

Interleukin-33 in inflammation and wound repair – an experimental study with special reference to endothelial cells and myofibroblasts

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To Birgitte, Ingrid and Eira

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Abbreviations

aa	amino acid
ab	antibody
bp	base pair
BSA	bovine serum albumin
CAM	cell adhesion molecule
CD	cluster of differentiation
CD	Crohn's disease
CFU	colony forming unit
CRP	C-reactive protein
DAMP	damage associated molecular pattern molecule
DNA	deoxyribonucleic acid
<i>Drosophila</i>	<i>Drosophila melanogaster</i>
EC	endothelial cell
Fab	fragment, antigen binding
Fc	fragment, crystallizable
h	human
HKLM	heat-killed <i>Listeria monocytogenes</i>
HMGB-1	high-mobility group box 1
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IBD	inflammatory bowel disease
IL	interleukin
IL-1R1	interleukin-1 Receptor 1
ICAM-1	intercellular adhesion molecule-1
IFN(γ)	interferon(γ)
JNK	c-Jun N-terminal kinase
KLH	keyhole limpet hemocyanin
KO	knock-out
LPS	lipopolysaccharide
LTA	lipoteichoic acid
m	monoclonal
m	mouse
MAdCAM	mucosal vascular addressin cell adhesion molecule

MAPK	mitogen activated protein kinases
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PDGFβ	platelet derived growth factor β
PDGFRβ	platelet derived growth factor receptor β
PECAM-1	platelet-endothelial-cell adhesion molecule-1
PET	positron emission tomography
PGN	peptidoglycan
Poly (I:C)	polyinosinic:polycytidylic acid
r	rat
RNA	ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SCID	severe combined immunodeficiency
siRNA	small interfering ribonucleic acid
SMC	smooth muscle cell
TGFβ	transforming growth factor β
TIR	Toll/Interleukin-1 Receptor
TLR	Toll like receptor
TNF(α)	tumor necrosis factor (α)
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WT	wild type
vWf	von Willebrand factor

Publications included

I. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation

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II. Inflammatory bowel disease-associated interleukin-33 is preferentially expressed in ulceration-associated myofibroblasts

Sponheim J, Pollheimer J*, Olsen T*, Balogh J, Hammarstrøm C, Loos T, Kasprzycka M, Sørensen DR, Nilsen HR, Küchler AM, Vatn MH, Haraldsen G.

*) These authors contributed equally

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III. Interleukin-33 preferentially targets non-quiescent endothelial cells

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IV. Interleukin-33 mediates neutrophil recruitment in immunity against

Staphylococcus aureus

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1 Introduction

1.1 Inflammation

The human body is often confronted with infections and injuries resulting in tissue damage. The general response to this is a process called inflammation, in which the body tries to restore the healthy state. To guide the reader to the presented work I will start by giving an overview to these important events.

1.1.1 Historical background

In the medical literature the suffix *-itis* is frequently encountered, and it is used in concert with almost all parts of the human body. At present it usually indicates an inflammatory condition or disease, thereby distinguishing these subsets from other types of pathologies. Originally this suffix had a broader reference since it was the feminine form of the Greek suffix *-itēs* which turned a noun into an adjective. Since the word for a disease, *νόσος* (*nosos*), was feminine, any adjective describing a disease of a part of the body ended in *-itis*, e.g. *appendicitis* or *colitis* [*nosos*] ¹.

From ancient times humans have observed the timely changes that take place after an injury. Much attention was drawn to the emerging heat, and inflammation literally means *to set on fire* (Latin; *inflammare*). The set of cardinal signs of an inflammatory lesion *calor, rubor, dolor and tumor* (heat, redness, pain and swelling), which still indeed are recorded in everyday clinical assessments, were actually recognized more than 2000 years ago, and written down by the author of *In Medicinæ*, the Roman doctor and encyclopedist Aulus Cornelius Celsus.

The underlying processes to these clinical manifestations were subjected to continuing speculations. By the 18th century several theories were put forward, among them that excessive blood flow was the primary event, or that the local irritation was primarily driven by the corresponding nerve fibers ². It was not until the introduction of microscopy in the examination of tissues in the 19th century that made it possible for Virchow and others to look to the cellular level for causes of inflammation, ultimately leaving the long-lived concept of disease as a product of disequilibrium of the four humors. Rudolf Virchow (1821-1902) hypothesized that the tissues themselves produced the different pathologies. Although he did not uncover the contribution of microorganisms in infected lesions, he made substantial discoveries in the field of medicine including the description

of the fifth cardinal sign of inflammation, *functio laesa* (loss of function), and initiated a new era of modern pathology³. Augustus Waller observed in 1846 that white blood cells could emigrate selectively from blood vessels, and one of Virchow's students, Julius Friedrich Cohnheim, described in 1867 that accumulations of pus was the result of this process. Cohnheim also recorded that tissue damage induced changes in the vessel wall that were independent of the circulating blood, and hence suggested, well ahead of their later discoveries, that the endothelium underwent molecular changes during inflammation⁴.

After discoveries in the second half of the 19th century by among others Koch and Pasteur had established the microbial origins of many infectious diseases, the time was ripe to study the contribution of the host to the pathogenesis. Mechnikov recorded in his famous experiment in Messina in 1883 that after introducing rose thorns into transparent starfish larvae he could witness an accumulation of mobile cells at the point of damage trying to engulf the foreign bodies⁵. By his comparative approach he confirmed this observation in several species, and with his background as a developmental biologist he also noticed the similarities with the incomplete digestive tracts in simple single cell organisms. He designated the white corpuscles and other cells with this capability as *phagocytes*, i.e. devouring cells, and hence unraveled one of immunity's central functions: *phagocytosis*. Mechnikov explored further the concept and proof of natural, innate immune defense and he has later been referred to as *Father of natural immunity*⁵. In 1908, he shared the Nobel Prize in Medicine with Paul Ehrlich who was central in the discovery of humoral immunity (complement and antibodies), thus acknowledging both the cellular and humoral aspects of the host's immune system. In fact, Mechnikov introduced his Nobel Lecture with the words: *There is no need to be a doctor or a scientist to wonder why the human body is capable of resisting so many harmful agents in the course of everyday life*⁶, which is still a valid question to ask now more than 100 years later.

I will now take a closer look at the inflammatory response that is central to wound healing, and later on discuss how the host can recognize invading pathogens following an injury.

1.2 Wound healing

To maintain the integrity of an organism, the barrier towards the external environment must be retained. If this protective shield is broken, e.g. in a wound, the body urgently needs to replace the damaged tissues in a process called wound healing. This is a dynamic

program involving cells and other constituents from the blood, tissue resident cells, the extracellular matrix and soluble factors, that can be described in three overlapping phases: inflammation, tissue formation and tissue remodeling (reviewed in refs. nos. ⁷⁻⁹).

Promoting wound healing has been one of the central practices of medical personnel for all times. Healing of a wound by first intention is possible when the wound edges are brought together, e.g. by sutures, promoting the most efficient healing and the least of scar development. The ancient Egyptians recorded pulling together the wounded edges by linen strips ¹⁰, but this approach could not be applied successfully to contaminated wounds. Hippocrates perceived the importance of proper drainage from injuries producing pus, thus recognizing the wounds that should be left unclosed to heal by second intention ¹¹. Although the localization of an injury to the body can vary, the inflammatory response by the host is surprisingly similar to that observed during cutaneous wound healing ¹², making this model a basis for the understanding of tissue damage even to the specialized parenchyma of internal organs.

1.2.1 Cellular events in wound healing

Wound healing is a complex process that is brought about by many cell types that act together in a well-coordinated manner. Wounding is usually followed by an immediate disruption of blood vessels producing a local hemorrhage. The blood dilutes any concomitant microbes and also provides an instant supply of platelets, neutrophils and serum proteins, but hemostasis must be reestablished rapidly to prevent excessive blood loss. This is accomplished by plugging of platelet aggregates and activation of the coagulation cascade that eventually results in factor Xa mediated cleavage of prothrombin, which is a novel target for anticoagulation treatment ¹³. The active thrombin converts fibrinogen into fibrin that is polymerized to produce a clot interlaced with platelets that efficiently seals off the denuded tissue and makes a provisional scaffold where cells can migrate during the repair process ⁸. This mass develops into a granulation tissue as multiple vascular sprouts follow angiogenic cues and invade the clot. Both damaged and activated resident cells release functional molecules in the wound area. Moreover, additional cell types are recruited to the lesion according to the local demand, as will be discussed below.

1.2.1.1 Platelets

Platelets, or thrombocytes, are 2-3 μm small cell fragments generated from megacaryocytes in the bone marrow. Circulating blood normally contains $150\text{-}400 \times 10^6$ platelets per ml representing a large number of cells in the initial hemorrhage capable of plugging damaged vessels and facilitating blood coagulation. By degranulation they can release growth factors like PDGF β , TGF β and VEGF¹⁴, which are central molecules to several cellular actions during wound healing, as will be discussed below. However, even though experimental depletion of platelets has been shown to affect the content of inflammatory cells in wounds, the absence of platelets does not limit wound repair^{15, 16}, demonstrating that platelets are not indispensable for the later phases of wound healing.

1.2.1.2 Endothelial cells

Endothelial cells line all blood and lymphatic vessels and represent an interface between the tissue and the circulating blood and lymph. This unique localization allows them to inhibit the potent coagulation system¹⁷ and regulate the extravasations of serum proteins, leukocytes and immune cells from the circulation and into the tissues in both health (e.g. organ specific homing of circulating immune cells in high endothelial venules (HEVs), as reviewed in¹⁸), and in pathologic conditions such as a wound. The endothelial cells also regulate blood flow through the vasodilatory gas nitric oxide (NO)¹⁹. In the healthy adult vasculature the endothelial cells adhere in a monolayer to each other by means of adherens junctions containing adhesion molecules like vascular-endothelial cadherin (VE-cadherin), and tight junctions, controlling microvessel permeability²⁰⁻²². Increasing vascular permeability through opening of tight junctions allows serum proteins to enter the inflamed tissues. The more specialized tasks of repair call for recruitment of immune cells from the circulation. Extravasation of leukocytes upon demand is directed in a multi-step process that is divided into the sequential steps of tethering, rolling, activation, adhesion, diapedesis and migration across the basal lamina²³ (Figure 1).

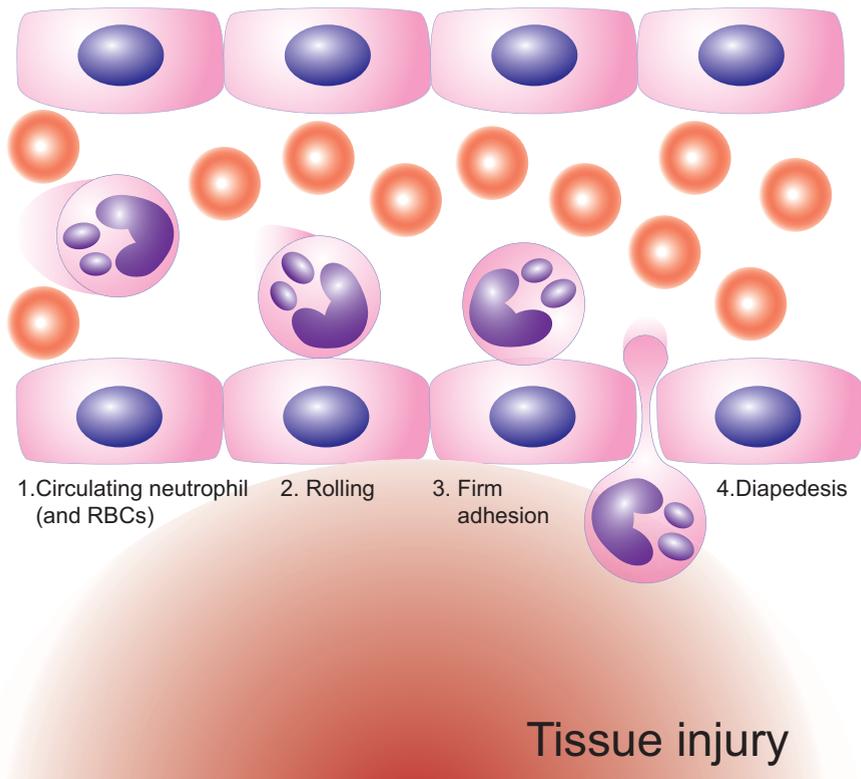


Figure 1. *Sequential steps mediate leukocyte recruitment during inflammation.* Leukocytes are captured within the vessel and begin to roll on P- and E-selectins upregulated by the endothelial cells at the site of inflammation. Leukocyte activation leads to firm adhesion before transmigration (diapedesis) either through the transcellular or, like shown here, the paracellular pathway. Adapted from ref. no. ⁴.

Unique molecules expressed on the endothelium are able to bind corresponding molecules expressed on the leukocytes, controlling each individual step of this process. Hence, as Cohnheim predicted, leukocyte trafficking is indeed directed by the endothelium. However, the vasculature is not only supplying the wounded tissue with serum proteins and immune cells. Tissue repair is a metabolically active process generating a high demand for oxygen and nutrients ²⁴, and during the first days post injury a granulation tissue develops in the wound cavity. This is characterized by numerous new blood vessels that are assembled through sprouting from the remaining functional blood vessels, a process termed angiogenesis (reviewed in refs. nos. ^{25, 26}), opposing the normal state of resting vessels where the endothelial cells are non-proliferating and termed quiescent ¹⁷.

1.2.1.3 Pericytes and vasculature smooth muscle cells

Pericytes are found around the microvasculature in adult tissues where they are crucial for vasculature function²⁷. They represent a cell type of high similarity to the vascular smooth muscle cells of larger vessels and share many molecular properties such as expression of α -smooth muscle actin (α SMA)²⁸ and platelet-derived growth factor receptor β (PDGFR β)²⁹. During onset of angiogenesis, when endothelial cells have formed primitive tubes, these structures recruit mural cells representing pericytes or vascular smooth muscle cells that spread longitudinally as the blood vessels grow³⁰. Platelet-derived growth factor (PDGF)-B is secreted by the endothelium and can signal via PDGFR β on the pericytes and vascular smooth muscle cells. Both of these proteins are critical for vascular stability as deletion of either molecule greatly reduces the number of pericytes, leading to microaneurisms and abnormal vasculature in the fetus²⁹. In fact, homozygous mutants for the ligand PDGF-B³¹ and the receptor PDGFR β ³² are both lethal at birth presenting with edemas and spontaneous hemorrhages. Blocking of PDGFR β during wound healing has demonstrated reduced number of proliferating pericytes and fibroblasts, resulting in delayed wound closure³³.

