cytokines. The intensive inflammatory response is balanced by Treg cells producing IL-10 and TGF-β1 as anti-inflammatory cytokines. Adapted from (Chang 2020).

## 1.5 Tumour microenvironment and inflammation

The tumour cells are in permanent contact with the cells and the structures of the surrounding tissue in which they are proliferating, together forming the tumour microenvironment (TM) (**Fig. 12**). This is a unique niche including tumour cells, immune cells, fibroblasts and other connective cells, blood and lymphatic vessels, pericytes, adipocytes, nerves, all cellular products including collagen and other extracellular matrix components. TM is also including the products of the various types of cells. Among immune cells, mainly T lymphocytes, B lymphocytes, NK cells, NKT lymphocytes and tumour-associated macrophages (TAMs) are the most abundant (Marelli, Sica et al. 2017). Tumour stroma is an important part of TM, since it can condition the behaviour of all cells including tumour cells (Vannucci 2015). The understanding of TM by morphological and molecular view is crucial for producing new insights to progress in diagnosis and therapies of tumours.

The presence of immune cells in the TM has a double-edged function - they are able either to suppress or to promote the tumour growth, depending on the stage of tumour development in which they are active. All tumours start development through three sequential phases defined by the three E theory: elimination, equilibrium and escape (Dunn, Bruce et al. 2002). In the elimination phase, immune cells are able to completely destroy the growing tumour clone. If the immune response is not enough valid to eliminate the tumour, it starts to develop in balance with the immune response (equilibrium) The tumour is nor growing nor reducing (e. g. tumour *in situ*). If the immune system is not able to reverse this state back to elimination and heal the tumour, the third phase called escape starts. In this phase, tumour cells escape immune surveillance mechanisms and start to grow uncontrolled, leading to more established cancer.

At the beginning of tumour growth, the immune system is trying to heal tumour. Immune cells with the anti-tumour capacities include neutrophils, macrophages and lymphoid cytotoxic T cells and Th1 cells which produce high amounts of IL-1β, TNF-α and IFN-γ (Shah, Divekar et al. 2005, Thibaut, Bost et al. 2020). The presence of NK and NKT cells in the TM is considered as sign of good prognosis (Hanna 1982, Liu, Cheng et al. 2017, Paul, Chhatar et al. 2019). One of the most important anti-tumour immune cells are CD8+ T cells (cytotoxic T lymphocytes), which decrease in the TM of CRC patients is predicting worse prognosis for the patients (Wang, Yu et al. 2020).

The equilibrium phase is characterised by the formation of tumour cell variants that has survived the elimination process and enter into a dynamic equilibrium. In this process,

lymphocytes and released IFN-γ increase the selection pressure on the tumour cells to transform to genetically unstable and rapidly mutating tumour cells. During this period of selection, many original variants of the tumour cell are destroyed, but new variants arise carrying different mutations that increase their resistance to immune attack. Normally, equilibrium is the longest of the three processes and may occur over a period of many years.

As the immune system fails to eliminate the newly growing transformed cells, tumour is becoming like a "wound that do not heal". The reason to use this definition is because the continuous remodelling activity of the tissues and the structural reorganisation continuously maintained by the chronic inflammatory background similar to the processes in a not healing tissue (Dvorak 1986, Ribatti and Tamma 2018). The presence of chronic inflammation has been proved as a typical phenomenon of the TM and this condition produce a change in the immune activity that becomes tumour-promoting (Balkwill and Mantovani 2001, Hanahan and Weinberg 2011). More studies showed that chronically inflamed tissues exhibit higher tumour incidence, thus inflammation is a promoter not consequence of tumorigenesis (Landskron, De la Fuente et al. 2014, Greten and Grivennikov 2019).

About 90 % of cancers develop from epithelial cells as carcinomas. Epithelial tissues are originally less vascularised therefore intensive angiogenetic processes is associated with carcinomas´ growth. Tumour cells are considered as major source of VEGF, while stromal cells within TM are the secondary VEGF producers (Lichtenberger, Tan et al. 2010). The VEGF expression and density of newly created microvessels is one of the markers of tumour growth. Normally, more malignant tumours help to develop more chaotic and branched network of new vessels. The loosening of cell junctions between pericytes and changes in the basement membrane causes that vessels are more leaky and accumulate more molecules than normal vessels. Each TM is in its principle hypoxic because of as tumour mass is growing, less oxygen and nutrition is delivered to all tumour cell in this mass. As a result, low oxygenation of tumour tissue contributes to the genetic instability due to defects in various DNA repair pathways, tumour cells become resistant to apoptosis and increased expression of hypoxia-inducible factor α (HIF-α) is present (Semenza 2013, Ioannou, Paraskeva et al. 2015). This induce the angiogenesis in the TM, which intensity can be considered as one of the factors of tumour malignancy (Thomas and Kim 2008, Hida, Maishi et al. 2018). Other processes are also activated such as ECM remodelling, e.g., increased expression of collagen IV enhances the survival of tumour cells and facilitates their growth in the liver (Burnier, Wang et al. 2011). Another mechanism supporting the evolution of metastases was found to be the Warburg´s effect, saying that some tumour cells use

anaerobic glycolysis and produce lactate which decreases the pH of TM (Hsu and Sabatini 2008). This condition supports the degradation of ECM and tumour cell migration.

Very specific functions in the TM plays also ECM with essential part of tumour-associated fibroblasts in producing structural proteins such as collagen or proteoglycans (Pietras and Ostman 2010). Abnormal ECM structure can also enhance angiogenesis and inflammation in the TM. The collagen scaffold can influence cell behaviour and function, for example, effector immune cells require direct intracellular contact for priming process, thus remodelling and changes in the architecture of collagen network may influence the successful immune cell priming (Egeblad, Rasch et al. 2010). The changes in the ECM architecture (collagen, fibronectin, vitronectin, tenascin-C, separation of phases-based activity reporter of kinase (SPARK)) are also significantly affecting the effectivity of anti-tumour therapy. The changed network of ECM proteins creates a physical barrier that makes tumour mass less penetrable for the drugs (Tang, Chen et al. 2018).

As described in previous chapters, macrophages are distinguished to two different populations, classic M1 and tumor-associated M2 (TAMs). In the context of TM, M2 macrophages are more important because of their tumour-promoting function. The accumulation of these macrophages in the TM was proved by many experimental models and clinical cases (Cassetta, Cassol et al. 2011). The main source of TAMs are monocytes from the bone marrow, they differentiate to TAMs in response to Th2 cytokines, such as IL-4, IL-10 and IL-13.

Cytokines involved in this transformation, mainly IL-4, IL-6, IL-10 and TGF-β1, inhibit the anticancer activity of Th1 cells and sustain Th2-driven B cell maturation, altogether affecting the reconstitution and homeostasis of damaged tissue (Huang, Wang et al. 1995, Sato, Goto et al. 1998, Filella, Alcover et al. 2000). The other lymphoid cells such as Treg cells play double role in the TM, regulating immune homeostasis and inhibiting the tumour cell replication in early phases of tumour growth (protective role), while inhibiting the immune responses against newly developing tumours in later phases of the tumour establishment (pathological role) (Saleh and Elkord 2019). Intratumoral Tregs are differentiating from naïve to highly activated CD25+/FOXP3+ cells, that secrete high amounts of TGF-β1 and IL-10 cytokines with immunosuppressive functions (Sakaguchi, Miyara et al. 2010). TGF-β1 together with IL-10 produce suppressive effects on CD8+ T cells, NK cells and CD4+ Th1 cells but stimulating the maturation of Tregs (Liu, Wong et al. 2007). These cells cooperate in TM with other immunosuppressive cells, namely TAMs, myeloid-derived suppressor cells (MDSCs), mast cells, cancer-associated fibroblasts and

regulatory B cells, furtherly producing TGF-β1 and IL-10 and establishing a permanent molecular circuit enhancing the tumour growth (Najafi, Farhood et al. 2019). MDSCs releasing IL-4, IL-6, IL-10 and TGF-β1 inhibit cytotoxic effects of T cells and NK cells and influence presentation of antigens by DCs (Cheng, Corzo et al. 2008, Hoechst, Voigtlaender et al. 2009, Ye, Yu et al. 2010). When anti-angiogenic drugs (anti-VEGF) are used for the treatment of tumours, MDSCs produce other angiogenic factors than VEGF that mediate resistance to treatment (Romano, Parrinello et al. 2018). Mast cells release factors that enhance proliferation of endothelial cells to promote tumour angiogenesis and M2 TAMs are releasing cytokines IL-1β, IL-6, IL-10, IL-23 -α, TNF-α and TGF-β1, which are considered to be pro-tumorigenic, affecting the response of tumours to therapy (Kessler, Langer et al. 1976, Petty and Yang 2017). MDSCs can also rapidly differentiate into TAMs in the TM (Chanmee, Ontong et al. 2014).

Among many cytokines involved in tumour growth, TGF-β1 plays a specific double role (Caja and Vannucci 2015). On one side, it is a suppressor of inflammation that is a pathological factor in tumour growth, on the other side, TGF-β1 is stimulating fibroblasts to produce collagen that is supporting the tumour growth (Tang, Chen et al. 2018).

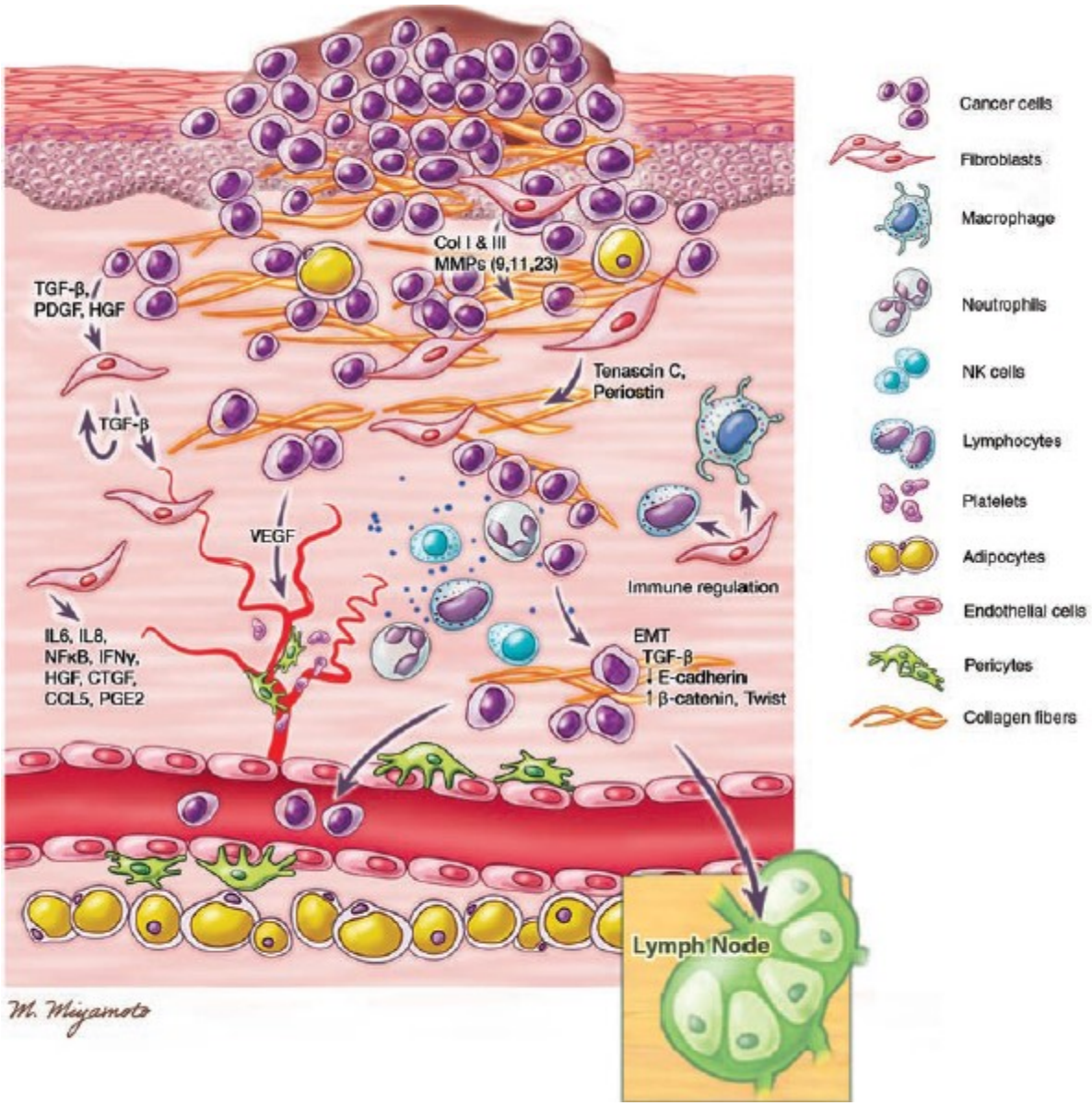


Figure 12: **Detailed scheme of tumour microenvironment showing cells involved.** Fibroblasts are recruited by cancer cells and activated in the local environment via TGF-β, PDGF, hepatocyte growth factor (HGF), and other signalling molecules. Cancer-associated fibroblasts (CAFs) secrete TGF-β, which on one hand promote the fibroblast activation, on the other hand suppresses the immune reactions in the TM. CAFs also secrete IL-6, IL-8, NFκB, IFN-γ, CCL5, and PGE2, as well as ECM molecules such as collagens, matrix metalloproteinases (MMPs), tenascin C and periostin. Neutrophils, lymphocytes, macrophages and NK cells are involved in immune responses to tumour by secreting cytokines. Both cancer cells and CAFs are also involved in stimulating angiogenesis. These new vessels are leaky and dysfunctional, which further suppress the immune access to the tumour. CAF signalling can stimulate EMT of tumour cells with downregulation of E-cadherin expression and upregulation of β-catenin and Twist, and escape through the basement membrane with access to vasculature for metastatic spread. Tumour cells can also metastasize through the lymphatics when collected to the local lymph nodes. Adapted from (Foster, Jones et al. 2018).

## 1.6 Fibrosis as a consequence of chronic inflammation

### 1.6.1 General aspects and mechanisms

The principles of inflammation were described in Chapter 1.3. As mentioned, one of the steps of inflammation is resolution and healing process by which injured, destroyed tissue is repaired. In normal conditions, new components of ECM are produced by various cells (endothelial, epithelial cells, fibroblasts, smooth muscle cells) and form support for new tissues. However, pathologic conditions occur if excessive production of ECM proteins is present. This is a typical feature of chronic inflammation and the fibrotic process leads to organ malfunction and even to death (e. g. in the lung, in the liver). The proteins such as collagen and fibronectin are produced in high quantities, leading to scarring, replacement of the original tissue structures and consequent organ malfunction (Wynn and Ramalingam 2012). This phenomenon is associated to many diseases, for example liver cirrhosis, idiopathic pulmonary fibrosis, autoimmune diseases including scleroderma, rheumatoid arthritis or IBDs. Fibrosis is also considered as a hallmark of tumour progression (Dhar, Baglieri et al. 2020). The collagen accumulation distorts the architecture of normal tissue, producing the stiffening and modification of the intracellular spaces, that lead to organ dysfunction, e. g. in the liver cirrhosis (Hoffmann, Djerir et al. 2020).

Among various pro-inflammatory cytokines, TNF-α and IL-1β were identified as important mediators of fibrosis (Zhang, Lee et al. 1993). Together, they induce production of IL-6 which is a growth factor for fibroblasts. TGF-β1 produced mainly by Treg cells plays both protective and pathologic roles in the fibrosis. TGF-β1 is the suppressor of inflammation as main cause of fibrosis but also one of the key drivers of fibrosis as it is a strong inducer of fibroblasts (Kitani, Fuss et al. 2003). Some clinical studies also showed that patients with higher TGF-β1 production displayed more progressive fibrosis of internal organs. Interestingly, TGF-β1 produced by classically activated M1 macrophages induced by IFN-γ plays more wound-healing and pro-fibrotic role (Wynn and Barron 2010). On the contrary, alternatively activated M2 macrophages were found to be protective in fibrotic process by competing with Th2 cells and fibroblasts for L-arginine that is necessary for biochemical synthesis of collagen (Shapouri-Moghaddam, Mohammadian et al. 2018).

Adaptive immunity contributes to fibrosis by Th17, Th2 and Th1 immune responses. Th17 cells are producing IL-17A which is the driver of fibrosis itself (Ruiz de Morales, Puig et al. 2020). Together with TGF-β1, IL-1β–IL-17A–TGF-β1 cytokine axis is an important

pathway in inflammation-driven fibrosis. Fibrotic properties of fibroblasts, epithelial and smooth muscle cells are further enhanced by IL-13-TGF-β1 cytokine axis (Li, Liu et al. 2020).

The other mechanisms that contribute to fibrotic process include: 1) myofibroblast activation by Wnt-β-catenin signalling pathway; 2) endoplasmic reticulum stress caused by excessive accumulation of misfolded or unfolded proteins is leading to apoptosis of cells (example: nonalcoholic steatohepatitis); 3) telomere shortening in mesenchymal cells caused by mutations in the gene encoding telomerase reverse transcriptase-leading to apoptosis of these cells; 4) microRNAs regulating fibroblast growth and activation, for example miR-21, associated with lung fibrosis. They regulate the collagen production directly or through other factors that initiate collagen production (TGF-β1, mitogen-activated protein (MAP) kinase signalling, Smad3, connective tissue growth factor, etc.) (Tsakiri, Cronkhite et al. 2007, Korfei, Ruppert et al. 2008, Liu, Friggeri et al. 2010, Baarsma, Spanjer et al. 2011).

### 1.6.2 Collagen

Collagens are the most abundant proteins in mammals, representing about 30 % of total protein mass. Until now (2020), 28 types of collagens have been identified (Ricard-Blum 2011). The common structural feature of collagens is the presence of a triple helix that is built mainly by collagen I (96 %) to less than 10 % by collagen XII. Collagens consist of three polypeptide chains called α chains, numbered with Arabic numerals. Except of 28 existing collagen types, further diversity occurs in the collagen family because of other several molecular isoforms for the same collagen type (Ricard-Blum 2011). Additional levels of diversity are due to proteolytic cleavage of several collagen types or by conformational changes induced in collagens by interactions with ECM proteins or glycosaminoglycans (Raspanti, Viola et al. 2008, Bella and Hulmes 2017). Collagen α chains vary in size from 662 up to 3152 amino acids and three α chains can form either homotrimers (formed by three identical chains, e. g. collagen type III assembling three α1(III) chains) or heterotrimers (formed by different chains, e.g., collagen type I is assembling two α 1(I) chains and one α 2(I) chain). Collagens are forming left-handed polyproline II helices twisted in a right-handed triple helix with a one-residue stagger between adjacent α chains (Shoulders and Raines 2009, Gordon and Hahn 2010). Very important is the stabilisation of triple helix by glycine as every third residue, giving higher flexibility to polypeptide containing high content of proline and hydroxyproline. The stability of collagen fibre is further enhanced by hydrogen bonds and electrostatic interactions between aminoacidic

residues (Fu, Case et al. 2015). Collagens are multidomain proteins, containing triple-helical domain but also thrombospondin, non-collagenous, von Willebrand domains and fibronectin III type repeats that may allow more interactions of the collagen with the microenvironment (van der Rest and Garrone 1990). Folding of the triple helix requires trimerization of domains necessary for proper alignment of collagen α chains. This allows the triple helix to fold in a zipper-like fashion. Different types of collagen can be organised in various structures: fibrils, networks, anchoring fibrils, beaded filaments or hexagonal structures. The biosynthesis of collagen was intensively studied by many groups. It is produced as procollagen fibre consisting of amino-terminal propeptide followed by a short N-telopeptide, a central triple helix, a C-telopeptide and a carboxy-terminal propeptide (Fessler, Doege et al. 1985). Pro-α chains of triple helix are intensively modified after the translation and more than 20 heat-shock proteins 47 (HSP47) are required for the stabilisation of triple helix (Sauk, Nikitakis et al. 2005). Both propeptides of procollagens are cleaved during the maturation process. Collagen fibres are flexible proteins able to deform reversibly and their degradation is mediated by matrix metalloproteinases (MMPs), zinc-dependent endopeptidases from metzincin superfamily (Klein and Bischoff 2011). Different types of MMPs degrade different types of collagens. Collagens are deposited in the ECM, but they participate in cell-matrix interactions via several receptor families. The list of all collagen types, their functions and diseases associated with defects in collagen structure or production are listed in the **Table 1**.

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Table 1: **The molecular composition, genetic background and tissue localization of various collagen types.** Adapted from (Gelse, Poschl et al. 2003).

| Type | Molecular composition | Genes (genomic localization) | Tissue distribution |
|---|---|---|---|
| *Fibril-forming collagens* | | | |
| I | $[\alpha1(I)]_2\alpha2(I)$ | COL1A1 (17q21.31–q22)<br>COL1A2 (7q22.1) | bone, dermis, tendon, ligaments, cornea |
| II | $[\alpha1(II)]_3$ | COL2A1 (12q13.11–q13.2) | cartilage, vitreous body, nucleus pulposus |
| III | $[\alpha1(III)]_3$ | COL3A1 (2q31) | skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.) |
| V | $\alpha1(V),\alpha2(V),\alpha3(V)$ | COL5A1 (9q34.2–q34.3)<br>COL5A2 (2q31)<br>COL5A3 (19p13.2) | lung, cornea, bone, fetal membranes; together with type I collagen |
| XI | $\alpha1(XI)\alpha2(XI)\alpha3(XI)$ | COL11A1 (1p21)<br>COL11A2 (6p21.3)<br>COL11A3 = COL2A1 | cartilage, vitreous body |
| *Basement membrane collagens* | | | |
| IV | $[\alpha1(IV)]_2\alpha2(IV)$; $\alpha1-\alpha6$ | COL4A1 (13q34)<br>COL4A2 (13q34)<br>COL4A3 (2q36–q37)<br>COL4A4 (2q36–q37)<br>COL4A5 (Xq22.3)<br>COL4A6 (Xp22.3) | basement membranes |
| *Microfibrillar collagen* | | | |
| VI | $\alpha1(VI),\alpha2(VI),\alpha3(VI)$ | COL6A1 (21q22.3)<br>COL6A2 (21q22.3)<br>COL6A3 (2q37) | widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc |
| *Anchoring fibrils* | | | |
| VII | $[\alpha1(VII)]_3$ | COL7A1 (3p21.3) | skin, dermal–epidermal junctions; oral mucosa, cervix, |
| *Hexagonal network-forming collagens* | | | |
| VIII | $[\alpha1(VIII)]_2\alpha2(VIII)$ | COL8A1 (3q12–q13.1)<br>COL8A2 (1p34.3–p32.3) | endothelial cells, Descemet's membrane |
| X | $[\alpha3(X)]_3$ | COL10A1 (6q21–q22.3) | hypertrophic cartilage |
| *FACIT collagens* | | | |
| IX | $\alpha1(IX)\alpha2(IX)\alpha3(IX)$ | COL9A1 (6q13)<br>COL9A2 (1p33–p32.2) | cartilage, vitreous humor, cornea |
| XII | $[\alpha1(XII)]_3$ | COL12A1 (6q12–q13) | perichondrium, ligaments, tendon |
| XIV | $[\alpha1(XIV)]_3$ | COL9A1 (8q23) | dermis, tendon, vessel wall, placenta, lungs, liver |
| XIX | $[\alpha1(XIX)]_3$ | COL19A1 (6q12–q14) | human rhabdomyosarcoma |
| XX | $[\alpha1(XX)]_3$ | | corneal epithelium, embryonic skin, sternal cartilage, tendon |
| XXI | $[\alpha1(XXI)]_3$ | COL21A1 (6p12.3–11.2) | blood vessel wall |
| *Transmembrane collagens* | | | |
| XIII | $[\alpha1(XIII)]_3$ | COL13A1 (10q22) | epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver |
| XVII | $[\alpha1(XVII)]_3$ | COL17A1 (10q24.3) | dermal–epidermal junctions |
| *Multiplexins* | | | |
| XV | $[\alpha1(XV)]_3$ | COL15A1 (9q21–q22) | fibroblasts, smooth muscle cells, kidney, pancreas, |
| XVI | $[\alpha1(XVI)]_3$ | COL16A1 (1p34) | fibroblasts, amnion, keratinocytes |
| XVIII | $[\alpha1(XVIII)]_3$ | COL18A1 (21q22.3) | lungs, liver |

### 1.6.3 Evaluation of collagen by microscopy

Various methodological approaches can be used to evaluate the collagen organisation by microscopy: confocal microscopy, multiphoton microscopy, polarized light microscopy, histochemistry, X-radiography and others. The histochemistry is a basic method by which collagen fibres can be stained by Masson´s trichrome or Picrosirius red by which qualitative evaluation is possible (Davis, Burnum et al. 2020).

More advanced method to visualize spatial organisation of collagen fibres is by multiphoton microscopy. This method provides high resolution images and can be used for direct collagen visualization (Dudenkova, Shirmanova et al. 2019). While polarized light microscopy is a method to visualize collagen in fixed paraffin sections, multiphoton microscopy allows to achieve high-resolution images of collagen both in fixed samples or in fresh, non-fixes, non-stained tissue, even in *in vivo* conditions (Gade, Robertson et al. 2019). This method is based on the intensive photon irradiation of the tissue, focusing the beam on a local region, which increases the axial resolution and provides visualization of the tissue spatial structure at the subcellular level (Konig 2000, Dunn and Young 2006). Another qualitative method for visualization of collagen is electron microscopy, substituting the conventional structure-determination techniques, which are not able to visualize noncrystalline, extensively cross-linked, and very large collagen fibrils (Starborg, Lu et al. 2008).

The biggest advantage of confocal microscopy is the possibility to obtain increased optical resolution and contrast of a images using a spatial pinhole to block out-of-focus light in image (Nwaneshiudu, Kuschal et al. 2012). This technique enables capturing the sections of 2D images at different depths in a sample giving also the possibility to reconstruct 3D structures within an object. The cell morphology and microstructure of the specimen can be visualized by two mods: reflectance and fluorescence. Reflectance confocal mode microscopy is based on the light reflection principle, used for imaging of cells and tissues at high definition and enhanced sensitivity compared to conventional bright-field microscopy (Prins, Velde et al. 2006), producing histology-like images. Fluorescence confocal mode microscopy visualizes cells or molecules based on either intrinsic one-photon autofluorescence or extrinsic fluorescent markers (Voytik-Harbin, Roeder et al. 2003, Gareau 2009).

The most precise method to visualize collagen structure is two-photon microscopy using second harmonic generation principle (SHG). This is an optical process based on

conversion of two incident photons into a single photon that has twice the energy of the wavelength of incident photons (**Fig. 13).** This method can be used also for visualization of structures without central symmetry like collagen. The benefits of SHG method include high resolution, direct, non-invasive visualization, reduced photobleaching and phototoxicity (Chernyavskiy, Vannucci et al. 2009, Campagnola 2011). Since 1986, this method is becoming more popular and better developed and today new approaches showed its application also in the clinical praxis, for example for imaging structures in organs such as the oesophagus or colon where conventional endoscopes are already used routinely in clinical imaging applications (Freund, Deutsch et al. 1986, Chen, Wang et al. 2014). By more progress of this technique, a real-time "optical biopsy" of various tissues will be available in the clinical and research field as done for the melanoma studies (Balu, Kelly et al. 2014, Wu, Hsieh et al. 2015).

The confocal microscopy images obtained by SHG mode presented in this thesis are proving for the first time that even limited inflammatory stimulation can quickly produce significant changes of the collagen scaffold in the colon mucosa.

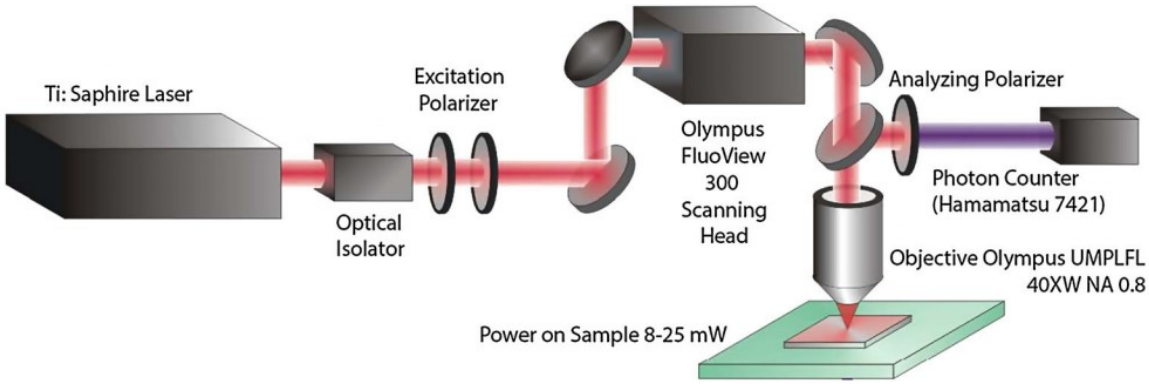


Figure 13: **Simplified illustration of SHG microscope.** Adapted from (Birk, Tadros et al. 2014).

