UNIVERSITY OF CALGARY

CD43 in the murine inflammatory response

by

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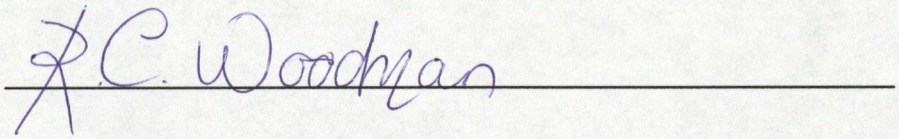

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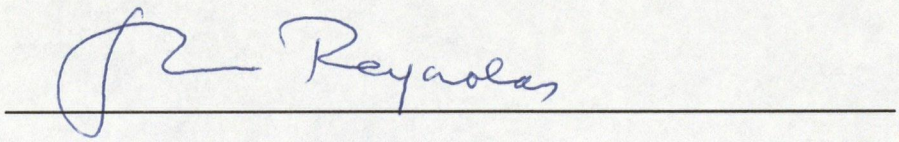

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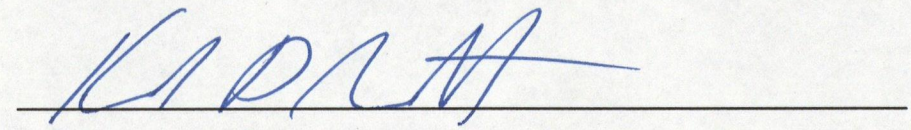

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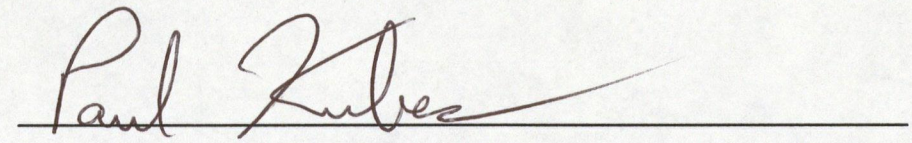

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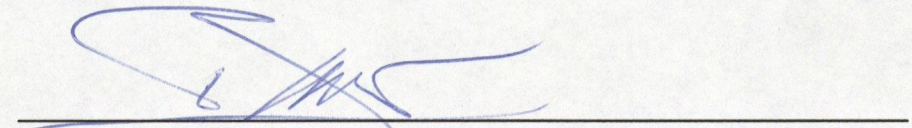

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Abstract

CD43 is a large, sialomucin present on most leukocytes. It has a well-defined anti-adhesive function and CD43-deficient leukocytes show increased adherence to endothelium. This does not always lead to increased recruitment as well, suggesting an additional role for CD43 in the promotion of leukocyte emigration.

For this thesis we utilized comparisons between CD43-deficient and normal animals to investigate the role of CD43 in an acute inflammatory process.

In a model of bacterial peritonitis, we show impaired host defense against a bacterial pathogen associated with CD43 deficiency. This is due to significantly reduced leukocyte recruitment. This reduction is, at least partially, due to excessive accumulation of CD43-deficient leukocytes in other than target tissue.

We show novel data demonstrating murine neutrophil CD43 shedding in response to inflammatory stimuli and recruitment. This illustrates important parallels with the human system and further validates the murine model for CD43 research purposes.

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AP-1	Activated Protein-1
APC	Antigen Presenting Cell
BPI	Bactericidal Permeability-Increasing Factor
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CGD	Chronic Granulomatous Disease
CLA	Cutaneous Lymphocyte Antigen
CR	Complement Receptor
DAG	Diacylglycerol
DC	Dendritic Cell
ds RNA	Double stranded RNA
Dv	Venular diameter
E.coli	Escherichia coli
EC	Extracellular
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ERK	Extracellular Regulated Protein Kinase
ESL	E-Selectin Ligand
FcR	Fc Receptor
FITC	Fluorescein Isothiocyanate
fMLP	formyl-Methionyl-Leucyl-Phenylalanine
GAP	GTPase-activating protein
G-CSF	Granulocyte Colony Stimulating Factor
GDI	Guanine nucleotide dissociation inhibitor
GEF	Nucleotide exchange factors
GlyCAM	Glycoprotein Cell Adhesion Molecule
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GTP	Guanine Triphosphate
HEV	High Endothelial Venules
HUVEC	Human Umbilical Vein Endothelial Cells
i.p.	Intraperitoneal
i.v.	Intravenous
IAP	Integrin Associated Protein (CD47)
IC	Intracellular
ICAM	Intercellular Cell Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IP3	Inositol 1,4,5-trisphosphate
IR	Ischemia-Reperfusion
IRAK	IL-1 receptor associated protein kinase
JAM	Junctional Adhesion Molecule
KO	Knockout

LAD	Leukocyte Adhesion Deficiency
LBP	Lipopolysaccharide-binding protein
LFA-1	Lymphocyte Function Associated Antigen-1
LPAM	Lymphocyte Peyer's Patch Adhesion Molecule
LPS	Lipopolysaccharide
LT	Leukotriene
mAb	monoclonal Antibody
MAdCAM	Mucosal Addressin Cell Adhesion Molecule
MAP kinase	Mitogen-Activated Protein kinase
MBL	Mannose Binding Lectin
MFI	Mean Fluorescence Intensity
MPO	Myeloperoxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear transcription Factor-kappa B
NK cell	Natural Killer cell
PAF	Platelet-Activating Factor
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
PECAM	Platelet Endothelium Cellular Adhesion Molecule
PH domain	Pleckstrin-homology domain
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PI3K	Phosphoinositide 3-kinase
PLN	Peripheral Lymph Node
PMA	Phorbol Myristyl Acetate
PNAd	Peripheral Lymph Node Addressin
PRR	Pattern Recognition Receptor
PSGL	P-Selectin Glycoprotein Ligand
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate
SCR	Short Consensus Repeat
SEM	Standard Error of Mean
SLe	Sialyl Lewis
SOD	Superoxide Dismutase
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor-Activated Factor
TSA	Tryptic Soy Agar
VCAM	Vascular Cellular Adhesion Molecule
VLA	Very Late Antigen
V_{WBC}	White Blood Cell Velocity
V_{RBC}	Red blood cell velocity
WT	Wildtype
XD	Xanthine Dehydrogenase
XO	Xanthine Oxidase

Chapter One

Introduction and Background

Introduction

Acute inflammation is the response of the immune system to tissue damage or infection by microorganisms. A key feature of this rapid response is the movement of leukocytes from the bloodstream to the affected site, a process known as leukocyte recruitment. The ability to rapidly accumulate sufficient numbers of leukocytes at sites of infection is crucial to host defense. The failure to do so results in severe recurrent infections as seen in patients with Leukocyte Adhesion Deficiency or neutropenia (100, 101).

The main effector cell type in the acute inflammatory response, the neutrophil, eliminates its target through the release of highly reactive molecules. The combination of the nonspecific nature of this innate inflammatory response and the toxic properties of these substances, can result in unwanted tissue damage. This is aptly demonstrated in clinical cases of ischemia-reperfusion injury, inflammatory bowel disease and rheumatoid arthritis in which excessive neutrophil influx is an important factor in the pathogenesis (102, 103, 104). Precise control of leukocyte recruitment is therefore a critical element in mounting an immune response that is both effective against pathogens and safe for the host.

In recent years considerable research has focused on the mechanism of recruitment of leukocytes from the bloodstream into tissues. This has led to the identification of many surface adhesion molecules involved in the so-called leukocyte recruitment cascade: a complex interplay between adhesion molecules expressed by the leukocyte and the endothelium lining the blood vessel wall, leading to leukocyte recruitment (105). Although many of the adhesion molecules that promote leukocyte recruitment have been identified, less is known about molecules that balance this inflammatory response by inhibiting recruitment.

One candidate molecule is CD43. CD43 is classically viewed as a passive inhibitor of cell-cell interactions, and therefore of leukocyte recruitment. There is now evidence however that in the complete absence of CD43, neutrophil recruitment is in fact severely impaired. This suggests a more complex role for CD43 in both anti- and pro-adhesive processes. The objective of this thesis is to characterize the phenotypic consequences of complete CD43 deficiency during acute inflammatory responses.

1.1. Leukocyte recruitment

Inflammation is a response to invasion by microorganisms and a wide variety of tissue damage. During inflammation, leukocytes are rapidly recruited to the affected area to clear the pathogen and its toxins, remove necrotic tissue and ultimately restore organ (or tissue) function. The different processes ultimately leading to recruitment are discussed below.

1.1.1. Margination

The physical forces that need to be overcome to bring a circulating leukocyte to a full stop are impressive. The process depends both on rheological properties of the vascular system and on chemical (adhesive) interactions between the leukocyte and endothelial cells. The initiation of adhesive interactions between leukocytes and the blood vessel wall is favored by a process called margination i.e. the concentration of leukocytes near the endothelial lining of the vessel. Margination is a direct consequence of the large amount of erythrocytes found in the blood (between 37 and 49 % of total blood volume in healthy individuals). Because these cells are also approximately half the size of leukocytes (diameter 6-8 μm for erythrocytes vs. 8-14 μm for leukocytes in humans), they are concentrated in the center of the blood vessel, forcing leukocytes closer to the blood vessel wall (11,131). This region is also the most turbulent due to the flow profile in the vessel, variations in vessel diameter and branching, additionally favoring proximation to the endothelium (46,106). Although only partially understood, these

combined rheological properties enable more leukocytes to potentially interact with the endothelium than observed in a system of only uniformly sized cells (11, 46).

The venular side of the vascular system favors leukocyte recruitment because of lower mean flow rates resulting in (non-linearly) reduced shear forces. In addition, only post capillary venules express adhesion molecules (discussed later), crucial to leukocyte recruitment. Because of the combination of low flow rate and adhesion molecule expression, post capillary venules are key sites of leukocyte extravasation.