1.2.1.4 Neutrophils

Numerous neutrophils arrive in a wound where vessel damage causes hemorrhage, and more of these highly mobile cells are rapidly recruited within minutes and hours to any injured area. The role of this cell type is predominantly to eradicate invading microbes, e.g. by phagocytosis before releasing bursts of reactive oxygen species (ROS) into the phagosome³⁴. Extracellular release of adenosine triphosphate (ATP) upon tissue damage is suggested to be an important initiating step for their recruitment, and interestingly neutrophils have recently been shown to approach the inflamed area also via intravascular migration, possibly to limit excessive neutrophil extravasation that can cause collateral damage, e.g. in sterile inflammation³⁵.

1.2.1.5 Monocytes and macrophages

Originating from myeloid progenitor cells, monocytes are released from the bone marrow and into the circulation. Migrating into the tissues, they transform into macrophages in virtually all parts of the body, e.g. histiocytes, osteoclasts, microglia and Kupffer cells. Macrophages perform homeostatic tasks like phagocytosis of damaged erythrocytes, but they also clear the interstitium of cellular debris that is generated upon injury and

during tissue remodeling. Cutaneous resident macrophages have been reported in healthy mice at a low density of only 1-2 per mm² ³⁶, but the numbers of F4/80-positive macrophages have been observed to increase during wound healing and peak around day 3 ³⁷. They clear debris and cellular fragments from the wound and release growth factors important for the formation of new tissue ⁷. This is also supported by the observation that specific depletion of macrophages in mice impaired wound healing, showing delayed re-epithelialization, impaired vessel formation and reduced collagen deposition ³⁸. In response to IL-4 released from mast cells, basophils and other cells during tissue injury, macrophages can transform into so-called *wound healing macrophages* which promote wound healing, e.g. by stimulating arginase activity that allow macrophages to convert arginine to ornithine, a precursor of collagen ³⁹.

1.2.1.6 Mast cells

Mast cells are localized most abundantly in the skin and gut, at the interface of the external barriers of the host. They are long-lived tissue residents, and release a spectrum of active substances upon degranulation, including the vasoactive substance histamine that increases both blood flow and permeability of the microvasculature, the cytokine TNF α and mast cell tryptases that together produce a strong proinflammatory response. Numerous actions have been suggested for this cell type, of which several are linked to host protection from trauma or to tissue remodeling (extensively reviewed in ref. no. ⁴⁰). Weller and coworkers have demonstrated that the mast cell-deficient *Kit^W/Kit^{W-v}* mice exhibit delayed wound closure, and that this phenotype could be fully rescued by reconstituting these transgenic mice with functional mast cells ⁴¹.

1.2.1.7 Epithelial cells

During the proliferation and formation of new tissue, approximately 2-10 days after injury, a key event is the proliferation and migration of keratinocytes over the injured dermis ⁹. The migrating front dissects under the eschar, which is made up of dead tissue, and keratinocytes behind the leading wave proliferate and mature to re-epithelialize and restore the barrier, which is crucial for maintenance of the homeostasis. Growth factors like hepatocyte growth factor (HGF), fibroblast growth factors (FGFs) and epidermal growth factors (EGFs) regulate this process ⁹. Widespread damage to full-thickness skin as seen in extensive burns still show high mortality rates if re-epithelialization is inadequate. Keratinocytes are also the source of the angiogenic factor, vascular

endothelial growth factor (VEGF). Mice lacking epidermal VEGF show fewer vessels in the upper layer of the skin, and after barrier disruption they show delayed angiogenesis compared to wild type controls ⁴².

1.2.1.8 Fibroblasts

The fibroblast is the most abundant cell type in connective tissue. They have a small and spindle-shaped appearance and are responsible for the turnover of extracellular matrix proteins, typically being quiescent in healthy adult tissue. Upon activation they can start proliferating and increase their size and number of processes. This is typically seen after wounding where fibroblasts quickly migrate into the provisional matrix and start producing extracellular matrix proteins, e.g. collagen and fibronectin in the developing granulation tissue, adding structural integrity to the wound ¹². However, the paucity of specific fibroblast markers has hampered the functional characterization of different subsets. Nevertheless, diversity has been demonstrated between fibroblasts from different sources ⁴³⁻⁴⁶. For now, I will concentrate on the myofibroblast.

1.2.1.9 Myofibroblasts

The myofibroblast is a cell type that is encountered in tissues subjected to trauma, such as the healing wound. They are involved in formation and repair of the extracellular matrix, and their central role in wound healing is viewed as an extension of their role in normal growth and development ⁴⁷. The myofibroblast has the appearance of a reactive cell with abundant rough endoplasmatic reticulum (RER), peripheral myofilaments and fibronexus junctions, and shares features with fibroblasts, pericytes and smooth muscle cells (SMCs). (For detailed characterization on myofibroblast (ultra)structure see ref. no. ⁴⁸). Much attention has been drawn to the origin of this cell type that is held responsible for excessive development of fibrosis following tissue damage, e.g. in the lung, kidneys and the liver ⁴⁹. Most myofibroblasts are thought to be derived from resident fibroblasts by changes in the connective tissue microenvironment into so-called proto-myofibroblasts, e.g. in early granulation tissue 2-4 days after an open wound ⁵⁰. This cell type is characterized by stress fibers ⁵¹ that express cytoplasmic actins, similar to those of fibroblasts cultured on a rigid surface, and can be differentiated further to become a myofibroblast expressing α -smooth muscle actin (α SMA), the most commonly used molecular marker ⁵² (Figure 2). The latter differentiation step is dependent *in vivo* on TGF β , ECM proteins like the ED-A splice

variant of fibronectin and high extracellular stress⁵¹, and mechanical tension has also been shown to correlate with α SMA expression in wounds⁵³.

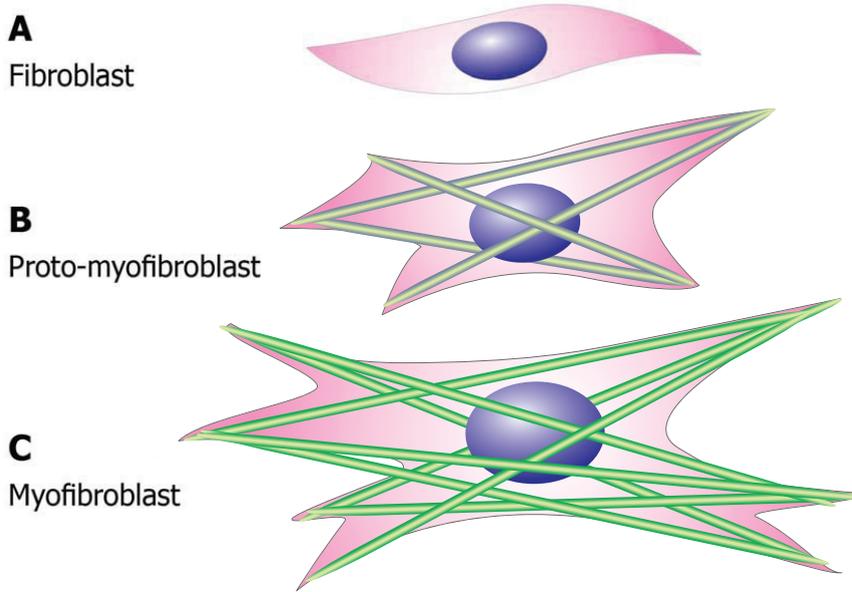


Figure 2. *Myofibroblast differentiation.* (A) Fibroblast in normal dermis. (B) Tissue matrix reorganization during wound healing leads to the assembly of cytoplasmic stress fibers in the proto-myofibroblast. (C) Differentiated myofibroblast with α SMA incorporated into the stress fibers enhancing the contractile properties. Adapted from ref. no. ⁶¹.

Alternative cell sources have been suggested for the generation of myofibroblasts, among them endothelial cells⁵⁴, epithelial cells⁵⁵, pericytes^{56, 57} and circulating fibrocytes^{58, 59}. Tracking these cells has not been straightforward since current myofibroblast markers are not exclusive for this cell type (reviewed in refs. nos. ^{52, 60}), and e.g. the classic marker α SMA is only induced late in healing wounds⁵³. Recently, proto-myofibroblasts linked together by *de novo* expression of adherens junctions (AJs) in early granulation tissue have been shown to express N-cadherin, whereas differentiated myofibroblasts were associated with the marker OB-cadherin⁶¹. Myofibroblasts promote wound closure by contraction of the extracellular matrix through integrins⁶², but it is most essential that this phase is brought to arrest in a well-timed manner since exaggerated contraction will lead to

scarring and obscure tissue remodeling. As for the recruitment of myofibroblasts, there are still open questions related to the mechanisms during resolution of wound healing. Most evidence suggests that the decreased cellularity is caused by apoptosis, when cells die without stimulating neighboring cells¹², which has also been shown to be important in resolution of experimental fibrosis⁶³. However, myofibroblasts are not exclusively linked to trauma or pathologic tissues; in the normal colon α SMA-positive pericryptal myofibroblasts are organized in a network with fibroblasts within the lamina propria⁶⁴. This pericryptal sheath provides support for the epithelial cells and is considered to take part in a broad range of functions including mucosal protection and intestinal motility⁶⁵⁻⁶⁷, and myofibroblasts have also been demonstrated to express Toll-like receptors enabling them to sense invading microbes and tissue damage⁶⁸.

1.2.2 Molecular players in wound healing

1.2.2.1 Cell adhesion molecules

The cell adhesion molecules are critical to the regulated extravasation of immune cells. They are transmembrane proteins that can bind to other cells or to the extracellular matrix. Several of them have been implicated in wound healing, and among them, the selectins are central in initiating leukocyte extravasation. Whereas L-selectin is constitutively expressed by leukocytes, both E-selectin (CD62E, ELAM-1, endothelial-leukocyte adhesion molecule 1) and P-selectin (CD62P) are upregulated by the endothelial cells at sites of vascular injury or upon proinflammatory stimulation^{69, 70}. They can interact with for example PSGL-1 (P-selectin glycoprotein ligand-1), which is enriched at the tip of microvilli protruding from circulating leukocytes, and they are shown to be essential in capturing leukocytes and initiating the rolling along the endothelium (reviewed in ref. no⁴). Although P-selectin-deficient mice demonstrate impaired neutrophil, macrophage and lymphocyte influx in inflamed tissues, there was no notable effect on wound healing in P-selectin- or E-selectin-deficient mice. However, the double knock-out showed impaired wound closure and delayed re-epithelialization⁷¹. Another class of adhesion molecules are the superimmunoglobulin family members ICAM-1 (intercellular adhesion molecule-1, or CD54) and VCAM-1 (vascular adhesion molecule-1). ICAM-1 is constitutively expressed at low levels on endothelial cells⁷², but is upregulated by proinflammatory cytokines⁷³. It is recognized by the β_2 integrins expressed on leukocytes,

among them LFA-1 (lymphocyte function-associated antigen-1), and their interaction promotes firm adhesion prior to the transmigration process. Mice deficient in ICAM-1 exhibit decreased leukocyte accumulation and impaired wound repair^{74, 75}. Whereas E-selectin and ICAM-1 bind all subsets of leukocytes, VCAM-1 binds to lymphocytes, monocytes, eosinophils and basophils, but not neutrophils, by means of the integrins VLA-4 (very late antigen-4/ $\alpha 4\beta 1$)^{76, 77} and $\alpha 4\beta 7$. Firm cell binding via integrins to the endothelium is a crucial step before the blood borne cells can penetrate the endothelial barrier via either the paracellular or transcellular route⁷⁸. The majority of cells appear to exit via the former route passing between the endothelial cells, where platelet-endothelial cell adhesion molecule 1 (PECAM1, also known as CD31) and CD99 are concentrated. In addition, they are expressed on leukocytes, enabling these proteins to interact in a homophilic manner. They appear to be crucial for diapedesis as blockage of either potentially reduces transmigration, and blocking of both nearly abolish diapedesis. Interestingly, blocking CD31 was recently shown to arrest leukocytes on the luminal surface of the endothelium while anti-CD99 treatment trapped monocytes within the interendothelial junctions, pointing to a sequential regulation⁷⁹.

1.2.2.2 Extracellular matrix proteins,

Cells in tissue are embedded in extracellular matrix (ECM), which has numerous effects including the provision of substrates for cell attachments and cues for migration during development and wound repair (reviewed in ref. no. ⁸⁰). Collagen I accounts for more than 80% of the normal ECM in the fibroblast microenvironment, and the levels of collagen I, III, VI, VII and XVI have all been reported to be upregulated or altered during tissue repair⁸¹. Fibronectin is a glycoprotein dimer linked by disulphide bridges that contain binding sites for integrins, fibrin, collagens and growth factors⁸², and different splicing can produce at least 20 isoforms of this molecule in humans⁸³. Fibroblasts have been suggested to use fibronectin conduits⁸⁴ to migrate into the provisional matrix of a newly formed wound cavity, and interestingly the fibronectin splice variant ED-A that has been linked to embryonic development is expressed *de novo* in healing wounds⁸⁵. This splice variant has also been shown to promote myofibroblast differentiation⁵¹. Indeed, specific deletion of this splice variant demonstrated normally developing mice, but during wound healing re-epithelialization was delayed⁸³.