## 1.7 Models of chronic inflammation in the bowel

Chronic inflammation of the bowel or IBD was studied on many various models using mainly rodents as experimental animals. The aim of these models was to mimic the conditions that are present in the human with IBD diagnose. Gnotobiotic animals were additionally used to study the roles of microbes in this disease. In general, these models can be divided to spontaneous colitis (cotton-top tamarins, C3H/HeJBir mice), inducible colitis, genetically modified and adoptive transfer models (Elson, Sartor et al. 1995, Kim, Shajib et al. 2012). The most common are chemically induced murine models - they are simple and many protocols of administrations can be designed (doses, number of cycles, concentration, combination with other chemicals or treatments). The most commonly used inducers are dextran sodium sulphate (DSS) and trinitrobenzene sulfonic acid (TNBS). The experience with those two chemicals showed that DSS with molecular weight from 40 to 54 kDa is more suitable inducer of colitis. It is possible to add it to drinking water and the induced chronic colitis more closely resembles IBD in patients (Kitajima, Takuma et al. 2000, Karlsson, Jagervall et al. 2008). The induction of colitis by DSS brings many advantages: it can be used in various animal models, resemble pathologies known in human, the price of DSS is acceptable. It is possible to apply this model in studies of resistance to drugs, evolution of inflammation in genetically engineered animals, regeneration processes in the bowel and development of dysplasia and colon adenocarcinoma (Elson, Sartor et al. 1995, Thaker, Shaker et al. 2012). Also other colitis animal models were developed earlier, now not applied, using chemicals such as indomethacin, carrageenan, PG-PS, Cyclosporin A or lymphogranuloma venerum-induced infection (Elson, Sartor et al. 1995). Transgenic models were used in later period with many various modifications, among them, the IL-10 KO mouse model became one of the most used (Kuhn, Lohler et al. 1993). In these mice, the lymphocyte development and antibody responses are normal, but their growth is retarded and suffer from chronic enterocolitis. This induced extensive mucosal hyperplasia, inflammation and aberrant expression of MHC II molecules on epithelia cells.

The first study using DSS as IBD inducer is from 1985 by Ohkusa et al. who used hamster as experimental model. When using a 54-kDa form, an acute colitis is induced by the administration of 3%-10% DSS in drinking water, normally in one cycle lasting 7-8 days (Perse and Cerar 2012). The symptoms of acute colitis include rectal prolapse, bloody diarrhoea, weight loss. At autopsy and histology, shortening of the colon, mucosal

ulceration, and neutrophilic infiltration into the left colon mucosa can be observed (Okayasu, Hatakeyama et al. 1990). The chronic inflammation can be induced by repeated administration of 2-3% DSS with molecular weight (MW) of 30-40 kDa, normally 3 cycles lasting 7 days are used, interrupted by 14 days of drinking water (Wirtz, Popp et al. 2017). This protocol is used for Swiss-Webster mice, optimisation is necessary when using other rodent strains. The accumulation of plasma cells, lymphocytes and foamy macrophages can be evidenced in the colon mucosa. Lymphoid aggregates can be found in the lamina propria and serosa with a patchy distribution of inflammation and fissuring ulcers (Cooper, Murthy et al. 1993). Long administration of DSS in rat models also can induce the formation of colonic polyps (Yamada, Ohkusa et al. 1992, Tamaru, Kobayashi et al. 1993). DSS directly inhibits the proliferation of epithelial cells, leading to formation of small lesions especially in the left colon and around lymphoid structures. DSS accumulates in GALT-associated macrophages which display increased lysosomes, full of DSS (Okayasu, Hatakeyama et al. 1990). The immune responses to DSS include increased production of thromboxane $B_2$, leukotriene $B_4$, IL-2, IL-4 and IL-6 (Elson, Sartor et al. 1995). The colitis was also found in immunodeficient mice (SCID), suggesting other than immune mechanisms are involved in its toxicity (Dieleman, Ridwan et al. 1994). The overview of IBD-induced animal models is summarised in **Table 2**.

Table 2: **The overview of IBD animal models, differentiated into separate groups according to used method.** Adapted from (Goyal, Rana et al. 2014).

| Animal models | | | | | | |
|---|---|---|---|---|---|---|
| Chemically induced models | Bacterial induced models | Spontaneous models | Genetically engineered models | Transgenic Mouse models | Mutation knock- in models | Adoptive transfer models |
| | | | Conventional | | | |
| DSS | Salmonella- induced | C3H/HejBir mice model | IL-10 −/− | IL-7 Tg mice | $TNF^{\Delta ARE}$ | CD45RB high-transfer model |
| TNBS | | | TGF-β −/− | STAT 4 | | |
| DNBS | | | IL-2 −/− | HLA-B27 | | |
| Oxazolone | Adherent invasive *E. coli* | | NOD2 −/− | | | |
| Acetic acid | | SAMP1/4it mice model | A20 −/− | | | |
| Carrageenan | | | MDR1A −/− | | | |
| Indomethacin | | | Gαi2 −/− | | | |
| Iodoacetamide | | | TCRα −/− | | | |
| | | | IL-23 −/− | | | |
| Immunologic model | | | Conditional | | | |
| DNCB | | | XBP1 −/− | DNN | | |
| | | | NEMO −/− | Cadherin Tg mice | | |

## 1.8 Models of carcinogenesis in the bowel

Colorectal cancer (CRC) represents a significant health problem in developed countries, thus understanding the biological and molecular mechanism of its development is still very important. To provide sufficient detailed information about the CRC development, various animal models using different carcinogens were designed (**Table 3**). Moreover, the role of microbiota in tumour pathology started to be studied in gnotobiological animal models since 70´years of 20$^{th}$ century. Models in mice or rats showed that GF animals develop less neoplasms or tumours than CV animals (McIntire and Princler 1969, Vannucci, Stepankova et al. 2008). Better anticancer immune response with increased NK, NKT, CTL, B cells and cytotoxicity in peripheral blood of GF rats may be explained as that lower antigenic challenge and the absence of the microbiota-induced "physiological inflammation". These conditions may enhance their capability to sense the danger signals and to develop more prompt anticancer immune responses. Also, differences in bile production by the GF animals and possible different metabolism of the carcinogen can contribute to different effects in the given inducers. A comprehensive review about spontaneous and induced tumours in GF animals was recently published by Mishra et al. (Mishra, Rajsiglova et al. 2021). The authors discussed that GF animals are less susceptible to develop tumours due to lack of bacteria, which activate carcinogens in CV animals. The similar trend was found in IBD animal models where GF animals are more resistant to induced colitis (Qian, Tonkonogy et al. 2005).

Since the spontaneous CRC in mice or rats is relatively rare with the incidence < 1%, chemically induced models of CRC were prepared (Kobaek-Larsen, Thorup et al. 2000). They started since 1955, when 20-methylcholanthrene was applied to induce colon tumours in mice and rats (Mori, Ichii et al. 1955, Rack, Kaufman et al. 1955). Another group of indirect carcinogens include heterocyclic amines, among which 10 were identified as potent carcinogens for rodents. 2-amino-1-methyl-6-phenylimidazol (4,5-b) pyridine (PhIP) has been identified as the most potent heterocyclic amine (Ito, Hasegawa et al. 1997). Later on, widely applied administration of 1,2-dimethylhydrazine (DMH) was used since 1973 to induce tumours in mice, which mimics the distribution in the distal portion of the colon seen in human CRC (Haase, Cowen et al. 1973). The principle of carcinogenic effect of DMH is based on its oxidation to azoxymethane (AOM) in the liver and this (AOM) is then oxidised to methylazoxymethanol (Tamaru, Kobayashi et al.). This is degraded to formalin and methylhydrazonium ion which is an alkylating agent of DNA, RNA and proteins in all

organs that are in contact with it, namely liver, kidney and colon (Venkatachalam, Vinayagam et al. 2020).

The more potent carcinogen than DMH that is frequently used in current research practice is called azoxymethane (AOM). Its higher potential to induce CRC tumours is based on different metabolic pathways than in DMH because AOM is closer substrate for MAM than DMH. The strongest advantages to use AOM as carcinogen include higher potency and enhanced stability in solution (Neufert, Becker et al. 2007). Occasionally, higher variability of tumour induction in response to DMH has been reported due to differences in hepatic metabolism between individuals (Moriya, Harada et al. 1982). In contrast to other models of CRC, tumours induced by AOM are very frequent in the distal part of the colon, which resembles the predominant localization of spontaneous CRC in human. They resemble histopathological features of CRC in human but rarely form metastases (Nambiar, Girnun et al. 2003). Tumours induced by AOM are similar to human CRC also by molecular aspects, in which similar sequence of genetic changes in APC and β-catenin genes appears (Maltzman, Whittington et al. 1997, Takahashi, Nakatsugi et al. 2000). The efficacy of tumour induction by DMH or AOM depends on animal model, administration type, age of animal, gender of animal and especially number of doses and used concentration (Kobaek-Larsen, Thorup et al. 2000). The usual route is subcutaneous (SC) administration, alternatively, oral, intrarectal or intramuscular administration can be used. Oral treatment results in variable low incidence of CRC (Thorup, Meyer et al. 1992), SC treatment may lead to high incidence of CRC (Rubio and Takayama 1994), however, low incidence of CRC is also reported after SC administration (Nordlinger, Panis et al. 1991). The overview of carcinogens used in CRC animal model is summarised in the **Table 3**.

In summary, our experiments showed that even in the early stages of DSS-induced colitis or AOM-induced carcinogenesis, smouldering inflammation is the promoter of both pathologic processes.

Table 3: **The overview of CRC animal models.** The species, used strain, type of induction and administration is showed, followed by colon tumour incidence statistics and latency period information. Adapted from (Kobaek-Larsen, Thorup et al. 2000).

| Species/sex | Strain (in-/outbred) | Chemical | Dose regimen | Colon tumor incidence | Latency period | Other observations |
|---|---|---|---|---|---|---|
| Rat/? | BD (?) | DMH | 21 mg/kg weekly SC | 100% | 231 days | small bowel |
| Rat/male | F344 (inbred) | DMH | 1 injection SC 10 mg/kg | 0% | 9 months after injection | NR |
| | | | 30 mg/kg | 10% | | |
| | | | 100 mg/kg | 39% | | |
| | | | 200 mg/kg | 65% | | |
| Rat/male | Sprague-Dawley (?) | DMH | 20 weekly injections SC 20 mg/kg | 100% | 28 weeks after 1st injection | NR |
| Rat/male | Sprague-Dawley (?) | DMH | 1 injection SC 21 mg/kg | 6% | NR | NR |
| | | | 12 weekly injections SC 21 mg/kg | 55.7% | NR | |
| | | | 24 weekly injections SC 21 mg/kg | 65% | NR | |
| Rat/male | Sprague-Dawley (?) | DMH | 27 weekly injections SC 21 mg/kg | 92.6% | NR | NR |
| Rat/male | BD-IX (inbred) | DMH | 23 weekly injections SC 20 mg/kg | 100% | 24 weeks | NR |
| Rat/male | Wistar (outbred) | DMH | 10 weekly oral doses 20 mg/kg | 14-30% | 52 weeks | 28-79% ACF |
| Rat/? | Sprague-Dawley (?) | DMH | 8 weekly injections IR 250 mg/kg | 0% | 34 weeks after 1st injection | mild hyperplasia colon |
| Rat/male | F344 (inbred) | AOM | 4 weekly injections IM 8 mg/kg | 0% | 8 weeks after 1st injection | liver/region lymph nodes |
| | | | 6 weekly injections IM 8 mg/kg | 0% | 10 weeks after 1st injection | metastases |
| | | | 9-12 weekly injections IM 8 mg/kg | 80% | 12 weeks after 1st injection | |
| Rat/male | Wistar (?) | AOM | 12 weekly injections SC 10 mg/kg | 35.2% | 26 weeks after 1st injection | 58% ear canal<br>52% small bowel<br>14% liver metastases<br>24% peritoneal carcinomatosis |
| Rat/male | Wistar (?) | AOM | 6 weekly injections SC 15 mg/kg | 100% | 20-38 weeks after 1st injection | 48% small bowel |
| Rat/male | F344 (inbred) | AOM | 2 weekly injections SC 15 mg/kg | 77.7% | 52 weeks after 1st injection | NR |
| Rat/male | Sprague-Dawley (outbred) | MNNG | 2 mg/rat IR twice a week for 2 weeks | 67% | 52 weeks | NR |
| Mice/female | Shrews | MNU | 16 doses of 1.5 mg/mouse biweekly | 100% | 31 weeks after 1st injection | 31% metastases<br>38% tumors in small bowel |
| | | | 24 doses of 0.5 mg/mouse biweekly | 100% | 31 weeks after 1st injection | 23% metastases<br>31% tumors in small bowel |
| Rat/male | F344 (inbred) | PhIP | 25 ppm in the diet | 0% | 104 weeks | no other tumors |
| | | | 100 ppm in the diet | 43% | 104 weeks | no other tumors |
| | | | 400 ppm in the diet | 55% | 52 weeks | no other tumors |
| Rat/female | F344 (inbred) | PhIP | 25 ppm in the diet | 0% | 104 weeks | 7% mammary tumors |
| | | | 100 ppm in the diet | 13% | 104 weeks | 47% mammary tumors |
| | | | 400 ppm in the diet | 7% | 52 weeks | 47% mammary tumors |
| Mice/ both sex | CDF1(?) | PhIP | 0.04% in the diet | 0% | 579 days | lymphomas |

# 2. AIMS OF THE THESIS

The aim of this thesis was to investigate the relationship between immunological changes in the colon mucosa and its collagen scaffold architecture in nontreated CV and GF rats, compared with DSS- or AOM-treated CV rats at one month after the induction. The systemic immunity was additionally analysed in all animals to investigate the impact of bacteria or treatment on this part of immune system.

The specific objectives of the thesis were:

1) to evaluate the local and systemic immune aspects by various methods (FACS, ELISA) in CV and GF Wistar-AVN rats.
2) to evaluate microscopic and macroscopic differences in the colon of CV vs GF rats in healthy conditions.
3) to induce either colitis by DSS and to start the carcinogenesis by AOM in CV Wistar-AVN rats.
4) to evaluate the local and systemic immune aspects by various methods (FACS, ELISA, RT-PCR) in healthy and treated CV Wistar-AVN rats.
5) to evaluate microscopic and macroscopic differences in the colon of healthy and DSS- or AOM-treated CV rats.

The additional aims of this thesis were:

1) to evaluate the immunological and structural changes in the colon mucosa of CV and GF Wistar-AVN rats induced by DSS.
2) to investigate genetic, immunological, and structural changes in the colon mucosa of CRC patients, which did not receive any chemical or radiological therapy, as a preliminary part of a comparative approach with the animal models.

# 3. RESULTS

**Immune activation by microbiome shapes the colon mucosa: comparison between healthy rat mucosa under conventional and germ-free conditions**

This study was designed to evaluate the immune aspects in the colon mucosa in conventional and germ-free Wistar-AVN rats and to answer if immunological patterns are reflected in the structural changes of the colon tissue. To prove our hypothesis that commensal microbiota has significant impact on the local and systemic immunity, we analysed immune parameters of colon mucosa, spleen and mesenteric lymph nodes samples by various molecular methods (ELISA, RT-PCR, FACS). The impact of microbiota on the structural organisation of the collagen scaffold in the colon mucosa was investigated using confocal microscopy. We observed that CV and GF rats significantly differed in the collagen scaffold structure in relation to a more activated (and regulated) immune environment. Our study confirmed the hypothesis that commensal microflora has modulatory capacity not only on the immune but also on the structural level.

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RESEARCH ARTICLE

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# Immune activation by microbiome shapes the colon mucosa: Comparison between healthy rat mucosa under conventional and germ-free conditions

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**ABSTRACT**

Germ-free animals (GF) are those without a microbiome since birth. This particular biological model has become one of special interest with the growing evidence of importance of the microbiome in the life, development, adaptation, and immunity of humans and animals in the environments in which they live. Anatomical differences observed in GF compared with conventionally-reared animals (CV) has given rise to the question of the influence of commensal microflora on the development of structure and function (even immunological) of the bowel. Only recently, thanks to achievements in microscopy and associated methods, structural differences can be better evaluated and put in perspective with the immunological characteristics of GF vs. CV animals. This study, using a GF rat model, describes for the first time the possible influence that the presence of commensal microflora, continuously stimulating mucosal immunity, has on the collagen scaffold organization of the colon mucosa. Significant differences were found between CV and GF mucosa structure with higher complexity in the CV rats associated to a more activated immune environment. The immunological data suggest that, in response to the presence of a microbiome, an effective homeostatic regulation in developed by the CV rats in healthy conditions to avoid inflammation and maintain cytokine levels near the spontaneous production found in the GF animals. The results indicated that collagen scaffold adapted to the immune microenvironment; therefore, it is apparent that the microbiome was able to condition the structure of the colon mucosa.

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## Introduction

The role of intestinal microbiota both in healthy conditions and in various human diseases, including inflammatory bowel diseases (IBD), cancer, autoimmune disorders and increased susceptibility to infections, has started to be intensively studied in recent decades. Many reports underline how the combined action of the various bacteria that colonize the bowel, through their antigens and metabolic products, can play multiple roles in the body, including immune regulation and possible prevention/acceleration of diseases (Guarner and Malagelada 2003; Tojo et al. 2014; Blander et al. 2017). Altered composition of the colon microflora, termed dysbiosis, is associated with modulation of differentiation, migration, and function of mucosal and immune cells in the large bowel that increase the risk of colorectal cancer or IBD (Ni et al. 2017; Roy and Trinchieri 2017). On the other hand, selected types of bacteria were found to have a positive impact on functions of gut-associated lymphoid tissue (Tlaskalová-Hogenová et al. 1983; Cebra 1999; Christensen et al. 2002; Umesaki 2014). Therefore, an evaluation of the microbiome and its effects on the tissues, organs and, globally, on the organism, represents an important field of investigation in human and animal physiopathology (Barko et al. 2018).

Under normal physiological conditions, the mucosa-associated immune system is continously activated in conventional animals (CV) because of the presence of commensal microflora. This is demonstrated by the constant cellular infiltration in the CV bowel mucosa (also indicated as a "physiological inflammation"), not present at that level in the mucosa of germ-free (GF) animals (Tlaskalova-Hogenova et al. 2005). To prevent damage to a host's own tissues, the mucosal system has an ability to maintain local homeostasis by impeding commensal bacteria translocation through the mucosa (which, in turn, would generate a classic inflammatory response). This homeostasis also permits the tolerance of immunogenic products of non-pathogenic microflora and so mitigates effects at both the local and systemic levels (Haneberg et al. 1995; Maaser and Kagnoff 2002; Tlaskalová-Hogenová et al. 2004, 2011; Dubois et al. 2005). Reflective of this is that in the first days after the birth of CV animals, the lymphatic tissue is intensively activated––as evident by the increasing presence of immune cells in the appendix and in Peyer's patches (Carter and Collins 1975; Waksman and Ozer 1976). In turn,

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cells from Peyer's patches migrate in the mucosa, ultimately leading to formation of the so-called "gut-associated lymphoid tissue (GALT)" (Mestecky et al. 1978; 1980; Hall 1980).

A different route of evolution of GALT occurs in GF animals due to the absence of a microbiome (Sterzl 1979). In GF animals, Peyer's patches are underdeveloped and show a lower intra-mucosal lymphocyte infiltration due to the lower antigenic stimulation compared to that in CV animals (Tlaskalová-Hogenová et al. 1983). Further, compared to under CV conditions, in these GF hosts, the lymph nodes, spleen and liver display lower weight, while the cecum is much larger and with a thin wall (Khlystova 1976; Vannucci et al. 2008). The stimulation of GF animals using known antigens or bacterial strains, with consequent activation of immunity, has become an important tool to studying the adaptation of immune structures and the development of immune responses under the selected conditions (i.e., gnotobiology) (Pollard and Sharon 1970; Sterzl et al. 1987).

Interestingly, since the early years of GF animal preparation and study, it appeared that when a bacterial colonization was occurring, the bowel acquired an aspect and activity like in the CV animals. This illustrated the importance of microbiota in sustaining bowel structure and function, effects that at the beginning were considered secondary to metabolic modifications (Freter and Abrams 1972; Pesti 1979; Koopman et al. 1984). At present, with the continuously growing knowledge about the mucosal immune system, the importance of investigating the relationships between structures and immunity in the colon appears more relevant.

Accordingly, we hypothesized that immune stimulation chronically induced by commensal microflora may directly influence local tissue structure, with effects on both gross morphology of the bowel and on the microscopic structure of the mucosa, in particular influencing collagen organization. The latter (re: deposition and re-organization) is involved in tissue re-modelling under inflammatory conditions (especially chronic inflammation), as well as in wound healing, and cancer (Diegelmann and Evans 2004; von Lampe et al. 2000; Madsen et al. 2017). The studies reported here document that commensal microflora in CV rats increase the complexity of the collagen scaffold. These studies also, for the first time, show that the collagen architecture significantly differs in CV rats from that in GF rats, and links the differences to the microbiome-activated immunity in the bowel mucosa of CV animals.

## Materials and methods

### Experimental animals

All experiments were performed using inbred male Wistar-AVN (F89) rats, reared in the animal colony of the Institute of Physiology of the Czech Academy of Sciences (CAS) in Prague under pathogen-free conditions (Zidek 1968; Jurcovicova et al. 1984; Stepankova et al. 1995). Part of these rats was used to produce germ-free animals in the Laboratory of Gnotobiology of the Institute of Microbiology of the CAS (Nový Hrádek, Czech Republic). The CV rats were housed under normal conditions in the animal facility of the Institute of Microbiology of the CAS in Prague. Nutrition by standard ST-1 pellet diet and water intake were without restrictions. The GF rats were obtained by the standard procedure developed by the Laboratory of Gnotobiology (Khlystova 1976; Vannucci et al. 2008). In brief, this consisted of the harvest of the uterus of a pregnant rat female at term, under general anesthesia and with subsequent sacrifice. After washing in antiseptic solution, the uterus was transferred to a sterile transparent Trexler-type plastic incubator. Here the new-borns were delivered by Cesarean section and then immediately passed into a communicating sterile incubator (definitive place of their rearing). Initially, the rats were hand-fed every 3-4 hr with a specifically formulated milk diet; this was subsequently substituted with a standard pellet diet with controlled endotoxin content that had been sterilized by irradiation (59 kGy). Throughout the entire experiment, the GF rats were maintained inside the sterile incubators at a constant 60% relative humidity and temperature of 22 °C, under filtered air flux. The GF rats had *ad libitum* access to the diet and filtered water. Weekly tests for sterility were regularly performed on samples of feces/other materials that were collected and cultivated for the presence of bacteria and fungi (Stepankova 1979; Sterzl 1979; Vannucci et al. 2008). All experiments were performed according to the rules of the European Convention for the Care and Use of Laboratory Animals as approved by the Czech Animal Care and Use Committee and by the Institutional Ethical Committee.

### Design of experiment

At 6-8-wk-of-age, rats (≈150 g BW; 15 CV and 10 GF) were euthanized by exanguination under general anesthesia (0.8% chloralium hydrate). The collected blood was used for mononuclear cell separation and flow cytometric (FACS) analysis of lymphocyte sub-populations. Gross examination of internal organs was performed and spleen and mesenteric lymph nodes (MLN) were harvested. Part of each sample was used for obtaining immune cells for FACS analysis and part was snap-frozen in liquid $N_2$ for later tissue analysis. The large bowel was isolated, removed, longitudinally opened to remove stools, and washed with cold saline. Colon samples were taken either for immediate confocal microscopy evaluation on fresh tissue or fixed in 10% buffered formalin for standard histology. Colon mucosa samples were collected by gentle scraping with a glass slide and put into cryo-centrifuge tubes, snap-frozen in liquid $N_2$ and stored at −80 °C for use later in protein analysis.

### Peripheral blood mononuclear cell (PBMC) and splenic mononuclear cell preparation

Spleen and heparinized peripheral blood samples were collected at sacrifice and kept on ice before isolation of leukocytes. Spleen samples were homogenized in 3 ml of FACS PBS (PBS + 0.1% gelatin and 0.02% sodium azide) solution. All samples, including blood, were then centrifuged in QUICK mode (1000 rpm) and supernatants were removed. The pelleted cells in each sample were then washed three times with H-MEMD (10 ml) and then centrifuged at 1550 rpm for 5 min. The pelleted cells were re-suspended in H-MEMD and then separated over a Telebrix-Ficoll mix by centrifugation (2080 rpm, 25 min, 25 °C). The layer/buffy coat containing lymphocytes was removed, washed three times in H-MEMD, and then re-suspended in 5 ml H-MEMD. The cells were then counted using Turk's solution (50 µl suspension to 950 µl Turk's solution) and counted in a Bürker chamber.

### Fluorescence-activated cell sorting (FACS) analysis

Aliquots of the cell suspensions were placed in wells of 96-well plates (at $5 \times 10^5$ cells/well). FACS-PBS containing 10% rat serum autologous (10 µl) was added to each well and the plates

were incubated for 20 min on ice. The plate was centrifuged (1680 rpm, 2 min, 25 °C) and resulting supernatants removed. The pellets were then treated with a panel of primary monoclonal antibodies against rat CD3, CD4, CD8a, and CD25 (BD Pharmingen, San Diego, CA) as well as NKRP-1 (Invitrogen, Boston, MA), each with distinct fluorescent labeling. After a 30-min incubation on ice, the plates were centrifuged (1680 rpm, 2 min, 25 °C), well super-natant was removed, and the pelleted cells in each well washed three times each with 200 µl FACS PBS. For labeling with a secondary antibody, the same steps were performed, followed by three washings. Finally, the pellets in each well were re-suspended in 60 µl FACS PBS before 10 µl propidium iodide (PI; Sigma, St. Louis, MO) was added to visualize dead cells before flow cytometry was performed on a Becton-Dickinson LSR II system (Becton-Dickinson, Princeton, NJ) using system associated software. A minimum of 50,000 events/sample was acquired.

For the evaluation of lymphocyte sub-populations, all single cells were gated for live cells; lymphogate was then applied by scattering cell type identification, and ultimately the cells were then gated for CD3± and NKR-P1± cells. Sub-populations were then gated and analyzed for specific marker expression as: NK cells ($CD3^{-}NKRP\text{-}1^{+}$), $CD4^{+}$ NKT cells ($CD3^{+}NKRP\text{-}1^{+}CD4^{+}CD8^{-}$), all T-cells ($CD3^{+}NKRP\text{-}1^{-}$), T-helper ($T_H$) cells ($CD3^{+}NKRP\text{-}1^{-}CD4^{+}CD25^{-}$), T-cytotoxic $CD8^{+}$ cells ($CD3^{+}NKRP\text{-}1^{-}CD4^{-}CD8^{+}$), and T-regulatory ($T_{reg}$) cells ($CD3^{+}NKRP\text{-}1^{-}CD4^{+}CD25^{high}$).

### Confocal microscopy analysis

Second harmonic generation (SHG) technique was used for investigation of collagen fiber structures. For this, a Leica TCSSP2 AOBS confocal laser scanning microscope (based on a DM IRE2 inverted microscope) equipped with an argon laser (Leica Microsystems, Wetzlar, Germany) was employed. A 20X magnification by water immersion plan apochromatic objective HC PL APO CS (WD 5 250 lm, NA 5 0.7) was applied; this yielded sufficient resolution while offering a relatively large field of view for general structure evaluation. A 43X apochromatic objective was used for obtaining images at higher magnification. In some cases, to further analyze particular fields of interest found during the 20X observations, a digital zoom was used. For visualization of reflection (reflectance mode-RM) in the tissues, excitation wavelengths of 488, 514, and 633 nm were applied, singularly or in various combinations. The triple combination permitted to obtain histology-like images from unstained, fresh tissue. The reflected signal was detected in up to three corresponding channels, using a detection wavelength range of 478-498, 504-524, or 623-643 nm, respectively. Auto-fluorescence of non-stained tissues was detected by 2-photon microscopy at 860 nm, which implied an expected detection of SHG signal at a 430 nm wavelength (Chernyavski et al. 2009). The detection of an SHG signal was checked by changing the excitation wavelength to 800 nm, and then detecting the emitting signal by setting up a band pass filter for 400 nm.