1.1.2. The paradigm of the leukocyte recruitment cascade

This cascade describes the now generally accepted concept of several distinct steps in the recruitment of leukocytes: Rolling, followed by activation/firm adhesion and transmigration out of the vasculature (reviewed in 105, 107, 122). Perhaps the most valuable concept described by this paradigm is that recruitment requires a complex interplay between leukocytes and endothelium; neither activated leukocytes nor activated endothelial cells alone support all stages of cellular recruitment. Combined with the large degrees of redundancy and promiscuity of the molecules involved, this leads to several levels to regulate leukocyte recruitment. It is this precise control that enables differential recruitment of the diverse leukocyte subtypes to the target site, in the correct numbers. The different steps in the cascade are discussed separately below (See figure 1). For a list of the adhesion molecules and their ligands involved in leukocyte recruitment see table 1.

1.1.2.1. The leukocyte recruitment cascade: Rolling

Leukocytes rolling can be defined as 'leukocytes moving slower than erythrocytes along the blood vessel wall' and is considered a prerequisite for all other steps of the recruitment cascade. It allows leukocytes to interact with the endothelium and the different pro-inflammatory molecules it may express. The specialized, long molecules primarily responsible for the transition of a circulating leukocyte to a rolling state are the selectins (17,108). Three selectins that can mediate rolling have thus far been identified: P, E and L-selectin. They all share a similar extracellular structure with a Ca^{2+} -dependent amino terminal lectin domain, an epidermal growth factor (EGF)-like domain and a varying number of short consensus repeats (SCRs) that determines the length of the molecules. P, E and L-selectin possess nine, six and two SCRs, respectively (17, 124).

A general characteristic of selectins is the transient nature of interaction with their ligand (195). It is this quality, combined with their length, that make selectins ideal as first contact molecules. Multiple engagement and disengagement interactions between selectins and a marginated leukocyte effectively impede the velocity of the cell and causes rolling along the endothelium. This brings the leukocyte closer to the endothelium and allows for further interactions (111). This can be considered a critical step in the recruitment cascade. If the correct activation signals (discussed later) are not received, the cells remain within the bloodstream. Under non-inflammatory conditions many leukocytes will briefly roll on venular endothelium only to disengage moments later, returning to the circulation (130).

P-selectin ("platelet selectin") is prestored in the α-granules of platelets and in Weibel-Palade bodies of endothelial cells. This pre-storage enables endothelial P-selectin surface expression to occur within minutes, suggesting it is a crucial element in acute inflammation. Although P-selectin is constitutively expressed, its expression can be increased dramatically in response to inflammatory mediators such as thrombin, cysteinyl leukotrienes, histamine, reactive oxygen intermediates (ROIs), and complement fragments (113, 128, 200-202). P-selectin expression is typically maximal 10-20 min. after endothelial activation but enhanced expression may persist up to 2-10 hours (127, 128). In Human umbilical vein endothelial cells (HUVEC), IL-4 and IL-13 were recently shown to increase P-selectin expression on HUVEC up to 48 hours (262).

To achieve and sustain maximal P-selectin expression, endothelial cells synthesize P-selectin *de novo*. Crucial differences exist between the human and murine system however, specifically in the response to Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) and Lipopolysaccharide (LPS). *In vitro* stimulation with these mediators was shown to increase P-selectin mRNA production in mice *in vitro* and *in vivo* (252,253,254). In human endothelial cells however, these mediators do not increase P-selectin mRNA production (250, 255).

The differential responses to TNF-α, IL-1β and LPS appear related to differences in promotor sequences that regulate gene expression. Although many similarities in these regions exist, the murine promotor sequence contains several binding sites (for transcription factors NF-κB and ATF-2) that are absent in the human sequence (257). Experiments in mice in which these regions were mutated, revealed they are important (but not the only) regulators of gene transcription in response to TNF-α and LPS (256).

Despite these differences, some agonists such as oncostatin M upregulate both murine and human P-selectin mRNA several-fold (250,255).

Consistent with its role in acute inflammation, P-selectin-deficient mice have a specific phenotype characterized by peripheral neutrophilia and typically show no increased rolling or adherent leukocytes up to 1 or 2 hours after exposure to inflammatory stimuli (114, 115, 116). In experimentally induced acute inflammation, the recruitment of neutrophils into the peritoneum of P-selectin deficient mice is also delayed (116).

E-selectin ("Endothelial selectin") expression on endothelial cells absolutely requires *de novo* protein synthesis and therefore has a delayed response time (4-8 hours) (205). In contrast to the differences discussed for P-selectin, *in vitro* and *in vivo* studies show that both murine and human E-selectin are upregulated in response to LPS, TNF-α, and IL-1β (251). Both species contain E-selectin promotor sequences similar to those discussed for murine P-selectin (NF-κB and ATF-2 binding sites). It appears that the human P-selectin promotor is unique in the species studied (rat, mouse, cow and dog) in that it appears to have lost its sensitivity to TNF-α, LPS and IL-1β (250).

Convincing evidence that E-selectin can mediate leukocyte rolling was generated *in vitro* (132) although its involvement *in vivo* has been more difficult to demonstrate (116, 133). This may be partly due to the degree of redundancy in the mediators of recruitment. The fact that P-selectin can be synthesized *de novo* (preventing possible depletion of intracellular stores) and that expression can persist up to 48 hours (262), suggest that P-selectin may complement E-selectin-mediated recruitment even in later stages of

inflammation. Reduced peritoneal recruitment after 48 hours in P-selectin-deficient mice supports this hypothesis (115).

Other molecules may also be involved: *in vivo* observations of mice deficient in both P- and E-selectin revealed reduced leukocyte-endothelium interactions and recruitment after 4-6 hours (134) but normal recruitment at 24 hours (135, 136). It was demonstrated that this normal recruitment was also L-selectin-independent and, in fact, involved α_4-integrin (136). These results suggest that recruitment under some circumstances is not dependent on selectins and that when redundancy exists, selectin deficiency may be compensated for by other adhesion molecules. This may be an important consideration when studying the effects of complete deficiency in knockout models. In this light, antibodies and/or inducible knockout systems may add valuable information regarding redundancy in the setting of leukocyte recruitment.

L-selectin ("Leukocyte selectin") is constitutively expressed by most leukocytes except by resting memory T-cells (163). As with all selectins, its large size makes it suitable as a first contact molecule. The selective expression of L-selectin on the tips of the finger-like microvilli of leukocytes may serve to extend its reach even more and has been shown to be necessary for its proper functioning (165,166).

Neutrophil L-selectin is more heavily glycosylated. In humans this results in a 90-110 kDa glycoform on neutrophils vs. a 74 kDa form on lymphocytes (173, 174). The role of L-selectin in recruitment has been confirmed *in vitro* and *in vivo* although results again vary with the particular inflammatory stimulus used (133, 139). L-selectin-deficient mice

showed reduced peritoneal recruitment both at early (4 hours) and late stages (4-72 hours) (163).

The regulation of L-selectin expression is unique among the three selectins in that it is not internalized (as P- and E-selectin are (137)), but shed from the membrane upon cell activation. This occurs on all cell types and involves proteolytic cleavage by metalloproteases. Such rapid downregulation is thought to facilitate further (selectin-independent) interactions by decreasing steric hindrance (14, 85, 110). Alternatively, it has been proposed that shed L-selectin fragments act as anti-inflammatory molecules: L-selectin fragments remain biologically active and may balance the inflammatory response by competing with leukocytes for L-selectin ligands on endothelial cells (176).

The field of selectin research is continuously changing. Besides their established direct role as adhesive molecules, new evidence suggests selectins are important signal transducers facilitating leukocyte and adhesion molecule activation (108, 112, 109). Crosslinking of L-selectin has been shown to induce adhesion molecule (Mac-1) expression on neutrophils and an accompanying transition from a rolling to an adherent state (119,121). In addition, crosslinking of L-selectin can prime (increase sensitivity) neutrophils for formyl-methionyl-leucyl-phenylalanine (fMLP) or TNF-induced respiratory burst (120).

Selectin ligands are generally heavily O-glycosylated proteins (mucins) that carry the sialyl-Lewis x (SLe^x) epitope or its isomer SLe^a. These are oligoaccharides containing sialic acid and fucose residues linked to N-acetylgalactosamine (125, 140). Under the

right circumstances, SLe^x binds to all selectins and is considered the prototype selectin ligand (167). The importance of such fucosylated carbohydrates for selectin- ligand interactions is best illustrated in patients suffering from Leukocyte Adhesion Deficiency II (LAD II) (138).

A genetic defect in fucose metabolism severely compromises leukocyte recruitment in these patients, leading to immunodeficiency.

Sulfation of sLe^x may alter the affinity for selectins. It was shown that sulfation of a tyrosine residue in P-selectin glycoprotein ligand (PSGL-1) was essential for P-selectin binding. L-selectin was also found to bind with increased affinity upon sulfation of sLe^x although sulfation does not appear to affect E-selectin binding (125). Other selectin ligands have been reported including glycolipids, proteoglycan, integrins, heparan sulfate and glycosaminoglycans (140). The biological significance of some of these is unclear as information on their interaction under appropriate shear conditions is presently lacking. As shown in table 1, both the selectins and their ligands are promiscuous, indicating a high degree of redundancy.

Since rolling is, in most circumstances, a requirement for subsequent adhesion and emigration, inhibiting this process has important therapeutic anti-inflammatory potential. *In vivo* animal experiments suggest however, that rolling events have to be reduced by at least 90 % to significantly affect the number of adhering cells and total recruitment (90, 136, 164). Unfortunately, such a high degree of inhibition is likely to be difficult to achieve clinically.

1.1.2.2. The leukocyte recruitment cascade: Activation and adhesion

The interactions described above result in leukocytes rolling along the blood vessel wall. As mentioned, this can be considered a pivotal phase in recruitment: If the correct activation signals are not received, the cells remain within the bloodstream.

The molecules involved in binding of leukocytes to the endothelium are the integrins. Integrins are heterodimeric molecules composed of an α and β chain. They are divided into five subfamilies ($\beta_{1,2,3,4,7}$). Within a subfamily, members have a different α chain but a similar β chain. The β_1 (CD29), β_2 (CD18) and β_7 (CD nomination not defined) integrins mediate leukocyte recruitment.

Neutrophil adhesion is primarily mediated by members of the β_2 subfamily, particularly Mac-1 (CD11b/CD18) and to a lesser extent Lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) and p150,95 (CD11c/CD18). LFA-1 is an important mediator of lymphocyte recruitment (210, 211, 222). Mac-1 and p150,95 are pre-stored in neutrophil granules, available for rapid surface expression in response to inflammatory signals (such as fMLP, LPS, TNF-α)(38).

The importance of the β_2-integrins is dramatically demonstrated in patients suffering from a genetic defect in the common β-chain (CD18) of this complex. This deficiency results in Leukocyte Adhesion Deficiency I (LAD I) characterized by severe recurrent infections that would normally be resolved by neutrophils (100).

Integrin ligands belong to the immunoglobulin superfamily (145). Important members for leukocyte recruitment are the Intercellular Cell Adhesion Molecules (ICAM-1, ICAM-2 and ICAM-3); the Vascular Cellular Adhesion Molecule (VCAM-1); Platelet Endothelium Cellular Adhesion Molecule-1 (PECAM-1) and Integrin Associated Protein (IAP or CD47).

Tissue and leukocyte-specific expression pattern of selectins, integrins and their ligands play a major role in which cell types are recruited, and to which tissue. An example of this is the localized expression of Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1), the high affinity ligand for $\alpha_4\beta_7$ integrin, or lymphocyte Peyer's patch adhesion molecule-1 (LPAM-1) which regulates T-cell homing to secondary lymphoid tissue in the gut (147, 148, 149).

Generally, integrins have to be activated in order to interact with their ligands. Activation leads to conformational change and increased affinity. Activation may additionally induce clustering leading to higher avidity (143, 144, 210). The activation signal is generated by separate receptors that are activated through the binding of Platelet-activating factor (PAF) or other lipid mediators, chemokines or chemoattractants. Signaling further activates the leukocyte and induces integrin activation. The requirement for a conformational change in integrins before a rolling cell can become adherent, illustrates that adhesion is not simply an extrapolation of rolling but rather a discrete step in the recruitment cascade. It has to be actively induced and creates therefore another level of tight control over leukocyte infiltration.

1.1.2.3. The leukocyte recruitment cascade: Transmigration

Once tightly bound to the endothelial surface, leukocytes undergo a shape change; they flatten and spread out (213). *In vitro* studies suggest that different densities of ICAM-1 on the endothelium subsequently guide leukocytes towards inter-endothelial junctions (156,157). After arriving at these junctions, the cells extend pseudopods between them and 'squeeze through' (diapedesis). To accomplish this, emigration involves a variety of molecules including integrins, CD31 (PECAM-1) and CD47 (IAP).

Members of the β_1 and β_2 integrins are especially important in inflammation (292). Although β_2 integrins are expressed on all leukocytes, different leukocyte subtypes utilize different forms. Lymphocytes primarily express LFA-1 whereas neutrophils, monocytes and Natural killer (NK) cells express all three forms (LFA-1, Mac-1 and p150,95) (280,281).

For neutrophils, the β_2 integrins are particularly important as demonstrated in LAD I patients. In these patients neutrophil recruitment is defective whereas mononuclear leukocyte trafficking and cell-mediated immunity remain largely intact (280,282). Lymphocytes from these patients are presumably recruited through the β_1 integrin Very Late Antigen-4 (VLA-4 or $\alpha_4\beta_1$)(283). Because of the defects in neutrophil accumulation in LAD I patients, it was believed neutrophils were incapable of expressing VLA-4. It was subsequently shown however, that human neutrophils can utilize this integrin in a CD11/CD18 independent pathway of recruitment (284,285). Integrins play a pivotal role after transmigration through the endothelial barrier as well, both during and after the

penetration of the basal lamina because of their ability to bind Extracellular matrix (ECM) components such as fibronectin, collagen, laminin and vitronectin.

Since firm adhesion is a prerequisite for subsequent transmigration, interfering with the function of integrins that have a role in transmigration as well as in adhesion (such as LFA-1 and Mac-1) impacts strongly on cellular recruitment. This is a complicating factor in the experimental separation of the specific contribution of some of these molecules to the transmigration process itself.

CD31 is a 130 kDa transmembrane protein belonging to the immunoglobulin superfamily. It is constitutively expressed on platelets, most leukocytes and endothelial cells. On the latter, it is concentrated at junctions between opposing cells (286,288,293). Consistent with an adhesive function, CD31 is structurally related to ICAM-1 and VCAM-1 (287). In addition, crosslinking of CD31 generates an intracellular (IC) signal that upregulates CD11/CD18 complexes on leukocytes (290,291).

CD31 mediates two different stages in leukocyte extravasation. First, homophilic interactions between CD31 on the leukocyte and CD31 on the endothelium mediate the emigration of the leukocytes across the endothelial cell layer. After this, heterophilic interactions of CD31 with ECM components play a role in the movement across the underlying basal membrane. It is believed different immunoglobulin domains of CD31 mediate these different steps (293).

The basal lamina does not contain CD31 and its ligand in this event is believed to be heparan sulfate glycosaminoglycan which is abundantly present at this site. The addition

of heparan sulfate has been reported to interfere with this step of transmigration in experimental models (243). However, CD31 does not bind heparan sulfate directly, suggesting its role in CD31-basal lamina interactions may be indirect (289, 293). CD31 antibodies or purified CD31 inhibit neutrophil emigration across TNF-α stimulated endothelium *in vitro* (293). Similar antibodies prevented neutrophil recruitment in diverse *in vivo* animal models including thioglycollate-induced peritonitis, mesenteric inflammation and ischemia-reperfusion injury (295,296,297). The literature provides intriguing examples illustrating that the requirement for CD31 may vary however depending on the inflammatory stimulus. Although neutrophil emigration in response to fMLP, IL-8, Leukotriene B_4 (LTB_4) and *Escherichia coli* (*E.coli*) is CD31-independent, IL-1β and TNF-α induced emigration does require CD31 (294).

CD47 is a heavily glycosylated membrane protein belonging to the immunoglobulin superfamily and present on all leukocytes. It associates with β_1, β_2 and β_3 integrins and affects their ability to bind their ligands (245). CD47 expression on neutrophils was also shown to be directly correlated to the amount of recruitment (151, 152).

Many details concerning leukocyte emigration and its regulation remain ellusive. The underlying molecular mechanisms are a matter of intense research and are expected to yield further valuable clues for therapeutic intervention in this crucial step in the inflammatory process.

1.2. The neutrophil

The neutrophil is the principal effector cell in the inflammatory responses in the experimental models that were investigated for this thesis. This potent leukocyte and its effector mechanisms are discussed below.

1.2.1. Neutrophil structure

The primary function of neutrophils is the ingestion and/or digestion of unwanted materials such as necrotic host tissue, microbes and toxins. Neutrophils are often referred to as "the first line of defense" in the immune response to microbial pathogens. This is best illustrated by clinical cases of prolonged neutropenia or inherited defects in neutrophil function, which usually lead to recurrent opportunistic infections (169). In humans, neutrophils are the most abundant circulating leukocyte, accounting for 60% of cells (approximately 5000/μl blood)(126).

Neutrophils arise in the bone marrow and can first be positively identified in the myeloblast stage. After further development through the promyelocyte, myelocyte and metamyelocyte stages, the neutrophil reaches the 'banded' form, at which stage the synthesis of all granular contents is completed. Further segmentation of the nucleus into the characteristic lobes is the final step in the maturation process. The output of mature neutrophils from the bone marrow can be doubled within a week and even tripled within 14 days under the influence of Granulocyte colony stimulating factor (G-CSF) or

Granulocyte-macrophage colony-stimulating factor (GM-CSF) (171,172). The bone marrow pool represents approximately 90% of all neutrophils in the body with the remaining 10% in the peripheral blood. It is estimated that of the latter fraction, only half is circulating freely while the rest are interacting with endothelial cells (rolling or adhering) mainly in organs such as the lungs, liver and spleen (206,236). Under inflammatory circumstances, the number of peripheral neutrophils increases due to increased hematopoiesis, accelerated release from the bone marrow and mobilization of this rolling/ adherent pool.

Mature neutrophils will circulate for 6-8 hrs. before entering tissues and, in contrast to lymphocytes, do not return to the blood. Under inflammatory circumstances the time spent in the peripheral blood is even shorter. They become apoptotic after an additional 1-2 days before being cleared (153, 206).

1.2.2. Neutrophil priming

A feature of neutrophils that enhances their effectiveness against pathogens is the priming effect: After exposure to one substance (priming agent), their sensitivity and response to a different subsequent agonist is synergistically increased. Examples of priming molecules include G-CSF, GM-CSF, LPS and certain cytokines such as IL-1 (179, 95). Each of these increases the sensitivity for agonists such as fMLP and augment effector functions like superoxide production. Priming is also thought to have an anti-apoptotic affect, prolonging the lifespan of the primed cell (170, 177). It is believed that the mechanism

responsible for priming is modulation of the signaling pathways involved and not increased affinity/avidity of the receptors (178, 206). Interestingly, certain pathogens (e.g. *Baccilus antracis*) interfere with priming, adding to their pathogenicity (180).

1.2.3. Neutrophil chemotaxis

Chemotaxis is the process whereby an immune cell detects, and moves toward, higher concentrations of a chemoattractant. A spectrum of chemoattractant molecules induce chemotaxis in neutrophils including PAF, C5a, LTB_4, IL-8 and fMLP. Exposure to such chemoattractants rapidly polarizes neutrophils, giving rise to a forward extension (pseudopod) and a uropod (tail). The actin-rich pseudopod attaches to the surface through β_1, β_2 and β_3-integrins (192) and essentially stretches the cell forward. The cell then contracts, pulling forward, in the direction of the pseudopodium and the chemoattractant source (15, 154).

Polarization is achieved through the action of chemoattractant receptors. These generally belong to the seven-transmembrane helix receptor family. When a chemoattractant binds to its receptors, the associated heterotrimeric G proteins are activated (263, 264, 265) Of the chemotaxis-inducing pathways described, the PI3K pathway is the best characterized. In this scenario, subunits of the G protein complex activate phosphoinositide 3-kinase (PI3K). This enzyme converts phosphatidylinositol 4,5-biphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3) (271, 268). The importance of these events is demonstrated in mice deficient in p110γ (The PI3K isoform

specifically associated with chemoattractant receptors) (273). p110γ deficiency leads to severe, albeit not complete, defects in neutrophil and macrophage chemotaxis.

Downstream from PI3K, several Guanine triphosphate (GTP)-binding proteins can regulate cytoskeletal reorganization during chemotaxis in leukocytes. The Rho family of GTP-binding proteins is particularly important. These proteins are part of the Ras superfamily and include Rho, Rac and Cdc42. They are linked to cytoskeletal activation by promoting cytoskeletal contractility and actin polymerization (274,275). In addition, indirect links to cell motility are suggested by their interaction with proteins with known cytoskeletal activation properties (276,277,268)

Recent experimental evidence from neutrophils derived from the myeloid cell line suggests Rac plays an important role in accumulating PIP_3 and actin at the leading edge (pseudopod) of neutrophils (267). Since PIP_3 results in further Rac activation, this results in a positive feedback loop. Cdc42 was shown to mediate proper cell polarization by regulating the stability and position of the leading edge. These results have also been confirmed in macrophages (268).

The activation of Rho, Rac and Cdc42 is regulated by at least three different types of proteins. Nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) promote Rho,Rac and Cdc42 activation while guanine nucleotide dissociation inhibitors (GDIs) reduce their activation (268). Activation of GEFs is thought to be linked to chemoattractant receptor activation by the binding of PIP_3. PIP_3 can bind the pleckstrin-

homology (PH) domain of three GEFs including the leukocyte-specific Vav and activation of Vav was shown to lead to Rho, Rac and Cdc42 activation (278).

Neutrophils can respond to very subtle gradients of chemoattractants. A 1% difference in concentration of fMLP between opposite sides of the cell may cause polarization and chemotaxis (154). Polarization is not caused by the localized aggregation of chemoattractant receptors (these remain diffusely distributed over the membrane) but rather involves the localized recruitment of PIP_3-binding proteins to the leading edge. This accumulation results in a much sharper gradient than the chemoattractant gradient itself and in essence functions to amplify of the directional component of the chemoattractant signal (268, 272).

As suggested by the results discussed above in which p110γ deficiency leads to severe but not complete inhibition of chemotaxis, other pathways exist that are independent of PIP_3 formation. Upon activation, seven transmembrane chemoattractant receptors can generate a multitude of signals in leukocytes (266). In human and murine cells, the p38 Mitogen-activated protein kinase (p38 MAPK) pathway was shown to play an important role in neutrophil chemotaxis (269, 270). It was recently shown that the existence of different pathways in response to different chemoattractants may explain how neutrophils are able to properly migrate towards a site of infection when multiple chemotactic signals are present. In the complex *in vivo* environment, the initial site of recruitment in the blood vessel and the ultimate target site are often not the same. This implies that end-target chemoattractants (such as fMLP or C5a) generated at the site of infection have to

dominate over intermediary-target chemoattractants (such as IL-8 or LTB_4) emanating from the endothelium. Such prioritizations were demonstrated *in vitro* (28, 40). Recently, Heit et al (19) showed that IL-8 induced PI3K-dependent signaling, whereas chemotaxis towards fMLP was p38 MAPK-dependent in human neutrophils. Moreover, a signaling hierarchy was demonstrated, in which p38 MAPK signaling inhibited the PI3K pathway. Interestingly, the inhibition of the PI3K pathway did not depend on chemotactic properties of the end-target molecule: LPS also inhibited intermediary-target signaling, again with clear physiological benefits.

1.2.4. Phagocytosis by neutrophils

Phagocytosis is the binding and ingestion of foreign particles and necrotic host tissue (reviewed in 10). Receptors mediating phagocytosis can either bind the pathogen directly or indirectly. Indirect binding involves so-called opsonins (antibodies, mannose binding lectins (MBLs) or complement fragments).

Opsonin-independent receptors include Scavenger receptors and Lectins. These recognize a wide variety of ligands including modified host proteins, conserved microbial patterns such as cell wall components of (both gram-negative and gram-positive) bacteria (e.g. LPS, lipoteichoic acid) and bacterial carbohydrate molecules such as branched mannose and fucose oligosaccharides (20, 141).

Such receptors on phagocytes that are able to recognize pathogens independently of the adaptive immune system are termed Pattern Recognition Receptors (PRRs). They

recognize conserved microbial structures called Pathogen-Associated Molecular Patterns (PAMPs) (175). PAMPs are often critical to the survival of the microorganism producing them, ensuring continued effectiveness of the essentially rigid innate immune response. The direct recognition of microbial components by such receptors, bypassing the need for opsonins, also reduces the response time of the innate immune response. This makes it effective against often rapidly dividing pathogens and pathogens not previously encountered.

Opsonin-dependent receptors include complement receptors (CRs) and antibody receptors (FcγRs) that bind the target indirectly. While CRs bind fragments of activated complement such as C3b and iC3b, FcγRs bind opsonizing antibodies (mainly IgG and IgA). Although the affinity of the different FcγRs for these antibodies varies, they all bind to the (invariable) Fc domain. These receptors enable neutrophils to benefit from the flexibility of the adaptive immune system while utilizing only a few receptors themselves (181, 182).

Opsonization may affect the microbicidal capacity in more ways than facilitating phagocytosis: *E.coli* bacteria that are presented to neutrophils without the presence of opsonizing complement, are phagocytosed but actually survive within the phagolysosome (188).

After binding the target, the phagocyte membrane extends broad pseudopods to surround the pathogen. The pseudopods fuse together at their meeting point, forming a separate

vesicle. These processes show multiple similarities with cell movement during chemotaxis. As in chemotaxis, the Rho family GTPases are key signaling components for cytoskeletal rearrangement (22). Dependency on the type of Rho GTPases may differ depending on which receptor is used however. In macrophages Cdc42 and Rac were shown to be required for FcγR-mediated phagocytosis whereas CR3-mediated uptake was only dependent on Rho activity (34,123,209).

After fusion, further destruction takes place in what is now termed the phagolysosome (or *phagosome*). The destruction of the target within a specialized compartment allows for tight regulation of pH and toxic microbicidal molecule concentration while protecting the surrounding microenvironment from the harmful contents of the granules. There are circumstances in which neutrophils release their granule contents into the extracellular medium however. This may be a result of the nature or size of the target but can also be induced by certain humoral agents. This is often associated with undesired host tissue degradation and disease (6).

Some bacteria resist phagocytosis by heavy encapsulation (*Bacteroides fragilis*) or by depleting complement regulatory factors (*Streptococcus pyogenes*). Clearance of such infections often requires the generation of specific antibodies, which substantially increases the resolution time (186, 187).

Phagocytosis of necrotic cells or pathogens may lead to apoptosis in neutrophils and subsequent clearance by macrophages. Apoptotic neutrophils are recognized through the

expression of phosphatidylserine, desialylated proteins and opsonization by thrombospondin, a platelet product (183, 184, 185).

Other important PRRs belong to the family of Toll-like receptors (TLRs). Ten TLRs have been identified in humans and mice (203). The family of TLRs is capable of recognizing a wide variety of PAMPs including LPS, Lipoproteins, Peptidoglycan, Lipoteichoic acid, bacterial DNA (CpG DNA), flagellin, and viral elements such as Double stranded RNA (dsRNA) and viral envelope proteins (204, 203, 207). TLRs are involved in cell activation, the production of inflammatory mediators and (in the case of APCs) upregulation of costimulatory molecules such as CD40, CD80, CD86 (142).

An important immunostimulatory component produced by the *Escherichia coli* bacteria used in the experiments described in this thesis is lipopolysaccharide (LPS or endotoxin). LPS is an integral part of the cell membrane of gram-negative bacteria and consists of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A. The latter can activate phagocytes through TLR-4 (and perhaps partially through TLR-2) (204,207).

Lipid A is bound by the acute phase protein lipopolysaccharide-binding protein (LBP) present in plasma. This complex then binds CD14, a GPI-linked surface protein (204, 244). CD14-LPS-LBP is thought to activate TLR-4. An additional requirement for proper TLR-4 signaling is the binding of a secreted protein MD-2 to the EC domain of TLR-4 (207,212).

The IC domain of TLRs is closely related to that of the IL-1 receptors. Upon TLR activation, IL-1 receptor associated protein kinase (IRAK) is recruited to the receptor

complex, via adaptor MyD88. MyD88 is also a crucial component of IL-1R signaling. Therefore, without it, mice can no longer respond to IL-1, peptidoglycan, lipoproteins or CpG DNA (146, 204).

Activated (phosphorylated) IRAK forms a complex with tumor necrosis factor receptor-activated factor 6 (TRAF6), leading to the oligomerization of TRAF6. Ultimately this leads to activation of transcription factors (such as AP-1 and NF-κB) and immune response gene transcription (203, 204, 207). This pathway is conserved between the different TLRs. Additional pathways linked to activation of the different TLRs are being reported. New pathways differentiating between the different TLRs and their ligands is consistent with the notion that different pathogens may require different immunological responses.

An example was demonstrated in MyD88 mice. As mentioned, normal TLR signaling is absent in these animals. A response to LPS, albeit delayed, still persists. MyD88-independent LPS induced TLR-4 signaling was shown to induce not NF-κB but IRF-3 activation (150). If this is related to how the ligand (directly or indirectly) engages the EC domain of the TLR remains to be determined.

1.2.5. Neutrophil microbicidal function

The neutrophil cytosol is packed with 2 main types of granules referred to as primary (azurophilic) and secondary (specific or secretory) granules. They contain antimicrobial molecules, receptors (for complement fragments, antibodies, conserved microbial

components), ECM degrading enzymes and pro-inflammatory molecules. The most important molecules in these granules are listed in table 2. Some of these have direct and specific antimicrobial activity (reviewed in 25) but many are more general proteases or create products that are equally harmful to the host as to a pathogen.

Degranulation makes the contents of the granules available. It is the process in which cytosolic granules fuse with either the phagolysosome or the plasma membrane and release their contents. Next to its role in microbicidal activity, degranulation is essential for ECM degrading processes and is thought to play a role in endothelial transmigration.

Although both primary and secondary granules may deposit their contents in the phagosome or the EC matrix, the bulk of the secondary granules is targeted especially towards the latter. Strong stimuli (e.g. fMLP) generally lead to degranulation of both types of granules but certain (weaker) stimuli (LPS, TNF-α) can selectively mobilize only the secondary granules. This may be especially relevant during neutrophil recruitment when adhesion molecules and ECM degrading enzymes need to be released from these granules without causing complete degranulation at the endothelial surface (38).

Neutrophils possess both oxygen-dependent and oxygen-independent bactericidal mechanisms. Many cytokines, lipid mediators, antibodies, bacterial products, and growth factors can activate these pathways.

Oxygen-dependent killing is also known as the respiratory burst (reviewed in 197). These processes cause a sharp rise in cell metabolism and oxygen consumption. Aggressive, indiscriminate oxidizing agents such as ROIs and Reactive nitrogen intermediates (RNIs) are formed. The responsible enzymes are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), myeloperoxidase (MPO) and inducible nitric oxidase synthase (iNOS). The chemical reactions are depicted in figure 2.

Oxygen-independent killing is mediated by a variety of proteases, cationic proteins (defensins) and other antimicrobial proteins such as Bactericidal Permeability-increasing Factor (BPI) and lactoferrin(190, 193, 196). Some of these (e.g. defensins) affect mammalian cells as well, illustrating the need for phagosomal containment (194).
The combination of oxygen-dependent and -independent killing mechanisms, with their large diversity in effector molecules, ensures effectiveness against a wide array of pathogens. Although there is considerable functional overlap between the discussed molecules, redundancies do not always exist and deficiencies in some may have consequences for host defense. This is most clear in patients that have defects in the NADPH-oxidase system (Chronic Granulomatous Disease or CGD). This disease is characterized by severe recurrent infections by bacteria and fungi (199).

1.3. CD43

CD43 (leukosialin) is expressed on T cells, monocytes, granulocytes (including neutrophils), hematopoietic stem cells, platelets and some B cells, but is absent on erythrocytes (7, 51). CD43 belongs to the family of mucins, which also includes Glycoprotein cell adhesion molecule-1 (GlyCAM-1), MAdCAM-1, CD34 and PSGL-1. Their mucin domains are regions with a high content of threonine, proline and/or serine that are O-glycosylated. CD43 is considered a prototype mucin, deriving over 50 % of its molecular weight from a large number (85-90) of O-linked oligosaccharides (249). There are two known glycoforms of CD43 with a molecular weight of approximately 115 kDa and 130 kDa. Because of the different glycosylation, the two forms differ in size, shape and net negative charge and may serve different functions (37, 44). Regulation of the CD43 gene is thought to be mediated by methylation (preventing transcription) and by transcription factors that regulate the gene through a promoter sequence (21, 32, 52). Recently, active repression by nuclear factors (hnRNP-K and Pur-alpha) on the transcriptional activity of the CD43 promoter was described (239).

CD43 is implicated in numerous processes that require signal transduction including: T lymphocyte proliferation (42, 69, 247), monocyte-dependent T-cell proliferation (65), mast cell activation (33), apoptosis (62, 68), hematopoiesis (64), dendritic cell (DC) and lymphocyte maturation (63, 67), monocyte activation (70) and NK cell activation (74, 75). In all of these processes, the precise mechanism by which CD43 acts is unknown.

CD43 has a relatively long cytoplasmic tail (123 amino acids in humans) (29, 36) and several groups have shown that after cross-linking of CD43, multiple phosphorylation events occur in this domain. It has been consistently demonstrated that the first phosphorylation event involves members of the Src family of tyrosine kinases (Fyn/Lyn seem particularly important) (31, 59, 75, 83). Several authors have reported Diaclyglycerol (DAG) and IP3 production resulting in the release of calcium from intracellular stores (78, 82). As a second mechanism, activation of the MAP kinase ERK2 through the Ras pathway has been described. This pathway leads to the induction of nuclear transcription factors NF-AT, AP-1 and NF-κB and subsequently to IL-2 production (31, 80). Because of these targets, the Ras protein is an important regulator of growth and differentiation in many cell types and may explain the involvement of CD43 in cell proliferation and maturation. Most of this data was generated in T-lymphocytes. In neutrophils, CD43 signaling has been associated with Src-kinase activation and respiratory burst initiation (83, 96).

1.3.1. CD43 as an anti-adhesive molecule

CD43 is classically viewed as an inhibitor of cell-cell and cell-matrix interactions (For a review see reference 3). There is both direct and indirect evidence for this:

1. Structural properties and physical properties When fully glycosylated, CD43 extends approximately 45 nm from the cell surface making it one of the largest molecules on leukocytes (53).

Since CD43 is also very abundant (over 10^5 copies per cell for human neutrophils) it is postulated to interfere with adhesion molecules (29, 30). In addition, the mucin domains of CD43 carry negatively charged sialic acid residues. Since cells in general are inherently negatively charged due to the nature of their outer lipid membrane, CD43 expression will hinder cell-cell-interactions due to same charge repulsion. The importance of the sialic acid residues has been confirmed experimentally by desialylation of neutrophil CD43, which showed strong correlation between a reduction in negative charge and the increase in cell-cell and cell-matrix interactions (61, 242).

This anti-adhesiveness may not be as passive as it appears: The IC domain of the molecule may well be an important element in decreasing the anti-adhesive forces, although the mechanism for this is unknown. It may be that the IC domain is crucial to the proper redistribution of the molecule on the membrane. In experiments comparing CD43-deficient cells and cells transfected with only the EC or IC domain of CD43, the same (high) degree of homotypic aggregation was observed (77). This suggests that the passively expressed, negatively charged EC domain alone is insufficient to effectively mediate anti-adhesiveness.

2. <u>CD43 can decrease homotypic aggregation of T cells.</u> Using a murine model in which the CD43 gene was made inactive by targeted disruption, it was shown that without CD43 expression, T cells show an enhancement in homotypic aggregation (41, 45). The same principle was demonstrated when peripheral B cells were transfected to over-express CD43. These cells showed a higher threshold for activation by T-helper cells,

which resulted in immunodeficiency (2, 4). Also, lymphocytes lacking CD43 show stronger adherence to fibronectin-coated surfaces (41).

3. CD43 decreases heterotypic cell-cell interactions: CD43 can protect cells from cell-mediated cytolysis (9, 79), which may explain increased survival of certain tumors over-expressing CD43 (66).

The heavier 130 kDa glycoform of CD43 seems to be primarily responsible for the anti-adhesive properties of the molecule (8). This form is also implicated in selection of immature T cells in the thymus; during positive selection (in which cell-cell contact is crucial for cell survival) this form of CD43 is downregulated (47).

4. CD43 is excluded from the site of interaction in many cell-cell (or cell-matrix)-contact situations: T-lymphocytes and neutrophils interacting with a monolayer of endothelial cells or a protein-coated surface redistribute their CD43 to a cellular uropod (76, 234). Monoclonal antibodies against CD43 or chemotactic peptides induce capping or redistribution of CD43 (1, 72, 73) that excludes CD43 from the site of contact. A similar redistribution occurs in the contact of a T lymphocyte with an APC, in which CD43 becomes part of the distal pole complex (5, 54, 231).

1.3.2. CD43 in leukocyte recruitment

As discussed below, there is both evidence to suggest CD43 inhibits and promotes leukocyte recruitment.

The strong anti-adhesive effect of CD43 combined with the notion that leukocyte recruitment requires intimate cell-cell contact, suggests the need to downregulate this effect and/or molecule. On neutrophils and monocytes, this occurs through proteolyic downregulation (shedding). Shedding is a common phenomenon to rapidly change the expression of membrane proteins. Examples include FcγRs, CD44 and L-selectin (85, 86, 88, 89). There is convincing evidence CD43 expression is also modulated in this manner during leukocyte recruitment:

CD43 shedding occurs on activated cells and has been demonstrated in neutrophils after stimulation with Phorbol myristyl acetate (PMA) and with the physiological mediators PAF and fMLP (35, 48, 49). *In vitro*, as much as 80 % shedding was reported (61). Supporting *in vivo* data comes from the comparison between circulating and emigrated neutrophils from patients with rheumatoid arthritis and bacterial peritonitis. In all cases, neutrophils at the inflammatory site have significantly reduced CD43 expression compared to unactivated, circulating cells (14, 84).

Additionally, *in vitro* studies have shown that CD43 shedding is associated with increased neutrophil adhesive interactions with ECM components, other neutrophils and endothelial cells (213, 241, 61, 240)

Although additional shedding may occur after arrival at the inflammatory site (84), the presence of EC domain fragments of CD43 in human plasma suggests that at least part of the shedding takes place before neutrophils emigrate through the endothelial cell layer (94).

Enzymes responsible for shedding are metalloproteases, especially the family of serine proteases (chymotrypsin, elastase and trypsin) (35, 48, 55, 56). Exactly which serine protease(s) mediate CD43 shedding may be influenced by the type of mediator that is used to activate the cell. CD43 appears to be especially sensitive to elastase, resulting in the release of approximately 60 % of the EC domain in two shedding fragments (246).

In contrast to neutrophils and monocytes, lymphocytes do not shed CD43. It is unknown whether some other mechanism of downregulation (such as internalization or capping) occurs.

The development of a CD43 deficient (KO) mouse strain by Manjunath et al. (45, 235) has made significant contributions to CD43 research. This group convincingly demonstrated the anti-adhesive properties of CD43 *in vivo*: intravital microscopy of lymphocyte homing to High endothelial venules (HEVs) revealed both increased lymphocyte rolling and adhesion in CD43 KO animals. Decreased viral clearance in CD43 KO animals was also demonstrated but the responsible mechanism was not clarified. CD43 was shown to interfere with L-selectin function.

Evidence for a pro-adhesive role of CD43 in leukocyte-endothelial interactions recruitment has also been reported and several ligands have been suggested.

Selectins are postulated as candidate CD43 ligands; many selectin ligands belong to the mucin family and some (like PSGL-1) show a remarkable similarity with CD43 with regards to their EC domain (53). Furthermore, particular glycoforms of CD43 may express sLe^a and (to a lesser extent) sLe^x epitopes (13, 261). An *in vitro* study revealed that cancer cells overexpressing SLe^x increased their adhesion to E-selectin and that this adhesion could be inhibited by soluble CD43 (13).

McEvoy et al. showed that binding of a CD43-specific mAb can block lymphocyte, neutrophil and monocyte adhesion and extravasation. This antibody was able to prevent disease in a murine model of diabetes but *in vitro* did not block leukocyte binding to surfaces that were coated with purified Peripheral lymph node Addressin (PNAd), MAdCAM-1 or ICAM-1, suggesting these are not CD43 ligands (43, 57, 238). The authors suggest this mAb interferes with CD43-mediated signaling.

Our research group utilized the CD43 KO mouse strain to perform *in vivo* experiments of PAF-mediated neutrophil recruitment in murine cremaster tissue. Increased leukocyte adhesion in CD43 KO animals confirmed the anti-adhesive effect of CD43. It was hypothesized that increased adhesion would result in increased recruitment as well. Unexpectedly however, total leukocyte recruitment was actually reduced, suggesting an additional role for CD43 in the promotion of leukocyte recruitment. Reduced recruitment

was confirmed by measuring neutrophil and mononuclear cell recruitment into the peritoneum in response to an injection of oyster glycogen; again the CD43 KO animals recruited significantly fewer cells than their wild type (WT) counterparts (18).

These results fit a hypothetical model in which CD43 serves to prevent leukocyte infiltration under non-inflammatory circumstances. When an inflammatory stimulus is present, neutrophils and monocytes partially downregulate CD43 through proteolytic shedding to facilitate and optimize further interactions with the endothelium. The residual CD43 molecules act to promote emigration.

1.4. Research objectives and hypothesis

The discussed experiments suggest that in addition to its role as an anti-adhesive molecule, CD43 may function to promote leukocyte emigration and recruitment. These results are limited in that the inflammatory responses examined were generated in a system with a single specific inflammatory stimulus (PAF, fMLP or oyster glycogen). It is uncertain therefore, if these observations can be expanded to a biologically more relevant inflammatory response and if so, if the effect is significant enough to induce phenotypic consequences in terms of host defense.

To investigate this, we studied the effect of complete CD43 deficiency in two murine models of acute inflammation: ischemia-reperfusion (IR) injury and bacterial peritonitis. These models were chosen because they are well characterized and induce an acute inflammatory response in which early neutrophil infiltration is a key feature.

Ischemia-reperfusion injury occurs after blood flow is restored to previously obstructed vasculature. This may for example occur due to the resolution of a blood clot or the clinical opening of a blocked coronary. Paradoxically, the restoration of blood flow causes more damage to the tissue than the initial ischemia.

Upon reperfusion the damaged tissue starts to express adhesion molecules and releases large quantities of inflammatory mediators. These include IL-8, PAF, LTB_4, oxidants and activated complement factors (C5a, C3a) (117, 118). These are all strong neutrophil

chemotactic factors and in addition cause neutrophil degranulation. There is convincing evidence that neutrophils are the main mediator of tissue damage in post-ischemic events:

* Neutrophils infiltrate reperfused tissue (214, 215, 217, 219).
* Depletion of the circulating neutrophil pool, significantly reduces tissue damage (87, 217, 219, 226)
* Interfering with neutrophil-endothelial interactions reduces neutrophil recruitment and tissue damage (215, 216, 221, 223, 224).

Upregulation of adhesion molecules leading to recruitment is thought to be triggered by the formation of ROIs by the endothelial metalloflavoprotein xanthine oxidoreductase (191, 218, 220). During ischemic periods, the normal form of this enzyme, xanthine dehydrogenase (XD), is proteolytically converted into xanthine oxidase (XO). Both forms mediate the oxidation of purines but whereas XD uses NAD^+ as electron acceptor (yielding the harmless NADH), XO uses (reintroduced) O_2, resulting in the formation of superoxide. Partial conversion of superoxide into H_2O_2 by SOD is therapeutic and the introduction of additional SOD had been shown to substantially reduce postischemic damage (223, 225). Administration of ROI scavengers was also effective in a similar model (226, 227). The importance of the conversion of XD into XO was shown *in vivo* by inhibiting this event, reducing neutrophil infiltration and tissue damage (198).

There is increasing evidence that ROIs can act as signaling molecules to induce adhesion molecule upregulation (189, 191). Illustrative of such alternative mechanisms of adhesion

molecule upregulation during reperfusion is a report in which ICAM-1 and VCAM-1 upregulation after ischemia was normal in TNF-R1 and IL-1 R1 deficient mice (228).

In E.coli-induced peritonitis a foreign stimulus (the bacterium) causes inflammation. Clinically, this may occur due to intestinal damage and/or perforation. The immune system responds by the directed movement of cells into the affected area by homing in on inflammatory signals produced by the host and foreign molecules produced by the pathogen. Neutrophils are the first cells to be recruited to sites of microbial infection (159, 169). Their recruitment follows the release of pro-inflammatory mediators (IL-4, IL-6, TNF-α) mainly by tissue resident mast cells (12, 27). TNF-α together with other pro-inflammatory cytokines causes upregulation of adhesion molecules both on the leukocyte and the endothelium, leading to recruitment. In later stages of bacterial infection, monocytes / macrophages are recruited as well, partly due to the release of chemoattractants by neutrophils (PAF, LTB_4) that had been recruited earlier in the inflammatory response (16, 206).

Hypothesis

CD43 limits leukocyte-endothelial cell interactions. During an acute inflammatory response, neutrophil CD43 is partially downregulated, leading to increased adhesion. The residual CD43 molecules play a role in promoting emigration. Complete CD43 deficiency will therefore increase adhesive interactions but decrease total cellular recruitment. This decrease will impair host defense and influence neutrophil bactericidal capabilities.

To test this hypothesis we examined the following:

1. The effect of CD43 deficiency on leukocyte recruitment kinetics (rolling, adhesion and emigration) in a murine model of IR injury.
2. The consequences of CD43 deficiency on total leukocyte recruitment and host defense in a murine model of bacterial peritonitis.
3. The ability of normal and CD43-deficient murine neutrophils to perform chemotaxis, phagocytosis and bacterial killing activities *in vitro*.
4. The amount of CD43 downregulation in response to recruitment. This was done by comparing CD43 expression of peripheral and recruited neutrophils by flow cytometry.

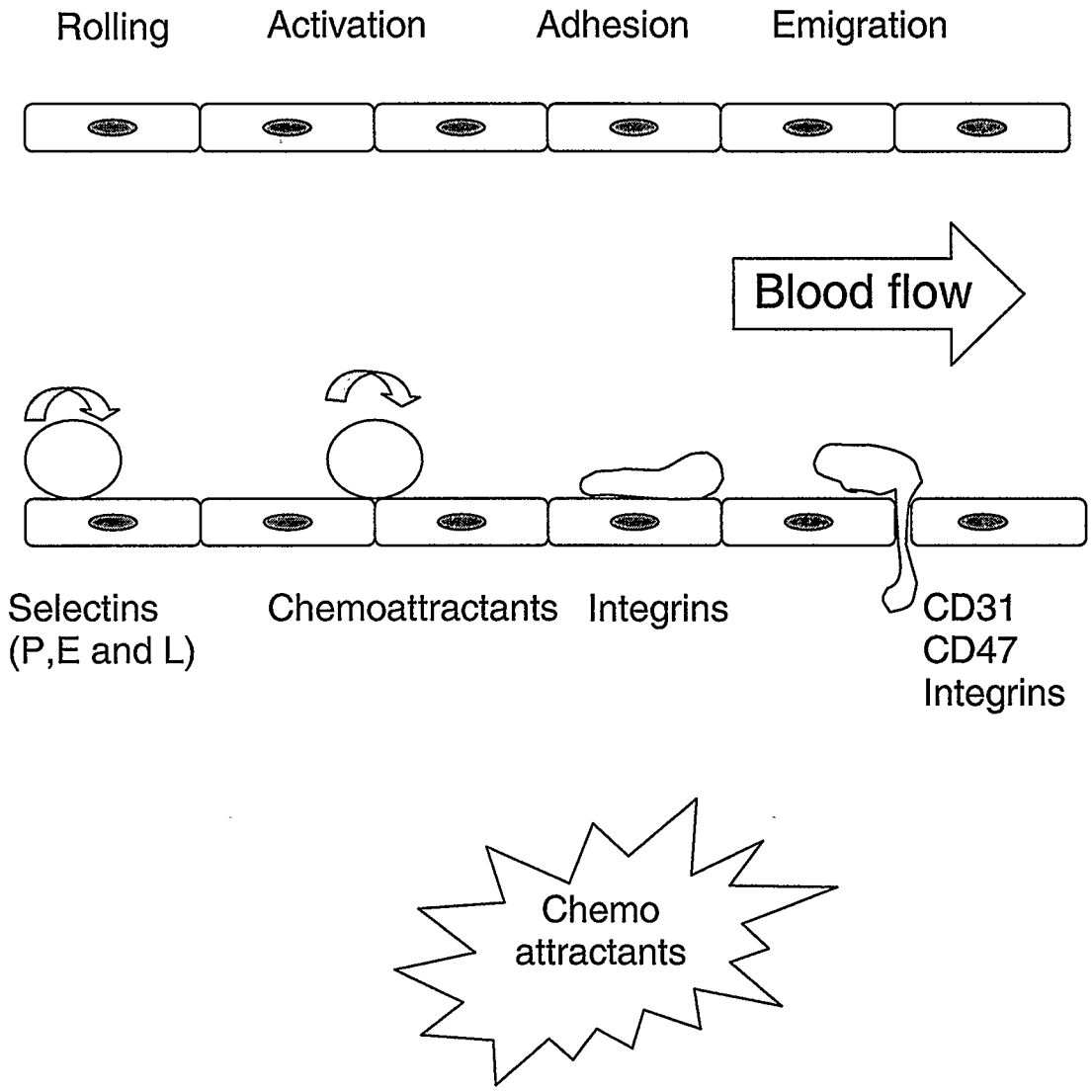

Figure 1. Schematic representation of the leukocyte recruitment cascade in a postcapillary venule (not to scale). The ligands for the adhesion molecules are not depicted. First, selectins (P- and E-selectin on the endothelium and L-selectin on the leukocyte) enable free flowing leukocytes to tether and roll. Next, inflammatory signals on the endothelium stimulate the leukocyte to express its integrins in a high affinity state. Engagement of the integrins with their ligands on the endothelium allows the cell to become adherent. This is followed by emigration out of the vasculature (primarily mediated by CD31, CD47 and integrins). Leukocytes migrate towards the site of insult by following gradients of chemoattractants produced by affected host tissue and/or pathogens.

Table 1. Important adhesion molecules in leukocyte recruitment during inflammation (Adapted from ref.167,168).

Adhesion molecule	Ligand
L-Selectin	PSGL-1 CD34 MadCAM-1 GlyCAM-1 PNAd
P-Selectin	PSGL-1 CD24
E-Selectin	PSGL-1 (low affinity) ESL-1 (murine) CLA L-selectin
LFA-1 (CD11a/CD18)	ICAM-1 ICAM-2 ICAM-3
Mac-1 (CD11b/CD18)	ICAM-1 Fibrinogen Fibronectin
P150,95 (CD11c/CD18)	Fibrinogen ICAM-1 ? ICAM-3 VCAM-1
VLA-4 ($\alpha_4\beta_1$)	VCAM-1 Fibronectin $\alpha_4\beta_7$
CD31 (PECAM-1)	CD31

Table 2. Contents of human neutrophil granules
(Adapted from reference 38).

Primary granules	**Secondary granules**	**Tertiary granules**
Lysozyme	Lysozyme	
Alkaline phosphatase	Alkaline phosphatase	
Defensins		
Azurocidin		
Proteinase-3		
Glucosidase		
Arginase		
B-glucuronidase		
MPO		
BPI		
Cathepsin G		
Elastase		
Neutral serine proteinases		
Acid phosphatases		
Acid hydrolases		
	Gelatinase	Gelatinase
	C3bi receptors	
	Collagenase	
	Lactoferrin	
	Complement receptors	
	fMLP receptor	
	CD11b/CD18 (Mac-1)	
	CD11c/CD18 (p150,95)	
	monocyte chemoattractant	
	Histaminase	
	Plasminogen activator	
	Vit B_{12} binding protein	
	Cytochrome b_{558}	

Figure 2. Respiratory burst reactions that create ROIs and RNIs. Reactions have been simplified for clarity. Components with strong antimicrobial activity are depicted in **bold** (197).

Reactive oxygen intermediates

$$2\,O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2\,O_2^- + NADP^+ + H^+$$

$$2\,O_2^- + 2\,H^+ \xrightarrow{SOD} \mathbf{H_2O_2} + O_2$$

When iron is present the Haber-Weiss reaction can follow:

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow \mathbf{.OH} + OH^- + Fe^{3+}$$

$$O_2^- + H_2O_2 \longrightarrow \mathbf{.OH} + OH^- + O_2$$

(This reaction may also yield singlet oxygen $^1\mathbf{O_2}$)

Myeloperoxidase catalyses the formation of hypochlorous species in:

$$H_2O_2 + Cl^- \xrightarrow{MPO} H^+\mathbf{OCl^-} + H_2O$$

Reactive nitrogen intermediates are formed by the reaction of HOCl with nitrogen containing compounds and through the action of iNOS:

$$HOCl + R\text{-}NH_2 \longrightarrow R\text{-}NHCl \rightleftharpoons R\text{-}NCl_2$$

$$L\text{-arginine} \longrightarrow L\text{-Citruline} + \cdot\mathbf{NO}$$

$$O_2^- + NO\cdot \longrightarrow \mathbf{ONOO^-} + H^+ \longrightarrow NO_2^- + \mathbf{.OH} + \mathbf{NO.}$$

Note that some hydroxyl radicals are rendered harmless in:

$$NO_2^- + .OH \longrightarrow NO_3^- + H^+$$

Chapter 2

Materials & Methods

Chapter 2. Methods & Materials

2.1. Animals.

CD43-deficient mice (CD43 KO) were purchased from The Jackson Laboratory (Bar Harbor, ME). They were generated by homologous recombination in embryonic stem cells in a mixed background of 129/SvEv × C57BL/6 as previously described (41). WT mice from the same background were used as controls. Animals were bred and housed in specific pathogen-free facilities and used between 6 and 12 wk of age.

2.2 Intravital Microscopy on murine cremaster muscle

For this assay, the mice were anesthetized with a mixture of Xylanine/Ketamine and the cremaster muscle exteriorized as previously described (18, 90). Leukocyte-endothelial interactions in single unbranched cremasteric postcapillary venules (25-40 μm in diameter) were observed through a microscope. Rolling flux was determined by counting the number of leukocytes that passed a specific point in the vessel per minute. Leukocytes are considered adherent to the endothelium if they remain stationary for a period > 30 s. Leukocyte rolling velocity is calculated from the mean time it takes 10 leukocytes to each roll a distance of 100 μm. Venular diameter (D_v) is measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Red blood cell velocity (V_{RBC}) is also measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute). The number of emigrated leukocytes was defined as all leukocytes outside the vasculature within 1 field of view. All observations were recorded on a VCR for subsequent playback analysis. See figures 3 and 4 for a schematic representation of the intravital microscopy setup and an example of the obtained VCR images.

2.3. Bacterial culture

Escherichia coli (#25922 ATCC Rockville MD) were grown overnight at 37 °C to a thick suspension in Mueller-Hinton broth (Sigma). The culture was spun down (3000g, 15 min, RT) and the pellet resuspended in PBS containing 0.1 % glycerol. 100 µl volumes of this were aliquoted and stored at -70 °C. All bacterial assays were done with cultures generated from fresh aliquots of this stock. Aliquots were not reused. To obtain a culture, a 10 µl volume of the stock was streaked on a tryptic soy agar (TSA) plate (Sigma) and grown overnight at 37 °C. A few colonies were transferred into Mueller-Hinton broth and grown to sufficient density in 3-4 hours at 37 °C. The culture was then spun down (3000g, 15 min, RT), washed with, and suspended in, PBS containing 0.1 % gelatin (pH 7.4). The bacteria were further diluted based on photospectrometric absorbance at 620 nm. For every experiment, control plates were grown to confirm actual counts. This strain was made ampicillin resistant by introducing the commercially available Bluescript vector (Stratagene) using a standard temperature shock protocol. This made selection against potential bacterial contamination possible. Culture media always contained the selecting agent (50 µg/ml ampicillin), were made fresh before each experiment and stored at 4 °C to avoid degradation of the antibiotic. Manipulations involving bacteria were done using conventional sterile laboratory techniques.

2.4. Murine model of bacterial peritonitis

Paired groups of KO and WT animals were injected intraperitoneally (i.p.) with 10^7 colony forming units (CFUs) *E. coli* in sterile saline. Before injection, the abdomen was wetted with 70% ethanol and allowed to dry. After 4 or 24 hrs. the animals were anesthetized by inhalation of methoxyflurane (Metofane, Janssen, Toronto, Canada) and

killed by cervical dislocation. The abdomen was sterilized with 70% ethanol and the peritoneum lavaged with 10 ml ice-cold sterile PBS. Total cell counts were performed with a hemacytometer and differential counts with Wright-Giemsa stained cytospins. Lavage fluids were serially diluted and viable CFUs counted after overnight incubation at 37 °C on TSA agar plates containing 50 μg/ml ampicillin

2.5. Collection of peritoneal neutrophils.

Mice were given a 1 ml i.p. injection of 1% oyster type II glycogen (Sigma) in sterile saline as previously described (18). After 2 hrs. cells were harvested from the peritoneal cavity by lavage with 10 ml sterile ice-cold sterile PBS (containing 5 U/ml heparin) and spun down (150g, 6 min, 4 °C). Contaminating erythrocytes were lysed by suspending the pellet briefly in 0.2 % saline. Isotonicity was restored by the addition of an equal volume of 1.6 % saline. Remaining leukocytes were spun down (150g, 6 min, 4 °C) and counted on a Coulter apparatus. Differential counting was performed with Wright-Giemsa stained cytospins. Cell viability was determined by trypan blue staining and was typically $\geq$ 95%. Differential counts were similar for the 2 strains and showed $\geq$95 % purity for neutrophils with few contaminating mononuclear cells. In our experience a 2 hrs. incubation time was optimal both for purity and quantity of the neutrophil population.

2.6. Collection of peripheral blood neutrophils

Murine

Mice were anesthetized by inhalation of methoxyflurane (Metofane, Janssen, Toronto, Canada) and killed by cervical dislocation. Peripheral blood was collected by cardiac puncture. Erythrocytes were lysed with 0.2 % saline and isotonicity was restored using an

equal volume of 1.6 % saline. The resulting leukocyte mixture was further purified by centrifugation over Ficoll-Histopague (Sigma). The resulting neutrophil suspension was ≥95 % pure as determined by differential counts of Wright-Giemsa stained cytospins. Cell viability was determined by trypan blue staining and was typically ≥95%. Contaminating leukocytes consisted of lymphocytes.

Human

30 ml of whole blood was collected from healthy volunteers and anti-coagulated with ACD. Dextran was added after which the red blood cells were allowed to sediment. Cells in the supernatant were spun down and remaining red cells lysed after which isotonicity was restored. The resulting cell population was separated with a Ficoll-Hypague (Sigma) centrifugation. The pellet containing ≥ 98 % pure neutrophils (based on cytospin and Wright-Giemsa staining) was resuspended in PBS and cell viability (typically ≥ 95%) checked with Trypan blue. Total cell count was determined with a Coulter Counter.

2.7. Chemotaxis assay

To assay chemotaxis, under agarose assays were performed using a method similar to that described by Lineaweaver et al. (24). Briefly, 10^7 neutrophils isolated from the peritoneum were inserted in the center well of a thin agarose layer (see figure 5). Two wells on either side of this center well contained the chemoattractant fMLP or a control solution (HBSS). In this manner, each plate is its own control. After 2 hrs. incubation at 37 °C, the number of cells that had moved towards the chemoattractant source (directed migration) or to the control solution (random migration) was counted using an inverted microscope with a 40X objective. Cells that had migrated away from the center well were identified on the basis of their distance to the center well, their shape in comparison to

resting cells in the center well and their focal plane as they moved under the agarose layer.

2.8. Phagocytosis assay

This was done using a flowcytometric assay as previously described (23, 50). Fluorescein isothiocyanate (FITC) labeled *E.Coli* bioparticles (BODYPI Molecular Probes Inc, Eugene, OR, USA) were opsonized with WT or KO serum, aliquoted and stored at –70 °C. For every assay a fresh aliquot was used. 10^7 bioparticles and 10^6 neutrophils were combined in 1 ml PBS containing 0.1% gelatin, 0.5 mM $MgCl_2$, 7.5 mM sucrose and 0.9 mM $CaCl_2$ (PBS^{+++}gel). Incubation took place at 37 °C for 1 hr. under slow rotation. 50 µl samples were drawn at 0, 30 and 60 min., diluted in 300 µl 0.02 M acetate buffer (pH 5.8) and analyzed by FACScan (Becton Dickinson, San Jose, CA). The Mean fluorescence intensity (MFI) of 10,000 cells was determined. Cells that showed an increase in fluorescence compared to zero time were considered to have bound or phagocytosed bioparticles (see figure 6). As negative control, cells without bioparticles were used.

To exclude possible opsonization or serum differences, additional samples were measured in which WT cells phagocytosed bioparticles opsonized with KO serum and vice versa. To distinguish between cell-adherent and intracellular bacteria, duplicate samples were measured in 0.02 M acetate buffer (pH 5.8) containing 5 mg/ml trypan blue (Sigma) to quench the fluorescence of cell-adherent bioparticles (39,58) (See figure 7). Since this protocol is well established in our laboratory for human cells, these were used as positive controls.

2.9. Bacterial killing assay

The capacity of isolated peritoneal neutrophils to kill bacteria was assayed using a modification of the method of Quie et al. (26). Fresh *E.coli* (10^6 CFUs) were opsonized by incubation with PBS^{+++}gel and 10% autologous serum for 15 min. at 37 °C. 10^7 isolated neutrophils were then added and incubated for 2 h. at 37 °C in PBS^{+++}-gel under slow vertical rotation. Samples were drawn at 0, 60 and 120 min. Neutrophils were lysed through incubation in sterile distilled water containing 0.1% gelatin for 10 min. After vortexing, an aliquot from this suspension was transferred into 10 mM Tris buffer containing 0.1% gelatin (pH 7.4). Viable bacterial counts were determined by spreading an aliquot of this onto TSA plates and counting the number of colonies after overnight incubation at 37 °C. The observed decrease in CFUs over time indicated neutrophil-mediated killing of *E.coli*. To exclude possible serum defects, additional samples were measured in which WT neutrophils were combined with KO serum and vice versa. Since this protocol is well established in our laboratory for human cells, these were used as positive controls.

2.10. MPO assay

This assay is based on a colorimetric reaction mediated by MPO (97). Briefly, the tissue is homogenized in a hexadecyltrimethylammoniumbromide (HTAB) detergent buffer after which hydrogen peroxide is added as substrate for MPO. The resulting hypobromous products reduce *O*-dianisidine, forming a colored compound, the formation of which can be monitored photospectrometrically. One unit of MPO activity is defined as the degradation of one micromole of peroxide per min. at 25 °C.

2.11. Flow cytometric analysis of murine neutrophil CD43

To measure relative CD43 expression, we used Phycoerythrin (PE) -labeled rat-anti-mouse IgG_{2a} mAbs specific for the 115 or the 130 kDa glycoform of CD43 (BD Sciences 01605B and 558762 respectively). A matched isotype control from the same company was used to control for nonspecific binding. Peripheral neutrophils were measured in whole blood drawn via cardiac puncture. 50 µl blood was incubated with the antibodies for 30 min. at RT. Erythrocytes were then lysed with Optilyse (BD Sciences) and distilled water. After washing, cells were fixated with 1 % formaldehyde. Measurements were done on a FACScan using Cell Quest software (Becton-Dickinson). Gating, based on size and granularity, was used to distinguish neutrophils (from whole blood and lavage fluids) from contaminating erythrocytes and monocytes/macrophages. Since the remaining subpopulations could still contain some lymphocytes, a FITC-labeled anti-mouse Ly-6G/Ly-6C was used (BD Sciences 553126). These are granulocytic markers expressed by monocytes and neutrophils. Although subsets of lymphocytes may also express Ly-6G, neutrophils have been shown to express 10-100 times more (98,99). We corrected for the small increase in the PE (FL-2) range by the FITC. CD43 expression was measured on subpopulations that were Ly-6G/Ly-$6C^{high}$. CD43 expression was determined by measuring the mean fluorescence intensity (MFI) of 10,000 cells.

2.12. Statistical analysis

All data are presented as mean ± SEM. The results of the *in vitro* assays were analyzed using an unpaired *t*-test. Since bacterial counts differed between experiments, results of the peritonitis model were analyzed using a paired *t*-test. P values ≤ 0.05 were considered significant.

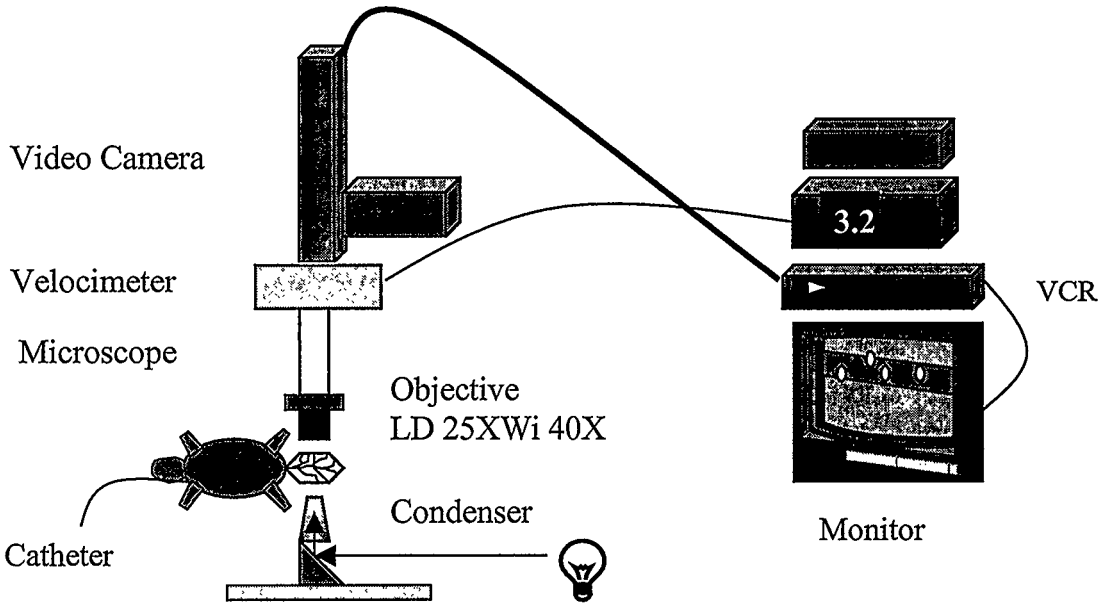

Fig 3. Schematic representation of the intravital microscopy setup. