Role of nuclear receptor Nur77 during inflammation
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CHAPTER 1

General introduction and Scope of the thesis

Section I
Inflammation, Peritonitis & Sepsis, Inflammatory Bowel Disease

Section II
Nuclear Receptors in atherosclerosis: A superfamily with many ‘Goodfellas’.
Mol Cell Endocrinol 2013, 368:71-84

Section III
NR4A nuclear receptors in immunity and atherosclerosis
Curr Opin Lipidol 2013, 24:381-385
General Introduction

The subject of this thesis is to understand the role of the nuclear receptor Nur77 in acute and chronic inflammatory disease. To provide a complete overview of the study subjects, this general introduction is composed of three sections. Section I presents a short overview regarding the history of inflammation and an introduction to the etiology and pathogenesis of sepsis and Inflammatory Bowel Disease. Section II introduces the superfamily of nuclear receptors as well as the development of atherosclerosis in the vessel wall. Subsequently, our current knowledge on the function of various nuclear receptors in atherosclerosis is summarized with a strong focus on the modulation of cellular functions in endothelial cells, smooth muscle cells and macrophages inside the vessel wall. In the third and last section of this general introduction the nuclear receptor Nur77 is described in detail as one of the members of the subfamily of NR4A nuclear receptors. The available data on the function of Nur77 in atherosclerosis and inflammation are presented with emphasis on the pivotal role of Nur77 in the myeloid cell compartment and T-cells.

Section I

I-1 Inflammation

Inflammation, derived from the Latin inflammare meaning “to set on fire”, is a defense mechanism to injury or destruction of tissues, or abnormal stimulation caused by a physical, chemical or biological agent; which serves to destroy, dilute, or wall off both the injurious agent and the injured tissues. For example, peritonitis is caused by invasion of bacteria into the germ-free peritoneal cavity and atherosclerosis can be seen as an inflammatory response towards oxidated low density lipoprotein (oxLDL) particles. The 4 classical signs of inflammation, rubor et tumor cum calore et dolore, redness and swelling with heat and pain, were first described and documented by the Roman Aulus Cornelius Celsus (1st century AD).\(^1\)

The prominent Roman surgeon and philosopher Claudius Galen, circa A.D. 130–200, believed that pus formation was necessary for wound healing and this theory persisted for almost 15 centuries.\(^2\) Much later, a fifth classical sign, loss of function, functio laesa, was added by Rudolf Virchow in 1871, who viewed inflammation as purely pathological and recognized that there were various inflammatory processes.\(^1,3\)

Two of his scholars, von Recklinghausen (1833-1910) and Cohnheim (1839-1884) can be seen as great contributors to our modern understanding of inflammation. Von Recklinghausen discovered the presence of mobile cells with varying shapes in human pus, nowadays known as leukocytes, and concluded that these cells could have migrated from another place of origin than the inflamed tissue itself.\(^4\) Cohnheim confirmed these observations and discovered that round shaped cells were recruited via the blood vessels and migrated across the vessel wall into the injured tissue.\(^4\)
Tissue resident cells produce chemotactic proteins attracting different leukocyte populations, neutrophils and macrophages, to migrate from the bone marrow, the spleen and lymphoid tissues to the wounded area where they engulf foreign material and cell debris. This process of phagocytosis was first described by Metchnikoff in the 1890s and the concept of cell-mediated immunity as a defense mechanism against pathogens was developed. In the 1940s, soluble factors derived from the white blood cells in pus of patients with an infection were found to have effects on the body. It wasn’t until the 70s that Stanley Cohen proposed to call these “soluble factors” cytokines. Two cytokines tumor necrosis factor-α (Carswell and Old 1975) and Interleukin-1 (Dinarello and colleagues 1981) are now known to play an important role in sepsis and other systemic inflammatory states. After the initial pathogenic trigger is removed by the immune system, the inflammation is resolved and tissue homeostasis restored.

The process described above is recognized as an acute inflammatory response, a controlled and self-limiting process. In 1880 Carl Weigert was the first to recognize that the immune system can also be harmful to the body by overperforming in its response to fight a pathological agent. Mild forms of chronic inflammation are now important in many diseases traditionally not seen as inflammatory diseases, such as atherosclerosis or obesity. Next to a persistent injury or infection, chronic inflammation can also be caused by a response towards auto-antigens. Autoimmunity plays an important role in several common and debilitating chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis.