Finally, image analysis was performed to obtain objective measurement of the collagen scaffold organization (Levy et al. 2015; Gao et al. 2018). The intensity of each pixel in a given microscopic field was analyzed using Image J software (NIH, Bethesda, MD). The analysis was applied for defining various parameters (integrated density [ID], inter-cryptal space, distance between centers of crypts, and skewness) characterizing the mucosal structure and the collagen scaffold. At least five representative fields were examined in each image; a minimum of five samples were taken for each colon.

### Isolation of proteins and ELISA assay

Protein lysates were prepared according to the manufacturer's protocol. In brief, 400 µl of tissue extraction buffer T-PER (Thermo Scientific, Waltham, MA) containing the protease inhibitors Complete Mini and Pierce Protease Inhibitor to protect samples from spontaneous degradation (1 tablet/10 ml T-PER buffer [Roche Diagnostics GmbH, Mannheim, Germany and Thermo Scientific). Tissue samples were then homogenized on Lysing Matrix D ceramic balls (MP Biomedicals, Irvine, CA) using a FastPrep-24 Instrument (MP Biomedicals) in two cycles of 60-sec homogenization at 6.5 m/s. The vials were then centrifuged (15,000 rpm, 15 min) and resultant supernatants collected and stored at −80 °C for later analysis.

The analyses were performed according to manufacturer protocols in a reaction volume of 100 µl/well and measured in a doublet. For this, all samples were weighed and lysed with 400 µl lysing buffer/mg sample. The concentration of proteins in the lysate was checked using a UV/VIS NanoPhotometer P300 system (Implen GmbH, Munich, Germany) and adjusted by addition of lysing buffer to achieve a final concentration of 2.5 mg/ml. Results for each sample were then extrapolated from standard curves, and presented as pg/ml. The ELISA kits used were for rat: interleukin (IL)-1α (eBioscience, San Diego, CA), IL-6 (R&D Systems, Minneapolis, MN), transforming growth factor (TGF)-β1 (Thermo Scientific), and interferon (IFN)-γ (PeproTech, Cranbury, NJ). All plates were analyzed using a Multiskan Ascent ELISA plate reader (Thermo Scientific).

### Histology and immunohistochemistry

Samples were fixed with in 10% buffered formalin solution for 24 hr before paraffin embedding. Slices (3-5-µm thick) were cut from each sample using a microtome, transferred to standard slides (Bamed, Litvínovice, Czech Republic), and stained with haematoxylin-eosin solution (DiaPath, Martinengo, Italy). Serial slices were cut and transferred to special silanized positive slides (DAKO, Glostrup, Denmark). Collagen structures were detected using picrosirius staining (Sirius Red kit, DiaPath). All final preparations were evaluated by two independent certified pathologists using an Olympus Provis BX50 light microscope (Tokyo, Japan); pictures were taken using an Olympus DP 70 camera with QuickPhoto Micro 23 program (Promicra, Prague, CZ).

### Statistical evaluation

All results are expressed as means ± SEM. Differences in distributed parameters between groups were compared using a non-parametric unpaired Mann-Whitney test with a two-tailed P-value calculation. Differences were considered statistically significant at $p \leq 0.05$. All data were analyzed using Prism software (v.5.0, GraphPad, San Diego, CA).

## Results

### Microscopic aspects of the Colon mucosa in GF versus CV rats

Differences in the colon mucosa of rats reared under either GF or CV conditions were evaluated using classical histology,

including picrosirius staining to visualize the collagen matrix in the samples and by direct imaging of unstained fresh samples using laser scanning confocal microscopy. In this case, the reflectance mode (RM) in 1-photon confocal microscopy was used to obtain histology-like images; the second harmonic generation (SHG) in multi-photon confocal imaging was used to assess the collagen mucosal scaffold. The results showed the different impact of microbiome on the constitution of the colon mucosa. In RM imaging, looking to the most superficial aspect of the mucosa not washed of the mucus (immediately in contact with the glass) it was possible to distinguish a presence of bacteria within the mucus in the CV rat (Figure 1(A)) that was not present in the GF hosts (Figure 1(B)). The different mucosal environment induced differences in the immune cell distribution and in the stroma organization (Figure 2). In the inter-cryptal spaces, a larger number of immune cells could be seen in the colon with microbiota in comparison with the section of the axenic colon mucosa, with a ratio around 3:1. The picrosirius staining evidenced a better organized and more complex network of the collagen scaffold under CV than under GF conditions where it appeared to be less regular and with lower complexity. Using the RM technique in confocal microscopy on fresh tissue, it was possible to see similar aspects of the crypts—with a larger opening subtly layered by mucus in the GF mucosa (Figure 2(D)). The inter-cryptal spaces were better defined and more symmetric in the mucosa samples from the CV hosts than from the GF ones.

Better confirmation of the histological findings was obtained by further evaluation of the collagen scaffold characteristics using the SHG in multi-photon confocal microscopy to evaluate freshly harvested, unfixed, and unstained samples (Figure 3(A–D)), and subsequent image analysis (Figure 4(A–D)). The images confirmed a well-developed and regular organization of the collagen scaffold in the mucosa of the CV rats, while less elaborated structures were seen in samples from the GF rats. In CV rats, the collagen architecture appears constituted by a tiny and complex network of collagen fibers producing a diffuse sustain around the crypts. The structure was also organized to create slots that could allow positioning of immune cell inside inter-cryptal spaces. The scaffold organization in the GF mucosa similarly appeared as a fiber networking around and within the crypts, but with less complexity in its general architecture, with a more rough constitution (Figure 3(A,B)). This disparity is well illustrated also by different aspect of the largely developed and complicated framework of the intra-mucosal lymphatic follicles in the CV rat versus a less developed and structurally less elaborated architecture in the GF rats. This could be put in relation to the larger accumulation of lymphatic cells in the CV rat colon than in the GF colon (Figures 3(C–H)).

The organization of the collagen scaffold in the CV rat mucosa can offer a suitable support to the locally active immune cells (Figure 3(I)) and the subdivision of follicles in small chambers could allow grouping of a limited number of cells together. The images suggest the importance that the structural organization of the collagen net may have to facilitate activated cell migration as well as cell-cell interactions (Figure 3(J)). It would seem that the relationship between immunity and tissue structure development appears to allow for more efficient immune cell activity and interactions.

Image analysis confirmed the microscopy observations showing significant values for the collagen fiber signal (integrated density) and its matrix complexity (skewness), both being greater in the CV vs. in the GF rat mucosa (Figure 4(A,C)). Tighter inter-cryptal spaces and consequently a reduced distance between the centers of the crypts indicated a more compact organization of the colon mucosa in the CV rats (Figure 4(B,D)). These results indicated that the continuous presence of bacteria and the constant immune activation due to their presence have influence on the structural organization of the colon mucosa.

### *Baseline production of cytokines in Colon mucosa, spleen, and mesenteric lymph nodes*

Select cytokines involved in inflammatory (e.g., IL-1α, IFNγ, IL-6) and regulatory (e.g., TGFβ1) responses—as well as involved in induction of tissue re-modelling, were evaluated by ELISA. Specifically, these analyses sought to quantify the spontaneous, natural, unstimulated production of cytokines in the healthy animals. Proteins were extracted from samples of the right and left colon mucosa, spleen and mesenteric lymph nodes (see Methods). Full mucosa samples were used to globally evaluate the local microenvironment. Comparison between right and left colon in CV and GF hosts was done to detect possible constitutive differences unrelated to any presence of a local microbiome.

### *Colon mucosa*

The production of the evaluated cytokines (Figure 5) did not show significant differences between the right and left colon

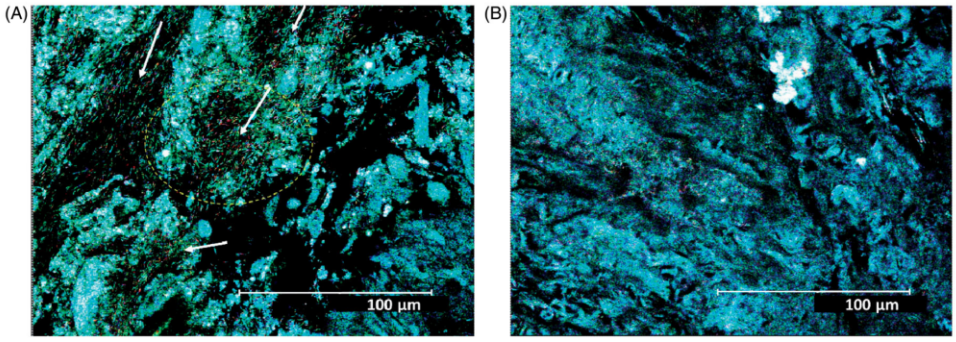


Figure 1. Surface of rat colon mucosa with mucus in CV- and GF-reared animals. (A) CV rat colon mucosa with evidence of bacteria (also evidenced in circle and by arrows). (B) GF rat colon mucosa with no evidence of bacteria. Confocal microscopy was performed on fresh samples using RM imaging. Original magnification = 43X.

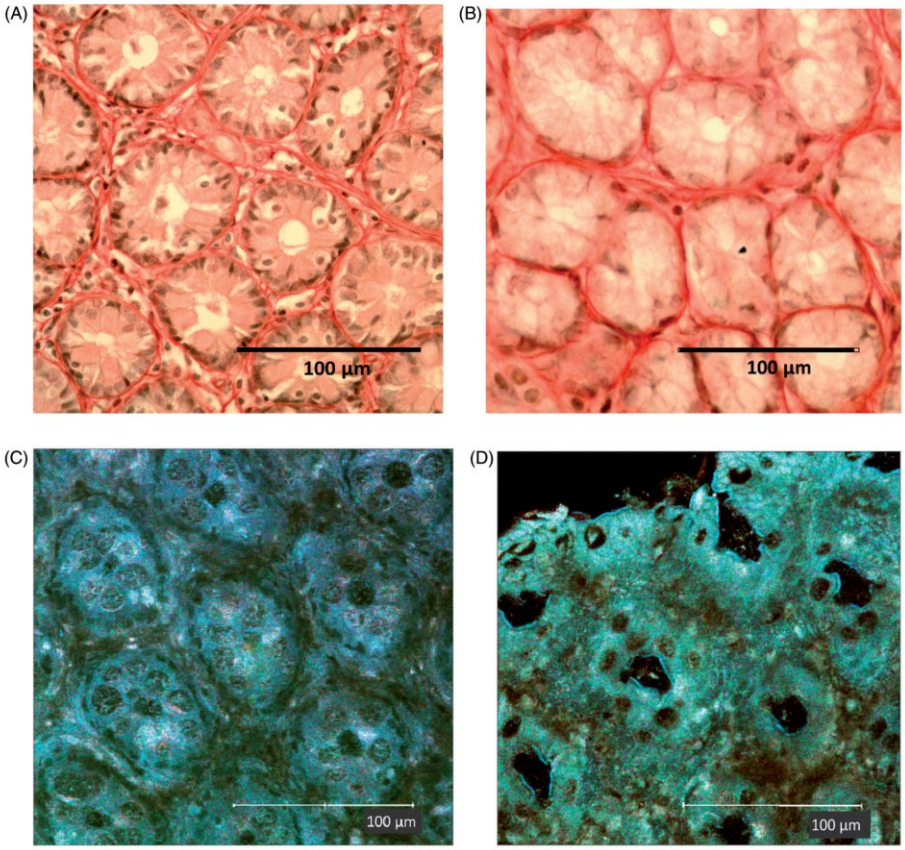


Figure 2. Transversal section of colon mucosa in (A, C) CV and (B, D) GF rats. The represent-ative images show differences in the inter-cryptal spaces and organization of the collagen fibers (see text). The immune cells in the spaces within the crypts (A,B: dark dots) are more represented in the CV rat mucosa (in accordance with a presence of commensal microflora) than in the GF rat mucosa. (C,D) Aspect of mucosa in fresh samples examined by confocal microscopy reflectance mode technique. (A and B) Samples are colon mucosa sections with picrosirius staining of collagen (red). Magnification = 40X.

mucosa either under CV or GF conditions, except for the IL-6 in the CV rats. In that case, the IL-6 level in the CV left colon was lower than in the right colon, but the difference was not significant. In contrast, when comparing the CV and GF rats, levels of IL-1α were significantly higher in the CV right colon. Levels of IFNγ were down-regulated in the CV in comparison to GF rats in both compartments, but the change did not reach significance. Compared to in the GF rats, IL-6 was found at similar levels in the right colon, but significantly down-regulated in the CV left colon ($p = 0.0006$). TGFβ1 was found at almost the same levels in both sites and under both conditions, albeit though that it was seen to be significantly increased in the CV right colon vs. in the GF counterpart ($p = 0.0305$).

### *Spleen*

Levels of IL-1α, IL-6, IFNγ, and TGFβ1 were also in the spleen homogenates (Figures 6(B,D,F,H)). While no significant differences were found in levels of the pro-inflammatory cytokines between the CV and GF rats, a significant difference was found for TGFβ1, with much higher levels in the CV than in the GF rats ($p = 0.0016$). The elevated TGFβ1 production could explain a non-increase in pro-inflammatory cytokines (especially IL-6) in the CV hosts, despite any ongoing stimulations by intestinal microflora. Consequently, it could be suggested that the spleen might be involved in the general homeostatic regulation that permits a co-existence of the microbiome with the host organism.

### *Mesenteric lymph nodes (MLN)*

Levels of IL-1α, IL-6, and TGFβ1 evaluated in MLN isolated from the rats did not reveal any significant differences between the CV and GF rats (Figure 6(A,C,E,G)) even though there was more variability among their levels in the CV than in the GF rats. A sign of immune activation by microbiota resulted in increased IFNγ production in the CV rats as compared with a negligible production seen in the GF hosts ($p = 0.0211$). The levels of IL6 in the CV rat MLN were relatively higher than in GF animals, though the difference was not significant.

## FACS analysis

### *Blood*

The main differences in lymphocyte sub-populations in the peripheral blood (Figure 7) of CV and GF rats was a prevalence of

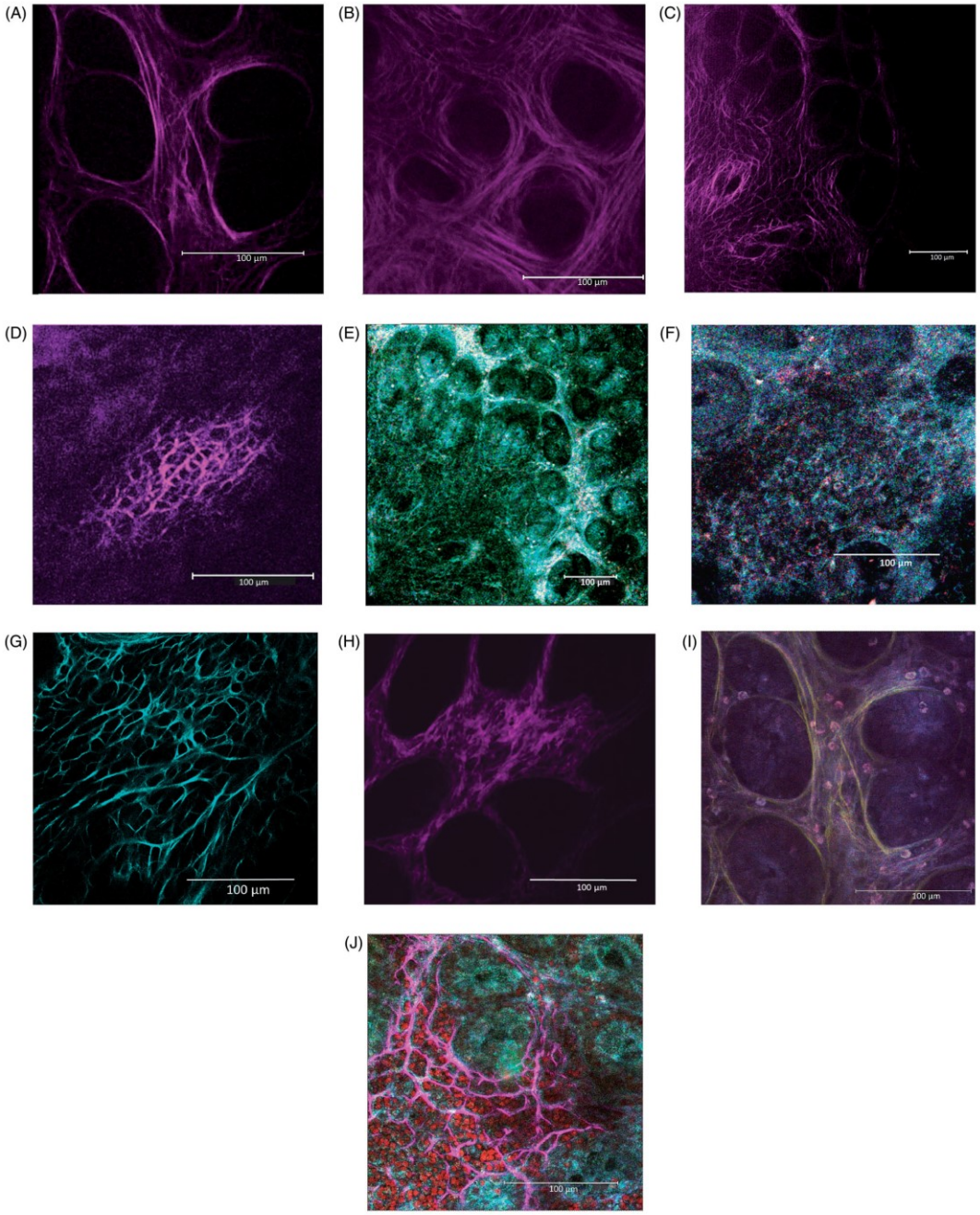


Figure 3. Collagen scaffold of left colon mucosa in (A, C) CV and (B, D) GF rats using SHG in multiphoton confocal microscopy. (A,B) Structure of collagen scaffold sustaining crypts is shown. (C,D) Intra-mucosal lymphatic follicles are shown. Different complexity and collagen fiber conformation is evident between microbiome-trained mucosa vs. axenic mucosa. (E,F) Same place in RM in 1-photon confocal microscopy. (G,H) Other intra-mucosal follicles confirming the lower complexity and dimension of the structure in (H) GF rat mucosa are shown. (I) Sample in A is imaged by RM, and then merged with SHG image (here in yellow). Degree to which immune cells are in relation with/oriented by disposition of the scaffold fibrous structure is evident. (J) Immune cells can find a place in limited numbers and in tight contact inside slots created by complex structure of well-developed intra-mucosal follicles of CV rats. (A, I) CV left colon mucosa, magnification 43X. (B) GF left colon mucosa (43X). (C, E) CV Left colon follicle (20X). (D, F) GF Left colon follicle (43X). (G) CV Right colon follicle (20X). (H) GF right colon follicle (43X). (I) RM imaging, 1-photon confocal microscopy merged with SHG image (original magnification 43X). (J) Merged RM, SHG imaging and fluorescence detection of Draq5 nuclear staining of immune cells performed on fresh tissue: particular of a lymphatic follicle in CV left colon mucosa (original magnification 63X).

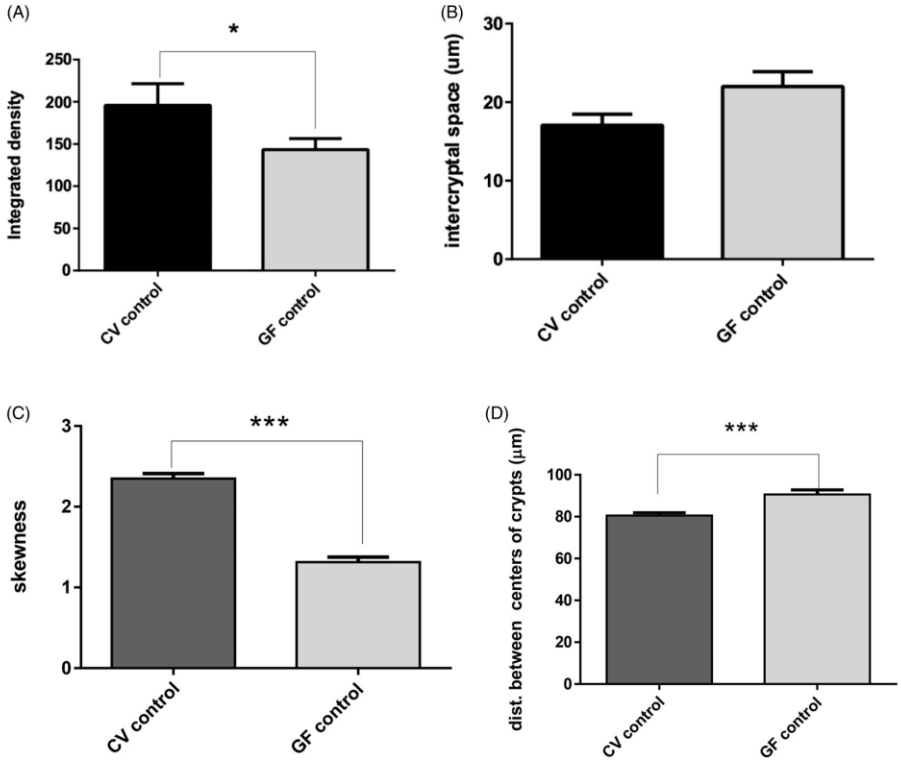


Figure 4. Imaging results. Confocal imaging was analyzed by various criteria, from which integrated density values were found to be significantly higher in CV rats ($p = 0.0209$). Distances between centers of the crypts were significantly higher in GF rats ($p = 0.0007$). The opposite trend was found in skewness, which was higher in CV rats ($p < 0.0001$). Differences in inter-cryptal spaces between CV and GF rats were non-significant. Significance: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

NK and NKT cells in the GF animals ($p = 0.0191$ for NK cells). In the T-cell compartment, no differences were found in $CD3^{+}$ cell population (All T-cells), while $CD8^{+}$ cells were slightly more represented in the CV rats ($p = 0.0465$) and $CD4^{+}$ cells were significantly higher in the GF rats ($p < 0.0001$). No significant differences were found for $CD4^{+}CD25^{high}$ $T_{reg}$ cell values.

### *Spleen*

Compared to in the spleens of CV rats (Figure 8), GF rats presented with a significantly higher number of NK cells ($p = 0.0381$) and NKT cells ($p = 0.0221$). No significant differences were found at the T-cell level, with the exception of $CD8^{+}$ cells ($p = 0.0095$). The levels of $T_{reg}$ cells were greater in spleens of the CV animals, though the difference did not reach statistical significance (still suggesting a role for immune homeostasis in the presence of microbiome).

## Discussion

The anatomical differences in the bowel under CV and GF conditions suggest that dynamic relationships between the intestinal microbiota and both local and systemic immunity are able to influence the structures of the intestinal wall. Previous observations by various authors showed changes in the GF bowel anatomy after bacterial colonization, with conversion to CV animal characteristics. Therefore, the presence of the commensal microflora was considered an efficient factor for shaping the bowel anatomy (Freter and Abrams 1972; Pesti 1979; Koopman et al. 1984). The idea of a relationship between different bacterial strains and collagenolytic activity was also recently reported by Guyton et al. (2019). In the present study, whether in GF or CV hosts immunological conditions may have a possible role in shaping the colon mucosa structure was investigated.

The current study also looked at possible immunological differences between the right and left colon, due to their different physiology and microflora (Donaldson et al. 2016; Flynn et al. 2018; James et al. 2020). Spontaneous cytokine production in the colon mucosa of CV and GF rats only displayed mild differences. Similar results were found in the comparisons between the two parts of the colon under each condition. The most relevant variation noted was a down-regulation of pro-inflammatory cytokines IL-6 and IFNγ especially in the CV left colon mucosa and the higher production of TGFβ1 in the CV spleen. What was new and significantly different here were the findings about the organization of the collagen scaffold, i.e., it was less structured in the colon of GF rats than in CV rats, especially at the level of intra-mucosal lymphatic follicles. Taken together, these results indicated that the microbiome could influence cytokine production at the local and systemic level (spleen) and had effects on the organization of the collagen scaffold in the colon mucosa. Moreover, the mild differences found between the two

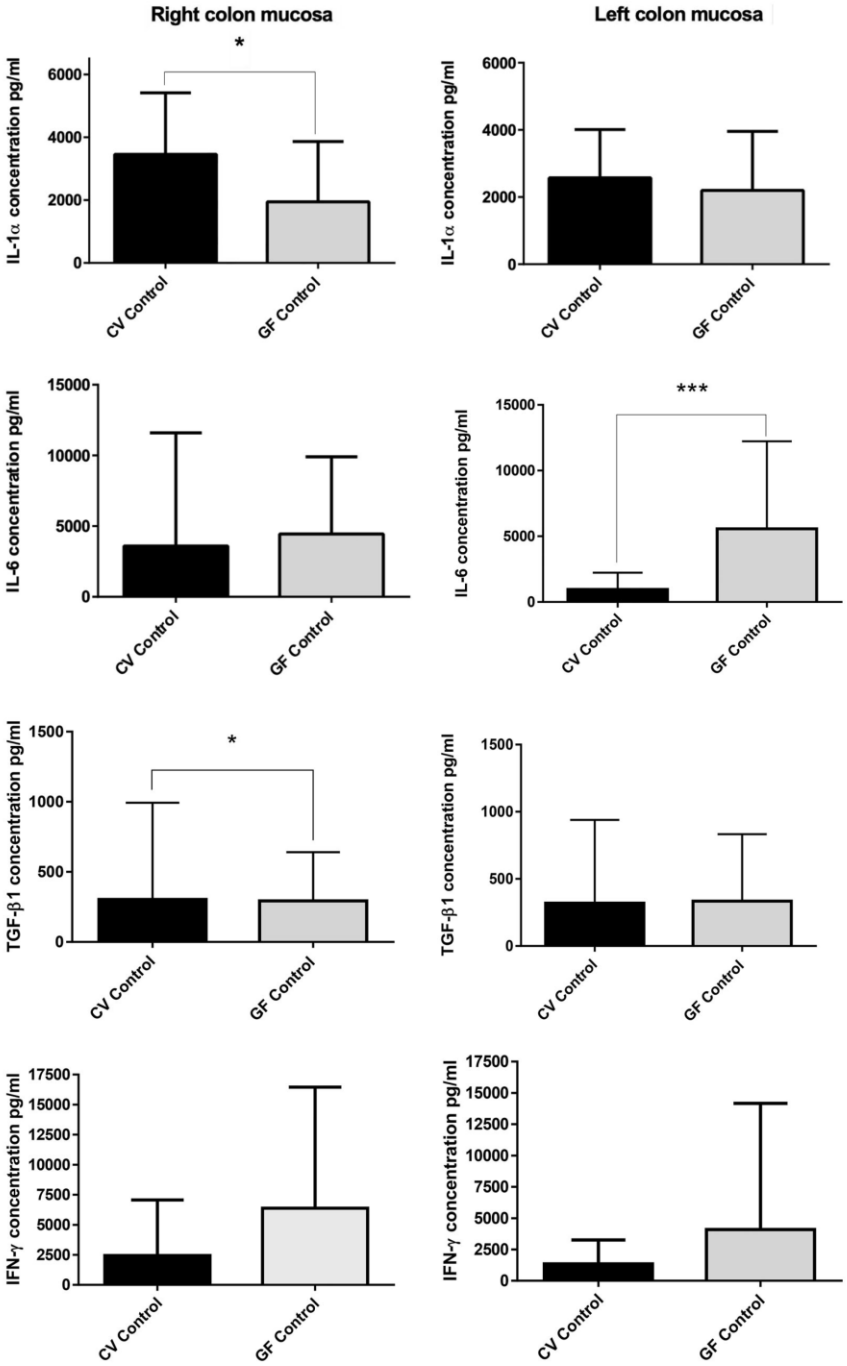


Figure 5. Spontaneous cytokine levels. Right and left colon of CV and GF rats (full mucosa samples). IL-1α production was significantly higher in right colon mucosa of CV rats ($p = 0.0131$); same trend was found in TGFβ1 production ($p = 0.0305$), while IL-6 was significantly higher in left colon mucosa of GF rats. Differences in the production of other cytokines were non-significant. Data reported as pg/ml. Significance: $^{*}p < 0.05$, $^{**}p < 0.010$ and $^{***}p < 0.001$.