1.2.2.3 Pattern recognition receptors

Recognition of pathogens and/or cellular damage is pivotal to initiating a proper immune response. Pattern recognition receptors (PRRs) detect structures conserved among microbial organisms, so-called pathogen-associated molecular patterns (PAMPs), or molecules released from damaged cells, which are termed damage-associated molecular patterns (DAMPs). Four different classes of PRR families have been identified: the *transmembrane* proteins Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as the *cytoplasmic* proteins Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (reviewed in ref. no. ⁸⁶). The Toll-like receptors were named after the homologous *Drosophila* Toll protein, and the first human variant was cloned in 1997 (TLR4) ⁸⁷. Among the ten currently identified human Toll-like receptors, TLR2 bind several molecular components from bacteria, fungi and viruses, forming heterodimers with TLR1 or TLR6. Known ligands to TLR2 include peptidoglycan (PGN), which is a structural component of cell walls in bacteria, especially abundant in Gram positives (*e.g. Streptococci* or *Staphylococci*), lipoteichoic acid (LTA), lipoproteins/lipopeptides (*e.g.* from the tuberculosis causing *mycobacterium tuberculosis* ⁸⁸ and the tick-bite transferred *Borrelia Burgdorferi* ⁸⁹), glycolipids and zymosan (the latter derived from yeast cell wall). TLR2-deficient mice are indeed highly susceptible to *Staphylococcus aureus* bacteremia ⁹⁰, a condition with high mortality frequently encountered in hospital intensive care units. TLR4 is best known to sense the endotoxin lipopolysaccharide (LPS), a component of the outer membrane of Gram negative bacteria, but also endogenous molecules like high-mobility group box 1 (HMGB1) that can be released from necrotic cells ⁹¹, fibrin ⁹², and several of the heat shock proteins (HSPs) can stimulate both TLR2 and TLR4. TLR5 senses flagellin *e.g.* from the pathogenic *Listeria* and *Salmonella* species. Other members of the TLR family (TLR3, -7, -8 and -9) are localized to the endosome and detect nucleic acids derived from viruses, bacteria or those released endogenously from damaged cells. TLR3 is known to recognize double stranded (ds)RNA and also the synthetic molecule polyinosinic polycytidylic acid (poly I:C) ⁹³. Binding of the ligand dsRNA to the receptor leads to dimerization of two TLR3 molecules. In addition, TLR3 activation is also driven by host mRNA released from damaged cells ⁹⁴. Single stranded (ss)RNA from viruses is recognized by TLR7/8, and TLR9 senses unmethylated CpG motifs commonly found in bacterial and viral DNA, whereas ligands for TLR10 are not yet identified. Activation of the Toll-like receptors lead to recruitment

of several adaptor molecules resulting in activation of the transcription factor NF- κ B and the MAP kinases p38 and Jun amino-terminal kinase (JNK) (reviewed in ref. no. ⁹⁵).

1.2.2.4 Interleukin-1 family of cytokines

There are currently 11 members of the interleukin-1 family of cytokines designated IL-1F1-11 ⁹⁶. *Interleukin (IL)-1 α (IL-1F1)* is found in keratinocytes and epithelial cells, and proinflammatory activation with the TLR4 ligand LPS has been demonstrated to translocate IL-1 α to the nucleus where it can induce transcription of proinflammatory cytokines ⁹⁷. This cytokine is normally not secreted extracellularly, but following a significant injury, IL-1 α can be released from dying cells and then potently activate neighboring cells through binding to the IL-1 receptor IL-1RI ⁹⁸, which shares the TIR (Toll-interleukin -1 receptor) domain of the Toll-like receptors (discussed later in the section *1.3 ST2, A Member of The Interleukin-1 Receptor/Toll-Like Receptor Superfamily*). In this manner, IL-1 α can act as an alarmin ⁹⁹, priming the immune system when there is significant tissue damage.

Another member of the IL-1 family is *interleukin-1 β (IL-1F2)* that also binds to IL-1RI, but in contrast to IL-1 α , this cytokine can be secreted, and is produced by activated leukocytes, in particular macrophages but also fibroblasts and endothelial cells. Cloning of the IL-1 β molecule in 1984 identified this molecule as being the endogenous pyrogen that was sought after ever since the discovery in the 1940ies that leukocyte supernatants were pyrogenic. Patients with familial Mediterranean fever (FMF) who experience episodic fever and abdominal pains, have been shown to secrete more IL-1 β ¹⁰⁰, and this molecule is capable of inducing fever in humans at concentrations of only 1-10 ng/kg body weight ¹⁰¹, demonstrating the need for a tight control of its release. Generation of biologically active IL-1 β that can be secreted is dependent on cleavage of the inactive IL-1 β precursor by caspase-1 ¹⁰², a process that is controlled by an assembly of proteins including the NALP3 (NOD-like receptor protein 3) or other members of the NLR family of pattern recognition receptors, and this protein complex is referred to as the inflammasome ^{100, 103}. Caspase-1 belongs to the inflammatory caspases, whereas e.g. caspase-3 and -7 are effectors of apoptosis (reviewed in ref. no. ¹⁰⁴).

Regulation of IL-1 β activity is also controlled at the receptor level, because the presence of a homologous IL-1 molecule, IL-1Ra (IL-1 receptor antagonist, or IL-1F3) efficiently

blocks the receptor and thereby prevents signaling¹⁰⁰. This endogenous inhibitor has been shown to cause some growth retardation in mice when deleted, and lack of IL-1Ra induces spontaneous arthritis in the BALB/cA strain¹⁰⁵. Administration of recombinant IL-1Ra intravenously to healthy volunteers has not shown alterations in biochemical or hematologic parameters even at high doses, suggesting that IL-1 does not have a role in the regulation of body temperature or hematopoiesis in health¹⁰⁶. Indeed, this recombinant molecule (anakinra, Kineret®) has been approved for clinical use in inflammatory conditions like systemic onset juvenile idiopathic arthritis and refractory Still's disease¹⁰⁰. Yet another level of regulation of the interleukin-1 system is mediated by IL-1RII which also binds IL-1 β , but fails to produce a signal and is therefore considered to be a decoy receptor. Upon binding of IL-1 to the IL-1RI, this complex makes a heterodimer with the accessory protein IL-1RAcP¹⁰⁷ to facilitate signaling, presenting yet another level of possible modulation to IL-1 activity. IL-1RI deficient mice show a reduction in inflammatory cell infiltration and accelerated re-epithelialization following wounding, suggesting that IL-1 signaling can impede tissue repair¹⁰⁸.

The third well-characterized member of the interleukin-1 family of cytokines is *Interleukin-18 (IL1F4)*, which is also synthesized as an inactive precursor and requires caspase-1 cleavage for processing to the active form. IL-18 is produced by macrophages, dendritic cells and epithelial cells¹⁰⁹, and shares many of the proinflammatory properties with IL-1 β and signals through the IL-18 receptor. Moreover, it affects the polarization of T helper cells and is known to induce production of interferon (IFN)- γ or IL-4, depending on the presence or absence of IL-12¹⁰⁰. The action of IL-18 is inhibited by the high affinity IL-18 binding protein (IL-18BP) that is present in excess compared to IL-18 in serum from healthy humans, and this mechanism is exploited by some of the poxviruses, e.g. in the *Molluscum contagiosum* lesions that are commonly long-lived in young children¹⁰⁰. During skin wounding, IL-18 protein has been shown to be strongly upregulated, in part localizing to infiltrating cells in the granulation tissue¹¹⁰. IL-18-deficient mice are resistant to experimental arthritis, but with increasing age they become obese and develop a phenotype similar to the metabolic syndrome seen in humans (reviewed in ref. no.¹⁰⁰).

1.2.2.5 Interleukin-6

Interleukin (IL)-6 is produced by keratinocytes, endothelial cells, B-cells, T-cells, monocytes and fibroblasts. Hepatocytes respond to IL-6 by producing acute phase proteins such as fibrinogen and C-reactive protein (CRP), and (also well in line with a pro-inflammatory role) IL-6 deficient mice are resistant to experimental arthritis (on a DBA/1J background) (reviewed in ref. no. ¹¹¹). IL-6 has also been shown to be localized in neutrophils, macrophages and fibroblasts in cutaneous wound healing ¹¹², and wound healing is impaired in IL-6 deficient mice ^{112, 113}.

1.2.2.6 Platelet derived growth factor

Platelet derived growth factor (PDGF) is a rapidly introduced to a wound by degranulating platelets. This growth factor can be secreted as a homodimer of two A-, B-, C- or D-chains, or as a heterodimer (A- and B-chain), where the B chain represents a potent mesenchymal mitogen ¹². As discussed above, PDGF is crucial for pericyte coverage and vessel stability during development as demonstrated with the lethal phenotypes for both the PDGF-B and the PDGFR β deficient mice. Administration of imatinib mesylate (aka Gleevec®), an inhibitor to PDGFR β , during wound healing in mouse skin has been reported to potently inhibit proliferation and migration of both pericytes and fibroblasts ³³.

1.2.2.7 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is best known for its angiogenic properties, the most important molecule being VEGF factor A (VEGFA) that signals through the VEGF-receptor 2 (VEGFR2, KDR, FLK1)²⁵, but has also been demonstrated to activate the endothelium by inducing selectins and adhesion molecules and by promoting exocytosis of Weibel-Palade-bodies (WPB) ¹¹⁴. During wound healing, analyses of wound fluid from surgical patients have suggested that VEGF is crucial for formation of new blood vessels in the proliferative phase of wound healing, but that other molecules, e.g. basic fibroblast growth factor-2 (bFGF-2), can be more important for angiogenesis during the first three days post wounding ¹¹⁵. Using *in situ* hybridization, Brown et al demonstrated markedly elevated VEGF mRNA levels in keratinocytes at the wound edge 1 day after wounding and abundant expression in epithelial cells that later on migrated in to cover the wound, as well as in mononuclear cells in the wound bed ¹¹⁶. The role of epithelial cell-derived VEGF was further corroborated in studies of transgenic mice (see section on keratinocytes above). The mononuclear cells observed by Brown et

al¹¹⁶ correspond well to wound associated macrophages that have been shown to be crucial for vessel formation in wound granulation tissue³⁸.

1.2.2.8 Transforming growth factor β (TGF β)

The pleiotropic growth factor TGF β plays a central role in wound healing, were it can be released by activated macrophages, platelets, fibroblasts and keratinocytes¹¹⁷, and promote myofibroblast differentiation. The three isoforms found in mammals, TGF β 1, - β 2 and - β 3, have all been shown to be strongly upregulated after injury¹¹⁸ and are mitogenic for fibroblasts, but inhibit proliferation in most other cell types, e.g. endothelial and epithelial cells¹¹⁸. Evaluation of the wound phenotypes in TGF β 1 knock-out mice has been challenging because these mice develop a spontaneous systemic inflammatory response. However, when this deletion was crossed onto the immunocomprimized *SCID* (Severe combined immunodeficiency) mouse background to prevent the inflammatory component, a substantial delay in wound healing was recorded compared to the single *SCID* knock-outs (reviewed in ref. no. ¹¹⁷). In contrast, blocking TGF β signaling specifically in keratinocytes has been shown to accelerate re-epithelialization in skin wounds¹¹⁹. Both the isoforms TGF β 1 and TGF β 2 have been implicated in cutaneous scarring, whereas TGF β 3 has been suggested to limit scar development¹²⁰, and the latter is upregulated in embryonic wound healing that heal without scars. Subcutaneous injections of recombinant TGF β 3 (avotermin, Juvista®) in surgical wounds have been tested in clinical trials, but recently this approach failed to show efficacy in a phase III trial.

1.2.2.9 Tumor necrosis factor (TNF) α

TNF α is produced by many cell types, including macrophages, keratinocytes and fibroblasts, in response to tissue injury¹²¹. This cytokine has been shown to inhibit TGF- β 1 induction of α SMA by destabilizing α SMA mRNA¹²². Also IL-1 and IFN γ have been shown to reduce α SMA-expression in cultured fibroblasts, suggesting that these inflammatory mediators have the capacity to suppress myofibroblast differentiation in the early phase of a healing wound⁶⁰, delaying contraction and repair until the initial inflammatory phase is resolving. Well in line with these *in vitro* findings is the demonstration of reduced infiltration of neutrophils and accelerated re-epithelialization in mice deficient in the TNF-receptor type 1 (TNFR1, p55)¹²³.

1.2.2.10 Heat shock protein 47

Heat shock proteins (HSPs) represent a class of proteins that are amplified by sublethal elevation of temperature or other elements of stress, such as inflammation, toxins or hypoxia. HSPs are named according to their molecular weights, hence HSP47 is a 47 kDa large protein that acts as a chaperone for procollagen in the endoplasmatic reticulum of collagen secreting cells^{124,125}. It's synthesis is induced by transforming growth factor (TGF) β and IL-1 β ¹²⁶ and HSP47 has been linked to the development of fibrosis¹²⁷, as well as demonstrated to be a marker for activated fibroblasts¹²⁸.

1.3 ST2, A Member of The Interleukin-1 Receptor/Toll-Like Receptor Superfamily

The protein Toll in *Drosophila*, the Toll-like receptors and the receptors of the IL-1 family all share a similar cytosolic domain that was therefore termed the Toll-IL-1R (TIR) domain¹²⁹. Members of the IL-1 receptor family also share in common a variable number of extracellular immunoglobulin-like domains. In the latter subgroup we find among others the IL-1RI (the natural receptor for both IL-1 α and IL- β), the IL-18R, the IL-1RAcP (IL-1 receptor accessory protein) and ST2. ST2 was first cloned as a soluble receptor from growth-stimulated BALB/c-3T3 murine fibroblasts (from the clone ST2) in 1989¹³⁰. Later, the membrane-bound ST2 (ST2L) was identified, showing a sequence identity of 28% compared to murine IL-1R1¹³¹, and both the soluble and membrane bound homologues (Fit-1S and Fit-1M, respectively) were also identified in rat fibroblasts¹³². These two proteins are different splice variants sharing the extracellular portion, but soluble ST2 lacks both the transmembrane and cytosolic part. The high degree of similarity to the IL-1R1 suggested that ST2 could be another IL-1 binding receptor, but IL-1 failed to be confirmed as the natural ligand to ST2¹³³ and ST2 remained until recently an orphan receptor with no known ligand. Nevertheless, membrane-bound ST2L was shown to be selectively expressed on Th2 lymphocytes (which secrete mainly IL-4, IL-5 and IL-13, in contrast to Th1 lymphocytes that mainly produce IFN γ and IL-2, reviewed in ref. no. ¹³⁴). ST2 was also reported to be more abundantly expressed in human than in murine tissues, but the soluble form was readily induced by proinflammatory stimuli such as TNF, IL-1 α , IL-1 β and phorbol 12-myristate 13-acetate (PMA)¹³⁵, and overexpression of ST2 has later been shown to inhibit both IL-1 and LPS signaling and in this manner proposed to maintain endotoxin tolerance¹³⁶. ST2 deficient mice have been reported to thrive normally but interestingly failed to develop pulmonary eosinophilic

granulomas around *Schistosoma mansoni* eggs compared to wild type controls¹³⁷. ST2 has also been reported to be upregulated in vessels in biopsy specimens from patients with systemic sclerosis¹³⁸, a disease that is associated with early endothelial damage that can result in progressive fibrosis of the skin and internal organs. The interpretation of some of these studies are hampered by the fact that discriminating between the soluble (s)ST2 and the membrane-anchored ST2L, which might have opposing or different effects, can be experimentally challenging.