I-2 Peritonitis & sepsis
Peritonitis is an infection caused by bacteria in the normal germ free area of the peritoneal cavity. The predominant symptom is abdominal pain. Such an intra-abdominal infection can rapidly turn into a life-threatening sepsis, the leading cause of death in critically ill patients in developed countries. The most common causes of peritonitis in industrialized countries are bowel obstructions and Inflammatory Bowel Disease leading to intestinal perforations. In the developing countries infections predominantly cause intestinal perforations. A peritoneal infection can be caused by multiple bacteria, but Escherichia coli (E. coli) is the most common found pathogen in peritonitis (60%). Both host and bacteria may contribute to the development of the disease after E. coli infections. When a bacterium enters the peritoneal cavity it activates the innate immune system via its constituents such as polysaccharide, lipoproteins and DNA.

The innate immune system protects the host against bacterial infections by pattern recognition receptors (PRRs) which recognize these bacterial components; also known as pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are important families of PRRs and have a major role in the host defense against bacteria. TLR4 and 3 are of crucial importance since they recognize the most common PAMP’s; like lipopolysaccharide (LPS) from Gram- bacteria and lipoproteins from both Gram- and Gram+ bacteria,
respectively. Van ‘t Veer et al.\textsuperscript{20} showed that TLR4/TLR2 double knockout mice have decreased neutrophil infiltration and increased mortality compared to wild-type (WT) mice during an \textit{E. coli} peritonitis. Upon PAMP-binding, the PRRs activate signal transduction pathways which in turn activate latent transcription factors such as nuclear factor kappa B (NFkB) and activator protein 1 (AP-1) family members. Upon activation, these transcription factors translocate to the nucleus to induce the expression of a large number of genes that initiate the inflammatory response, e.g. tumor necrosis factor-\( \alpha \) (TNF\( \alpha \)), interleukin (IL)-1b and cyclooxygenase-2 (COX2) and exert antimicrobial activities by generating Reactive Oxygen Species (ROS). Also chemokines are produced via this route to attract additional phagocytes to the site of infection that will engulf and kill the microbes, as well as adaptive immune cells.\textsuperscript{21} Patients with septic shock had an elevated NFkB activity correlation with severity and predicting the clinical outcome.\textsuperscript{22, 23} Neutrophils and macrophages play essential roles in this early response to invading pathogens. Macrophages phagocytose and kill the invaders and subsequently release mediators to start local inflammation, hence playing a major role in cross talk between the innate and adaptive immune systems.\textsuperscript{24} Next to inflammatory mediators, macrophages are also critical for woundhealing and remodelling. Neutrophils migrate rapidly from the blood to the site of infection in response to the chemoattractants IL-8 and leukotriene B4 secreted by monocytes, (resident) macrophages, mast cells and endothelial cells. Neutrophils combat the infection by phagocytosis, the formation of neutrophil extracellular traps (NETs) and through ROS production. NETs comprise histones, chromatin and antimicrobial proteins and are involved in trapping and destroying pathogens.\textsuperscript{25} As one of the first immune barriers in peritonitis, the complement system is activated by bacterial products and activated cells. Complement activation in turn generates chemotactic factors, act as opsonic factors facilitating phagocytosis and can assemble a membrane attack complex on bacteria that lyses them.\textsuperscript{26} Failure of the immune system to eradicate the bacteria may lead to dissemination of the infection and sepsis; directly derived from the Greek sepo meaning “I rot”.\textsuperscript{27} When the initial peritoneal infection enters the bloodstream it has become a life threatening disease, with a mortality rate up to 80%.\textsuperscript{28} Early in sepsis there is excessive activation of phagocytes (macrophages and neutrophils) damaging cells and organs, which may lead to organ failure and death. Also, in response to pathogens the innate immune cells release multiple inflammatory cytokines, but this can become uncontrolled – known as a cytokine storm – leading to leakage from capillaries, tissue edema, organ failure and shock. This is called the systemic inflammatory response syndrome. As sepsis progresses, the recruited inflammatory cells frequently become dysfunctional. Multiple studies in animal models and patients show an impaired neutrophil function, reduced clearance of bacteria, diminished ROS production and decreased migration to the local infection.\textsuperscript{29, 30} Also, circulating neutrophil numbers are increased in sepsis patients, most likely caused by a combination of delayed apoptosis and massive recruitment from the bone marrow.\textsuperscript{31} Monocytes from patients show a diminished ability to release pro-inflammatory mediators and a decrease in antigen presentation,
whereas their release of anti-inflammatory factors, predominantly IL-10, is unchanged or even increased.\textsuperscript{32, 33} Increased apoptosis of macrophages occurs in sepsis, resulting in immune dysfunction and may lead to multiple organ dysfunction syndrome.\textsuperscript{34} In addition, increased numbers of Regulatory T-cells (Tregs) have been seen in patients with septic shock\textsuperscript{25} associated with a decrease in effector T-cell proliferation and function\textsuperscript{36}. Also the coagulation system is altered in sepsis, frequently leading to disseminated intravascular coagulation significantly contributing to organ damage and death in patients.\textsuperscript{37}