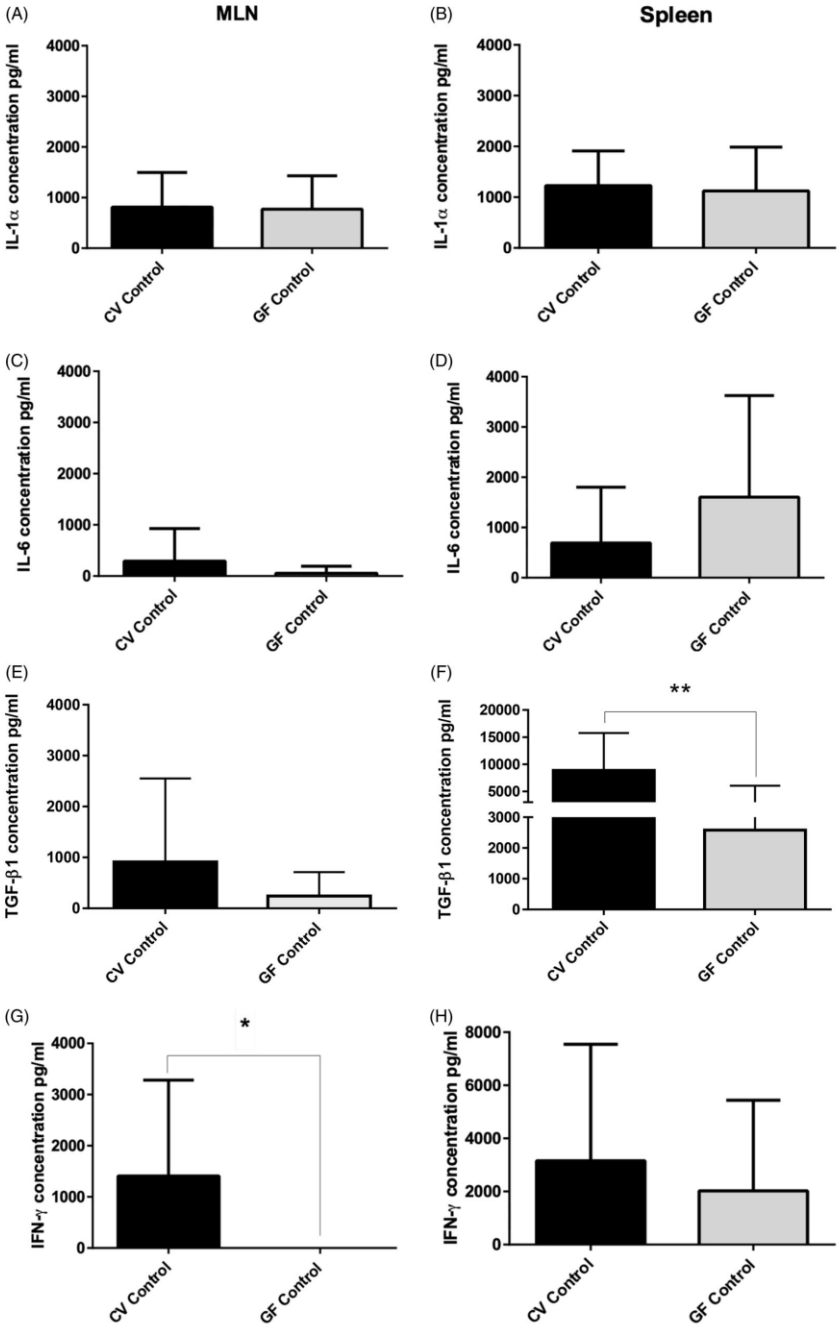


Figure 6. Spontaneous cytokine levels. (A, C, E, G) Mesenteric lymph nodes and (B, D, F, H) Spleen of CV and GF rats. Significant differences in production were found for TGFβ1 and IFNγ; TGFβ1 was higher in spleen of CV rats ($p = 0.0016$) and IFNγ was higher in MLN of CV rats ($p = 0.021$). Data reported as pg/ml. Differences in production of cytokines in other tissues were non-significant. Significance: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

conditions suggested intensive regulatory activity in the healthy CV colon that is able to avoid inflammation (approaching the basic cytokine production seen in the GF rats).

Many studies have described how gut microflora can impact on the immunological asset of the bowel mucosa, even with effects on the whole organism (Cebra 1999; Haneberg et al. 1995;

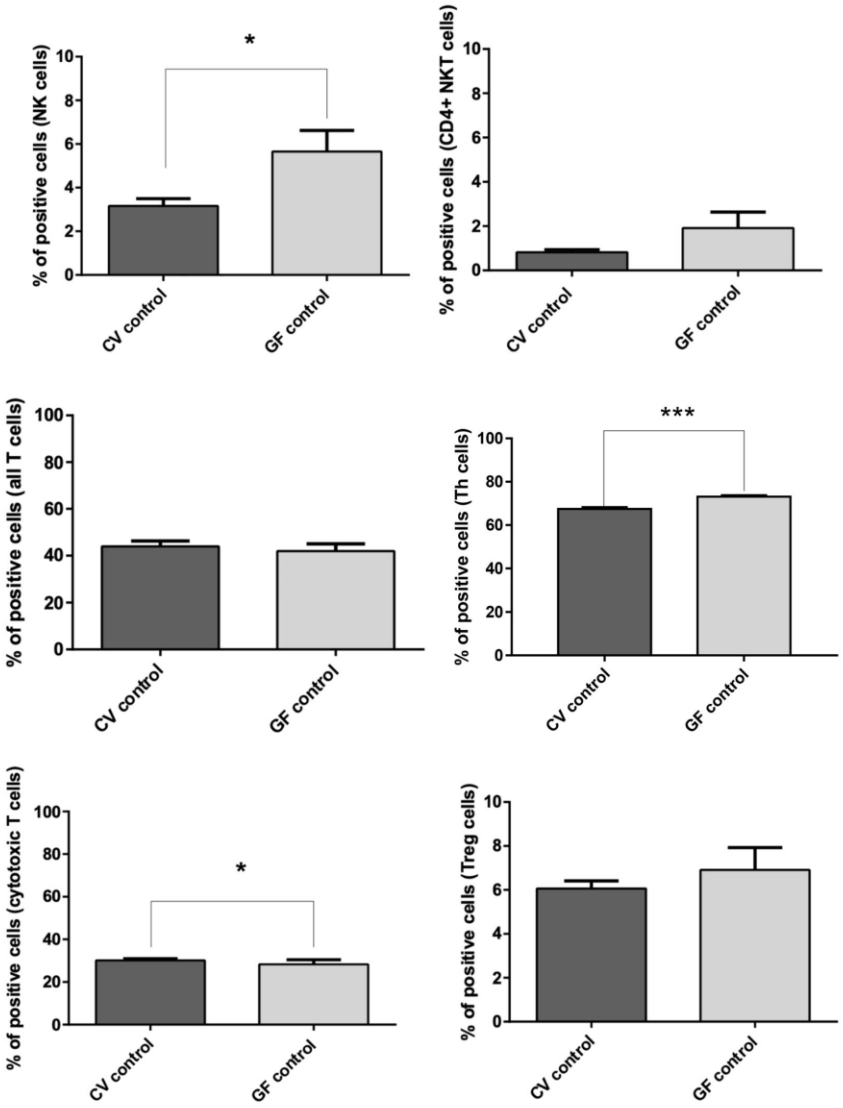


Figure 7. FACS analysis of immune cell sub-populations in peripheral blood of CV and GF rats. Analysis of immune cells showed significantly higher populations of NK cells in GF rats ($p = 0.019$); same trend was found for $T_H$ cells ($p < 0.0001$), while levels of cytotoxic T-cells were slightly higher in CV rats ($p = 0.0465$). Differences in other immune cell sub-populations were not significant. Significance: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Biancone et al. 2002; Pickard et al. 2004; Tlaskalová-Hogenová et al. 2004). Gut immune maturation depends on the composition of microbiota (Chung et al. 2012). Collagen is an important component of the mucosa structure and furnishing a scaffold able to be re-modelled by microenvironmental changes, including inflammatory processes that, when chronic, can bring about fibrosis. A difference in collagen synthesis between CV and GF rats was already described in a bowel anastomosis model by Mastboom et al. (1989). After performing an ileal anastomosis, those authors found hydroxyproline concentration was increased in CV but not in GF rats, where it was even reduced. One may hypothesize that metabolic and signaling products of the bacteria were able to influence fibroblasts in the colon mucosa, which are the main source of collagen fibers (Hooper et al. 2012 Narayanan et al. 1989; Wang et al. 2019; Jurgensen et al. 2020). Bacteria and their strain can differently affect the intestinal fibrosis process and fibroblast activation as shown by Jacob et al. (2018) in an experimental model where stool from mice with defined microflora was transplanted into GF mice. This model also indicated the importance of the microbiome in influencing collagen deposition and how it may be associated with immunological modulation.

The second harmonic generation (SHG) in multiphoton confocal microscopy technique has contributed greatly to the evaluation of collagen (and elastin) components in tissues (Chernyavski et al. 2009). SHG permits a direct approach to

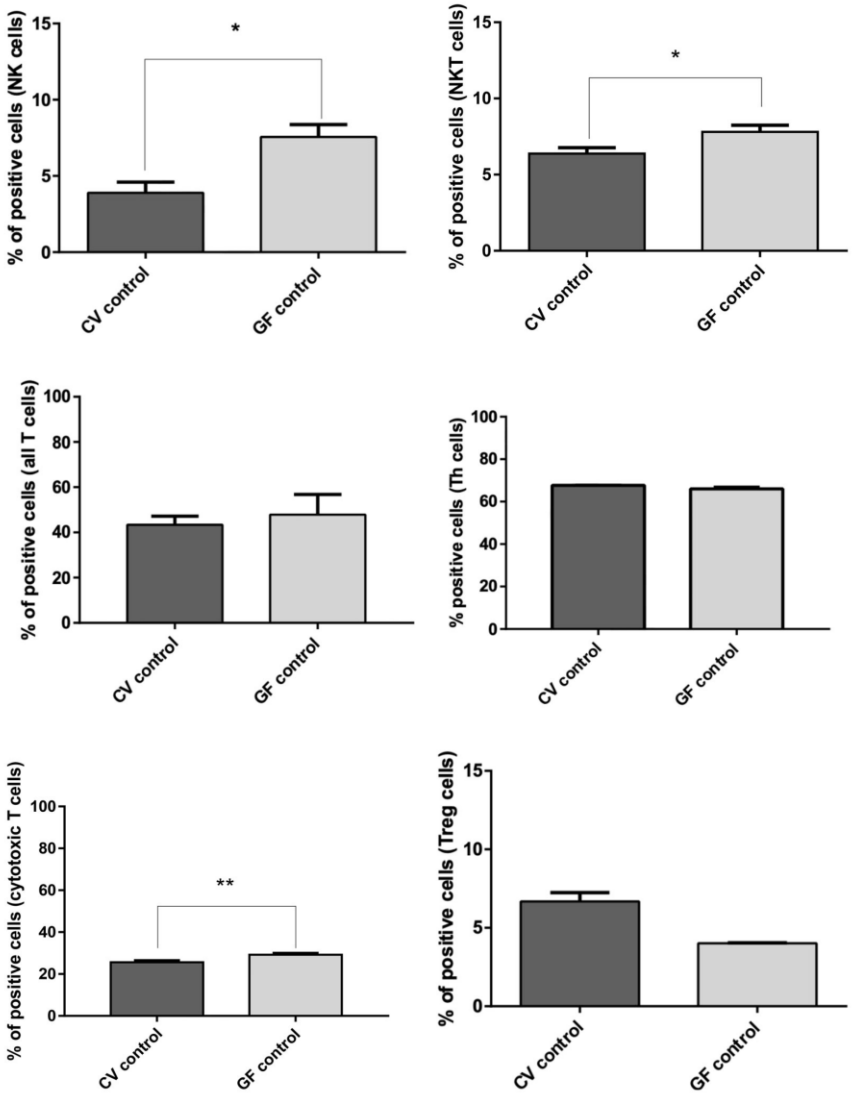


Figure 8. FACS analysis of immune cell sub-populations in spleens of CV and GF rats. Analysis of immune cells revealed a significantly higher population of NK ($p = 0.038$) and NKT ($p = 0.022$) cells in the GF rats. The opposite trend was observed for cytotoxic T-cells, i.e., higher in CV rats ($p = 0.0095$). Differences in other immune cell sub-populations were non-significant, even though $T_{reg}$ cells appeared basically more represented in CV rats. Significance: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

fresh (unfixed and unstained) samples to obtain more realistic picture of structures in their natural context. By image analysis, it is also possible to reveal measurable differences in collagen scaffold organization (Strupler et al. 2007; Campagnola 2011). Applied to the samples in the present study, the analyses confirmed there was a significantly lower complexity of collagen architecture in the GF rat colon mucosa compared to the more elaborated structures in the CV rats (already suggested by picrosirius staining in regular histology). Interestingly, the SHG imaging revealed that the collagen fibers in the CV rats created a fibrous network with free spaces, like slots, in which a limited number of immune cell infiltrates could evolve. These structures, never described before, were less represented in the GF intercryptal spaces and were more evident in the well-developed CV lymphatic follicles (see Figure 3). These novel data further underscore that the intestinal microbiome and activated immunity can have an impact on colon tissue development and structural organization. It is plausible to hypothesize these spaces may allow more efficient migration and homing of immune cells in CV rats, possibly enhancing cell-cell interactions and providing a support to the immune responses elicited by microbes that are present.

A greater presence of IL-1α was found in the CV colon mucosa, while that for TGFβ1 was almost similar in both CV and GF rats. The opposite trend was found regarding IL-6 and IFNγ levels—which were lower in CV than in GF rats, indicating

a down-regulation of these inflammatory cytokines in the presence of commensal microflora. These differences in cytokine expression in the CV rats may be explained even by a direct effect of bacteria which play an important role in regulation of immune responses (Levy et al. 2015; Gao et al 2018). Interestingly, the presence of these cytokines in distal immune organs (spleen, MLN) was higher in the CV rats; the only exception appeared to be IL-6 in the spleen (values were lower in CV than in GF rats samples). Such data suggest that IL-6, important in gut inflammation, is intensively inhibited in CV animals; the same explanation may be applied to IFNγ expression in the colon mucosa. Based on the present results, the mechanism of this local inhibition does not appear directly dependant on the presence of TGFβ1, as might be expected; therefore, a more complex network remains to be clarified.

The levels of TGFβ1—an important regulatory cytokine with inhibitory effects—were similar in both the CV and GF rat mucosa. This suggests to us that the regulatory network controlling "physiological inflammation" in the mucosa of CV rats required a more integrated intervention by other cytokines and molecular processes than only TGFβ1 (e.g., IL-10, IL-4, IL-13). Nevertheless, the higher TGFβ1 levels measured in the spleen of CV rats indicated this organ was involved in systemic immune regulation that allowed for a co-existence of the microbiome in the host. Lastly, no significant differences in cytokine presence between the left and right colon mucosa were noted, except for IL-6, which was lower in the CV left colon than in the right side. This may be associated to the microbiota colonizing the left colon, since in GF rats no differences at all were observed between the two compartments. The influence of how different compositions of the microbiome in the left and right colon could give rise to variations in local levels of cytokines has previously been reported (Samanta et al. 2012; Donaldson et al. 2016; Flynn et al. 2018; Tropini et al. 2017; James et al. 2020).

The FACS analyses performed here confirmed there was a higher proportion of NK, NKT and $CD4^+$ cells in the blood of GF rats, indicating a more prevalent innate cell immunity. Another Wistar-AVN model displayed the same trend in higher NK cells population in GF rats (Vannucci et al. 2008). This observation may be explained by the fact that GF animals have less stimulated adaptive immunity in the absence of continuous challenge by microbiota and related metabolites. As some authors have noted, microbiota can regulate the immune and inflammatory responses of diverse cellular compartments in the gut both directly and indirectly (Kabat et al. 2014; Palm et al. 2015).

## Conclusions

The data here show for the first time that the presence of bacteria in the bowel mucosa of CV rats has a direct impact on microscopic scaffolds in the tissue in relation to the local immune environment. They also suggest that in this rat model, there is a presence of an effective immune regulation in the colon mucosa of CV animals likely required to avoid host inflammatory responses to the local microbiota. These findings can help investigators to better understand the microflora-induced modulation of the local immunity in association with any impact on colon tissue structure, both under physiologic and pathologic conditions. The interaction of the colon mucosa with the intestinal microbiome in CV rats—since birth, the continuous challenge by bacterial antigens and, consequently, a constant immune activation in the mucosa, may "educate" both local immunity and the collagen scaffold (with evident effects on its structure). It would be reasonable to suggest that the collagen scaffold complexity in CV rats could provide for better mucosal barrier functions, and even support more efficient immune cell homing and interactions.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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**Local immune changes in early stages of inflammation and carcinogenesis correlate with the collagen scaffold changes of the colon mucosa**

The aim of this study was to compare the immunological and structural characteristics of the colon mucosa between healthy and treated conventional Wistar-AVN rats. The treatment was based on DSS to induce colitis and AOM to induce carcinogenesis. In both cases a pathological inflammation is rising in the colon mucosa that differs from the continuous immune activation (physiological inflammation) due to the presence of the commensal microbiota. More specifically, we focused on the immunological changes induced by DSS or AOM in animal models at one month after the end of the inductions and possibly related remodelling of the mucosa collagen scaffold. This is an intermediate and still scarcely studied period between the end of acute effects of the inducers and the appearance of the fully pathological alterations of the mucosa and to answer the question if the pathological inflammation in the colon mucosa has modulatory effects on the colon tissue architecture. We found significant changes in the production of cytokines in the colon mucosa between healthy and treated rats with particular role for IL-6 and downregulation by AOM of IL-1α, IFN-γ and not detectable TGF-β1 in the left colon. The confocal microscopy confirmed that also the collagen architecture in the colon mucosa was changed after the treatments. This study confirmed our hypothesis that pathological inflammation in the colon mucosa, induced either by DSS or AOM, has significant impact on the immune and structural processes in this tissue, even though the pro-inflammatory signals are downregulated consensually to an important reduction of the TGF-β1 level, resulting in a smouldering inflammation.

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# Local Immune Changes in Early Stages of Inflammation and Carcinogenesis Correlate with the Collagen Scaffold Changes of the Colon Mucosa

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**Simple Summary:** Chronic colitis and colon cancer develop for alteration of the mucosa homeostatic regulation, also involving TGF-β1. Dextran sulphate sodium (DSS)-induced colitis and azoxymethane (AOM)-induced colorectal carcinogenesis animal models allow for the investigation of the pathological evolution steps. Since chronic inflammation is a common factor, we aimed to explore in rat models the colon mucosa immunological and structural conditions at one month after the end of the inductions, a transition period between acute effects and established lesions. We found, in comparison to healthy controls, downregulation of inflammatory cytokines (except IL-6) and of TGF-β1. At the same time, the collagen scaffold was significantly remodelled in both groups. We conclude that the pro-inflammatory cytokines, in front of a downregulated TGF-β1, sustained a smouldering inflammation with structural changes preparing the niche of both pathologies (ulcerative colitis with fibrosis; tumour). The collagen scaffold changes pointing to an unnoticed inflammation may be suggested as a possible pre-neoplastic condition marker.

**Abstract:** Continuous activation of the immune system inside a tissue can lead to remodelling of the tissue structure and creation of a specific microenvironment, such as during the tumour development. Chronic inflammation is a central player in stimulating changes that alter the tissue stroma and can lead to fibrotic evolution. In the colon mucosa, regulatory mechanisms, including TGF-β1, avoid damaging inflammation in front of the continuous challenge by the intestinal microbiome. Inducing either DSS colitis or AOM colorectal carcinogenesis in AVN-Wistar rats, we evaluated at one month after the end of each treatment whether immunological changes and remodelling of the collagen scaffold were already in development. At this time point, we found in both models a general downregulation of pro-inflammatory cytokines and even of TGF-β1, but not of IL-6. Moreover, we demonstrated by multi-photon microscopy the simultaneously presence of pro-fibrotic

remodelling of the collagen scaffold, with measurable changes in comparison to the control mucosa. The scaffold was significantly modified depending on the type of induced stimulation. These results suggest that at one month after the end of the DSS or AOM inductions, a smouldering inflammation is present in both induced conditions, since the pro-inflammatory cytokines still exceed, in proportion, the local homeostatic regulation of which TGF-β1 is a part (inflammatory threshold). Such an inflammation appears sufficient to sustain remodelling of the collagen scaffold that may be taken as a possible pathological marker for revealing pre-neoplastic inflammation.

**Keywords:** colorectal cancer; DSS-induced colitis; chronic inflammation; collagen; tissue scaffold; AOM; tumour niche; IL-6

## 1. Introduction

In the last 20 years, the concept of tumour microenvironment has continuously developed, becoming an effective working model. It can permit the proper reconstruction of the relationships between tissue components and transformed cells and allows for a better understanding of the events and mechanisms of the tumour evolution. This is important to properly design and elaborate anticancer interventions and more appropriate clinical approaches [1–4]. The tumour microenvironment consists in the co-presence of transformed cells together with normal cells of the original tissue, stromal cells and their products (e.g., collagen), neovascularisation, nerves, lymphatics, and immune cells and their products. The immune cells are an important component of this network, and inflammation is recognised to have a relevant part in the carcinogenesis process and tumour development. The activation of innate immunity elicited by tissue stress signals under the pressure of the developing tumour clone stimulates a local inflammatory Th1 response activating natural killer (NK) cells and macrophages and further involving the adaptive immunity [1,2,5]. The failure of the anticancer response is associated with the shift of immunity towards an immune-suppressive and tissue remodelling Th2 response and the accrual of T regulatory (Treg) cells, M2 macrophages, and of myeloid derived suppressor cells (MDSC) that deliver in the microenvironment immune suppressive molecules (e.g., TGF-β, IL-10, arginase-1) [6]. Stromal structures are also involved in the process [7,8]. The interactions between immunity and fibroblasts shaping the chronically inflamed tissues, such as in the inflammatory bowel diseases (IBD), have progressively attracted the interest of researchers for their importance both in neoplastic and non-neoplastic pathologies and for the active role of fibroblasts in these contests. A fibrotic reaction with increased collagen deposition is often found in various types of tumours [7–9]. In relation to this, the IBD as well as the colorectal carcinoma (CRC) offer a particularly valuable model to study and compare the mechanisms and effects of inflammation, since these different pathologies affect the same structures in the same organ. Moreover, the bowel mucosa is a composite structure and can be considered as the most important immunological organ in the body due to its rich and diffused mucosa-associated lymphocyte population. For its particular characteristics, including the continuous interaction between mucosal immunity and commensal microflora, the bowel mucosa can be considered an excellent model for evaluating inflammation and its regulation [10–14].

Chronic inflammation resembling IBD or cancer-promoting conditions can be easily induced in mouse and rat models using dextran sodium sulphate (DSS) for IBD-like colitis or azoxymethane (AOM) for colorectal carcinogenesis [15–18].

DSS is a branched poly-α-D-glucoside in a sodium sulphate formulation that permits solubility and stability in water, and, at 30–50 kDa MW, has colitogenic effect. It induces a strong intestinal inflammation when orally administered in drinking water to experimental animals, for an established period, at the concentration of 1–5%, depending on the chosen experimental animal model. It produces the disruption of epithelial barrier function, increasing permeability and favouring trans-mucosal penetration of bacteria [15,18]. They can interact with bacterial molecular pattern Toll-like receptor (TLR-2, TLR-4), activating

pro-inflammatory cytokine production, mainly by the macrophages. Release of alarmins may derive by damage of colonocytes due to nano-lipocomplexes of DSS and medium-chain-length fatty acids (MCFAs) that can fuse with the cell membrane producing cytotoxic effects [19]. The consequent acute inflammation leads to further mucosal cell damage, infiltration of activated immune cells into the colon epithelia, and tissue alterations with also loss of mucosa continuity (erosions, ulcers). DSS-related acute tissue injury can progress to IBD-like chronic inflammation if repeated cycles of the polysaccharide are applied. In this process, IL-6 plays an important role, even in the progression to fibrosis [18,20–25].

AOM, the most commonly used colorectal carcinogen, is an active derivative of 1,2-dimethylhydrazine. Cytochrome P450 2E1 (CYP2E1) transforms AOM in methylazoxymethanol that induces O6-methylguanine adducts in the DNA, leading to G→A transitions [16]. Mutations in the KRAS gene with activation of the PI3K/Akt pathway, β-catenin mutations, accumulation and decrease of TGF-β active form, and inhibition of TGF-β pathway are all mechanisms involved in the AOM colorectal carcinogenesis process [26]. Moreover, increased TLR-4 was also found to be associated with the AOM-induced tumorigenesis [27]. AOM induces formation of aberrant crypt foci in the colon tissue with following sequence from adenoma to carcinoma after repeated administrations. Its activity is enhanced by cancer promoters such as secondary biliary acids and induced inflammation [28–30].

Even though DSS colitis and AOM carcinogenesis models are widely applied, studies are still ongoing as to their mechanisms of action and effects at cellular and immunological levels. A lack of information remains about the documentation of biological and immunological features in the transition period between the acute and the fully established outcomes of each type of induction.

Therefore, in this study, we selectively focused on the events at one month from the end of DSS and AOM induction in rat model in order to describe possible structural and immunological changes in the colon mucosa characterising this transition period. The results indicated that evident collagen structure remodelling and local immune changes are already present in both models at this time point (Figure 1). The immune network appeared deregulated and even partially suppressed, but with relative pro-inflammatory cytokines in comparison to the regulatory TGF-β1 levels. These new results indicate that the modification of an "inflammatory threshold" maintaining the immunological homeostasis in the colon mucosa may permit the persistence and resilience of inflammation even under apparently controlled conditions (as a latent inflammation).

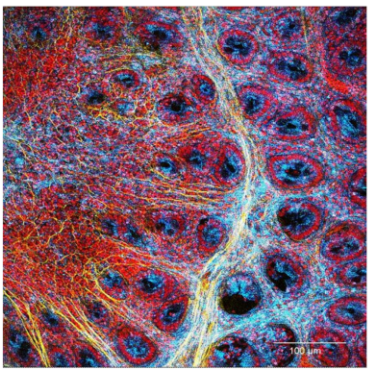

**Figure 1.** Lymphatic follicle in the colon mucosa at one month after the end of DSS colitis induction. The collagen structure surrounding the follicle (yellow) appears thickened. Increased signal of the connective tissue is present also in the inter-cryptal spaces (lower right in the figure, light blue and yellow colours). Merging of Draq5 nuclear staining (red, confocal microscopy), mucosa (blue, reflectance confocal microscopy—RCM), and collagen (yellow, second harmonic generation—SHG by multi-photon confocal microscopy); objective HC PLAPO 20×/0.70 lMM CS with water immersion.

## 2. Materials and Methods

### *2.1. Experimental Animals and Design of the Experiment*

Male inbred AVN-Wistar (F89) rats, 150 g medium body weight, were purchased from and housed in the Institute of Physiology of the CAS, v. v. i. in Prague, Czech Republic [31–33]. The rats were reared under standard conditions, with 12 h light/darkness cycle and without food and water restrictions. All experiments were designed to include minimally 5 animals per group in each used method. The housing conditions and the experimental procedures respected the rules of the European Convention for the Care and Use of Laboratory Animals (2010/63/EU) as approved by the Czech Animal Care and Use Committee and by the Institutional Ethics Committee.

Dextran sodium sulphate (DSS, MW = 36,000–50,000 Da, cat. no. 160110 lot. no. 5237K, MP Biomedicals, Irvine, CA, USA) was used to induce chronic colitis, and azoxymethane (AOM, Sigma-Aldrich, St. Louis, MO, USA) was used to induce colon carcinogenesis.

Colon inflammation was induced by 3% DSS sterilised water solution, supplied to the rats by drinking ad libitum during three treatment cycles of 7–10 days each. DSS solution administration was interrupted at the appearance of acute symptoms of colitis (anal oedema, diarrhoea, and/or rectal prolapse or bleeding) and substituted with drinking water ad libitum during the recovery period of ~1 week before the following cycle (Figure 2). Animal scoring for disease activity index (DAI) was performed at the end of each cycle and at the end of the experiment, i.e. at one month after completing the DSS treatment. The scoring system was changes in weight loss (score: 0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; and 4, >20%), stool consistency (score: 0, formed; 1, mildly soft; 2, very soft; and 3, watery stool), and bleeding (score: 0, normal; 1, brown; 2, reddish; and 3, bloody).