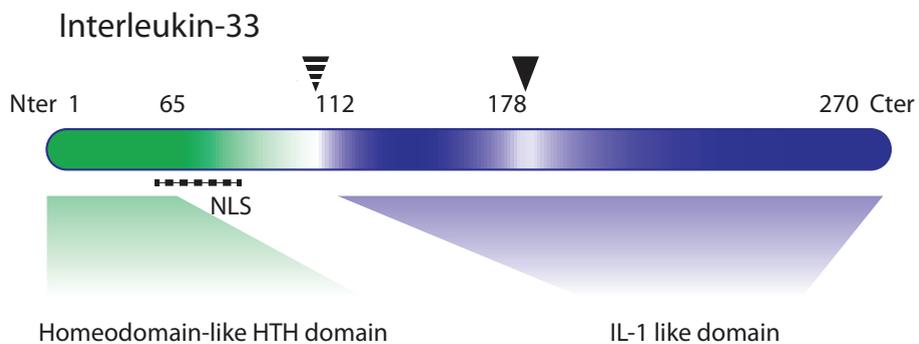
Soluble ST2 has been shown to be markedly induced in cardiac myocytes upon mechanical strain and is increased in serum after myocardial infarction¹³⁹ and suggested as a diagnostic marker for both mortality and heart failure development following ischemic myocardial damage¹⁴⁰. Elevated levels have also been reported in patients with moderate to severe chronic heart failure (NYHA III-IV)¹⁴¹, aortic stenosis¹⁴², during acute asthma exacerbations¹⁴³ and sST2 levels have also recently been associated with disease activity in systemic lupus erythematosus (SLE)¹⁴⁴. Administration of soluble ST2 in experimental airway inflammation reduces the production of IL-4, IL-5 and IL-13¹⁴⁵. In yet another inflammatory condition, administration of exogenous sST2-Fc fusion protein limits experimental arthritis¹⁴⁶, suggesting that sST2 might be exploited therapeutically as an anti-inflammatory agent.

1.4 Interleukin-33

Interleukin(IL)-33 was first characterized in 1999 as a highly upregulated transcript (DVS27) in vasospastic arteries in a canine model of subarachnoidal hemorrhage¹⁴⁷, and in 2003 as a nuclear factor of high endothelial venules (NF-HEV)¹⁴⁸. In 2005, Schmitz and coworkers published that exactly the same molecule was identified through a computational screen for new FGF and IL-1 family relatives. It was shown to bind and signal through the orphan receptor ST2, and the molecule was termed interleukin-33¹⁴⁹, becoming the 11th member of the IL-1 family of cytokines (IL-1F11), in which IL-18 is the most closely related by sequence. IL-33 enhanced the expression of IL-5 and IL-13 in polarized Th2 cells (naïve T-cells which were stimulated with IL-4) *in vitro*, and intraperitoneal administration of IL-33 resulted in splenomegaly in mice as well as eosinophilia and lymphocytosis¹⁴⁹. Moreover, the serum levels of IL-5, IL-13, IgA and IgE increased upon IL-33 treatment, and interestingly, pathological features were noted in the respiratory and gastrointestinal tract. Infiltrates of eosinophils and mononuclear cells were observed beneath the endothelium in lung vessels, and infiltrates of eosinophils,

neutrophils and mononuclear cells were seen in the lamina propria of the esophagus. The intestinal lumen contained more mucus upon IL-33 treatment, and goblet cell hypertrophy was also noted ¹⁴⁹. Another study found that IL-33 facilitated removal of intestinal parasites by induction of the Th2-associated cytokines IL-4, IL-9 and IL-13 ¹⁵⁰. IL-33 was also shown to be induced by mechanical stress in cardiac fibroblasts, and treatment with recombinant IL-33 reduced hypertrophy and fibrosis in a model of aortic stenosis in mice ¹⁵¹. An additional protective effect to the cardiovascular system was reported in 2008 by Miller et al with the demonstration of reduced size of atherosclerotic plaques in mice treated with IL-33 ¹⁵².

The identification of a nuclear localization sequence (NLS) ¹⁴⁸ neighboring the homeodomain-like Helix-Turn-Helix (HTH) in the Nter part of the molecule suggested that endogenous IL-33 could be localized to the nucleus, which also has been demonstrated ^{147, 148} (Figure 3).



Figur 3. Schematic drawing of the human interleukin-33 protein. The Nter part (aa 1-65) contains the homeodomain-like Helix-Turn-Helix (HTH) domain, and the Cter part (aa 112-270) contains an IL-1 like domain. The nuclear localization sequence (NLS) includes aa 61-78. Initially, caspase-1 was suggested to cleave IL-33 after the residue Ser 111 ¹⁴⁹. More recently, caspase-1 was shown rather to cleave IL-33 after residue Asp 178, and the apoptotic caspases-3 and -7 have also been shown to cleave at the latter site ¹⁵³⁻¹⁵⁵. Modified from refs. nos. ^{148, 156}.

In the nucleus, IL-33 has been shown to be associated with heterochromatin and to exhibit transcriptional repressor activity ¹⁵⁶, and to attach specifically to nucleosomal histone H2A-H2B dimers ¹⁵⁷. Interestingly, the acidic pocket formed between H2A and H2B

is also the target of the latency-associated antigen (LANA) protein from Kaposi's sarcoma-associated herpes virus (KSHV) ¹⁵⁷.

Initially, IL-33 was shown to be cleaved by caspase-1 *in vitro*, suggesting that this was essential for the generation of an active mature IL-33 form ¹⁴⁹, similar to the activation of IL-1 β and IL-18. However, more recent work have now demonstrated that full-length IL-33 is the bioactive form *in vivo*, and that cleavage of the apoptotic caspase-3 and -7 rather suppress its bioactivity ¹⁵³⁻¹⁵⁵.

The release of IL-33 upon damage to cells positioned at critical barriers in the organism has been suggested to function as an alarmin to enhance the immune response ^{158, 159}. Secretion of IL-33 has been demonstrated from astrocytes and fibroblasts (reviewed in ref. no. ¹⁵⁹), and importantly, bioactive IL-33 can be released from necrotic cells ¹⁵³. In such a manner IL-33 can have a dual function, both as a nuclear protein and as a secreted molecule. Macrophages have also been suggested to represent a source for IL-33 ¹⁶⁰. Extracellular release of IL-33 enables the assembly of and signaling through the IL-33 receptor complex ¹⁶¹ which contains heterodimerized ST2L and IL-1RAcP. Several cell types respond to IL-33 (reviewed in ref. no. ¹⁶²), among them mast cells, that are driven to maturation by IL-33 ¹⁶³. Moreover, Xu and colleagues demonstrated that ST2-deficient mice exhibited less disease activity in an experimental arthritis model, and that this phenotype was mast cell-specific by subsequently engrafting wild type (WT) mast cells into ST2-deficient animals ¹⁶⁴. Moreover, Kaieda and coworkers have suggested that IL-33 represents a fibroblast-derived factor that regulate tryptase expression in mast cells ¹⁶⁵, and IL-33 has been suggested to be a key component for mast cell activation following cell injury ¹⁶⁶. Furthermore, IL-33 has been shown to upregulate IL-8 in lung tissue ¹⁶⁷, and to promote allergic airway inflammation ¹⁶⁸. Increased neutrophil influx into the peritoneal cavity has also been described after intravenous administration of IL-33 in an experimental peritonitis model, and interestingly, this treatment reduced the ensuing sepsis-associated mortality ¹⁶⁹.

As discussed, IL-33 has been shown to exhibit both cardioprotective effects and to be pro-inflammatory in various inflammatory models. Moreover, repeated injection in mouse skin induced subcuticular fibrosis in an IL-13- and eosinophil-dependent manner ¹⁷⁰.

1.5 Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of inflammatory bowel disease (IBD) ¹⁷¹⁻¹⁷³. These are chronic inflammatory disorders of the intestine affecting approximately 850 new cases in Norway annually. A majority of patients progress to a relapsing and chronic disease ¹⁷⁴ making substantial impact on quality of life in a large number of individuals, most often presenting in early adult life. The etiology is still largely unknown, although several disease susceptibility genes have been identified ¹⁷⁵. In addition, environmental factors, particularly the intestinal microbiota, are thought to contribute to disease development ¹⁷⁶. In patients, Crohn's disease can affect all segments of the gastrointestinal tract, but most often the terminal ileum and colon are involved. Typically, the inflamed areas can be focal and discontinuous, and the inflammation is usually transmural ¹⁷⁷. Associated features include intestinal granulomas, deep fissures and fistulas (when the intestinal lumen communicates throughout the intestinal wall and onto another organ, e.g. to the skin) and fibrotic strictures, the latter typically producing abdominal pains (Figure 4).

In ulcerative colitis the inflammation usually affects only the mucosa and submucosa except in the most severe cases. Active disease disrupts the important barrier function of the mucosa and makes it more friable, often resulting in bloody stools. Ulcerative colitis is confined to the colon, and typically the distal part is involved with varying degrees of proximal involvement in a continuous distribution. Regarding colonic disease, discriminating between the two can represent a diagnostic challenge, e.g. in patients presenting with acute pancolitis, when the full length of the colon is involved. The diagnosis is based on a joint assessment of clinical characteristics, biochemistry, endoscopic features and histology.

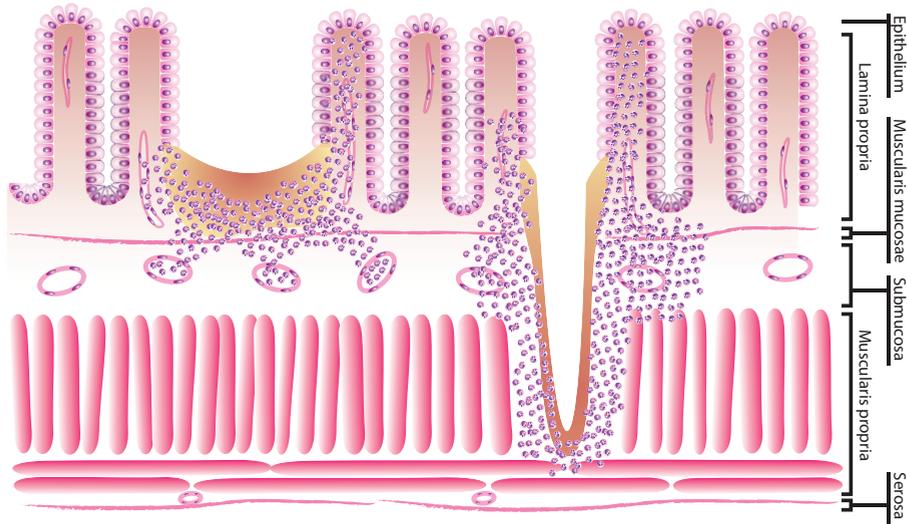


Figure 4. Schematic drawing of the bowel wall with a mucosal ulcer (left) and a fissure (right). Facing the luminal side (up), the mucosa consists of the lining epithelium, the lamina propria and muscularis mucosae, overlying the submucosa and the muscularis propria with its circular and longitudinal layers. The serosa faces the peritoneum. The *ulcer* on the left can be found either in ulcerative colitis or in Crohn's disease, whereas the deep *fissure* on the right is typical for penetrating Crohn's disease. Note the accompanying transmural cellular infiltrate. If the fissure extends throughout the intestinal wall and onto a neighboring organ, e.g. the skin, the lesion is called a *fistula*.

Assessment of fluctuations in disease activity may be performed by the use of endoscopic¹⁷⁸ or clinical activity indexes, like ulcerative colitis disease activity index (UCDAI)¹⁷⁹, and Crohn's disease activity index (CDAI)¹⁸⁰ or Harvey Bradshaw clinical activity index¹⁸¹ in Crohn's disease. Especially in patients with ulcerative colitis, colorectal cancer is more frequently encountered. Risk factors that have been identified are severe inflammation¹⁸², and extensive and long standing disease¹⁸³, which emphasize the need of efficiently arresting the chronic inflammatory reaction. Some patients even present with an acute severe colitis, needing rapidly effective anti-inflammatory treatment for this potentially life threatening condition. However, traditional anti-inflammatory treatment with corticosteroids raises safety concerns with respect to the development of osteoporosis, hypertension, dyslipidemia and diabetes. On the other hand, immunosuppressants like azathioprine, that can replace corticosteroids, frequently show myelotoxic effects on the bone marrow and may not be tolerated due to idiosyncratic reactions. The introduction of

TNF α blocking antibodies has been welcomed as an effective treatment option for patients with moderate to severe inflammatory bowel disease ¹⁸⁴, but also this strategy has shown severe infectious side-effects (e.g. tuberculosis, systemic fungal or herpes virus infections) that traditionally have been associated with immunocompromized patients, and also raised safety concerns related to infusion reactions and development of malignancies ^{185, 186}, underlining the fact that our understanding of the cellular events and cytokine interplay in IBD is far from complete ¹⁸⁷.

1.5.1 Cytokines and cellular players in inflammatory bowel disease

Active inflammatory bowel disease is characterized by a pronounced infiltrate in the lamina propria of both innate immune cells (e.g. neutrophils, macrophages) and adaptive immune cells (B cells and T cells) ¹⁷⁷. Lacking a yet identifiable pathogen, this enduring and fluctuating inflammation can be truly harmful for the affected individuals. So, which are the initiating steps leading to such an apparent autonomous inflammatory response? This question has been challenging to studies in humans, as most patients have had symptoms of their disease for quite some time before they come to a diagnostic endoscopic examination, presenting with an established and complex inflammatory condition. Nevertheless, data from experimental colitis studies have provided a lot of information on intestinal inflammation, but the true initiating factors still remain obscure. IBD patients have activated both innate and acquired immune responses and demonstrable loss of tolerance to enteric commensal bacteria ¹⁷². Tolerance has been shown to be mediated by TGF β , IL-10 and IFN α/β . Even though several anti-cytokine approaches have been tested so far, neutralization of TNF α has proven to be the currently most efficient cytokine blocking strategy in the treatment of moderate to severe IBD ¹⁸⁴. It is effective in Crohn's disease and ulcerative colitis, pointing at an essential role for TNF α in the pathophysiology for both of these conditions. However, there are some differences in the cytokine profiles between Crohn's disease and ulcerative colitis. Both show elevated levels of IL-1 β , TNF α , IL-6, IL-8 and IL-18, whereas Crohn's disease lesions contain elevated levels of IFN γ , IL-12, IL-17, IL-21, IL-23 and IL-27, and ulcerative colitis lesions exhibit more IL-5 and IL-13 ¹⁷². Recently the autophagy related gene ATG16L1 has been linked to Crohn's disease. Interestingly, depletion of ATG16L1 in mice leads to overactivation of the inflammasome, resulting in increased production of bioactive IL-1 β and IL-18 ¹⁸⁸, pointing at a potential mechanism to explain how a genetic predisposition can enhance

pro-inflammatory cytokines. TNF α , IL-1 β and IL-6 are considered to be important for augmenting and maintaining the inflammatory response ¹⁸⁹, and such inflammatory cytokines upregulate cell adhesion molecules like ICAM-1, VCAM-1 and VLA-4, promoting recruitment of neutrophils and monocytes ¹⁷². Another cell adhesion molecule is the mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) expressed on high endothelial venules (HEV) in intestinal Peyer's patches. Via binding to the integrin $\alpha 4\beta 7$ this CAM is thought to direct lymphocytes specifically to mucosal sites like the intestine ^{190, 191}, and appears to be a promising therapeutic target in ulcerative colitis ^{192, 193}.

1.5.2 Mucosal healing in inflammatory bowel disease

Inflammation can be graded according to several mucosal characteristics, including the presence of erythema (rubor), edema, loss of vascular pattern (due to thickening of the lamina propria reducing mucosal transparency), friability, erosions, ulcerations and spontaneous bleedings. Mucosal healing is assessed by endoscopic examination of the colon in ulcerative colitis and also the terminal ileum in Crohn's disease. Whereas complete mucosal healing (MH) represent healing of all inflammatory and ulcerative lesions, endoscopic mucosal healing of the gut mucosa in Crohn's disease and ulcerative colitis includes the absence of friability, bleeding, erosions and ulcers in all segments examined ¹⁹⁴. Traditional treatment with corticosteroids can induce clinical remission, but accumulating evidence has shown that treatment with immunosuppressors or biological agents (e.g. anti-TNF α) seem more potent in inducing long-term healing of the intestinal mucosa. This concept has recently gotten more attention, expanding the optimal treatment goal from clinical remission (symptom free patients), to also achieve mucosal healing, reasoning that even low grade inflammation is potentially harmful to the intestine and that such activity more easily can proceed to new flares of active disease and ensuing complications. A study by Frøslie and colleagues, analyzing a large population-based cohort of incident cases of patients with inflammatory bowel disease (IBSEN I), demonstrated that mucosal healing after 1 year of treatment indicated reduced subsequent disease activity ¹⁹⁵.

2 Aims of the study

The main objective of this study was to examine the role of interleukin-33 in inflammation

Specific objectives

1. Characterize interleukin-33 in endothelial cells and map the molecular mechanisms important for the regulation of IL-33
(Paper I)
2. Explore the cellular sources of interleukin-33 in inflammatory bowel disease
(Paper II)
3. Examine the effect of recombinant interleukin-33 on endothelial cells
(Paper III)
4. Explore the function of IL-33 in inflammation and wound healing in an interleukin-33 knock-out animal model
(Paper IV)

3 Methodological considerations

3.1 Patient specimens

In paper II, surgical and endoscopic biopsies from a total of 41 patients with inflammatory bowel disease, and from 28 controls patients, were analyzed according to protocols approved by the Regional committees for Research Ethics and the Norwegian Social Science Data Services. Endoscopic biopsy specimens from untreated UC patients ($n = 25$) and controls ($n = 22$) undergoing flexible sigmoidoscopy or colonoscopy for diagnostic purposes were used for the quantitative PCR analysis. The ulcerative colitis diagnosis was based on established clinical, endoscopic, and histological criteria ¹⁹⁶. The indication for colonoscopy in the control group was intestinal symptoms, without signs of endoscopic or histologic inflammation. Only subjects with normal colonoscopy, both macroscopically and histologically, were included in the control group. The disease activities for the UC patients included in the PCR analyses were assessed using the Ulcerative Colitis Disease Activity Index (UCDAI), which is based on clinical signs (score 0–12), and on endoscopic evaluation of the distal colon during colonoscopy (grade 0–3) ¹⁷⁹. Surgical specimens from patients with either UC ($n = 6$) or CD ($n = 10$), as well as control specimens obtained from patients who underwent bowel resection for nonmalignant conditions ($n = 6$) were used as a source for immunohistochemical studies. The original pathology examination records of UC and CD patients ranged from mild chronic to severe chronic active inflammation and the corresponding hematoxylin and eosin (H&E) stained sections were reexamined by a pathologist (Clara Hammarström). Specimens from each IBD patient included areas preferably ranging from mild to severe inflammation. Control samples showed no signs of macro- or microscopic inflammation. The clinical characteristics of the IBD patients and controls are listed in paper II, Table 1. In paper I, surgical specimens from normal skin, small intestine, umbilical vein, lung, colon, mammary gland and kidney, as well as from the following tumors: colon adenocarcinoma, ductal mammary carcinoma, renal clear cell carcinoma and renal papillary carcinoma were all used for assessment of endothelial IL-33.

3.2 Immunofluorescence staining

The principle of immunostaining is to take advantage of the stable and specific binding of antibodies to antigens present in cells or in tissue. It is possible to locate the protein of interest within the tissue when the incubating antibody is directly or indirectly labeled

with e.g. a fluorescent dye. By the use of selective filters it is possible to detect light emitted from different fluorochromes, e.g. FITC, Cy2, Alexa 488 (green), Cy3 (red), AMCA and Alexa 350 (blue). By attaching different fluorochromes to different antibodies it is possible to perform e.g. double- or triple-stainings, where two or three proteins, respectively, can be stained for at the same time. This is a valuable method for the localization of proteins at the cellular or even subcellular level.

3.2.1 Fixation and antigen retrieval

Cells were grown in Lab-Tek chamber slides (Nalge Nunc International, Hereford; UK) and fixed in MeOH or paraformaldehyde 4%. Biopsy specimens were either fixed in formalin 4 % or methanol at 4°C for 24 hours, before processing and embedding in paraffin. Thin (2-5 µm) sections were cut and placed on polysine or Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and dried at 37°C overnight. The sections were deparaffinized and rehydrated prior to staining. The tissue fixation preserves the morphology, but this can be at expense of excessive cross-linking of proteins. Since formalin fixation induces cross-linking of amino acid residues by methylene bridges, antigen retrieval is usually needed to re-expose the proteins to the detecting antibodies (reviewed in ref. no. ¹⁹⁷). The most common form is heat-induced antigen retrieval, and the slides were warmed in various buffers to 100°C in a water bath for 20 minutes before cool-down and subsequent incubation with relevant primary antibodies. Some antibodies will detect proteins by their three-dimensional structure and this is best achieved by using a gentle fixative of the tissue, such as cryo fixation or periodate-lysine-paraformaldehyde (PLP) ¹⁹⁸, whereas other antibodies can better detect the denaturated form of a protein, implying that both the fixation method and antigen retrieval protocol must be planned carefully in accordance to the antibodies' requirements. In general, increasing heat will expose more of the antigen (signal), but also increase the unspecific background staining (noise). Additionally, the selection of buffer medium and pH value can have great impact on retrieval success ¹⁹⁹. Protocols should be optimized to produce a high signal-to-noise ratio. Many antibodies can be useful with immunofluorescence techniques if optimal fixation and staining conditions can be identified.

3.2.2 Similarities and differences between species

Different species have unique, but often similar amino acid sequences and protein and immunoglobulin variants. This fact can represent challenges, but can also be exploited

methodologically. An antibody raised towards e.g. the antigen CD31 in the mouse can often detect the homologue protein in other species (human, rat), but on the contrary, an antibody that detects an unequal part of the protein will therefore fail to work in tissues from other species. After binding of a primary antibody to an antigen in tissue, a common technique is next to incubate with a secondary antibody that is conjugated with a fluorochrome allowing for subsequent detection. This secondary antibody should have a specific affinity towards the Fc region of the corresponding primary antibody without binding to endogenous immunoglobulins within the tissue or to other primary or secondary antibodies in multiple staining setups. This level of cross-reactivity is minimized as all the polyclonal secondary antibodies used in this study are cross-absorbed to subtract the clones that bind to other relevant species, but notably this can be at the expense of losing high affinity clones.

3.2.3 Antibodies and controls

Antibody producing cell lines from mice and rats, and recently also from rabbits can be immortalized to produce identical clones of antibodies, so-called monoclonal antibodies. E.g. the monoclonal Nussy-1 antibody (mouse IgG1) is generated towards the C-terminal part of IL-33. High affinity polyclonal antibodies can be produced by immunization in e.g. rabbits, goats and donkeys. The IL-33Nter rabbit polyclonal antibody was generated by Eurogentec (Seraing, Belgium), using a peptide antigen derived from the first 15 amino acids of human IL-33 (MKPKMKYSTNKISTA). Other primary antibodies used in this study and all working concentrations are specified in Table 1. The individual working concentrations of both the primary and secondary antibodies must be optimized to yield a good signal-to-noise ratio. The signal can be enhanced by adding an extra step, e.g. by exploiting the high affinity binding between biotin and streptavidin.

In general, concentration- and species/isotype-matched irrelevant antibodies were applied in parallel to serve as controls for unspecific background staining, e.g. antibody clones with affinity to the peptide carrier Keyhole Limpet Hemocyanin (KLH) and therefore a byproduct in the antibody production. When performing stainings with more than one primary antibody, care must be taken to apply a specific detection system. The chance of cross-reactivity should be minimized also at the level of the secondary antibodies, and should be checked for by omitting all but each one of the primary antibodies. Preferably, one should use secondary antibodies from the same species, e.g. a Cy2 (green) labeled donkey anti-mouse IgG in parallel with a Cy3 (red) labeled donkey anti-rabbit IgG

(if mouse and rabbit IgG primary antibodies were to be detected), as this will practically omit cross-reactivity between the secondary antibodies.

3.2.4 Immunoenzyme stainings

Replacing the fluorochrome in the detection system with an enzyme like horseradish peroxidase (HRP) introduces the possibility to oxidize a diaminobenzidine (DAB) substrate to produce a dark brown precipitate on the tissue slides. This can be followed by another primary antibody that can be coupled to an alkaline phosphatase (AP) enzyme producing e.g. a red color on the tissue slides, allowing for double stainings also with this technique. In contrast to immunofluorescent stainings, multiple stainings with this technique are done in serial. This can be exploited when a double staining with two primary antibodies of both the same species and isotype is set up within the same tissue. However, as a precipitate is laid down on the tissue with this technique, covering the underlying antigens, the staining sequence can have impact on the result, and this technique is therefore less suitable for doublestainings within the same cellular compartments. The slides can then be counterstained with hematoxylin for better orientation of the tissue. These colored precipitates are less inclined to bleach and evaluation is by light microscopy, making this technique also suitable for diagnostic purposes, e.g. classifying tumors.

3.2.5 Microscopic evaluation

The fluorescence stainings were evaluated on a Nikon Ellipse E800 microscope equipped with Nikon Plan-Fluor objectives and an F-VIEW digital camera controlled by a Cell[^]P imaging acquisition software and the immunoenzyme stainings were assessed by an Olympus BX51 microscope with an Olympus U-TVO.5XC camera controlled by AnalySIS 3.2 software (Soft Imaging System). In general, a positive signal was compared to the signal produced by the irrelevant control antibody. In paper I, grey filters with progressive light absorbing properties were used for semiquantitative analysis of the fluorescence signal. Cells staining positive for interleukin-33 in the ulcerative colitis lesions were stained with a broad repertoire of other markers in paper II to identify these cells. In paper III, endothelial cells responding to exogenous IL-33 by *de novo* expression of E-selectin were characterized on the basis of confluence and presence of relevant markers. In paper IV, IL-33 positive cells were identified in a murine inflammation and wounding model.

Table 1. Table of primary antibodies used in this study

Specificity	Clone	Species and subclass	Working Concentration	Source
hIL-33	Nessy-1	Mouse IgG1	1 µg/ml	Alexis
hIL-33	IL-33Nter	Rabbit IgG	1 µg/ml	Eurogentec
hIL-33	ALX-210-447	Rabbit IgG	1 µg/ml	Alexis
hIL-33	ALX-210-933	Rabbit IgG	1 µg/ml	Alexis
hIL-33	AF3625	Goat IgG	1:1000	R&D Systems
mIL-33	AF3626	Goat IgG	1:500	R&D Systems
mIL-33	396118	Rat IgG2a	1:150	R&D Systems
Actin	sc-8432	Mouse IgG1	1:500	Santa Cruz
αSMA	1A4	Mouse IgG2a	1:100	DAKO
BrdU	BU33	Mouse IgG1	2 µg/ml	Sigma
CD31	HEC7	Mouse IgG2a	1 µg/ml	Fitzgerald
CD31	RB-10333	Rabbit IgG	0.4 µg/ml	Labvision
CD31	JC/70A	Mouse IgG1	1:10	DAKO
CD34	QBend/10	Mouse IgG1	1:100	Novocastra
CD45	135-4C5	Mouse IgG2b	3 µg/ml	LabVision
CD45	2B11+ PD7/26	Mouse IgG1	1:100	DAKO
CD68	PG-M1	Mouse IgG3	1:100	DAKO
Cytokeratin	AE1 & AE3	Mouse IgG1	1:20	DAKO
Cytokeratin	MNF116	Mouse IgG1	1:100	DAKO
Desmin	D33	Mouse IgG1	1:100	DAKO
E selectin	BBIG-E4	Mouse IgG1	1 µg/ml	R&D Systems
E selectin	E-1E4	Mouse IgG1	10 µg/ml	PDL
Histone H1	sc-10806	Rabbit IgG	1 µg/ml	Santa Cruz
ICAM-1	84H10	Mouse IgG1	1:500-1000	AbDserotec
IL-8	500-M08	Mouse IgG1	1:250	Peprotech
hHSP47	sc-5293	Mouse IgG2a	1:200	Santa Cruz
rHSP47	M16.10A	Mouse IgG2b	1:200	Stressgen
IL-1R1	129304	Rat IgG2b	10 µg/ml	R&D Systems
Irrel.control	MOPC21	Mouse IgG1	Concentration matched	Sigma
Irrel.control	42/2	Mouse IgG2a	Concentration matched	R. Burns, Edinburgh
Irrel.control	MOPC-141	Mouse IgG2b	Concentration matched	Sigma

Irrel.control	H0892	Rabbit IgG	Concentration matched	Sigma
Irrel.control	I5256	Goat IgG	Concentration matched	Sigma
Irrel.control	KLH/G2a-1-1	Rat IgG2a	Concentration matched	SouthernBiotech
Ki67	MIB-1	Mouse IgG1	10 µg/ml	DAKO
Ki67	ab15580	Rabbit	5 µg/ml	Abcam
Ly-6G	1A8	Rat IgG2a	1:100	BD Pharmingen
Lymphatic EC	D2-40	Rabbit	0.26 µg/ml	DAKO
MC tryptase	AA1	Mouse IgG1	1:2 000	DAKO
PCNA	PC10	Mouse IgG2a	1.25 µg/ml	DAKO
PDGFRβ	28E1	Rabbit mAb	0.15 µg/ml	Cell Signaling
Phospho-Rb	#9308	Rabbit	1:100	Cell Signaling
VE-cadherin	ALX-210-232	Rabbit	1:10 000	Alexis
VE-cadherin	BV9	Mouse IgG2a	10 µg/ml	Hycult Biotech.
VE-cadherin	4.8.G	Mouse IgG1	1:1000	O. Bakke
Vimentin	3B4	Mouse IgG2a	1:200	DAKO
ST2	AF523	Goat IgG	5 µg/ml	R&D Systems

3.3 Animal models

3.3.1 The 3Rs

Russel and Burch developed the concept of *the Three Rs* in the 1950s as: “*Replacement* means the substitution for conscious living higher animals of insentient material. *Reduction* means reduction in the numbers of animals used to obtain information of a given amount and precision. *Refinement* means any decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used”²⁰⁰. This concept is important in everyday planning and performing experiments involving animals. The work included in this thesis has implemented the 3 *Rs* in the way that e.g. cells, rather than animals, has been used for screening purposes checking for biologic responses (replacement), and that a limited number of animals have been used for validation of biologic effects *in vivo* (reduction). These animal experiments have been performed according to institutional and national guidelines, with special care to humane handling of the animals (refinement), e.g. procedures involving injecting, cutting or punching the skin in mice or rats, were only performed on animals that were in deep sedation.

3.3.2 Transgenic animals

Interleukin-33 knock-out mice were shipped by air from Merck Research Laboratories (formerly Schering-Plough Biopharma), Palo Alto, CA. Originally, they were generated by Caliper Biosciences (Taconic, Hudson, NY, USA) on a C57BL/6 background as described in ref. no. ¹⁶⁸. Murine IL-33 is encoded in a 7-exon-6-intron gene located on mouse chromosome 19. With homologous recombination technique the entire 7-exon-6-intron stretch of genomic DNA of mIL-33 was replaced with a targeted transgene containing a neomycin resistance gene flanked by two flip recombinase target sites and homology regions. Since spontaneous homologous recombination is a rare event, successful events were selected for using neomycin treatment of embryonic stem cells. The neomycin resistance gene was then excised using recombinant flip recombinase. This is performed in cultured embryonic stem cells that were harvested from preimplantated mouse blastocysts. The selected embryonic stem cells with the targeted gene alteration can then be injected into a blastocyst stage embryo. If cells are derived from two different sources the offspring can be separated by fur color, but these IL-33 knock-out mice were generated with stem cells also coming from a C57BL/6 background. In the offspring a PCR-based strategy was used to discriminate between wildtype (WT) and mutant alleles at Merck with the following primers: 5' GGCATTAACACTAAGACTACTCAGCCTCAG 3' and 5' GCG-TATGTTTGGTTTGGTGC GA 3'; producing a WT amplicon of 280 bp, and 5' GGC-ATTAACACTAAGACTACTCAGCCTCAG 3' and 5' CGGGGAAATCTTGAGTT-GGAATACT 3', resulting in a mutant amplicon of 450 bp. Identified heterozygotes were mated to produce animals homozygous for the IL-33 deletion. However, in our lab we had some difficulties in performing certain genotyping with these primers. With the help of Finn-Eirik Johansen and Linda Solfjell new primers were designed, and the following ones performed excellent: wt fwd: 5' CAGCCTCAGATTTCTCTGTGC 3', wt rev: 5' TCA-GGTTTCTGTGGATTGA 3' (producing a wt amplicon of 217 bp), mutant fwd: 5' CAGCCTCAGATTTCTCTGTGC 3', mutant rev: 5' TGCAACAATGATGC-ACTGG 3' (producing a mutant amplicon of 343 bp).

3.3.3 Anesthesia and analgesia

A combination of fentanyl/fluanisone (Hypnorm, Janssen) and midazolam (Dormicum, Roche) was injected subcutaneously inducing surgical level anesthesia and analgesia for 30-45 minutes when used in concentrations of fentanyl 160-240 µg/kg, fluanisone 5-7.5 mg/kg and midazolam 2.5-3.8 mg/kg in the rat and fentanyl 400-800 µg/kg,

fluanisone 16.7-25 mg/kg and midazolam 8.3-12.7 mg/kg in the mouse (ref. *Course in anesthesia of small rodents, FHI, Jan 2010*, Henrik Rasmussen, DVM). When skin punches or incisions were performed, the experimental setup was further refined by administrating long-acting bupivacaine (Marcaine, AstraZeneca) directly into the wounds for additional analgesia. Experiments were performed by trained personnel (FELASA category C researchers). In paper II, rat skin incisions were performed by the late chief veterinarian Dag Sørensen. In paper IV, deep sedation was induced in the mice by gas anesthesia with isoflurane (Isoflurane, Baxter), at concentrations of 5% for the induction of sedation and 2-2.5% for the continuation of sedation. The analgesic bupivacaine was applied directly into the wounds when skin punches were made.

3.3.4 Wound healing models

To assess endothelial IL-33 during wound healing, a boat-shaped excision of 1 x 0.5 cm was made between the ears of inbred BD-IX rats using scissors and closed with one suture in paper I. Biopsy specimens were harvested at 1,2,4,6 and 11 days after injury and fixed in formalin for 24 hours at 4°C before processing and embedding in paraffin. For the wound healing time-course presented in paper II we did not have enough material left for analyses of the kinetics and cellular sources of non-endothelial IL-33. Taking advantage of a parallel study by Dag Sørensen, full-thickness, 10 mm midline skin incisions were made with scissors on the back of the neck and then closed with two sutures (Vicryl, 3-0, Ethicon), before harvest of a 10 x 5 mm full-thickness piece of wounded skin at day 0, 1, 2, 3, 4, 6 and 9, before resuturing. In paper IV, 2-5 mm skin biopsy punches were made on deeply sedated mice to standardize the initial injury, aiming for even more accurate and reproducible experimental injuries, allowing for subsequent comparisons between wild-type and IL-33 deficient mice. Initial small pilots demonstrated that the mouse skin was quite elastic, and the injured area often became oval. This was overcome by performing small 2 mm punch biopsies in the ear, where the central sheet of cartilage makes a scaffold for the wound.

3.3.5 Evaluation of injected cytokines, growth factors and bacterial compounds *in vivo*

In paper I, carrier-free recombinant rat TNF α , VEGF or PBS controls were injected subcutaneously on the back. To trace the site of injection, Indian fountain ink (1:200) was added to the vehicle before injection. Leukocyte infiltration was used as a control for the

TNF α injections, and extravasations of Evans Blue dye served as a positive control for the VEGF injections. In paper III, carrier-free recombinant murine IL-33 (0.3 or 3 μ g) or IL-1 β (2 or 200 ng), in PBS 30 μ l, was administered by subcutaneous injection in the inner thigh of C57BL/6 or BALB/c mice. Skin biopsies were fixed in formalin, embedded in paraffin, cut in 4 μ m thin sections and evaluated for infiltrating cells after H&E staining, or for neutrophils, assessed by immunohistochemical staining for Ly-6G (clone 1A8, BD Pharmingen), a marker for neutrophils. In paper IV, 20-100 μ g peptidoglycan (PGN), a structural compound abundantly expressed in Gram positive bacteria known to be a Toll like receptor (TLR)2 agonist, was injected subcutaneously in the dorsal skin of age and sex matched C57BL/6 wild-type or IL-33-deficient mice. The skin was first shaved and the remaining hair in the area of interest was removed by the use of depilation cream, and this was also performed under gas anesthesia. Care was taken to inject superficially in the skin, aiming to deposit the volume of 50 μ l superior to the *panniculus carnosus* muscle layer. Suspensions of *Staphylococcus aureus* (2.5-10 x 10⁶ colony forming units, CFUs) were injected subcutaneously in both wild-type and IL-33 deficient mice, and the developing lesion sizes were recorded.

3.4 Cell culture

In papers I and III, human umbilical vein-derived endothelial cells (HUVECs) were isolated as described by Jaffe and colleagues²⁰¹. Umbilical cords were from the Department of Gynecology and Obstetrics at the Oslo University Hospital Rikshospitalet, following a protocol approved by the Regional Committee for Research Ethics (S-05152). The HUVECs were cultured in MCDB 131 containing 7.5% fetal bovine serum, epidermal growth factor 10 ng/ml, basic fibroblast growth factor 1 ng/ml, hydrocortisone 1 μ g/ml, gentamicin 50 μ g/ml, and fungizone 250 ng/ml. The cells were kept at 37 °C in an incubator with 5% CO₂. The cultures were used at passage one to five. The medium was changed when the cells reached confluence 3-4 days after splitting, and the stimulations with various cytokines and growth factors were started 1 day later when the HUVECs were superconfluent. In paper II, human foreskin fibroblasts (NHDF-c, Promocell, Heidelberg, Germany) were cultured in Fibroblast Growth Medium (C-23010, Promocell) with SupplementMix (C-39315) containing insulin 5 μ g/ml and basic fibroblast growth factor 1 ng/ml, and stimulated with cytokines, growth factors and TLR ligands for 12-24 hours.

In Paper III, growth curves were generated by staining endothelial cell nuclei with crystal violet as described by Gillies et al ²⁰² and improved by Kueng et al ²⁰³. Cells were cultured in microtitre plates with detachable 8-well strips that were removed daily and fixed in PFA. Upon termination of the experiment, nuclei were stained with 0.08% crystal violet in PBS for 5 min followed by repeated rinsing in water, dye elution with 33% acetic acid and recording of OD, read at 570 nm. Three independent experiments were performed. To assess the fraction of cells in S phase, HUVECs were seeded at a density of 2×10^4 cells per well in gelatin-coated chamber slides. After 24 hours, they were washed twice with endothelial cell basal medium (EBM) and incubated for 5 hours in EBM containing 1% fetal bovine serum (FBS). Cells were treated with various concentrations of IL-33 for 36 hours followed by the addition of 10 μ M BrdU for 6 hours, washed in PBS and fixed in 4% PFA for 20 minutes.

For nuclear fractionation, HUVECs were lysed in a buffer containing Nonidet P-40 0.1%, NaCl 10 mmol/l, MgCl₂ 5 mmol/l, NaH₂PO₄ 10 mmol/l at pH 7.4, Na-orthovanadate 65 mmol/l (S-6508, Sigma-Aldrich), and protease inhibitor cocktail (P-8340, 1:100; Sigma-Aldrich), and centrifuged at 900 g for 10 minutes at 4°C. Supernatant fluid containing the cytoplasmic fraction was harvested and the pellet containing the nuclear fraction was resuspended in a buffer containing EDTA 1 mmol/l, sodium dodecyl sulfate 3.5%, glycerol 10%, and Tris 70 mmol/l at pH 6.8. Both nuclear and cytoplasmic fractions were sonicated (4 \times 10 seconds, pulse duration 0.8/second and 20% output of 400 W). Samples were boiled in loading buffer (Tris-HCl 300 mmol/l, pH 6.8, sodium dodecyl sulfate 2%, bromphenol blue 0.1%, glycerol 10%, and dithiothreitol 50 mmol/l), and 20 μ g of protein was separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

3.5 Cell cycle inhibition

To study the effect of cell cycle inhibition in confluent cells in paper I, monolayers were either treated with fumagillin in complete medium for 16 hours or full growth medium was replaced by growth factor- and serum-free MCDB 131 for 24 hours. To inhibit proliferation in subconfluent cells, they were seeded in complete growth medium at a density of 1.9×10^4 cells/cm², either in the presence of fumagillin (16 hours) or cells were allowed to attach for 4 hours before replacing the complete medium by growth factor- and serum-free MCDB 131 and incubating them for another 24 hours. To assess the percentage of cells positive for cell-cycle markers or IL-33, fixed cells were double stained for IL-33

and Ki-67, were the latter is present during all active phases of the cell cycle, or hyperphosphorylated retinoblastoma protein (ppRb), which allows progression from G₁ to S phase.

3.6 siRNA knockdown

In paper I and III, small interfering (si)RNA for IL-33 and negative controls were obtained from Ambion. Singel cell suspensions of HUVECs harvested at subconfluence were incubated with a pre-complexed mixture of siPORTAmine and 40 nM siRNA in Opti-MEM reduced serum medium (Invitrogen Life Technologies), and plated at a density of 7.2×10^4 cells/cm². Medium was replaced by regular growth medium at 6 hours and changed at 24 hours. The cells were harvested or fixed 48 hours posttransfection. siRNA amounts used were below the toxicity level judged from assessment of the control oligos.

3.7 Western blot

Total protein was harvested from HUVEC (paper I and III) or fibroblast (paper III) cultures by lysing them in 2x Laemmli buffer (SDS 4%, glycerol 20%, 2-mercaptoethanol 10%, bromophenol blue 0.004%, Tris HCl 0.125 M) protease inhibitor cocktail (P-8340, 1:100, Sigma-Aldrich), diluting the lysates 1:1 in phosphate buffered saline (PBS) and subsequently boiling the samples for 10 minutes. Protein concentrations were analyzed by the RC/DC Protein Assay kit (BioRad, Oslo, Norway) and up to 20 µg of protein was loaded per lane. The protein was blotted onto a nitrocellulose membrane (Hybond-ECL, RPN303D, Amersham Biosciences). Staining with Ponceau S solution (0.1% (w/v), 5% acetic acid in ddH₂O) was used for visualization of the protein loading. After washing once in ddH₂O the membrane was transferred to a shaking board and blocked in PBS, Tween and 5% non-fat dry milk. After incubation with the relevant primary antibodies, these were coupled to horseradish peroxidase via a conjugated secondary antibody or via an additional biotin-streptavidin bond (detailed in methods, Paper I). The horseradish signal was detected by chemoilluminescence and analyzed on a Kodak Image Station 4000R (Eastman-Kodak, Rochester, NY).

3.8 Cellular enzyme-linked immunosorbent assay (cELISA)

In paper III, the total cellular expression of E-selectin and ICAM was quantified by a cellular enzyme-linked immunosorbent assay (ELISA). HUVECs were cultured and stimulated in 96 well plates before fixation in 0.5% periodate-lysine-paraformaldehyde (PLP) for 10 min in room temperature, and air-dried over night. Fixed monolayers were

incubated with anti-E-selectin (clone BBIG-E4, 5D11, 1 µg/ml, R&D systems) or anti-ICAM (84H10, 0.1 µg/ml, AbDserotec) and then goat anti-mouse Ig (A3673, 1/3000), followed by peroxidase-conjugated swine anti-goat Ig and diaminobenzidine substrate. The color reaction was measured as optical density (OD), read at 492 nm by a plate reader. Wells were subsequently washed in PBS and the cell nuclei were stained with crystal violet, allowing adjustments for variations in cell numbers between wells²⁰³. In parallel wells the primary antibody was replaced by an isotype- and concentration-matched antibody to check for background signal. The peripheral wells were not included in the analyses, but they were filled with sterile liquid, omitting the potential variation within the same plate caused by excessive evaporation in the plate periphery.

3.9 Flow cytometry

Single-cell suspensions obtained after gentle treatment of endothelial cell cultures with 5 mM EDTA were incubated with primary antibodies for 30 min on ice and subsequently with biotin-conjugated donkey anti-goat (6.5 µg/ml, 705-065-147, Jackson) and PE-conjugated streptavidin (2.5 µg/ml, 7100-09, SouthernBiotech) or Alexa 488-conjugated donkey anti-mouse (2 µg/ml, A21202, Invitrogen). Cells were incubated with each secondary antibody or reagent for 20 min and washed twice after each incubation, making the final suspension in medium containing propidium iodide 2.5 µg/mL. The samples were analyzed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson). Irrelevant isotype- and concentration-matched antibodies served as negative controls.

3.10 PCR

Total RNA was isolated from cells using the RNeasy mini kit with on-column DNase treatment (Qiagen, Oslo, Norway). Total RNA was reverse-transcribed using Oligo(dT) and Superscript III reverse transcriptase (Invitrogen Life Technologies). Gene transcripts were quantified by real-time PCR using the Mx3000p system (Agilent Technologies, La Jolla, CA). Transcript levels were normalized against transcript levels for GAPDH and HPRT. In paper II, biopsies were taken from the most severely inflamed colonic mucosa and immediately immersed in RNAlater (Applied Biosystems, Ambion Inc, Austin, TX). Total RNA was extracted according to the Trizol method (Invitrogen, Paisley, UK) and quantified at 260 nm in a U-1500UV/Vis spectrophotometer (Hitachi Instruments Inc., San Jose, CA). RNA integrity was assessed in an Agilent 2100 Bio-analyzer on RNA 6000

Nano chips. All samples included were found to have RNA integrity number (RIN) ≥ 8 . Reverse transcription of total RNA was performed by iScript (Bio-Rad, Hercules, CA). Levels of mRNA of IL-33 and β -actin were determined by real-time quantitative PCR using TAqMan chemistry (Applied Biosystems, Foster City, CA). The stability of β -actin as a housekeeping gene in the present context has been verified earlier²⁰⁴. The PCR efficiencies were found to be close to 2 for both the IL-33 and β -actin cDNA using serial dilutions of the templates, allowing the use of the comparative C_T method²⁰⁵ to calculate the IL-33 transcript levels relative to those of β -actin. The laboratory investigators were blinded to the clinical data. A Pearson correlation coefficient was calculated from logarithmically transformed cytokine expression signals using SPSS 16 (SPSS Inc., Chicago, IL).

3.10 VE-cadherin blocking experiments

Single cell suspensions were seeded at 5×10^4 cells per well of Lab-Tek chamber slides (Nalge Nunc International) coated with 1% (w/v) gelatin type A from porcine skin and cultured for 48 hours until reaching confluence in the presence of monoclonal mouse anti-human VE-cadherin (BV9, 10 μ g/ml, Hycult Biotech) or vehicle (PBS). In paper III, cells were activated by adding rhIL-33 (100 ng/ml) for 5 hours before fixation in methanol. Fixed cells were stained for E-selectin (E-1E4, 1 μ g/ml), IL-33 (IL-33Nter, 1 μ g/ml), and VE-cadherin (ALX-210-232, 1:3000) or CD31 (RB-10333, 0.4 μ g/ml), detecting IL-33 and VE-cadherin, or CD31, with the same secondary antibody, and yet be discriminated since these antigens reside in different cellular compartments.

3.11 Microarray analysis of gene profiles

In paper III, HUVECs pooled from 10 donors were grown to superconfluence and stimulated for 4 hours with medium, IL-1 β (0.5 ng/ml) or IL-33 (50 ng/ml). Total RNA was isolated using the RNeasy Mini Kit with on-column DNase treatment according to instructions of the manufacturer (Qiagen) and processed for microarray analysis by the Norwegian Microarray Consortium at Radiumhospitalet. Each experimental condition was performed in two independent experiments, the intraclass correlation coefficient between these experiments exceeded 0.996 (calculated on SPSS 16 software (SPSS Inc., Chicago, IL)).

3.12 Positron emission tomography (PET)

In paper IV, a total of 16 mice were used for quantification of lesion sizes during wound healing and after injection of the TLR2 ligand PGN. Two animals got 4 mm skin punches as previously described, and assessed at day 1. Two more animals were assessed at day 5 post wounding, but regrettably they both died during the PET recording. A total of nine animals were injected with subcutaneously with a volume of 50 μ l of PGN in the range of 2-2000 μ g/ml, or LPS 200-500 μ g/ml. Animals were free to drink, but food was removed 4 hours prior to the PET assessment to reduce hyperglycemic dilution and to increase the uptake of the [18 F]-labeled fluorodeoxyglucose ([18 F]-FDG) substrate. Before PET assessment, the mice were put in deep sedation according to weight by a combination of zolazepam, tiletamine, xylazine and fentanyl. Intravenous access was established by positioning a small BD Vasculon Plus 26 GA 0.6x19 mm (Beckton Dickinson, Franklin Lakes, NJ) peripheral vein catheter filled with heparin 10 IE/ml, into the tail vein. The mice were then positioned into a small animal PET scanner (microPET Focus 120, Siemens Medical Solutions, Erlangen, Germany). 10 MBq [18 F]-FDG per animal was injected i.v. (or i.p., when positioning of the peripheral catheter failed), and data was collected for 90 minutes. Accompanying computer tomography (CT) data were collected with a small animal CT scanner (microCAT, Siemens Medical Solutions, Erlangen, Germany). A multi-modality bed was used to collect data from both the PET and CT scanner without repositioning the animals relative to this cast. To mark the point of wounding or injection, first 280 nm (M-280) or 450 nm (M-450) Dynabeads (DynaMagnetic Beads, Invitrogen) were injected in a volume of 50 μ l in a concentration of up to 6.5×10^8 and 1.4×10^8 beads/ml, respectively. However, as this was not detected by the CT, small titanium clips were used to mark the lesions. The animals were euthanized at the end of the experiment while still in deep sedation. Skin lesions were also harvested and analyzed separately on a Wallac Wizard 1470 gamma counter (Perkin Elmer).

4 Summary of results

4.1 Paper I

Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation.

Küchler AM, Pollheimer J, Balogh J, Sponheim J, Manley L, Sørensen DR, De Angelis PM, Scott H, Haraldsen, G.

IL-33 has been demonstrated to act via the ST2 receptor and also been proposed to act as a transcriptional repressor in endothelial cells. In paper I we showed that IL-33 is expressed in endothelial cell nuclei of blood vessels in healthy tissues, and was induced in endothelial cell cultures grown to confluence and ceasing to proliferate, but lost when the cells were released from confluence and allowed to migrate. We asked if this could be explained by contact inhibition, but IL-33 induction was at least not prevented by antibody-mediated blocking of the endothelial-endothelial interacting VE-cadherin, and knockdown of IL-33 did not have any noticeable effect on the endothelial cell distribution of VE-cadherin or CD31. We then tested if inhibiting cell cycle progression could increase the levels of IL-33 in subconfluent cultures, and found that neither cell starvation nor treatment with fumagillin induced IL-33. However, stimulation with tumor necrosis factor- α (TNF α) or vascular endothelial growth factor (VEGF) down-regulated nuclear IL-33 both *in vitro* and *in vivo*, consistent with the observation that vascular IL-33 was downregulated in angiogenic vessels during wound healing. Furthermore, we found that IL-33 was almost absent in tumor vessels.

Conclusion of the study:

Endothelial IL-33 is present in vessels of healthy tissues, but is lost during experimental wound healing and in tumor vessels. Likewise, IL-33 is induced in endothelial cell cultures when they reach confluence, but is lost when such cells are allowed to migrate, or in response to *in vitro* treatment with TNF α and VEGF. The cytokines have the same effect *in vivo*. In conclusion, downregulation of IL-33 may alleviate the proposed transcriptional repressor function and thereby facilitate endothelial cell activation.

4.2 Paper II

Inflammatory bowel disease-associated interleukin-33 is preferentially expressed in ulceration-associated myofibroblasts

Sponheim J, Pollheimer J*, Olsen T*, Balogh J, Hammarström C, Loos T, Kasprzycka M, Sørensen DR, Nilsen HR, Kückler AM, Vatn MH, Haraldsen G

*) the authors contributed equally

Interleukin-33 has been demonstrated to induce mucosal pathology *in vivo* by inducing goblet cell hypertrophy and increased mucus. In paper II, we aimed to investigate a potential role of IL-33 in inflammatory bowel disease. First, we observed a 2.6-fold up-regulation of IL-33 mRNA levels in biopsy specimens from untreated ulcerative colitis patients compared to controls. To explore the cellular sources of IL-33, we next immunostained surgical specimens from inflammatory bowel disease patients, observing that large IL-33⁺ cells were accumulated in the base of ulcers. These cells did not express markers of endothelial- or hematopoietic cells, but were positive for vimentin, the fibroblast marker heat shock protein 47, platelet-derived growth factor receptor (PDGFR) β , and, in part, the myofibroblast marker α -smooth muscle actin (α SMA), leading us to conclude that these IL-33⁺ cells were ulceration-associated myofibroblasts. However, this did not seem to be a unique feature for inflammatory bowel disease lesions, as we could identify such IL-33⁺ cells also in the granulation tissue of gastric ulcers and in cutaneous wound healing in the skin. In contrast, IL-33-positive myofibroblasts were almost absent near the deep fissures seen in Crohn's disease. In fibroblast cell cultures IL-33 was induced by tumor necrosis factor- α (TNF α) and IL-1 β , but we found that the TLR3 agonist poly (I:C) induced even higher expression levels, and that it synergized with TGF β , also known to boost myofibroblast differentiation. To investigate the recruitment of IL-33⁺ cells to granulation tissue of ulcers, we collected samples from healing skin wounds in rat skin, and observed *de novo* induction of IL-33 in pericytes. Moreover, we observed the occurrence of scattered, tissue-resident IL-33⁺PDGFR β ⁺ α SMA⁻ cells that were either migrating pericytes or activated fibroblast-like cells, in either case an event that preceded the later appearance of IL-33⁺PDGFR β ⁺ α SMA⁺ cells.

Conclusion of the study:

IL-33 is present in ulceration-associated myofibroblasts in inflammatory bowel disease and in cutaneous wound healing, pointing to a novel role for IL-33 in mucosal healing and wound repair. The apparent paucity of IL-33⁺ cells in fissures of Crohn's disease might indicate a different microenvironment and ensuing myofibroblast phenotype in such lesions.

4.3 Paper III

Interleukin-33 preferentially targets non-quiescent endothelial cells

Pollheimer J*, Balogh J*, Sponheim J, Brox MJ, Loos T, Sundlisæter E, Vatn MH, K uchler AM, Haraldsen G.

*) the authors contributed equally

We found that IL-33 could upregulate the cell adhesion molecules E-selectin and ICAM-1 on endothelial cells similar to IL-1 β , and confirmed that IL-33 signals via the IL-33R α /ST2 receptor. In fact, cultured endothelial cells exposed to IL-33 induced virtually identical transcriptome-wide activation profiles compared to endothelial cells stimulated with IL-1 β . We observed that IL-1 β - or IL-33-induced expression of E-selectin were both abolished by inhibitors of NF κ B and reduced by inhibitors of p38 and JNK. We further demonstrated that IL-1 β activated virtually all cultured endothelial cells, whereas IL-33 selectively activated subconfluent, non-quiescent cells that partly expressed Ki67, ppRb and cyclinD1, but not quiescent cells that expressed nuclear IL-33, high levels of p27KIP, and VE-cadherin. In line with this, we observed that functional levels of the ST2 receptor (ST2L) were restricted to such subconfluent cells, and that injecting recombinant IL-33 in healthy murine skin only induced a modest infiltration of granulocytes compared to IL-1 β . We tried to modulate the contact inhibition of cell growth by blocking of junctional VE-cadherin with a monoclonal antibody or knock-down of endogenous, nuclear IL-33, but neither of these approaches altered the selective IL-33 responsiveness.

Conclusion of the study:

These findings suggest a novel concept of selective activation of endothelial cells potentially important in the context of endothelial cell activation and/or vascular remodeling after e.g. tissue damage, in which their level of quiescence appears to determine the responsiveness to IL-33.

4.4 Paper IV

Interleukin-33 mediates neutrophil recruitment in immunity against

Staphylococcus aureus

Sponheim J*, Loos T*, Rankin AL*, Sundnes O, Nilsen HR, Manley L, Scott H, Pflanz S, Haraldsen G

*) These authors contributed equally

To investigate the contribution of IL-33 to host defense we used a model of *Staphylococcus aureus* skin infection in mice made genetically deficient in IL-33. Substantially larger skin lesions with higher bacterial counts and impaired neutrophil recruitment developed in IL-33-deficient mice compared with wild-type mice following subcutaneous injection of live bacteria. However, co-injecting recombinant IL-33 did not restore this phenotype. First we addressed whether impaired wound healing could account for the observed discrepancy, but macro- and microscopic assessment of wound sizes during cutaneous wound healing did not uncover significant differences. Nevertheless, we demonstrated that 1-week old cutaneous wounds of IL-33^{-/-} animals contained higher transcription levels of IL-1 β , IL-6 and COX2/PTGS2 and the chemokines GRO γ and LIX, and lower levels of several collagen subtypes and tissue repair transcripts such as α -smooth muscle actin (α SMA), compared to WT controls. By immunostaining healthy murine skin we observed IL-33 in nuclei of keratinocytes, periglandular cells and in scattered fibroblast-like cells in skin. 6 hours after wounding IL-33 was almost undetectable at the edge of lesions, and reappeared at 24 hours, but was never seen in Ki67-positive, proliferating keratinocytes. In parallel, IL-33 was induced in scattered fibroblast-like cells. A similar development in IL-33 expression was observed upon

injecting a sterile prepared peptidoglycan (PGN), a Toll-like receptor 2 ligand and a structural component of bacterial walls. This produced a strong inflammatory response with rapid abscess formation in both IL-33-deficient and WT mice, which were not macroscopically distinguishable. However, upregulation of transcript levels for IL-1 β , L-selectin and PSGL1 were delayed in lesions in IL-33 deficient animals whereas mRNA levels for GM-CSF and E-selectin were similar to lesions in WT controls. Moreover, isolated murine dermal fibroblasts produced a large panel of proinflammatory cytokines and chemokines in response to recombinant IL-33 through ST2L.

Conclusion of the study:

IL-33 appears to play a role in the immediate immune response in the skin, possibly by release from e.g. keratinocytes after tissue damage secondary to wounding or infection.

5 General discussion

5.1 Endothelial IL-33

Paper I demonstrated that IL-33 is a nuclear factor generally expressed in resting endothelial cells in healthy tissues of most organs. Conversely, IL-33 is strongly downregulated in the course of the endothelial cell activation seen in wound healing and tumor growth. These changes have good correlates *in vitro* because induction of nuclear IL-33 in endothelial cells appears to require the following conditions: first, cells must organize in a superconfluent monolayer and second (but possibly a consequence of the first condition), they have to be nonproliferating, because we never observed double-positive endothelial cells for both IL-33 and markers of proliferation. Indeed, the inhibition of cycle progression by growth factor deprivation or fumagillin treatment in subconfluent cultures failed to induce IL-33. We therefore concluded that the non-proliferative cell cycle state is not sufficient but perhaps a requirement for IL-33 induction.

The apparent association of IL-33 expression with the induction of a superconfluent monolayer prompted us to assess the role of VE-cadherin, considered important for endothelial integrity²⁰⁶. However, our efforts to manipulate contact inhibition by addition of a blocking antibody to VE-cadherin did not down-regulate IL-33, but such inhibition can be considered incomplete compared to that obtainable under conditions of flow or *in vivo*^{206, 207}. Conversely, the lack of change in VE-cadherin or CD31 expression levels or cellular localization observed after IL-33 knockdown implies that IL-33 is not involved in the control of junctional integrity but the result may be attributable to the incompleteness of IL-33 knockdown. Taken together, the careful dissection of the interplay between density-associated signals, cell cycle, and the expression of IL-33 deserves future attention.

The strong down-regulation of IL-33 in angiogenic vessels in tumors and in the course of wound healing could be explained by a cytokine profile in wounds containing those found to down-regulate IL-33 levels *in vitro* and *in vivo* (paper I) and inflammatory mechanisms may also dominate the tumor microenvironment²⁰⁸ perhaps explaining the absence of vascular IL-33 in the tumors examined in this study.

Although nuclear, full-length/precursor IL-33 has been described to possess transcriptional repressor properties¹⁵⁶ and also to interact with several nuclear proteins²⁰⁹ at least *in vitro*, the precise role of IL-33 in endothelial cells remains unclear. After publication of Paper I, it has also been shown that IL-33 may interact with proinflammatory NF- κ B signaling in fibroblasts²¹⁰. Thus, taken together these data are compatible with nuclear IL-33 acting as a repressor of proinflammatory or proangiogenic activation, and that downregulation of endothelial IL-33 might facilitate such activation.

5.2 IL-33 in inflammatory bowel disease and wound healing

To further explore the cellular sources of interleukin-33 in inflammation we first mapped IL-33 in biopsies from inflamed intestines from patients with inflammatory bowel disease (paper II). We observed higher levels of mRNA IL-33 in ulcerative colitis lesions compared to controls, and that a dominant source of IL-33 may be ulceration-associated myofibroblasts. These cells were not unique to ulcerative colitis lesions since they could

also be identified in both gastric ulceration and experimental wound healing of the skin, allowing us to conclude that the accumulation of IL-33⁺ myofibroblasts may be a general feature of mucosal healing and wound repair.

We observed that the TLR3 ligand poly (I:C) was the most potent inducer of IL-33 in fibroblast cultures and that TGF β could boost this effect, well in line with the report from Sugiura et al demonstrating that TLR3 activation also promotes myofibroblast differentiation²¹¹. In fact, poly (I:C) has been shown to promote wound healing in rats and accelerate reversal of fibrosis after carbontetrachloride induced liver damage^{212, 213}. While TLR3 is commonly thought to sense the presence of virus-derived dsRNA, it is less well-known that it is also activated by host mRNA released from damaged or necrotic cells upon tissue damage. It is tempting to consider the recruitment of IL-33-positive fibroblasts to ulcers as a protective process since it is similar to the observations made during wound healing in the skin, and interestingly TLR3 activation has been shown to be protective in a dextran sulphate sodium (DSS)-induced colitis model²¹⁴. On the other hand, the lack of IL-33-positive myofibroblast accumulations near the commonly present deep fissures of Crohn's disease (CD) lesions may reflect an impaired response of these fibroblasts to local tissue damage and breaks in the epithelial barrier, perhaps allowing for the extension of such pathological fissures. The latter explanation might represent a general mechanism of ulcer formation in CD, or more specifically, related to the subgroup of CD patients with penetrating disease (fistula formation). Interestingly, TLR3 expression has been reported to be reduced in Crohn's disease²¹⁵, and while lamina propria mononuclear cells from ulcerative colitis patients have showed increased production of TGF β compared to controls, lower levels of TGF β were observed in CD samples²¹⁶. CD lesions are also known to contain elevated levels of IFN γ which inhibit TGF β signaling²¹⁷ and α SMA induction in fibroblasts²¹⁸. Our observation that IL-33 is lacking in active CD lesions is supported by a study from Kobori et al²¹⁹. To this end, Crohn's disease often displays a segmental distribution of inflamed intestine that can be surprisingly stationary, perhaps implicating resident stromal cells in the pathogenesis?

Uncovering the origin(s) of the myofibroblast has drawn much attention. It is thought that the myofibroblast can be derived from several other cell types, and while not the main aim of this study, we observed several interesting features of IL-33 relevant to this question. First, IL-33 appears well suited to enhance the resolution of myofibroblast characterization which still is challenged by the paucity of specific markers (reviewed in refs. nos. ^{52, 60}).

Second, the expression of IL-33⁺PDGFRβ⁺αSMA⁻ fibroblast-like cells was an early event preceding the later appearance of IL-33⁺PDGFRβ⁺αSMA⁺ myofibroblasts, implying that IL-33 may serve as a new early marker for myofibroblasts, knowing that the classic myofibroblast marker αSMA is only induced late in healing wounds⁵³.

The function of IL-33 in fibroblasts, pericytes, and myofibroblasts is currently unknown but the transcriptional repressor activities of IL-33 in recombinant systems discussed in section 5.1 may apply to these cell types as they do to endothelial cells. Indeed, these ulceration-associated myofibroblasts were situated superiorly in the ulcers, being prone to repeated tissue damage. Even though our efforts to measure IL-33 in the supernatant of cultured fibroblasts were not successful, IL-33 still can be expected to be released upon cell damage¹⁵³. It is possible that released IL-33 may promote angiogenesis²²⁰ and scar formation¹⁷⁰, perhaps by acting directly or indirectly on stromal cells. How IL-33 can affect neighboring cells, e.g. endothelial cells, is addressed in the following section.

5.3 Effects of IL-33 on endothelial cells

In paper III we observed that IL-33 acts via IL-33R/ST2L by using a blocking antibody to the receptor, and that recombinant IL-33 selectively targets proliferating, non-quiescent endothelial cells. By contrast, non-proliferating, quiescent endothelial cells were poor responders to recombinant IL-33 *in vitro*, and the modest granulocyte infiltration observed when IL-33 was injected in healthy skin could possibly be explained by the fact that most endothelial cells *in vivo* are quiescent. This is in line with data from Manetti et al on IL-33-positive vessels of healthy skin that showed undetectable vascular expression of ST2, whereas activated vessels in scleroderma lesions displayed the inverse phenotype¹³⁸.

This study does not answer the question about the *in vivo* relevance of the endothelial cells' selective response to IL-33 according to their state of quiescence, but it is tempting to speculate on the physiological advantage of making only the non-quiescent cells respond to soluble IL-33. If the principal route of extracellular release of IL-33 is by cell damage, maybe only a fraction of the endothelial cells should increase their pro-inflammatory activation, e.g. the presently activated and/or sprouting endothelial cells, while the gross number of stromal cells still should fulfill other tasks, e.g. maintaining

barriers and tissue integrity. If IL-33 does represent an important alarmin^{99, 159} *in vivo*, maybe such a selective responsiveness is a built-in protection against an uninhibited activation of endothelial cells that could otherwise lead to failure of the already existing vasculature, whereas subconfluent endothelial cells *in vitro* could possibly reflect vascular sprouts that need activating cues and indeed are assembled efficiently after tissue damage. Future work will shed more light on these matters.

5.4 IL-33 in host defense and wound healing

In paper IV, we could take advantage of a recently generated C57BL/6 mouse strain that was deficient in IL-33 (IL-33^{-/-}). These mice were breeding normally, but in a bacterial skin infection model we observed that their ability to resolve a subcutaneous *S. aureus* infection was impaired when compared to wild-type controls. We asked if this impairment was due to either defective wound healing or an inability to mount a proper immune response by the host. Ear punch wounds in the absence of IL-33 contained more proinflammatory transcripts and fewer transcripts related to tissue regeneration one week after wounding, perhaps pointing to a prolongation of a dysmodulated primary inflammatory response resulting in a delayed initiation of wound healing. Likewise, the delayed host response to the TLR2 ligand peptidoglycan (PGN) during the first hours after s.c. PGN-injections pointed to an inefficient early immune response including an impaired early leukocyte infiltration in the lesion. These findings are in line with a recent report from Oboki et al observing that deficiency in IL-33 in a DSS experimental colitis model resulted in both delayed local inflammation, possibly due to reduced levels of neutrophil-chemoattracting factors, and also a delay in the resolution of the damaged tissue²²¹.

The analysis of IL-33 expression in the wild-type mice during wound healing and the PGN-response revealed interesting patterns. First, we observed for the first time that keratinocyte expression is rapidly downregulated in the course of the tissue activation that follows injuries, perhaps representing release of IL-33 as seen in other cell types. Such release may serve to activate several cell types including fibroblasts that, as demonstrated here, express IL-6 and several inflammatory chemokines in response to IL-33. Released IL-33 may also activate mast cells, allowing subsequent release of preformed TNF α ¹⁶⁶. It is also possible that IL-33 is not released from keratinocytes,

unless they become necrotic in the course of e.g. an ulceration, and that a short half-life of protein combined with a downregulation of IL-33 transcription rather serves to release a nuclear repressor function as described in other cell types^{156, 210}. The fact that injection of recombinant IL-33 in IL-33-deficient mice had no effect on the phenotype in this study, may favor the latter hypothesis but it is also possible that increasing the number of injected bacteria in that experiment may have obscured the rescue effect. Nevertheless, either of these mechanisms may serve to explain, at least in part, the delayed response in mice devoid of IL-33.

The strong upregulation of IL-33 mRNA seen within 6 h may originate from the induction of IL-33 in fibroblast-like cells surrounding the inflamed area. These cells were organized around the lesion and were most likely myofibroblasts, based on co-expression of HSP-47, and therefore very likely the same cell population that was recruited to intestinal ulcers and wounds in rat skin in Paper II.

The experiments presented in this study do not presently identify the specific molecular mechanisms that account for the phenotype observed in response to dermal *S. aureus* infection, but the data obtained imply that both the wound healing process and innate immune response can turn out to be dependent on IL-33 to function optimally. A number of improvements can be made to more firmly identify critical IL-33 functions, such as expanding the wound healing experiments to include more time points, and in the PGN model include higher number of replicates. Likewise, both approaches need to confirm the delayed responsiveness at the protein level. Nevertheless, these data indicate that IL-33 is important for mounting an efficient early immune response and more experiments are needed to clarify this matter.

6 Conclusions and future aspects

We have seen that IL-33 is dynamically changed under inflammatory conditions such as in the process of wound healing and in inflammatory bowel lesions. Puzzlingly, inflammatory stimuli have opposing effects on nuclear IL-33 in endothelial cells and fibroblasts. Still, a conceivable common denominator is that nuclear IL-33 is expressed in tightly interconnected cells, e.g. quiescent endothelial cells lining blood vessels, activated myofibroblasts in a wound and in fibroblastic reticular cells (FRCs) in lymph nodes. In fact, complicated cytoskeletal rearrangements, e.g. in myofibroblasts during wound healing, is incompatible with cell division²²², and interestingly we never observed IL-33⁺Ki67⁺ double-positive cells in this study. This incompatibility is during *Drosophila* development resolved by the proteins Tribbles and Frühstart that prevents cells from entering the cell cycle^{222, 223}. The human homologues of these proteins are not yet identified, but theoretically nuclear IL-33 could favor such a pro-structural, anti-mitotic cell program, which is also in line with nuclear IL-33 reappearing abundantly in murine keratinocytes upon reepithelialization of skin wounds. With repeated trauma resulting in significant cell damage IL-33 can possibly be released, alarming neighboring cells about the protracted damage to important structures and thereby modulate the amplitude of the inflammatory response. Future studies should aim to establish the importance of IL-33 under such conditions and clarify if this can be exploited in a therapeutic manner.

A different but essential aspect of inflammation is the resulting fibrosis. Rankin and coworkers have demonstrated that repeated injections of soluble, recombinant IL-33 into murine skin induced cutaneous fibrosis¹⁷⁰ but it remains unknown whether IL-33 favors fibrogenesis when expressed as a nuclear protein. Interestingly, fetal wounds heal without scarring²²⁴, and contain unique features like “purse string pulling” rather than wound contraction, and less inflammation. Scar development is also reduced in the oral mucosa²²⁵. Much interest is put into designing anti-fibrotic drugs, yet none are considered successful. Mapping of IL-33 in such wounds could potentially provide more information about the relevance of IL-33 also in this respect.

And finally we come back to a later statement from Mechnikov’s Nobel Laureate Lecture from 1908: “*The problem of the inner mechanism of immunity is so delicate and complicated that it is not yet definitely resolved*”²²⁶.

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8 Errata

- 3.2.2 page 36, line 8: to other *primary or* secondary antibodies
- 3.3.5 page 42, line 2: IL-1 β (2 or 200 ng) in PBS 30 μ l.
- Paper III page 7, line 8: ~~rabbit~~ goat anti-mouse Ig (4 ~~μ g/ml~~ A3673, 1/3000)
- Paper III page 21, Fig 1D: rmIL-1 β 2 ng/~~ml~~, rmIL-33 300 ng/~~ml~~
- Paper IV page 18, line 27 *Scale bars are 100 μ m*