A considerable high mortality from severe sepsis and septic shock persists despite the improvement in health care. Treatment of patients still involves intravenous broad spectrum antibiotic therapy.\textsuperscript{38} Many promising new therapeutics have shown disappointing results in patients.\textsuperscript{39} A worldwide phase 3 trial of the new anti-TLR4 drug (eritoran tetrasodium) did not demonstrate a reduced mortality.\textsuperscript{40} Also, activated protein C (drotrecogin alfa; Xigris) did not prove to save more lives and was withdrawn from the market by its manufacturer.\textsuperscript{41} Patients are usually elderly and there are large genetic differences resulting in distinct inflammatory responses. Personalized treatment strategies might be more effective in treating sepsis. Two single nucleotide polymorphisms (SNPs) at position 299 of TLR4 and position 702 of NOD2 were found associated with greater risk for blood stream infections.\textsuperscript{42} In addition, the C-allele of the IL-6 promoter polymorphism -174 is associated with increased risk of septic shock.\textsuperscript{43} Elucidating the inflammatory processes interacting with metabolic failure leading to organ failure in sepsis at genetic and molecular levels may bring us closer to improving mortality.

I-3 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) represents a group of idiopathic chronic inflammatory intestinal conditions of which the two main diseases are Crohn’s disease (CD) and ulcerative colitis (UC). UC is a chronic superficial inflammation that is restricted to the colon and rectum, while CD concerns chronic segmental transmural inflammation of the intestine which can occur all over the gastro-intestinal tract; from mouth to anus. IBD can be painful and debilitating and sometimes leads to life-threatening complications. The highest incidences of CD and UC have been reported in northern Europe, especially the United Kingdom and Scandinavia\textsuperscript{44, 45} and North America\textsuperscript{45-47}. In Europe alone, an estimated 2.5-3 million people are affected by IBD.\textsuperscript{44} There are a number and variety of drugs that target the inflammatory processes. Treatment with immunosuppressant Azathioprine is the established treatment which is in clinical use for more than 50 years.\textsuperscript{48} Because of suggestions that TNFα might play a potential role in IBD, a prototypical anti-TNF agent Infliximab was developed; yet it seems that it will be withdrawn from the market due to sustained clinical remission.\textsuperscript{49} Unfortunately, around 1/3 of the patients are non-responders regarding this therapy. Serious complications of IBD are perforation of the large or small bowel, resulting in sepsis and death, and gastrointestinal cancers.\textsuperscript{50, 51} Gastrointestinal deaths are particularly increased in patients with CD and only
moderately in UC patients. IBD patients (both UC and CD) have an increased risk of developing colorectal cancer through inflammation-induced genetic mutations. The anti-inflammatory agent and active metabolite of Azathioprine, 6-MP, has been shown to prevent advanced colorectal cancer in patients with IBD. The digestive tract contains trillions of colonizing microorganisms, which interact with the host. The mucosal immune system plays a crucial role in maintaining this relationship at a particular equilibrium. It is thought that IBD results from an excessive immune response towards the normal gut microflora in genetically susceptible individuals exposed to environmental risk factors. Defects in innate immunity are at the center of both UC and CD. The intestinal epithelium acts as a protective physical barrier and is actively involved in immune cell regulation. The epithelium secretes antimicrobial peptides, called defensins, which are retained in the mucus layer produced by the goblet cells. Intestinal epithelial cells (IECs) are connected by intercellular junctions and defects in this structure have been reported in IBD patients to lead to increased permeability. Barrier dysfunction of the colon results in a shift of colonal antigens to the submucosa which elicits an inflammatory response. This will result in a cascade of events which causes a huge inflammatory reaction in the colon. IECs are also able to uptake antigen, deliver it across the cell and efficiently present it to, preferentially, gut dendritic cells. In addition, IECs can express a range of inflammatory cytokines and chemokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-8 to regulate a proper immune response when necessary. Next to the intestinal epithelium, both innate and adaptive immune cells play an important role in the pathogenesis of IBD, consisting of a complex interplay of the many intestinal immune cell types. Key immune cells that maintain intestinal homeostasis are macrophages, dendritic cells (DCs) and T-cells. Different types of macrophages and DCs form a central part of the mucosal barrier. Macrophages are abundantly present in the lamina propria, while DCs are present at very low numbers. In mice, most lamina propria macrophages appear to express

Figure 1. Anatomy of the colon. The colon wall is divided into 5 layers as follows: 1) muscularis externa consisting out of a longitudinal and a circular muscle; 2) the submucosa containing connective tissue, colonic patches comprised of B- and T-cells and blood vessels; 3) muscularis mucosa; 4) lamina propria containing connective tissue and SILT; and 5) specialized epithelium. Macrophages can be situated in submucosa, lamina propria and between the epithelial cells.