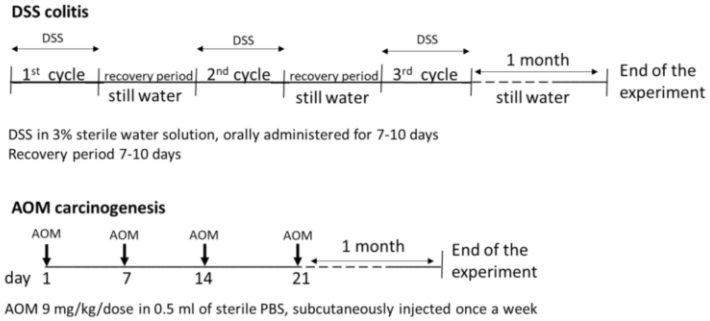


**Figure 2.** Design of the experiment.

Carcinogenesis was induced by subcutaneous injection of 4 doses of AOM, 1 dose weekly, 9 mg of AOM/kg/dose in 0.5 mL of sterile PBS (Figure 2). All rats were sacrificed one month after the last cycle of treatment by total bleed under general anaesthesia (0.8% chloralium hydrate). The necropsy was performed with gross examination of internal organs. Spleen and mesenteric lymph nodes (MLN) were harvested. The large bowel was isolated, longitudinally opened, and washed from stools with cold saline, and the total length was measured. Before the sampling of the mucosa, samples of fresh colon tissue were taken, partly for direct confocal microscopic analysis and partly to be fixed in 10% buffered pH 6.9 formalin for standard histology. Samples of colonic mucosa (right and left colon) were collected by gently scraping it with a sterile glass slide. Part of the obtained mucosa was immediately snap-frozen in liquid nitrogen and stored at −80 °C for further analyses, part was stored in RNA later (Life Technologies, Carlsbad, CA, USA) and at −80 °C, if not immediately processed, to be used for real-time polymerase chain reaction (RT-PCR) analysis (Life Technologies, Carlsbad, CA, USA). The fresh colon samples were directly transported to the confocal microscope for immediate evaluation. The specimens for histology were sent to the pathologist.

### 2.2. Histopathological and Histochemical Evaluation

All samples were fixed with a 10% buffered pH 6.9 formalin solution, preserved for 24 h and then embedded in paraffin, processed according to standard histological methods. Three to five micrometre-thick slices were cut from each sample and were stained with haematoxylin–eosin (HE). For histochemistry, other serial sections were cut from each sample and stained with picrosirius staining for collagen type I and reticulin using Sirius Red kit (DiaPath, Martinengo, Italy) according to the manufacturer's protocol. The final preparations were evaluated and described using an Olympus Provis BX50 microscope (Olympus, Tokyo, Japan). Images were acquired using an Olympus DP 70 Photo camera with QuickPhoto Micro 23 program (Olympus, Tokyo, Japan). Samples were independently evaluated by two expert pathologists (P.R. and P.M.).

Confocal Microscopy Analysis

All observations were performed with a Leica TCSSP2 AOBS laser scanning confocal microscope based on Leica DM IRE2 inverted stand and equipped with an argon laser. Images were acquired using a HC PLAPO 20x/0.70 lMM CS multi-immersion objective with water immersion (WD 0.250 mm). For higher magnification, we used either digital zoom of areas of interest or, in some cases, a HCX PL APO 63x/1.20 W Corr water-immersion objective (WD 0.220 mm). For visualisation of reflection (reflectance confocal mode—RCM) from all tissues under study, we applied three excitation wavelengths of 488, 514, and 633 nm with overlaying acquired images, resulting in a histology-like final image. The reflected signal detection was obtained in up to three corresponding channels, using detection wavelength ranges of 478–498, 504–524, or 623–643 nm, respectively. Each of the three different excitation wavelengths applied in reflection mode was valuable and brought specific information due to its specific scattering characteristics. The second harmonic generation (SHG) technique was used for investigation of collagen fibre structures. The autofluorescence of non-stained tissues was detected by 2-photon excitation microscopy at the wavelength of 860 nm, which implied expected detection of SHG signal at the wavelength of 430 nm, as previously described by Chernyavskiy et al. [34]. To confirm the detection of SHG signal, we checked if the signal disappeared when the excitation wavelength was changed to 800 nm, and then re-appeared after setting up a band pass filter for 400 nm. Image J software® (NIH, MA, USA) was used for analysis of the intensity of each pixel in a given microscopic field by various parameters: integrated density (ID), inter-cryptal space, and distance between centres of the crypts and skewness (symmetry and regularity of fibre distribution). For each examined image, analysis was performed of almost 5 representative fields per sample. For each colon, a mean of 5 samples was taken.

### 2.3. Isolation of RNA and RT-PCR Analysis

The colon mucosa samples were stored in RNA later (Life Technologies, Carlsbad, CA, USA) at −80 °C until further analysis. Total RNA was isolated from tissues using TRIZOL reagent according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Purity of RNA was measured using a UV–VIS NanoPhotometer® P300 (Implen, München, Germany). Only samples with OD260/280 between 1.8 and 2.0 were used for RT-PCR analysis. Quality of RNA was evaluated by denaturing agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA). DNAse I-treated total 2 µg of RNA were reverse-transcribed using Oligo(dT)20 primer and Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA), and then we used a real-time PCR (RT-PCR) reaction to determine the changes in the mRNA level. The level of expression of selected genes was evaluated by using the iCyclerTM iQ5TM RT-PCR detection system (Bio-Rad, Hercules, CA, USA). The list of studied genes and used primers is found in Table 1. The PCR reaction was performed in a volume of 25 µL, containing 4 µL of cDNA (10 ng/µL), 12.5 µL of SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), and 500 nM of each primer. Non-template controls were included in all experiments. The setup of RT-PCR reaction was as follows: 3 min at 95 °C, 40 cycles of 94 °C for 30 s, 59 °C for 40 s, and 72 °C for 70 s. The temperature

was then gradually increased to 95 °C to obtain the melting curves of the amplified fragments. The efficiency of primer pairs was estimated around 98%. All reference genes (Gadph, Pgk I, Ppia, Polr2A, Surf1, Tbp) were validated using Bestkeeper, Normfinder, and RefFinder algorithms, and Ppia gene was chosen as the most stable in the defined reaction conditions [35,36]. Reverse transcription was also performed without Superscript III for all samples (No-RT-control) and successful removal of gDNA was confirmed by PCR. Ppia gene was used in the assay. No PCR product was detected in all samples. Changes in the gene expression were shown as the normalised expression in treated rats compared to controls. Two independent experiments were performed. All parameters were measured in duplicates in each experiment. Changes in gene expression were calculated according to the 2-ΔΔCT (Livak) method [37]. Representative melting curves of the evaluated genes are reported in Supplementary Materials, Figure S1.

**Table 1.** List of primers used in RT-PCR analysis.

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *Il1a* | AAGACAAGCCTGTGTTGCTGAAGG | TCCCAGAAGAAAATGAGGTCGGTC |
| *Il1b* | CACCTCTCAAGCAGAGCACAG | GGGTTCCATGGTGAAGTCAAC |
| *Ifng* | ACTGGCAAAAGGACGGTAAC | ATCAGGTGCGATTCGATGAC |
| *Tgfb1* | TGAGTGGCTGTCTTTTGACG | TCTGTGGAGCTGAAGCAGTA |
| *Ppia* | TACAGGTCCTGGCATCTTGT | AGTTGTCCACAGTCGGAGAT |

### 2.4. Isolation of Proteins

Protein lysates were prepared according to the following protocol: each sample was placed in 400 µL of tissue extraction buffer T-PER (Thermo Scientific, Waltham, MA, USA). Protease inhibitors cOmplete Mini and Pierce Protease Inhibitor were used to protect samples from spontaneous degradation at 1 tablet/10 mL of T-PER buffer (Roche Diagnostics GmbH, Mannheim, Germany; Thermo Scientific, Waltham, MA, USA). Tissue samples were homogenised by FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). Lysing Matrix D ceramic balls were added to each vial to help homogenise the samples (MP Biomedicals, Santa Ana, CA, USA). The procedure was followed by two cycles of 60-s homogenisation at 6.5 m/s speed. All samples were held in cold conditions on ice. Then, the vials were centrifuged (HERMLE Z 383 K refrigerated centrifuge and HERMLE 220.87 V03/V04 rotor, HERMLE Labortechnik GmbH, Wehingen, Germany) at 4 °C and 15,000× *g* rpm for 15 min, and supernatants were collected and stored in −80 °C for further analysis.

### 2.5. ELISA Assay

All protein samples were analysed by sandwich ELISA method. The experiments were performed according to the manufacturer's protocols. The protein lysates, stored at −80° C, were used in a reaction volume 100 µL/well. All samples were diluted to the same concentration and measured in a doublet; concentration of protein lysates was measured by UV–VIS NanoPhotometer® P300 (Implen, München, Germany). The results were calculated according to a standard curve. The following ELISA kits were used: rat IL-1α Matched Antibody Pairs kit (eBioscience, San Diego, CA, USA), rat IL-6 DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN, USA), TGF-β CytoSet (Thermo Scientific, Waltham, MA, USA), rat IFN-γ ELISA Development kit (PeproTech, Rocky Hill, NJ, USA). ELISA plates were measured using Multiskan Ascent ELISA plate reader (Thermo Scientific, Waltham, MA, USA).

### 2.6. Statistical Evaluation

The results are expressed as means ± standard error of mean (S.E.M.). Differences in distributed variables between groups were compared by one-way ANOVA and subsequent

Kruskal–Wallis test using GraphPad Prism™. Statistical significance was considered for $p < 0.05$. Data related to the imaging analysis, ELISA and PCR evaluations are summarized in the Table S1.

## 3. Results

### 3.1. Evaluation of Changes in the Colon Mucosa Scaffold

AOM-treated animals did not present any alteration in functions and behaviour at any time during the entire experiment. A disease activity index (DAI) was assessed for the DSS-treated rats at the end of each cycle of treatment and at the end of the experiment (1 month after the end of DSS induction). Weight loss was mild (DAI score 1) during all experiments, occurring in 25% of animals after the first and second cycles, 8.33% after the third cycle, and 27.27% after one month. At the same observation time, bleeding was evident (DAI score 2–3) in 75%, 83.83%, and 58.33% of rats, respectively, while being absent after one month from the induction. Soft stools and, occasionally, fluid stools (DAI score 2–3) were observed after the first and second cycle, while only grade 2 was observed after the third cycle (with incidences of 75%, 100%, and 91.66%, respectively). After one month, about 63% of the animals still presented softened stools (DAI score 1–2), confirming the established chronic colitis.

All animals were sacrificed at one month after the end of the respective induction protocol. At the autopsy, the gross evaluation of the abdominal and thoracic organs did not show any notable modification or lesion. The colon mucosa, under both conditions, did not present macroscopically visible alterations, and only the colon of DSS animals appeared shortened in comparison to control rats.

Samples from the harvested colons were prepared for histological evaluations and for confocal microscopy analysis. Sections of paraffin-embedded, formalin-fixed tissue were used for classical diagnostic description as well as after picrosirius staining for evidencing the collagen structures. Confocal microscopy was used to evaluate unstained, freshly harvested samples, directly apposed with the mucosal side on the glass, using one-photon (reflectance confocal mode—RCM—for histological-like imaging) and 2-photon (second harmonic generation—SHG—for collagen visualisation) confocal microscopy. The results showed that, already at one month after the end of the inductions, both treatments were able to generate valuable modifications of the colon mucosa structures. Figure 3 displays the images of healthy colon mucosa obtained by classic histology and Figure 4 by 1- and 2-photon confocal microscopy. The figures document that, in healthy conditions, the colon mucosa had an orderly structure, with regular distribution and homogeneous shape of the crypts (Figures 3A and 4A,C). This is in relation with the well-organised architecture of the collagen scaffold, as demonstrated by the SHG imaging (Figure 4B,D). The picrosirius staining also evidenced how the immune cells in the inter-cryptal spaces are in relation with the delicate collagen net that sustains and symmetrically spaces the crypts (Figure 3C,D).

The histology of the DSS-treated animal samples, in comparison with the healthy colon, demonstrated that colitis was present, confirmed by the diffused parvocellular infiltrate in the mucosa and submucosa, variation in size and shape of crypts, the occasional appearance little ulcers (Figure 3E,F), and enhancement of the collagen structure, evidenced by the picrosirius staining (Figure 3G,H). After AOM induction, the histology showed a lower degree of alterations than for DSS, with mild increase of immune infiltrate and some variability in the diameter of the crypts as well as widening of inter-cryptal distance. The picrosirius staining highlighted enhancement of the connective structure (Figure 3I–L).

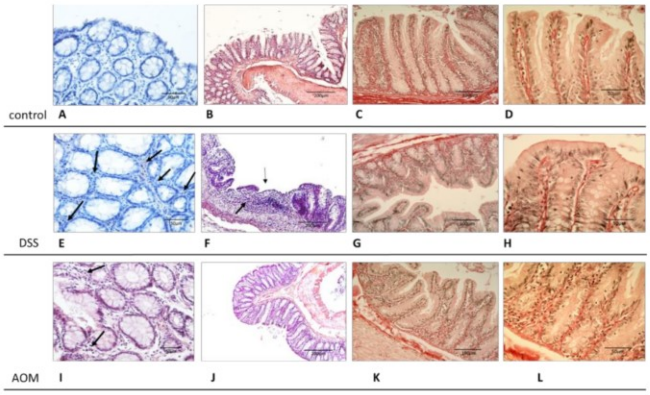


**Figure 3.** Histology of the colon mucosa from healthy, DSS-, and AOM-treated rats. The histological evaluation showed modification in the regular disposition and homogeneous shape of the crypts associated with increased cellular inter-cryptal infiltrate after DSS and AOM treatments in comparison to the control (**A**,**E**,**I**). The DSS-treated group showed evidence of colitis with parvicellular infiltrate ((**E**), arrows, and (**F**)); reduction of the crypt length; and, sporadically, little ulcerations (**F**). Mild changes in the crypts shape and cell infiltrate (**I**, arrows) resulted in the mucosa after AOM induction (**I**–**L**). The collagen structures appeared enhanced after each type of treatment (picrosirius stain, red; (**D**,**H**,**L**)). (**A**–**D**) healthy rats; (**E**–**H**) DSS-treated rats; (**I**–**L**) AOM-treated rats; (**A**,**B**,**E**,**F**,**I**,**J**) haematoxylin–eosin staining; (**C**,**D**,**G**,**H**,**K**,**L**) picrosirius staining; (**A**,**E**,**I**) and (**D**,**H**,**L**) 40× magnification; (**B**,**F**,**J**) 10× magnification; (**C**,**G**,**K**) 20× magnification.

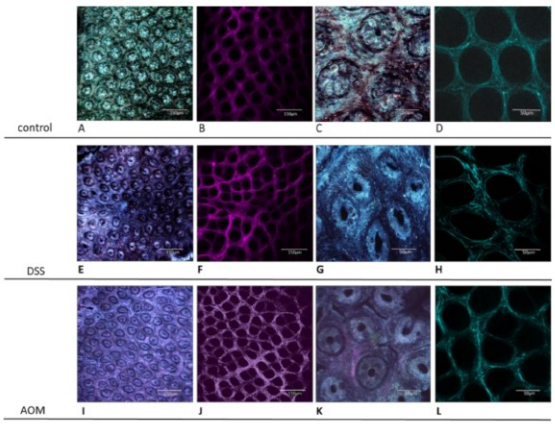


**Figure 4.** Healthy and treated rat colon mucosa evaluation by confocal microscopy. Confocal microscopy revealed more precisely the structural changes in the colon tissue related to the cryptal symmetry (**A**,**E**,**I**) and shape (**C**,**G**,**K**) and the collagen architecture (**B**,**D**,**F**,**H**,**J**,**L**). More irregular and dense organisation of collagen structures induced by both DSS (**F**,**H**) and AOM (**J**,**L**) in comparison with control animals (**B**,**D**). Inter-cryptal spaces were less regular and wider (**A**,**E**,**I**), and the shape of the crypts (**C**,**G**,**K**) resulted in being especially modified by the DSS treatment (**G**). (**A**–**D**) healthy rats; (**E**–**H**) DSS-treated rats; (**I**–**L**) AOM-treated rats; (**A**,**C**,**E**,**G**,**I**,**K**) reflectance confocal mode, 1-photon confocal microscopy; (**B**,**D**,**F**,**H**,**J**,**L**) second harmonic generation, multi-photon confocal microscopy. (**A**,**B**,**E**,**F**,**I**,**J**) HC PLAPO 20×/0.70 lMM CS objective with water immersion; (**C**,**D**,**G**,**H**,**K**,**L**) HCX PL APO 63×/1.20 W Corr water-immersion objective (WD 0.220 mm).

The RCM imaging by 1-photon confocal microscopy also showed that the DSS induction produced variations of crypts in their shape (from round to prevalently ellipsoid appearance, with lozenge-like central lumen) and in their symmetric distribution with irregular inter-cryptal distance (Figure 4E,G). Similar changes, but at a lower extent, were evidenced also after AOM induction (particularly in the lower part of the colon), as well as enlarged inter-cryptal spaces (Figure 4I,K).

The modifications that both treatments induced in the collagen scaffold appeared more relevant when evaluated in SHG. In the healthy colon, the collagen scaffold appears as a continuous net of fibres organised to obtain a cribriform texture. This is supposed to allow elasticity to the structure and spaces enabling immune cell location and transit (Figure 4B,D). Round and regularly spaced holes indicate the places where the crypts are sited, symmetrically distributed and at an almost constant inter-cryptal distance between each other. This regular condition is altered after administration of DSS (Figure 4F,H) and AOM (Figure 4J,L). In both cases, the thickening and loosing of orderly architecture of the collagen scaffold is clearly visible, accompanied, at various extents, by asymmetry and distortion of the general structure, more evident after DSS-induced colitis.

All the cited parameters, i.e., increase of scaffold thickness and complexity (integrated density), divergence of symmetry and regularity of fibrous architecture (skewness), inter-cryptal distance, and distance between the centres of the crypts, were measured and evaluated (Figure 5). Shift towards increased inhomogeneity and irregularity of the system was indicated by skewness value reduction under both treatments (about −25% after AOM and −35% after DSS in comparison to the control) especially under DSS-induced inflammation. Variations of inter-cryptal spaces modifying the crypt symmetric distribution were also documented but, more importantly, the integrated density showed that the level of collagen deposition and assembling can differently characterise the conditions either of chronic inflammation or pro-carcinogenic microenvironment induced by DSS or AOM administration, respectively.

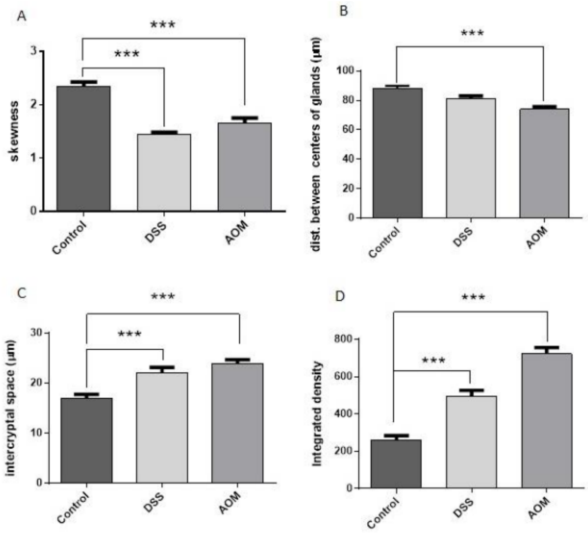


**Figure 5.** Evaluation of collagen distribution and colon tissue structures. In comparison to the control, the skewness variation (**A**) was significant in both DSS and AOM groups; the distance between centres of the crypts (**B**) was significant after AOM, while the inter-cryptal space wideness (**C**) was significant in both DSS- and AOM-treated groups. The integrated density (**D**) showed significant differences from the control and between the groups at $p < 0.001$. Significance by Kruskal–Wallis test: *** $p < 0.001$ in comparison to the untreated control.

### 3.2. Selected Cytokine Production and Gene Expression in the Colon Mucosa 1 Month after DSS or AOM Inductions

In consideration that even though the two inducers are different molecules that act with different mechanisms, the effects on the colon mucosa evidenced by the microscopic evaluation (pro-fibrotic evolution, remodelling of the collagen scaffold, and presence of parvocellular immune infiltrate) were similar, we assumed inflammation would be the common factor in both conditions. Detection of IL-1α, IL-6, IFN-γ, and TGF-β1 levels by ELISA and gene expression of *Il1a, Il1b, Ifng,* and *Tgfb1* by RT-PCR was performed on samples of full colon mucosa. In consideration that the left colon is the most involved in both chronic colitis (ulcerative colitis) and tumours, we evaluated the right colon and the left colon mucosa separately to evidence possible differences of the immune response in the two parts of the colon under the two inductions.

#### 3.2.1. ELISA Results

At the chosen time point, the production of cytokines evaluated by sandwich ELISA assay was similar in both the left and right colon mucosa after each type of induction (Figure 6). Interleukin-6 was increased by both inductions in comparison to the control (twofold higher than control after AOM and sixfold higher after DSS), especially in the left colon mucosa after the DSS administration. The production of the other cytokines instead resulted in being lower or not being modified in relation to the control levels after each type of treatment, with the exception of a slight increase of IFN-γ after DSS. One month after the end of AOM induction, IFN-γ production was decreased in the right colon mucosa and was at non-detectable levels in the left colon mucosa. In both parts of the colon, significant downregulation of IL-1α was also a common feature of both treatments (especially after AOM in the left colon, $p < 0.001$). The TGF-β1 production was significantly lower in the left colon mucosa than in the control mucosa. These results revealed an altered balance between regulatory and pro-inflammatory cytokines, still favourable to inflammation despite the generally reduced cytokine levels. This suggests the hypothesis of a phase in which the microenvironment attempts to recover and adapt after the acute response to the inducers. During this period, the progression to chronic inflammation may be sustained by the removal of an important regulator such as TGF-β1, with the effect of lowering the inflammatory threshold of the tissue. We suggest the existence of an "inflammatory threshold" that can be defined as the regulatory limit within which the inflammatory signals are tolerated without inducing pathological changes in a tissue. The increase of IL-6 in the DSS-treated group and, at a lesser extent after AOM, and altogether the persistence of IL-1α and IFN-γ at still valid levels in relation to TGF-β1, can cooperate to maintain the activity of the inflammation. This is particularly evident after DSS treatment and in the left colon, the target organ of colitis and cancer.

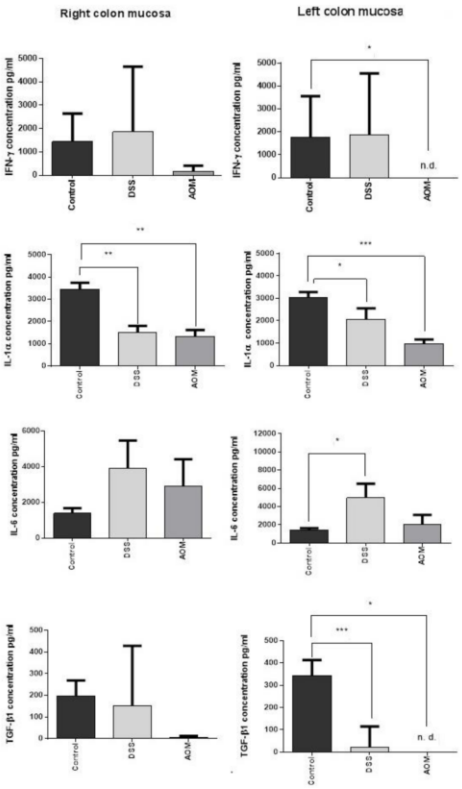


**Figure 6.** Cytokine production in the full colon mucosa of DSS- or AOM-induced rats at 1 month from the end of treatments. The production of all measured cytokines in the right and left colon mucosa showed similar patterns. The significant differences were found in the IL-1α production in both left and right colon between control and treated rats. Increase of IL-6 was particularly significant in the left colon mucosa after DSS colitis induction. Decrease of TGF-β1 was relevant after AOM in all colon mucosa and significant after both treatments in the left colon mucosa, the part of the colon known to be more affected by ulcerative colitis and cancer occurrence. IFN-γ production was slightly increased after DSS (not significant), whereas it was inhibited in the right colon mucosa and especially in the left colon mucosa (not at measurable levels) after AOM pro-carcinogenic treatment. ELISA values are expressed in pg/mL. Significance by Kruskal–Wallis test: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

#### 3.2.2. RT-PCR Results

The expression of *Il1a*, *Il1b*, *Ifng*, and *Tgfb1* was investigated in the right and left colon mucosa samples. At one month after the end of the induction with DSS or AOM, gene expression of pro-inflammatory cytokines resulted in generally being downregulated by both treatments in comparison to the expression in the mucosa of untreated rats (Figure 7). *Il1a* expression levels were much lower than the control in both the right and left colon. *Il1b* did not significantly change in the right colon, while it was significantly downregulated in the left colon after AOM ($p < 0.05$) but not after DSS induction. *Ifng* expression was reduced by both treatments in both parts of the colon.

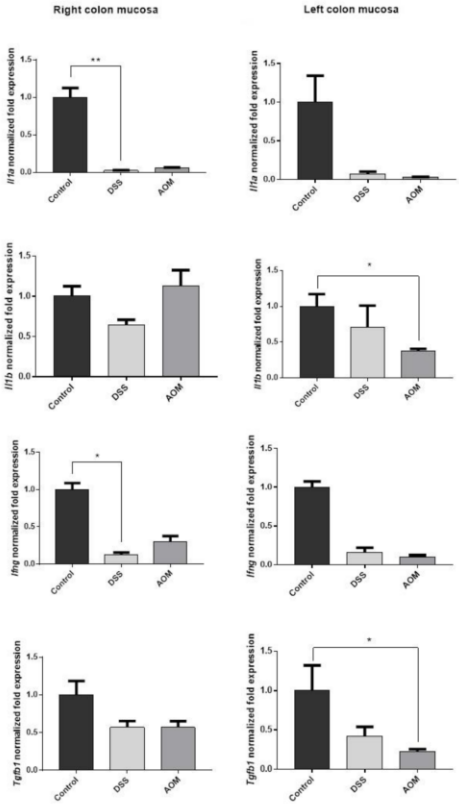


**Figure 7.** The expression of cytokines in the full colon mucosa of DSS- or AOM-induced rats at 1 month after the end of treatments. The expression of all measured genes in the induced rats, either after DSS or AOM, was reduced in comparison with control rats. The downregulation was present in all colons with exception of *Il1b* in the right colon, remaining at the control levels. Significant differences were found, particularly after AOM treatment in both left and right colon mucosa. Gene expression was evaluated according to expression of housekeeping gene *Ppia*. The expression stability of reference gene *Ppia* was confirmed using Normfinder, Bestkeeper, and RefFinder algorithms. Data were normalised to control groups. Significance by Kruskal–Wallis test: * for $p < 0.05$ and ** for $p < 0.01$.

About the regulatory cytokine TGF-β1, its gene expression was also significantly downregulated in the left colon, particularly after AOM treatment ($p < 0.05$). These data confirmed the presence of a downregulated immunological microenvironment during the evolution of chronic colitis and initial carcinogenesis processes at one month from the end of the inductions. They also indicated higher sensitivity of the left colon mucosa to the treatments. The downregulation was generally more significant in the AOM-induced rats.

3.2.3. Regional and Systemic Immune Responses: Mesenteric Lymph Nodes and Spleen

To investigate a possibly consensual systemic immune response (regional and distant) to the treatments targeting the colon, we also performed a preliminary evaluation of the levels of IL-1α, IL-6, TGF-β1, and IFN-γ on mesenteric lymph nodes (MLN) and spleen samples. The MLN collect the lymph drained from the colon, and the spleen is a systemic immune organ. At the MLN level, DSS induction did not produce effects on the IL-1α

and TGF-β1 cytokine levels. Even though the levels of IL-6 and IFN-γ appeared increased, no significance was found. The AOM induction, on one hand, produced increase of IL-6 with significance, and also a non-significant increase of IL-1α. On the other hand, decreased TGF-β1 and inhibited the IFN-γ production (Figure 8). This suggests inflammatory response at the regional level but of limited relevance. In the spleen, even though data did not reach significance, DSS and AOM decreased IL-1α and IL-6, and AOM, but not DSS, inhibited IFN-γ. The elevated level of TGF-β1, even in the untreated control, in comparison to the much lower production of the pro-inflammatory cytokines, suggests the spleen as a regulatory organ.

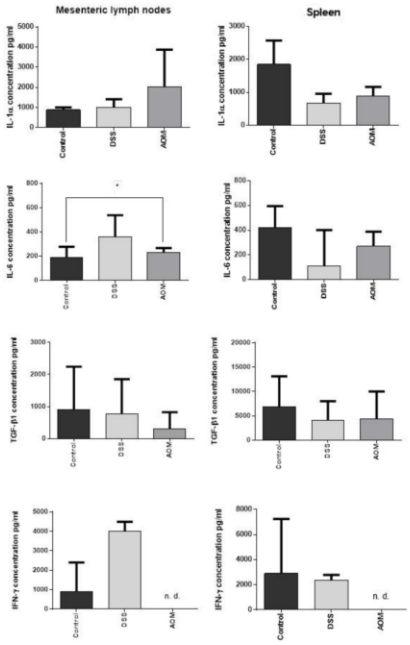


**Figure 8.** The production of IL-1α, IL-6, TGF-β1, and IFN-γ in mesenteric lymph nodes and spleen of DSS- or AOM-induced rats at 1 month from the end of treatments. The cytokine production in MLNs and spleen was indicative of the impact of DSS and AOM on immunity at systemic level. Signs of inflammatory activation were present in MLNs, even at a non-significant level, except for IL-6 after AOM ($p < 0.05$). Reduction (even though not reaching significance) of inflammatory cytokines and (at a lesser extent) TGF-β1 was observed in the spleen. IFN-γ resulted in being not detectable (n.d.) in AOM-treated groups. ELISA values are expressed in pg/mL. Significance was evaluated by Kruskal–Wallis test: * for $p < 0.05$.

## 4. Discussion

The rat model in the present study was evidenced the possible changes produced in the local immunity and in the collagen scaffold by the induction of DSS chronic colitis or AOM carcinogenesis at one month from the end of the treatments. This period is intermediate between the acute immune response immediately at the term of the inductions and the finally established conditions of tumour or chronic colitis, which appear after three or more months from the induction [15,17]. This transition period is almost neglected and not described in the literature. Therefore, we compared the induced chronic colitis and the induced carcinogenesis to evidence whether, at this time point, possible similarities in immunological and structural changes may confirm inflammation as a progressive and central event in cancer development and remodelling of the collagen scaffold. In fact, remodelling of the tissue scaffold is considered a factor that can promote changes in the

epithelial cell behaviour as well as in the local immunity [8,38,39]. Since both processes have the highest clinical relevance in the left colon, possible differences in the microenvironment of right and left mucosa were also considered.

In comparison to the control, similar changes in the evaluated cytokines under the two inductions were found in both parts of the colon, even though AOM appeared more active on the left colon mucosa. Notably, the findings revealed a downregulation of cytokine expression and production in comparison to the healthy mucosa, in particular of IL-1α and TGF-β1 by both inducers, while IFN-γ was especially affected by AOM administration, with relevant reduction in the left colon mucosa of the carcinogen-treated rats. Differently, the IL-6 cytokine increased in all colon mucosa under each of the treatments, particularly after DSS. This result is in accordance with the important role that IL-6 (involved in inflammation but also in tissue remodelling) has both in colitis and in tumour microenvironment development [40–44]. Even though the other inflammatory cytokines were downregulated, overall, they remained at higher levels in relation to the TGF-β1 regulatory cytokine that was decreased in the left colon by both inductions, especially AOM. TGF-β1 is a pleiotropic and regulatory cytokine [45]. It is considered as a double-edged sword in the carcinogenesis with a tumour-suppressing role in the early stages—e.g., in colon cancer development [46]. In later stages, it enhances tumour progression by contributing to the establishment of an immune suppressive microenvironment [47–49]. The TGF-β1 knockout animal models resulted in the elimination of advanced tumours compared to controls, suggesting importance of this cytokine in developed CRC [50,51]. Its reduction at one month after the carcinogenesis induction may underline one of the possible mechanisms favouring the establishment of the developing tumour in the colon. It was observed that AOM can negatively affect TGF-β1 and its pathway [52]. Interestingly, the cytokine production in both left and right colon mucosa was more affected after AOM than after DSS induction. After DSS, the inhibition was almost null in the right colon—generally less prone to colitis, than in the left colon, the target of colitis and even of sporadic or colitis-associated CRC. These differences may be secondary to differences in the local microbiome and its function in metabolising the inducers, especially the AOM that has a specific enterohepatic metabolism to be activated in its more toxic methylazoxymethanol [53–57].

The data about MLN and spleen cytokine variations indicate a possible immune activation at the regional (MLN) and systemic (spleen) levels. They suggest a pro-inflammatory response induced in the referent extra-colonic organ (regional lymph nodes), again sustained by IL-6 prevalence and apparently supported also by IFN-γ in the DSS treated group. On the contrary, non-detectable levels of IFN-γ were found after AOM induction, suggesting even a possible toxic effect on immune cells by the carcinogen. Differently, the spleen appeared to not be involved, indicating that the level of the pathologic process is still local. The relevant levels of TGF-β1 in the spleen, almost unchanged after the inductions, suggest a possible role in the maintenance of the systemic immune homeostasis. Splenic stroma-educated regulatory dendritic cells, for example, induce apoptosis of activated CD4 T cells via Fas ligand-enhanced IFN-γ and nitric oxide [57].

The described downregulation of the evaluated cytokines at one month from the end of the DSS and AOM inductions is a new insight that needs further investigations to understand the bases of these effects after the two types of induction. On the basis of these preliminary investigation results, we can hypothesise an attempt to recover homeostatic conditions after the acute effects of the treatments and/or an "exhaustion" of the local immunity. However, the direct toxicity of the two inducers (or their metabolic products) may also have a negative modulatory effect on immune cells, their viability, and their cytokine production [26,58,59]. Moreover, the responses to DSS or AOM treatment can differ between various rat and mouse strains, suggesting significant involvement of genetic background on the immune response [60,61]. Our observations also suggest the possible involvement of the microbiome in regulating the local immunity in the left or right colon, either due to changes of the microflora directly produced by the inducers or by different metabolic elaboration of the inducers, especially the carcinogen [12,62–65].

Very interesting and novel results were obtained from the microscopic analyses in multiphoton confocal microscopy using the SHG technique of imaging. Already at one month after both treatments, changes in the colon collagen structure were visible. Alteration of the collagen scaffold was already observed in other CRC-based studies using SHG microscopy for detection of changes in the structure of tissues [66–68]. Variation of symmetry as well as of collagen accumulation density in both conditions strongly highlight similar pathogenic mechanisms, i.e., response to inflammation and IL-6 activity. It is known from other studies that the immune activation in the colon can shape the collagen scaffold and that in the particular conditions of the tumour microenvironment, the tumour cells are able to set their own stroma to achieve tumour-promoting functions [69–72]. Moreover, the stiffening of a tissue due to the increase of the collagen accumulation and reorganisation can promote epithelial to mesenchymal transition and even affect the immune cell responses [69,73,74]. The processes of stromal remodelling that we observed in early period after the DSS and AOM inductions present different characteristics in symmetry of the structures and collagen deposition that can be evidenced and measured by SHG imaging. A relevant result is that the control, inflammation, and carcinogenesis groups showed significantly different integrated density (Figure 3), the values related to the signal obtained by the collagen deposited in the evaluated microscopic field. This early detection of collagen structure changes is very promising and can offer new possibilities to implement the pathological diagnosis as a new predictive marker, helping to discriminate between colon inflammation and cancer risk patients. Together with the evaluation of the local immunity, it could help in enhancing the possibilities of diagnosis, treatment, or prevision of these diseases [75,76].

The development of the tumour microenvironment is assumed to be strictly connected to its immune components and their modulation. At present, inflammation is indicated as one of the most important cancer hallmarks [77]. In its evolution, it is characterised by the paradoxical reversion of the original anti-tumour response into a chronic, tissue-remodelling and tumour-sustaining inflammation [1,6]. The establishment of these conditions are still under clarification and the progression of the immunological and biological events is generally described as a continuous process. According to our results, different levels of inflammation may occur in the transition between acute and chronic conditions. Apparently, the downregulation that we observed represents a discontinuity in the progression toward a fully established chronic inflammation. Nevertheless, it is the proportion between the levels of the various cytokines and the balance between pro-inflammatory and regulatory signals that may sustain a "latent" but still effective inflammation that is able to promptly restart under adequate stimulation (i.e., promoters). The situation described in this study documents this latent immune environment and supports its persistent activity as a smouldering inflammation, allowing the alterations in the collagen scaffold, already present in both chronic colitis and carcinogenesis conditions at the same time point [78]. Consequently, it is possible to suggest the existence of an "inflammatory threshold" that in the colon mucosa should be represented by the limit of immune regulation able to contain the immune activation due to the presence of the microflora without damaging inflammation. In case of reduction of the inflammatory threshold, even mild inflammatory signals can actively play and efficiently prepare the progression of the disease [79].

## 5. Conclusions

The observation of the immune activity and collagen scaffold changes at one month after the end of induction either with DSS or AOM has evidenced the possibility of transitory periods in which remodelling of tissue is assisted by a "remodelled" immune response. It can even be a downregulated response but one that is still able to maintain the inflammatory background, despite the apparent recovery from more intense inflammatory conditions. This is a proper example of smouldering inflammation that the changes in the collagen scaffold, first here described, can help to identify. These results provide support to a model of very plastic tumour microenvironment development, modulated and sustained by the vari-

ation of the mucosal inflammatory threshold, and propose new possible pathological markers for better distinguishing chronic and pre-neoplastic inflammation in the colon mucosa.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/cancers13102463/s1, Figure S1: Representative melting curves for the genes used in the study, Table S1: Values of ELISA, PCR and imaging analysis statistical evaluations.

**Author Contributions:** Conceptualisation, F.Č. and L.E.V.; methodology, F.Č., L.E.V., P.V., O.C., and M.L.; software, J.K.; validation, P.S., P.R., P.M. (Pavol Makovický), L.K., and J.D.; formal analysis, F.Č. and D.S.; investigation, F.Č., D.S., L.V., V.V., R.Š., P.M. (Peter Makovický), O.C., and L.E.V.; resources; data curation, F.Č., D.S., V.V., and J.K.; writing—original draft preparation, F.Č.; writing—review and editing, L.E.V.; visualisation, D.S.; supervision, L.E.V.; project administration, L.E.V., P.V., and L.K.; funding acquisition, L.E.V. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and The European Convention for the Care and Use of Laboratory Animals (2010/63/EU), as approved by the Czech Animal Care and Use Committee and by the Institutional Ethics Committee of Institute of Microbiology of the CAS, v.v.i. (protocol code 180/2009, 3.8.2009).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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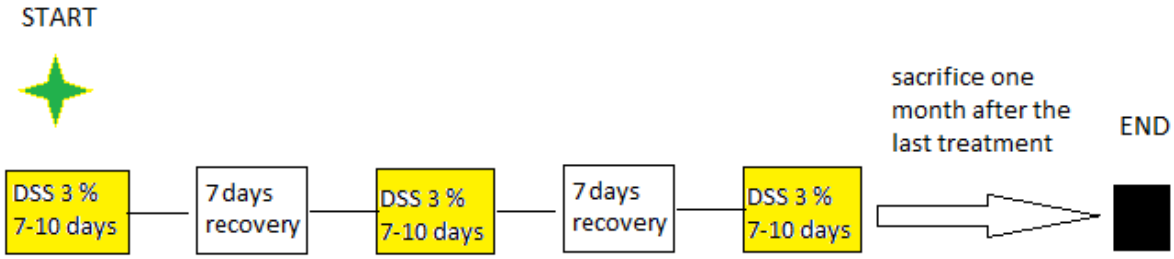
## A: Additional results for Doctoral thesis-part 1 (DSS induction in CV and GF rats)

The results shown in this chapter were obtained from preliminary experiments based on DSS-induced colitis in CV and GF Wistar-AVN rats. Our aim was to investigate how DSS-induced inflammation can modify the local immunity and mucosal structure in the two different conditions (with and without microbiome). The results can give also orientation on the role of microbiota in the DSS-induced colitis. The rats were sacrificed one month after the last dose of DSS. The simplified experimental design can be described as following:




Rats were reared either in conventional or sterile conditions. The colitis was induced using 3% solution of dextran-sodium sulphate (DSS) with 30-50 kDa MW (MP Biomedicals, Santa Ana, CA, USA). The experimental design followed this scheme: 1) colitis was induced by sterilized 3% drinking water solution of DSS per os ad libitum for about 10 days, until the appearance of acute symptoms of colitis (anal oedema, diarrhoea and rectal bleeding). 2) the recovery phase was following the induction phase, when rats were drinking water without the inducer. All rats were sacrificed one month after the end of the last treatment, using a lethal dose of chloralium hydrate. Autopsy was performed, and blood, spleen, mesenteric lymph nodes (MLNs) and colon samples were collected. Blood, MLNs and spleen samples, from which mononuclear cells were separated for FACS analysis. Colon was longitudinally opened and washed from stools with cold saline. Fresh colon tissue samples were taken for immediate confocal microscopic analysis (fixed by 4% paraformaldehyde solution) and representative colon samples were taken for regular histological diagnosis. For that purpose, the samples were fixed in 10% buffered formalin, then embedded in paraffin for further analyses. The colonic mucosa was collected by gently scraping with a glass slide. Samples of colonic mucosa (right and left colon) were collected into cryocentrifuge tubes for ELISA analysis, snap-frozen in liquid nitrogen and stored at -80 °C until the analysis was performed.

The detailed description of other methods (histochemistry, confocal microscopy, ELISA FACS) used for the analysis of samples in DSS-colitis model is described in publications Čaja et al., J. of Immunotoxicol., 2021, 18:1, 37-49 and Čaja et al., Cancers, 2021, 13(10), 2463, attached in the thesis. The preliminary results from above-described experimental model are shown in the following pages, with the description at the end of this chapter.

**Preliminary results from DSS-induced colitis in Wistar-AVN rats, reared in CV or GF conditions, sacrificed 1 month after the last dose**

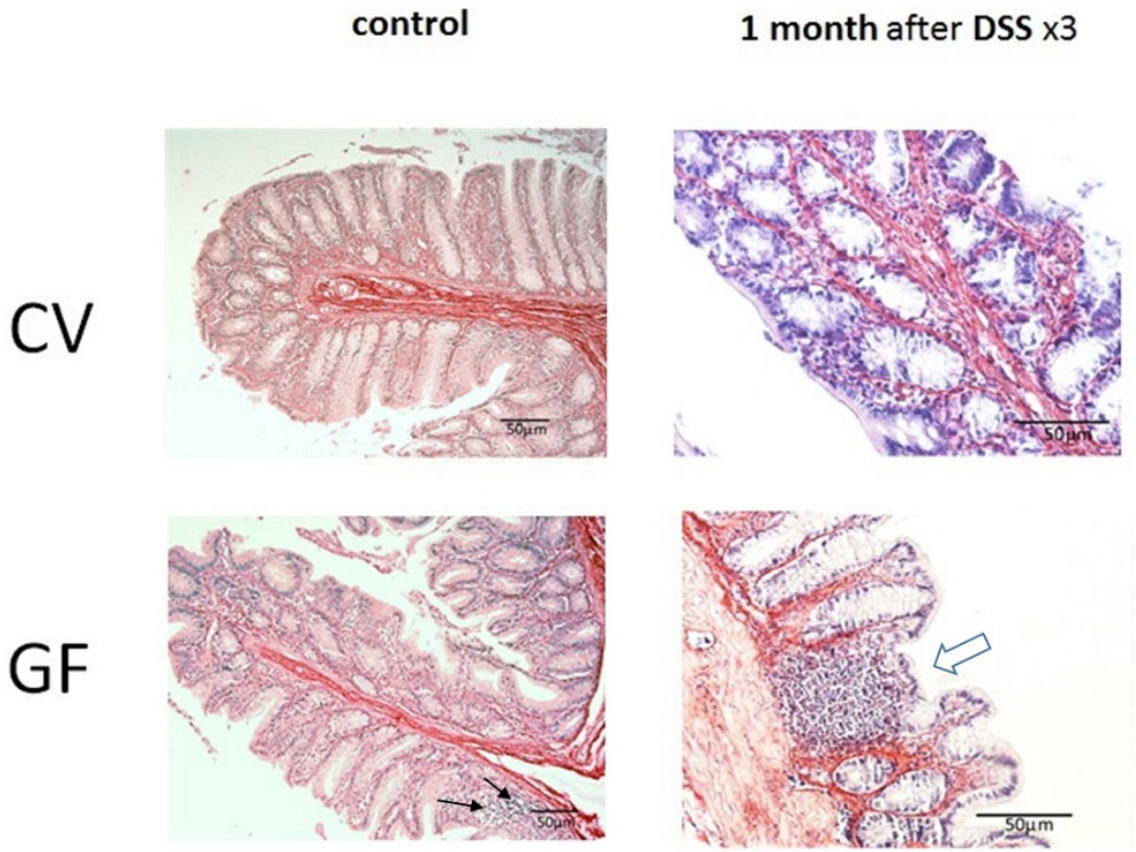



**Figure 1:** Histochemistry-microscopic analysis of colon tissue using Picrosirius red staining. CV, GF controls-10x magnification; CV DSS-induces colitis-40x magnification; GF DSS-induced colitis-20x magnification. Black arrows show a hypotrophic follicle in the GF control mucosa; empty arrow indicate expanded intramucosal follicle in the GF colon after DSS stimulation.

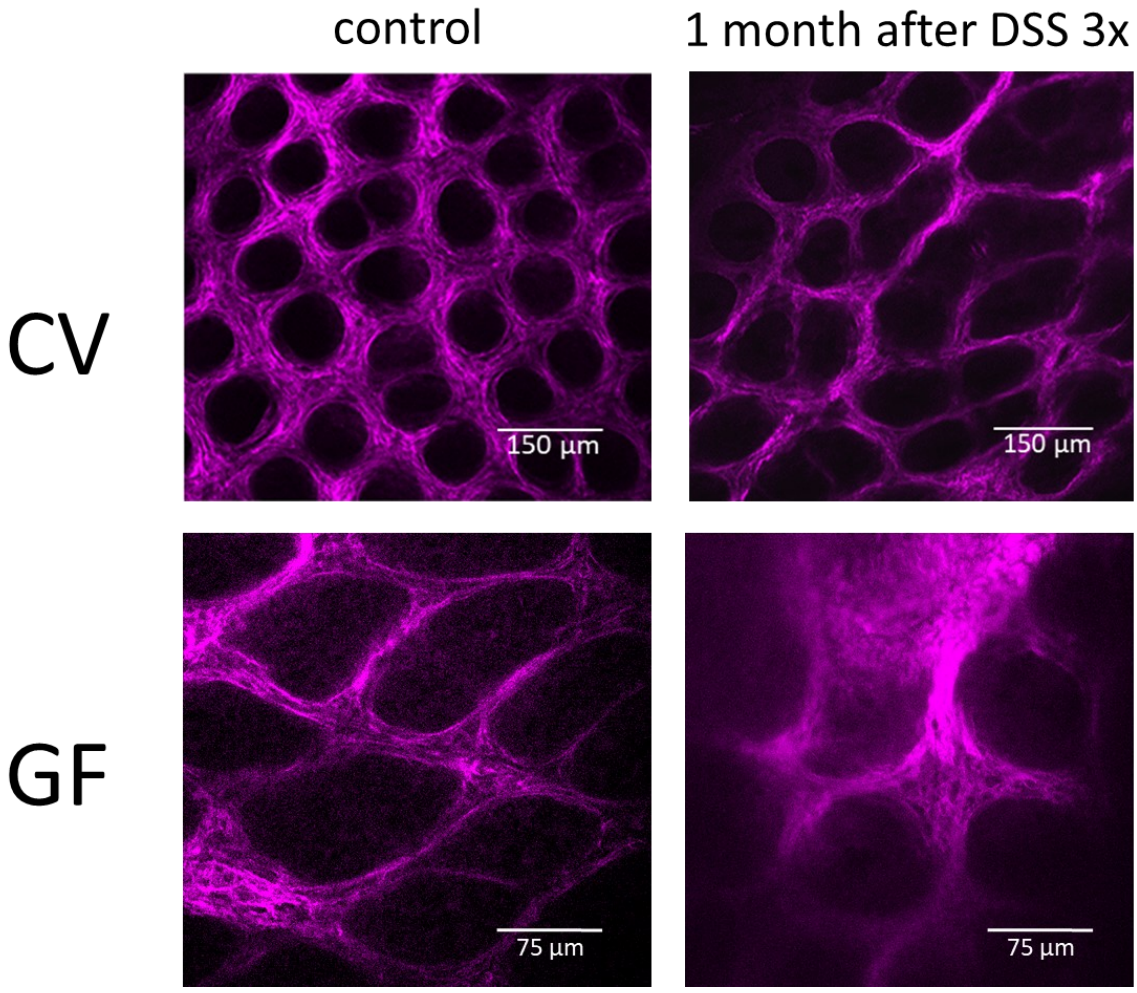



**Figure 2:** Multiphoton confocal microscopy in second harmonic generation mode. Visualisation of collagen fibres in the colon mucosa untreated and after DSS-induced colitis. CV - 20x magnification; GF DSS-induced colitis - 40x magnification.

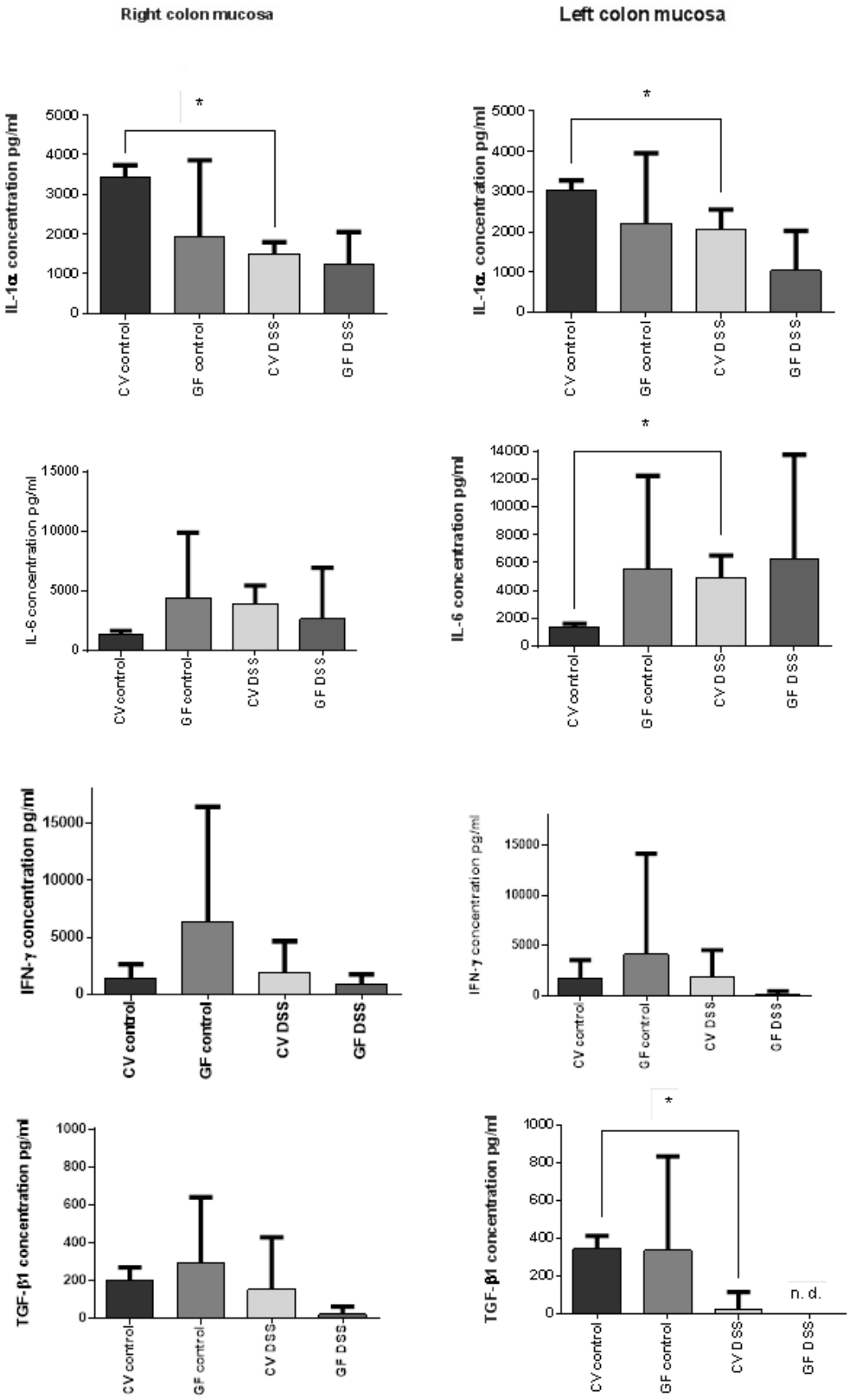



**Figure 3: S**andwich ELISA analysis of selected cytokines in the colon mucosa samples, showed in pg/ml; n. d. = not detectable

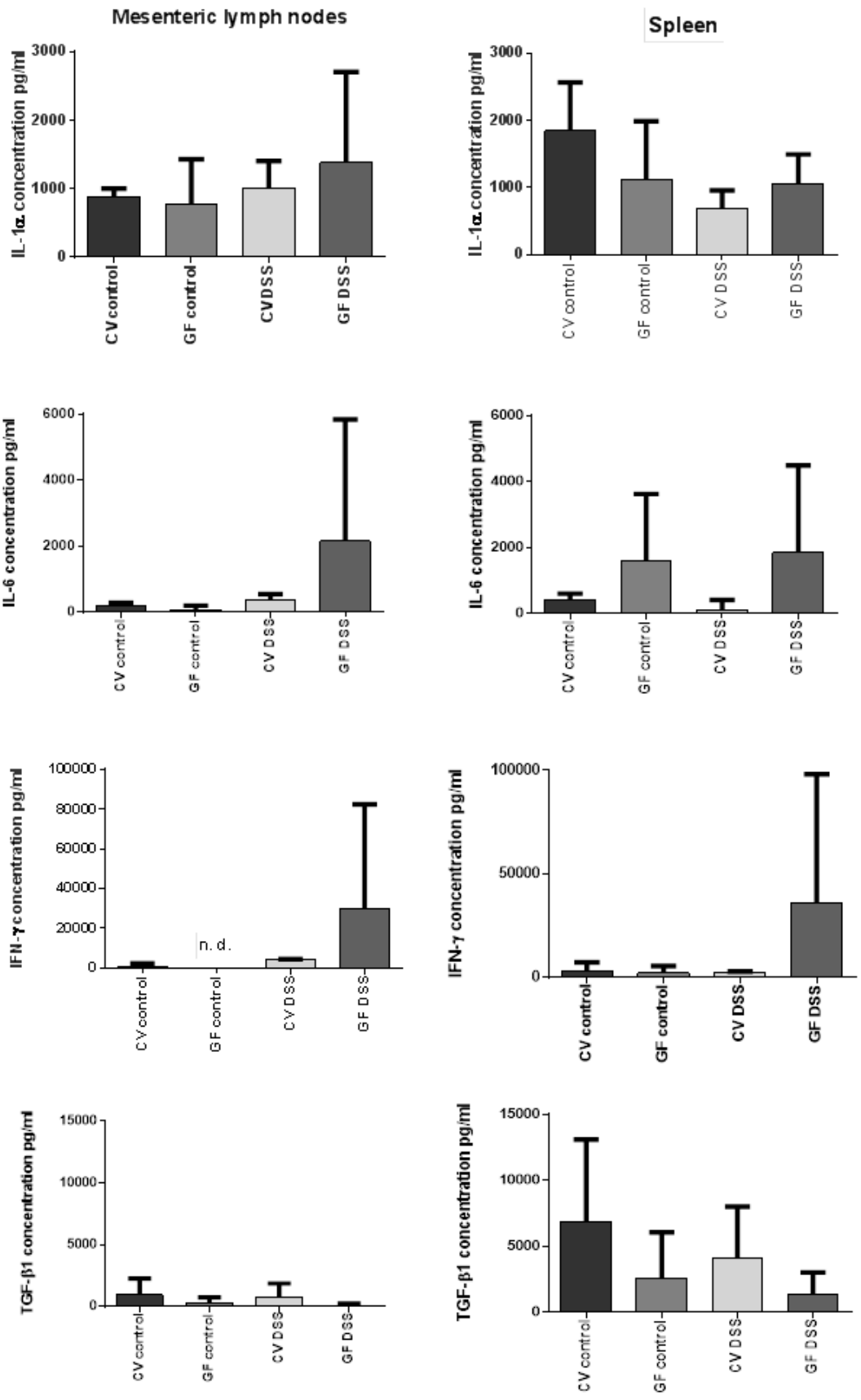



**Figure 4:** Sandwich ELISA analysis of MLN and spleen samples, shown in pg/ml; n. d. = not detectable

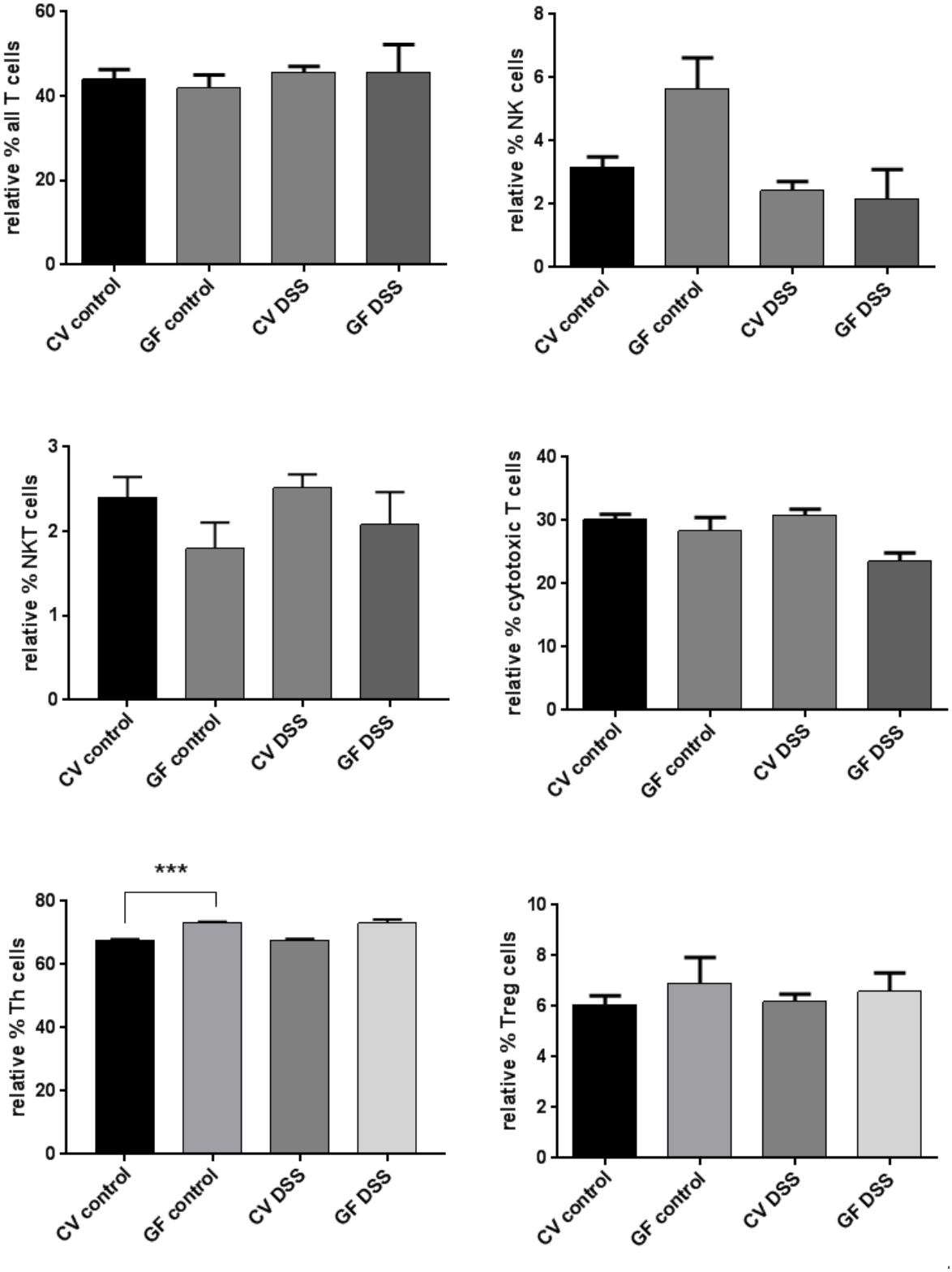



**Figure 5:** FACS-mononuclear cell relative abundance in the peripheral blood samples.

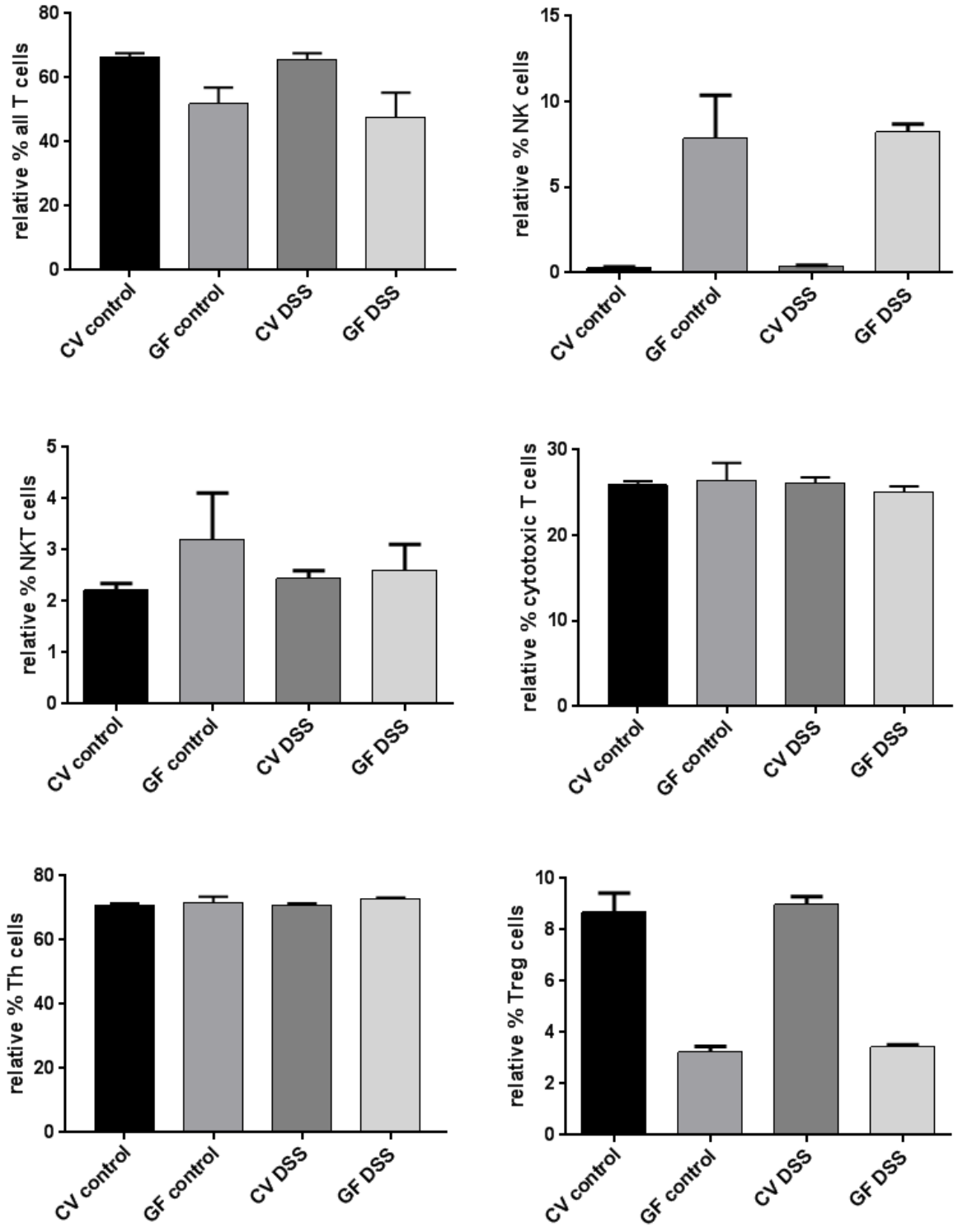



**Figure 6:** FACS-mononuclear cell relative abundance in the MLN samples

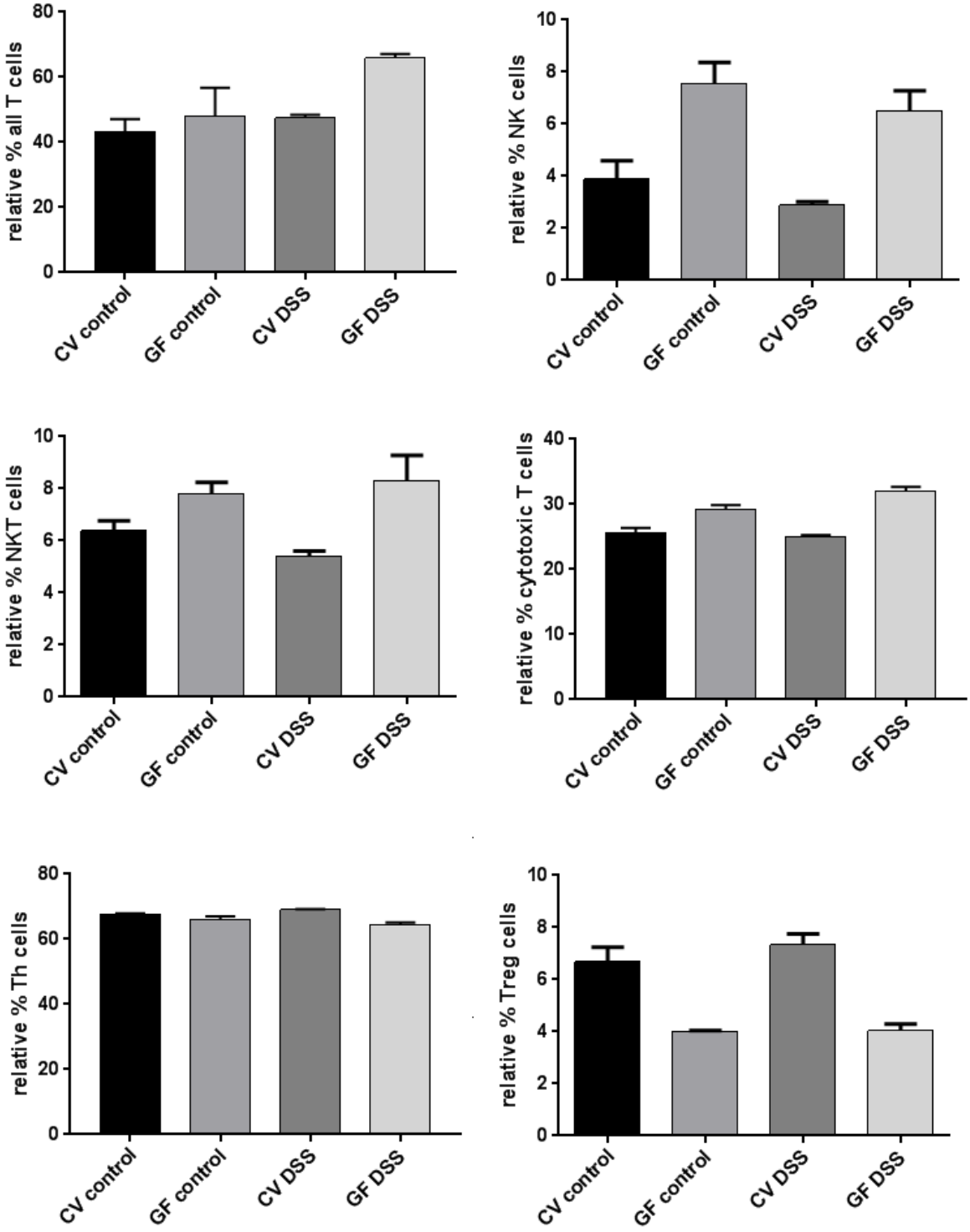



**Figure 7:** FACS-mononuclear cell relative abundance in the spleen samples

**Description of results**

The possibility to induce DSS colitis was evaluated in the presence or in the absence of microbiota to observe possible differences in the expression of inflammation at the immunological and at the structural level at one month after the last treatment.

**Fig. 1** and **Fig. 2** are showing the results obtained by histochemical staining by Picrosirius red and confocal microscopy imaging. The same type of treatment demonstrated that inflammation induced in CV rats produced enhancement of the collagen structures and diffused infiltration of lymphocytes (**Fig. 1**). GF rats also showed signs of inflammation with cellular infiltrate and expansion of lymphoid follicles. Even in these conditions, an increased collagen deposition stained by Picrosirius red was visible. When we evaluated the same tissues by SHG in multiphoton microscopy, the collagen scaffold in CV rats appeared to be altered in its symmetry and with increased deposition of collagen, while GF rats showed increased deposition of collagen, with enhanced fluorescent signal and much lower changes in the symmetry, like acquiring an aspect closer to the scaffold structure in the normal CV rats.

Our ELISA results from colon mucosa did not show any significant differences in control groups even though pro-inflammatory cytokines IL-6 and IFN-γ resulted to be tendentially more produced in GF rats. IL-1α resulted to be more produced in control CV rats, but it was decreased in both CV and GF treated rats (**Fig. 3**). The levels of IL-6 were significantly higher in the left colon mucosa as this is the target of colitis induced by DSS. Not significant changes in IL-6 were found between treated and untreated GF rats. Particularly no significant changes in IFN-γ production were found in CV rats, while IFN-γ and TGF-β1 production was found in treated GF rats. Finally, TGF-β1 did not show any differences between CV and GF rats in control group but after the DSS treatment, there was significant decrease in the CV rat left colon mucosa. No significant changes were found in GF group. Decrease of TGF-β1, involved in the regulation of immunity and extracellular matrix remodelling, can be hypothesized an involvement of other regulatory mechanisms (IL-10, IL-13, IL-4) that need to be further investigated. In any case, IL-6 appeared to be overproduced in this period of transition of inflammatory process. The increase of IL-6 in the left colon mucosa in CV rats in comparison with the GF rats indicate a possible role of the presence of microbiota inducing the inflammation in this compartment.

The IL-1α production in mesenteric lymph nodes (MLNs) resulted in no significant changes (**Fig. 4**), while a decreasing trend was found in the spleen of CV rats, but not in GF rats. Important increase of IL-6 was found in MLNs, but with no significant changes were found between CV and GF rats. Similar results were found also in the production of IFN-γ in MLNs that was reflected in the spleen of GF rats, but still not significant. No changes were found in the IFN-γ production in MLNs, the same in the spleen after DSS treatment in both CV and GF rats. Not significant changes of TGF-β1 production were fund after DSS treatment in both CV and GF rats.

No significant changes were present in the population of mononuclear immune cells except of NK cells decreased after DSS induction in GF rats (**Fig. 5**, **Fig. 6**, **Fig. 7**). Despite the limited number of observations, preliminary results showed here are still useful to show the first image of modifications in two different environments (CV vs GF).

In conclusion, we found the inflammation is induced in both CV and GF animals after DSS treatment. The pro-inflammatory cytokines did not significantly increase after the DSS induction but remained active due to the reduction of regulatory molecules like TGF-β1. This is particularly true in the left colon mucosa that is natural target in the human ulcerative colitis, supporting the validity of the model. The produced chronic inflammation appears to induce like a maturation of collagen scaffold in the GF rats while a more intensive

inflammation in the CV rats induces more significant alterations already in an early period after the induction.

**B: Additional results for Doctoral thesis-part 2 (CRC patients)**

The aim of this preliminary project was to investigate the immune-biological (ELISA of selected cytokines – TNF-α, IFN-γ, IL-10, TGF-β1) and structural changes of collagen scaffold in the colon mucosa (multiphoton confocal microscopy) of CRC patients, not receiving any chemical or radiological treatment. We hypothesised that inflammatory conditions present in CRC are resembling those induced by DSS or AOM. Our results are very promising and confirming the hypothesis that inflammation is a strong modulatory factor in various diseases.

Fresh colon tissue samples, obtained from 25 CRC patients cured in Thomayer University Hospital, were taken for immediate confocal microscopic analysis (fixed by 4% paraformaldehyde solution) and representative colon samples were taken for regular histological diagnosis. For that purpose, the samples were fixed in 10% buffered formalin, then embedded in paraffin for further analyses. The colonic mucosa was collected by gentle cutting from four locations in the colon - tumour, mucosa close (2 cm from the border of tumour), mucosa transitional and mucosa far (min. 10 cm from the border of tumour). These mucosa samples were collected into cryocentrifuge tubes for further ELISA analysis, snap-frozen in liquid nitrogen and stored at -80 °C until the analysis was performed.

The detailed description of histological, confocal microscopy and ELISA methods is described in the publication Čaja et al., J. of Immunotoxicol., 2021, 18:1, 37-49 and Čaja et al., Cancers, 2021, 13(10), 2463, attached in the thesis. The preliminary results obtained from CRC patients are shown in the following pages.

## Results

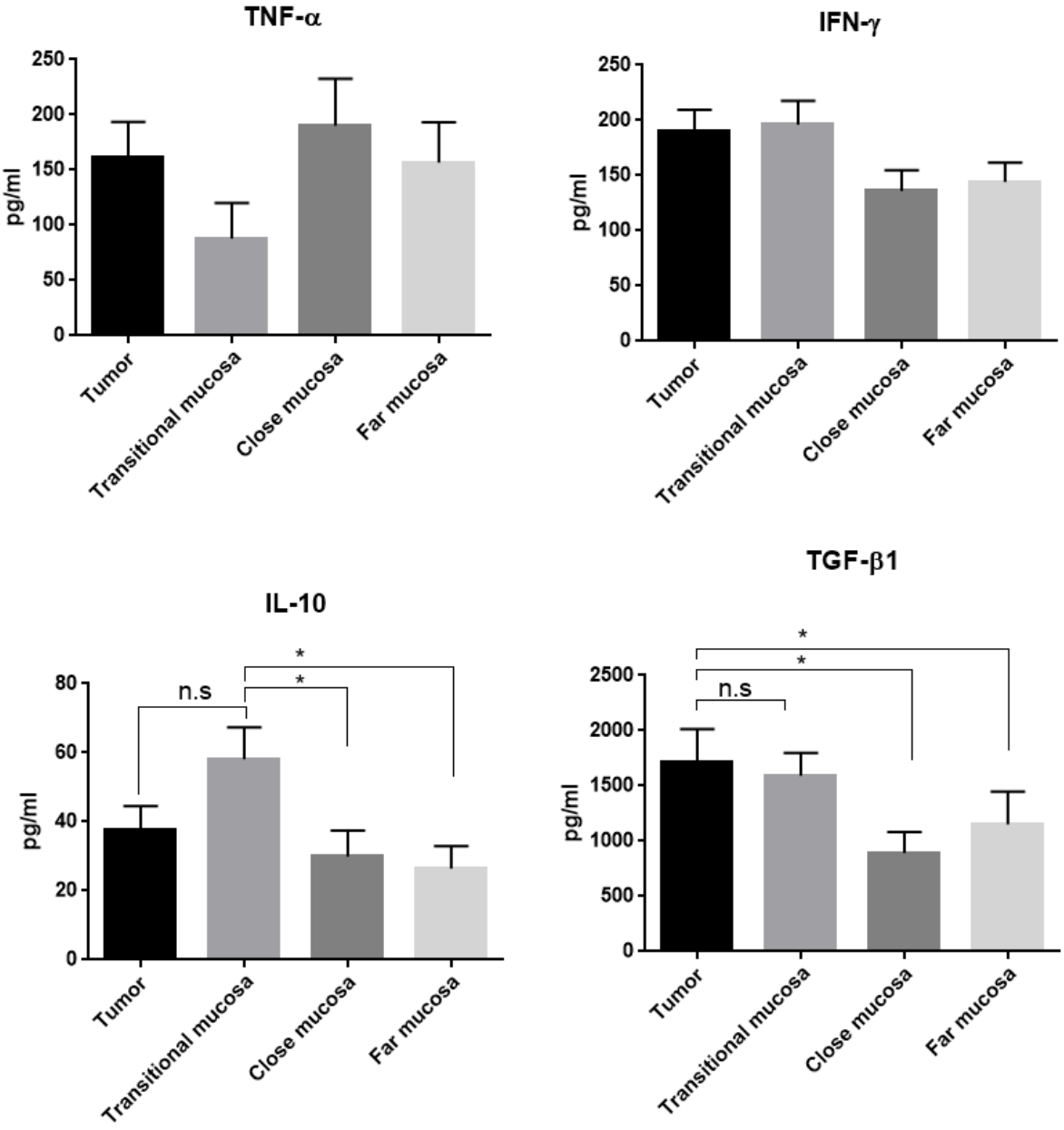



Figure 1: **The ELISA evaluation of pro-inflammatory and immunosuppressive cytokines in the colon mucosa of CRC patients.** Four locations in the colon were selected to compare the immune changes in the tumour and three different distances from the colon: close, transitional and far. All results are described in pg/ml. n. s. = not significant.

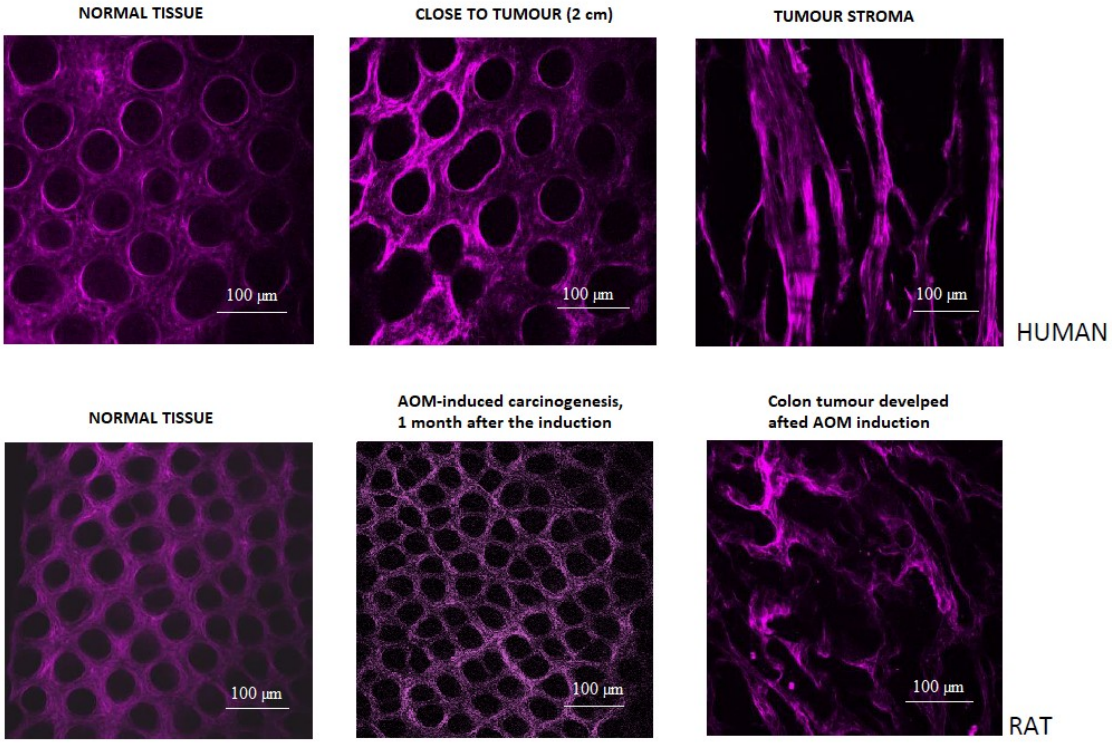



Figure 2: **Second harmonic generation confocal microscopy of the human colon mucosa in CRC patients compared with the results from rats induced by AOM.** Visualised by fluorescence confocal mode, based on autofluorescence of collagen fibres. Original magnification 20x.

## Description of results

The preliminary ELISA evaluation of cytokine production (**Fig. 1**) showed that all cytokines were increased in the tumour samples, all comparisons resulted non-significant except of the production of TGF-β1, which was significantly lower in the transitional and far mucosa samples when comparing to the tumour samples. A little higher resulted the production of IL-10 in the mucosa close samples, although non-significant, while the production of TNF-α was lower in the mucosa close.

The confocal imaging analysis confirmed very important fact that inflammation had strong modulatory effect on the collagen scaffold architecture as can be seen in the **Fig. 2**. The normal collagen structure in healthy colon tissue (more than 10 cm from the tumour) is progressively changing closer to tumour, finally totally disrupted in the tumour samples. These results showed that inflammatory microenvironment of the tumour stroma impacted the structure of the original tissue. This observation can be compared with the colitis- and carcinogenesis-induced rats showing very similar changes in the structure of collagen as a result of inflammatory microenvironment in both pathological conditions.

# 4. DISCUSSION AND CONCLUSIONS

The thesis presented here was focused on the study of relationship between inflammation and its effect on the tissue structures, specifically the collagen scaffold. As the most suitable model for that investigation, it was chosen the colon mucosa.

In the colon mucosa, by the use of animal models, is possible to evaluate the effect of the continuous immune activation produced by the presence of the microbiota, the development of IBDs (ulcerative colitis) as well as the development of cancer (CRC). This approach was looking to highlight immunological changes that in conditions of persistent inflammatory stimulus can lead to tissue remodelling and become useful marker for early diagnosis of the studied pathologies.

Thanking the availability of Wistar-AVN rats both under CV and GF conditions it was possible to design *in vivo* experiments according to the classical model of induction for chronic colitis by DSS, colorectal carcinogenesis by AOM and to directly evaluate the differences in healthy animals that the microbiota can induce by their continuous stimulation of the colon mucosa.

In consideration of the social impact that IBDs and CRC have due to a still problematic treatment and not yet available the efficient systems for very early diagnosis, the studies about possible new markers characterising changing tissues are of the highest necessity. From our experimental activity, it was becoming progressively evident that the local immunity can induce very quick modifications of the ECM, in particular of the collagen structures. In the last few years, various evidence from the literature is progressively supporting this field of investigation (Wang, Kou et al. 2012, Wong, Hutson et al. 2014, Cui, Fu et al. 2019). By the used models, translational studies can also be developed to better compare with the human illnesses. Especially, the study of carcinogenesis permit to try comparisons between the changes induced in the model and the alterations found in the samples from the CRC patients. An initial approach was also tried during our experimental work.

According to the aims and the results in the presented publications as well as in the additional materials, we can summarize our comments and conclusions as following:

1) we described immunological characteristics of CV and GF Wistar-AVN rats using different approaches (ELISA, FACS). We found that GF rats displayed significantly different characteristics in the production of selected cytokines (IL-1α, IL-6, IFN-γ,

TGF-β1), but also in the abundance of NK and NKT cells evaluated by FACS analysis. The production of IL-1α and TGF-β1 was higher in CV rat mucosa, while the levels of IL-6 and IFN-γ were higher in the colon mucosa of GF rats. Considering that the spontaneous production of cytokines in the mucosa of GF animals should represent the basic production by the mucosal system in the colon - due to lower antigenic stimulation by missing bacteria and their metabolites - the lower levels of IL-6 and IFN-γ in the CV animals suggest that the presence of the microbiota and their continuous challenge to the local immunity induce an intensive immune regulatory response. This should be constantly maintained in a range to avoid that inflammatory signals may easily activate a damaging inflammation. TGF-β1 appears, from these and the following data, only a part of the complex regulatory network that needs to be further investigated in its complexity and efficiency. The limit below which the inflammatory activity must be contained can be defined as the "inflammatory threshold". Variations in this threshold may permit the rise of pathological inflammatory conditions.

2) we successfully evaluated the microscopic characteristics of CV and GF Wistar-AVN rats in healthy conditions. Histochemical analysis by Picrosirius red highlighted the collagen structures showing a more diffused and regular net in the CV animals than in the GF ones. This was also associated with the different numbers of immune cells in the intercryptal spaces, higher in the CV rats. These results were confirmed by multiphoton confocal imaging, showing for the first time that the architecture of collagen scaffold in GF rats is less regular, developed and organized than in CV, with modifications also in the inter-cryptal spaces and distances between the centres of the crypts.

3) Using the same animal strain (Wistar-AVN) in CV conditions, we focused on the inflammatory processes in the colon mucosa after DSS-induced colitis and AOM-induced carcinogenesis evaluated at one month after the end of induction. This is an intermediate period, very poorly investigated, between the acute effects of induction and the appearance of the pathological alterations reported by the literature (Ibrahim, Hugerth et al. 2019, Yassin, Sadowska et al. 2019, Li, Chen et al. 2021). All DSS-induced rats showed the symptoms of colitis.

4) The immuno-biological characteristics of DSS- and AOM-treated rats were studied by various methods (ELISA, RT-PCR). We found the general trend that the expression of selected cytokine genes (*Il1a*, *Il1b*, *Ifng*, *Tgfb1*) in the colon mucosa was significantly reduced in the treated rats and this was approximately valid also for the production of selected cytokines (IL-1α, IFN-γ, TGF-β1), except of IL-6, which was more produced after DSS treatment, less but still higher than in control rats after the AOM treatment.

5) The histological evaluation of the colon mucosa structure and the confocal SHG imaging of collagen fibres was performed in DSS- and AOM-treated rats. We found that both DSS and AOM induction activated inflammation in the colon mucosa, which was reflected in the tissue architecture. The original colon tissue structure was altered, becoming more irregular and with evidence of collagen accumulation at the Picrosirius red staining. The DSS-treated group showed evidence of colitis with diffuse parvicellular infiltrate with sporadic ulcerations. The AOM-treated group showed only mild changes in the crypta shape and cell infiltration. Very interesting were the results obtained by multiphoton confocal microscopy, which showed irregularity of the collagen architecture in DSS-treated mucosa and alterations of symmetry and especially increased deposition of collagen also in AOM-treated rats. This is the first evidence that even in an early period after the end of induction, the changes in the collagen architecture can be observed with high resolution on the fresh tissue samples, can be measured and that both DSS and AOM induce inflammation with similar effect on the re-modelling of the original colon tissue. We can summarise our observations from DSS- and AOM-treated rats saying that in the colon mucosa, at one month after the end of the inductions and in the presence of a reduced TGF-β1, the pro-inflammatory cytokines were still able to sustain a smouldering inflammation despite their downregulation, reflected also in the structural changes of the collagen scaffold. The rats did not present any symptoms of inflammatory processes in the bowel, but the structural changes of collagen scaffold of the colon mucosa were already present. These changes can be suggested as an indicator of the local immunological environment and could be proposed as a pre-neoplastic condition marker for possible use in the clinical practice.

6) The additional preliminary experiments were focused on the induction of colitis in GF Wistar-AVN rats and to study the immune-biological and structural changes in the colon mucosa in the early period after the last treatment. We found that GF rats demonstrated that it is the antigenic but not bacterial challenge that produce the colitis because in the GF rats is the sufficient the toxicity produced by DSS to allow the filtration of antigens without checking, activating the inflammatory response. Bacteria are enhancing the inflammation and damage of the colon mucosa in CV rats. This observation was confirmed by multiphoton confocal imaging of collagen fibres showing that the initial remodelling becomes more intense and subverting the collagen original architecture in CV rats, but not in GF animals where the general structure remains almost preserved. We may suggest the inflammatory stimuli induced in GF rats activate the maturation of the collagen structures while in CV they produce the deregulation of the local homeostatic mechanisms, overcoming the inflammatory threshold. The ELISA evaluation of the colon mucosa showed that the production of IL-6, IFN-γ and TGF-β1 was higher in GF control rats than in CV ones but still decreased in treated rats. The peripheral immune organs showed completely different trends, where the GF rats displayed decreased production of IL-1α, IL-6, IFN-γ and TGF-β1 in control group, while IL-6 and IFN-γ were much higher in DSS-treated GF rats. The possible explanation for this observation could be the fact that the absence of permanent microbial stimuli in GF rats shifted the threshold of immune regulation to be more tolerant and the pro-inflammatory cytokines are thus produced in higher levels in GF rats. The FACS analysis showed one interesting phenomenon, the NK cells were much more abundant in the MLNs and spleen of GF rats and the opposite was found for Treg cells in these two organs. This is the clear evidence that GF rats display higher prevalence of innate immunity and the lack of microbial stimuli do not induce such Treg proliferation as could be found in CV rats.

7) We preliminary evaluated the immunological and structural characteristics of colon mucosa tissue samples obtained from the CRC patients not receiving chemical or radiation therapy. We found that samples collected from four locations in the colon (tumour, transitional, close to and far from the tumour mucosa) differed in the organisation of collagen scaffold of the colon and also in

the production of cytokines (TNF-α, IFN-γ, TGF-β1, IL-10). We found a progressive deformation of collagen scaffold from the normal tissue (far mucosa) towards the tumour location. The most important changes in cytokine production were found in the transitional mucosa (near the tumour border), where TNF-α was decreased, regulated by increased IL-10 production. Immunohistochemistry demonstrated presence of IL-6 production in the tumour invaded tissue. The plasticity of collagen scaffold resulting from local immunological changes could be observed also in human samples and IL-6 seem to be a key cytokine in the carcinogenesis and evolution of the tumour microenvironment as well as in the colitis inflammation, as found also in the animal models. Further comparison will be necessary to extend to AOM-induced animals with developed tumours.

# 5. LIST OF PUBLICATIONS

## 5.1 Publications

This thesis was prepared on the basis of the following publications:

**Čaja F.**, Stakheev D., Chernyavskiy O., Kubínová L., Křížan J., Dvořák J., Rossmann P., Štěpánková R., Makovický P., Makovický P., Vymetálková V., Souček P., Vodička P., Vodičková L., Levý M. and Vannucci L. Local immune changes in early stages of inflammation and carcinogenesis correlate with the collagen scaffold changes of the colon mucosa. Cancers 2021, 13(10), 2463.
PMID: has not been added to PubMed yet
**IF: 6.126**; original article

Contribution of the author: 80 %, my work in this project was focused on the preparation and analysis of protein samples using ELISA method. I assisted my colleagues with the sampling, preparation of samples for histology, with the analysis of samples by microscopy, FACS and RT-PCR methods.

**Čaja F.**, Stakheev D., Chernyavskiy O., Křížan J., Dvořák J., Rossmann P., Štěpánková R., Makovický P., Makovický P., Kozáková H. and Vannucci L. Immune activation by microbiome shapes the colon mucosa: comparison between healthy rat mucosa under conventional and germ-free conditions. J Immunotoxicol. 2021 Dec;18(1):37-49.
PMID: 33749490
**IF: 2.780**; original article, 1 citation (WoS)

Contribution of the author: 80 %, I participated mainly in the biochemical analysis of protein samples using ELISA method. I also assisted my colleagues with the sampling, preparation of samples for histology, with the analysis of samples by microscopy and I helped to evaluate the results from FACS analysis.

The other publications related to the theme of thesis:

*Original articles (co-author)*

Vodička P., **Čaja F.**, Vymetálková V., Procházka P., Vodičková L., Schwarzová L., Slyšková J., Kumar R. and Schneiderová M. A novel c. 204 Ile68Met germline variant in exon 2 of the mutL homolog 1 gene in a colorectal cancer patient. Oncol Lett. **2015** Jan;9(1):183-186.
PMID: 25435955
**IF: 2.311**; original article, 3 citations (WoS)

Contribution of the author: 70 %, I analysed mutations in *MLH1* gene in patients with CRC diagnosis, variant c. 204 Ile68Met was found as new germline variant, not yet described in *MLH1* gene.

*Reviews (first author):*

**Čaja F.**, Vodičková L., Král J., Vymetálková V., Naccarati A. and Vodička P. DNA Mismatch Repair Gene Variants in Sporadic Solid Cancers. Int J Mol Sci. **2020** Aug 3;21(15):5561. PMID: 32756484; **IF: 4.556**; review

**Čaja F.**, Makovický P. and Vannucci L. Immune Aspects of the Inflammatory Bowel Disease; Correlations with Celiac Disease. International Journal of Celiac Disease, 2016, 4(4), pp. 130–134. http://pubs.sciepub.com/ijcd/4/4/5
PMID: -; **IF: -**; review

**Čaja F**. and Vannucci L. TGFβ: A player on multiple fronts in the tumor microenvironment. J Immunotoxicol. Jul-Sep **2015**;12(3):300-7.
PMID: 25140864; **IF: 2.780** (IF 2015: 2.020); review, 28 citations (WoS)

*Reviews (co-author):*

Mishra R., Rajsiglová L., Lukáč P., Tenti P., Šima P., **Čaja F.**, Vannucci L. Spontaneous and Induced Tumors in Germ-Free Animals: A General Review. Medicina (Kaunas). 2021 Mar 11;57(3):260.; PMID: 33799911; **IF: 1.205**, review

Makovický P., **Čaja F.**, Vodičková L., Makovický P., Červinková M., Juhász A., Samasca G. and Vodička P. MutL protein homologue 1 (MLH1) in colon adenocarcinomas of the dog: minireview. Cesk Fysiol. **2016** Oct;65(2):88-93.
PMID: 29489087; **IF: -**; review

*Other publications:*

Makovický P., Makovický P., **Čaja F.**, Rímarová K., Samasca G. and Vannucci L. Celiac disease and gluten-free diet: past, present, and future. Gastroenterol Hepatol Bed Bench. Winter **2020**;13(1):1-7.
PMID: 32190218; **IF: -**; review

Vannucci L., Křížan J., Stakheev D., **Čaja F.**, Rajsiglová L., Horák V. and Saieh M. Immunostimulatory properties and antitumor activities of glucans; Int J Oncol. **2013** Aug;43(2):357-64.
PMID: 23739801; **IF: 2.657** (IF 2013:2.773); review, 108 citations (WoS)

### 6.2 Presented abstracts in congresses (first author)

**Čaja F.,** Stakheev D., Křížan J., Rajsiglová L., Dvořák J., Hudcovic T., Štěpánková R., Kozáková H. and Vannucci L. Cytokine production in the colon mucosa of conventionally vs germ-free reared animals under various conditions. Presented at 2nd Polish-Czech Probiotics Conference in Bielawa, Poland (24.-26.5.**2015**).

**Čaja F.**, Křížan J., Stakheev D., Rajsiglová L., Dvořák J., Chenyavskiy O. and Štěpánková R. Microbiome-mediated immune activation differently shapes the stroma of bowel both in healthy and pathological conditions. Presented at 4th European Congress of Immunology in Vienna, Austria (6.-9.9.**2015**).

**Čaja F.**, Stakheev D., Chernyvskiy O., Křížan J., Makovický P. and Vannucci L. Local immunity alteration reshapes the stroma of the colon mucosa in early steps of inflammation and carcinogenesis. Presented at 12th EFIS-EJI Tatra Immunology Conference in Štrbské Pleso, Slovakia (3.-7.9.**2016**).

**Čaja F**., Stakheev D., Křízan J., Chernyavskiy O., Makovický P., Sedláček R., Rajsiglová L., Hudcovic T., Štepánková R., Kozáková H., Dvořák J., Makovický P., Rossmann P., Šíma P., Tenti P., Lukáč P. and Vannucci L. Dextran sodium sulphate induced colitis induces maturation of the stroma in the mucosa of germ-free animals. Presented at International Conference Food, Microbiota and Immunity in Třešť, Czech Republic (7.-10.6.**2017**).

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ANNEX I

**A novel c. 204 Ile68Met germline variant in exon 2 of the mutL homolog 1 gene in a colorectal cancer patient**

This study evolved from the wide MLH1 gene mutation screening in the 102 colorectal cancer (CRC) patients with confirmed sporadic form. The evaluation was focused on finding new somatic mutations in the *MLH1* gene by using high-resolution melting mutational analysis. A new germline variant c. 204 Ile68Met was found in exon 2 of *MLH1* gene and the results were verified in a replication group of 1,095 patients and 1,469 controls. Sequencing of the patient's DNA helped to identify the heterozygous C>G variant at c.204, which resulted in an Ile68Met change in the amino acid, that SIFT and PolyPhen algorithms predicted to be affecting the proper function of MLH1. The other samples collected from CRC patients did not show any somatic mutations suggesting these are rare in sporadic form of CRC.

# A novel c. 204 Ile68Met germline variant in exon 2 of the mutL homolog 1 gene in a colorectal cancer patient

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**Abstract.** Mutations in the mutL homolog 1 (*MLH1)* gene are frequent in patients with hereditary non-polyposis colorectal cancer (CRC). The MLH1 gene was screened for mutations in patients with sporadic CRC. The nucleotide sequences for all 19 exons of *MLH1* were analyzed by high resolution melting and sequenced in a group of 104 sporadic CRC patients, and the results were verified in a replication group of 1,095 patients and 1,469 controls. Different melting profiles for exon 2 of the *MLH1* gene were observed in the germline DNA of one patient. Sequencing of the patient's DNA resulted in the identification of a heterozygous C>G variant at c.204, which resulted in an Ile68Met change in the amino acid. A detailed search of the National Center for Biotechnology Information and the 1000 Genomes databases indicated that the detected variant was unique. According to the SIFT and PolyPhen-2 algorithms, the substitution of Ile to Met was predicted to decrease the activity of the MLH1 protein. The newly identified, functional germline variant was not present in any other CRC patient or control. Thus, a novel germline variant in the *MLH1* gene was identified, representing a rare event in sporadic CRC. The occurrence and relevance of this mutation in other types of cancer requires additional investigation.

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*Key words:* MutL homolog 1, germline mutation, colorectal cancer

## Introduction

MutL homolog 1 (*MLH1*), a constituent gene in the mismatch repair pathway, carries germline mutations in individuals with Lynch syndrome, also termed hereditary non-polyposis colorectal cancer (HNPCC). The gene is reported to acquire 300 different germline mutations, which in addition to mutations in other genes involved in mismatch repair pathway, mainly mutS homolog 2 (*MSH2*), predispose individuals to the disease (1). MLH1, a key protein of the mismatch repair process, contains interaction domains for MutS homologs, including MSH2, MSH3 and MSH6, postmeiotic segregation increased 2 (PMS2), MLH3 and PMS1(2). The heterodimers formed by MLH1 recruit proteins for the excision and repair synthesis.

The germline mutations in *MLH1* in HNPCC include nucleotide substitutions, which result in missense, nonsense or splicing errors and also comprise insertions/deletions. A number of founder mutations, which account for a high proportion of mutations in families with HNPCC, which have been reported in patients with Lynch syndrome (3). In addition, certain non-pathogenic mutations in exons and introns of the gene have also been reported (4,5). The *MLH1* gene is highly polymorphic, with >1,600 variants reported to date (http://genecards.org/cgi-bin/carddisp.pl?gene=MLH1&search=mlh1%23snp).

In this study, a novel germline mutation in *MLH1* in a patient with sporadic colorectal cancer (CRC) is reported, which was detected during the whole genome mutational screening. In addition, a total of 1,095 sporadic CRC patients and 1,469 controls were tested for the detected mutation.

## Materials and methods

*Study population*. This study included a group of 104 newly diagnosed CRC patients with DNA extracted from their tumor tissues, adjacent healthy mucosa and peripheral blood tissues. A replication group included 1,095 CRC patients and

Table I. Characteristics of the study population.

| Characteristic | CRC cases (n=1095) | Control group I, CFCC (n=688) | Control group II, HBDV (n=781) | All controls (n=1469) | OR | 95% CI | P-value[a] |
|---|---|---|---|---|---|---|---|
| Tumor localization | | | | | | | |
| Colon | 725 | - | - | - | | | |
| Rectum | 370 | - | - | - | | | |
| Age (years) | | | | | | | |
| 47≤ | 94 | 164 | 427 | 591 | Ref. | | |
| 48-55 | 208 | 145 | 277 | 422 | 3.10 | 2.36-4.09 | ≤0.01 |
| 56-65 | 370 | 209 | 77 | 286 | 8.13 | 6.25-10.66 | ≤0.01 |
| >65 | 423 | 170 | 0 | 170 | 15.37 | 11.66-20.44 | ≤0.01 |
| Gender | | | | | | | |
| Female | 435 | 317 | 343 | 660 | Ref. | | |
| Male | 660 | 371 | 438 | 809 | 1.23 | 1.05-1.45 | 0.01 |
| BMI | | | | | | | |
| 23.7≤ | 184 | 154 | 215 | 369 | Ref. | | |
| 23.7-26.2 | 192 | 147 | 213 | 360 | 1.07 | 0.83-1.37 | 0.61 |
| 26.3-28.9 | 226 | 139 | 184 | 323 | 1.40 | 1.10-1.79 | 0.01 |
| >28.9 | 222 | 172 | 157 | 329 | 1.35 | 1.06-1.73 | 0.02 |
| Smoking history | | | | | | | |
| No | 536 | 364 | 451 | 815 | Ref. | | |
| Yes[b] | 501 | 254 | 327 | 581 | 1.31 | 1.12-1.54 | ≤0.01 |
| Family history of CRC | | | | | | | |
| No | 726 | 486 | 718 | 1204 | Ref. | | |
| Yes | 144 | 90 | 52 | 142 | 1.68 | 1.31-2.16 | ≤0.01 |
| Address | | | | | | | |
| City | 511 | 338 | 614 | 952 | Ref. | | |
| Suburbs | 128 | 118 | 53 | 171 | 1.39 | 1.08-1.79 | 0.01 |
| Countryside | 242 | 157 | 112 | 270 | 1.67 | 1.36-2.05 | ≤0.01 |
| Education | | | | | | | |
| Basic | 266 | 171 | 53 | 224 | Ref. | | |
| Medium | 469 | 327 | 492 | 820 | 0.48 | 0.39-0.59 | ≤0.01 |
| High | 138 | 114 | 231 | 345 | 0.34 | 0.26-0.44 | ≤0.01 |

[a]P-value for comparison of CRC cases and all controls. [b]Ex-smokers are included into this group. Basic education, completion of eight years of education at primary and secondary school; medium education, completion of 12 years of education at higher secondary school; high education, completion of 17 years of education at university. In cases where categories do not equal 100%, this is due to missing data. CRC, colorectal cancer; CFCC, cancer-free colonoscopy inspected controls; HBDV, healthy blood donor volunteers; OR, odds ratio, CI, confidence interval; BMI, body mass index.

1,469 controls from whom DNA was extracted from peripheral blood lymphocytes. The information regarding the CRC cases and controls included in the replication group is shown in Table I and has been described previously (6,7). Patients included in this study attended the General University Hospital (Prague, Czech Republic), Thomayer Hospital (Prague, Czech Republic), Central Military Hospital (Prague, Czech Republic), Faculty Hospital (Brno, Czech Republic), Regional Hospital Benesov (Benesov, Czech Republic), Regional Hospital Liberec (Liberec, Czech Republic), Hospital Na Plesi (Nova Ves pod Plesi, Czech Republic), Regional Hospital Pribram (Pribram, Czech Republic), Masaryk Regional Hospital (Ústí nad Labem, Czech Republic), Tomas Bata Regional Hospital (Zlín, Czech Republic) or Jihlava Regional Hospital (Jihlava, Czech Republic). This study was approved by the ethics commitee of the General University Hospital in Prague (Prague, Czech Republic) and written informed consent was obtained from all patients.

*Mutation screening in CRC patients*. DNA from tumor tissues, healthy mucosa and blood tissues was extracted using QIAamp DNA Mini Kit and QIAcube (Qiagen, Hilden, Germany). The extracted DNA of the 104 newly diagnosed CRC patients were subjected to mutation detection by high-resolution melting (HRM) using LightCycler® and a 480 High Resolution Melting Master® kit (Roche Diagnostics GmbH, Mannheim, Germany). Polymerase chain reaction amplicons were designed

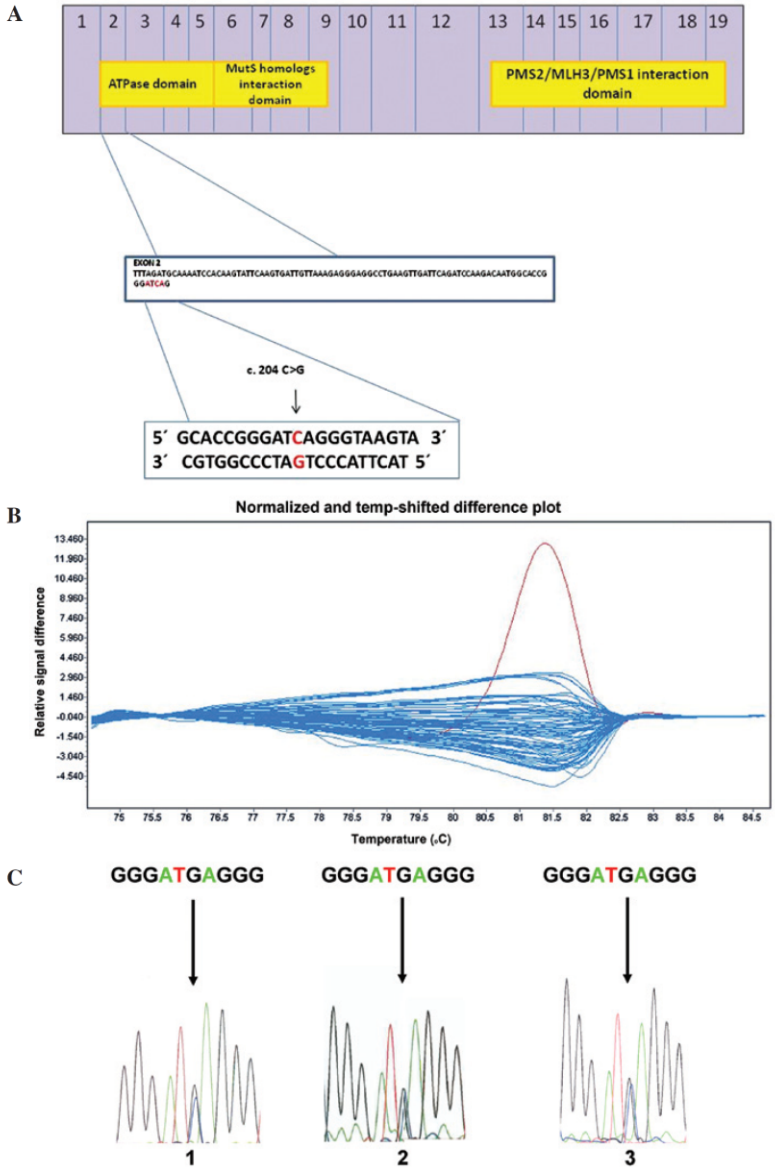


Figure 1. HRM analysis and DNA sequencing of exon 2 of the *MLH1* gene. (A) Diagram of the MLH1 protein in scale. Numbers inside the blue boxes indicate the numbers of exons from which each part of the protein is translated. The three yellow boxes inside represent the ATPase domain, the MutS homolog interaction domain and the PMS2/MLH3/PMS1 interaction domain. A new germ-line variant in exon 2 is located at position c. 204 C>G, p. Ile68Met, corresponding to the gene region coding a part of ATPase domain of MLH1 protein. The position c. 204 is shown by the black arrow. (B) HRM analysis of exon 2 in tumor DNA samples. The difference plot chart shows relativity between DNA melting temperature and relative intensity of emitted fluorescence. Each curve represents the melting of one DNA sample. The DNA sample bearing the novel gene variant exhibits a different melting profile, which is highlighted by the red color in comparison with the blue-colored melting profiles of wild-type DNA samples. This discrepancy of different melting curves is caused by nucleotide change in the analyzed DNA sequence. The red curve represents the melting of the DNA sample which bears the newly identified variant, c. 204 C>G, p. Ile68Met, in tumor tissue. To verify the possible germ-line origin of the variant, HRM was performed in healthy tissue and peripheral blood DNA samples. Both samples were positive for the same variant (data not shown). (C) Comparison of DNA sequencing plots of three different DNA samples obtained from the same patient bearing new heterozygous germ-line gene variant c. 204 C>G, p. Ile68Met. 1, tumor DNA sample; 2, healthy mucosa tissue DNA sample; and 3, peripheral blood DNA sample. MLH1, mutL homolog 1; PMS2, postmeiotic segregation increased 2;HRM, high-resolution melting.

to scan the *MLH1* gene using HRM analysis. All 19 exons in the *MLH1* gene were screened. The region containing exon 2 was amplified using primers with the following sequence: Forward, 5'-AGTTTGTTATCATTGCTTGGCTCAT-3' and reverse, 5'-TCCAGAACAGAGAAAGGTCCTGACT-3' (8). The 10-$\mu$l reaction mixture contained 20 ng genomic DNA, 0.4 mM of each primer and 3 mM $MgCl_2$. The reaction conditions were as follows: Activation step at 95˚C for 10 min followed by

45 cycles of 95˚C for 15 sec, 60˚C for 15 sec, 72˚C for 25 sec and 72˚C for 7 min. Samples with positive HRM signals were further analyzed by sequencing. Sequencing reactions were carried out in a total volume of 10 $\mu$l containing 2 $\mu$l Big Dye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Life Technologies, Foster City, CA, USA), 2.4 $\mu$l $H_2O$, 0.3 $\mu$l DNA template and 0.3 $\mu$l of one primer. The reaction products were purified by ethanol precipitation and sequenced by automated sequencing (Applied Biosystems® 3130 Genetic Analyzer; Applied Biosystems, Life Technologies). The reference sequence of the *MLH1* gene (NG_007109.1) was obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/nuccore/NG_007109.1?from=4863&to=62359&report=genbank).

*Genotyping of the replication group*. DNA samples from the replication group were genotyped for the novel germ-line variant. The DNA samples from CRC patients and controls were analyzed at the K Bio Science facility (K Bio Science UK Ltd., Hoddesdon, UK) (protocol available at http://www.kbioscience.co.uk/reagents/KASP_manual.pdf) under conditions described previously (6).

## Results

In the initial group, 1/104 sporadic CRC patients exhibited a single nucleotide variant at codon 204 within exon 2 of *MLH1* in tumor tissue and mucosa, as well as in blood lymphocytes DNA. A change of the base C to G resulted in Ile68Met change in the amino acid residue (Fig. 1). The carrier of the newly identified variant was a 59 year old female with rectal cancer (T3N0M0 stage). Although the family history of the patient was unavailable, the patient had been treated for schizophrenia for 15 years. The tumor was microsatellite-stable, the CpG sites on the *MLH1* promoter were not methylated and the expression of MLH1 protein was not altered in the tumor, when compared with the adjacent mucosa (data not shown). Genotyping of the replication revealed that there were no carriers of the newly identified variant Ile68Met in the CRC or control groups.

## Discussion

In this study, a unique variant c. 204 C>G, p. Ile68Met in exon 2 of the *MLH1* gene was identified in a patient with sporadic CRC. The mutation was germline and was also detectable in the DNA of tumor tissue, colon mucosal tissue and DNA of the peripheral lymphocytes of the patient. Predictive algorithms SIFT (9) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) indicated that the amino acid change from isoleucin to methionin may influence the functionality of the MLH1 protein. A 3D model of the MLH1 protein demonstrated that amino acid residue 68 isoleucin is located within the enzymatic core that interacts with ATP molecules (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=90223). Therefore, a substitution by methionine may decrease MLH1 activity. Similarly, a variant in an HNPCC patient at the position c.203 T>A, p. Ile68Asn was previously shown to be deleterious (10). Furthermore, a homologous substitution in yeast was found to cause loss of function in a mismatch repair assay (11).

The identification of a novel germline variant in *MLH1* with putative impact on the protein function is of significant importance in CRC. *MLH1* is the most frequently mutated gene in HNPCC and is also often altered in sporadic forms (12). The novel variant in *MLH1* gene presents a rare event in sporadic CRC, which was identified in 1/1,199 patients. The effect of the mutation, whether a causal germline variant or a rare polymorphism, remains to be determined. Due to the patient history, an association between the novel mutation and mental illness must not be excluded. Neurodegenerative diseases, such as Huntington's disease, are also known to exhibit alterations in mismatch repair genes (13).

In conclusion, in the present study a novel *MLH1* mutation was detected in a patient with sporadic CRC. The functionality of the novel c. 204 C>G, p.Ile68Met variant in exon 2 of *MLH1* gene remains to be determined experimentally, along with its occurrence and relevance in other cancer types.

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