SILT = solitary intestinal lymphoid tissue.
CX3CR1 (fractalkine receptor), which is a receptor involved in tolerance of these cells towards host bacteria.67 A subpopulation of macrophages is located just beneath the epithelium, extending its processes into the intestinal lumen to sample antigens.68 CX3CR1-deletion in mice results in a decrease of lamina propria macrophages and an increased severity of experimental colitis.67 Bain and colleagues69 have shown that newly arrived Ly6C<sup>high</sup> monocytes differentiate into IL-10 producing CX3CR1<sup>high</sup> expressing resident macrophages and that this process is disrupted during intestinal inflammation. They found an accumulation of a pro-inflammatory CX3CR1<sup>int</sup> TLR-responsive macrophage population after inducing acute colitis in mice. Dysregulation of macrophages and DCs leads to development of IBD through activation of colitogenic T-cell populations. CX3CR1<sup>high</sup> macrophages contribute to the maintenance and local expansion of Tregs in the gut through IL-10 production.70 CD has been associated with an exaggerated Th1/Th17 response, while in the healthy colon, these T-cell subtypes are homeostatically restrained by Foxp3<sup>+</sup> Tregs through the production of anti-inflammatory cytokines including IL-10 and transforming growth factor beta (TGF-β).71 A variety of functionally distinct CD4<sup>+</sup> T-cells reside in the mucosal tissues being Foxp3<sup>+</sup> Tregs, Th1, Th2, Th17, and follicular helper cells; representing the largest reservoir of memory/effector T-cells in the body.72 When antigen is presented by DCs, macrophages or IECs to gut-associated lymphoid tissue-resident T-cells or lamina propria T-cells; they get activated, clonally expand and take on an effector function which is largely determined by the subset and activation status of the antigen presenting cell and the location of presentation. Th1 cells produce high amounts of IFNγ, whereas Th2 cells secrete IL-4, IL-5 and IL-13.73 The Th17 cells are characterized by their production of IL-17, IL-21 and IL-22. Within the last era, it has become clear that next to immunological and environmental factors (altered luminal bacteria); genetic factors play an important role in the pathogenesis of IBD. The pathogenesis of CD and UC is directly related to a dysfunction of innate responses to bacterial products, and multiple genetic defects have been linked to immune dysfunction.74 5-20% of IBD patients have a positive family history.75 Certain polymorphisms of genes like NOD276, Muc1977 and IL23r78 have already been associated with IBD. The number of potential IBD-associated genes continues to increase.79

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Scope of the thesis

Introduction (Chapter 1)

Section I presents a short overview regarding the history of inflammation and an introduction to the etiology and pathogenesis of sepsis and Inflammatory Bowel Disease.

Section II reviews our current knowledge on the function of various nuclear receptors in atherosclerosis development, in particular their modulation of cellular functions in endothelial cells, smooth muscle cells and macrophages inside the vessel wall.

Section III reviews the role of NR4A nuclear receptors in atherosclerosis and inflammation, with emphasis on the myeloid cell compartment and T-cells.

Chapter 2 reports a role for Nuclear receptor Nur77 in Atherosclerosis. We show that bone marrow-specific Nur77-deficiency attenuates atherosclerotic lesion size by 2-fold with a larger necrotic core, and more macrophages, T-cells and Smooth muscle cells. Mechanistically, this can be explained by higher SDF-1α chemokine levels in these lesions.

In Chapter 3 we studied the role of Nuclear receptor Nur77 in a model of acute inflammation, namely *E.coli*-induced peritonitis and found that Nur77 modulates bacterial influx into the organs via increased vascular permeability, resulting in a more aggravated distant organ damage.

Chapter 4 reports a protective function for Nur77 in experimental colitis shown in two chemically-induced models. In addition, Nur77-overexpression suppresses the inflammatory status of both macrophages and gut epithelial cells.

Chapter 5 investigates the effect of the immunosuppressive drug 6-mercaptopurine on RAW murine macrophages and human endothelial Caco-2 cells, two important cell types in Inflammatory Bowel Disease. We show that 6-MP represses inflammation in both cell types and that this effect is partly mediated by Rac1 specific inhibition and partly through another GTP-dependent pathway.

Chapter 6 deals with the differential gene-expression patterns in Nur77-/- murine macrophages compared to Wild-type cells showing a modulated inflammatory response and reduced collagen content of extracellular matrix.

In Chapter 7 the General discussion section critically evaluates the findings presented in this thesis and gives directions for future research on this Nuclear Receptor.

The thesis is concluded with Summaries in English and Dutch, and appendices including the author’s Portfolio and Acknowledgements.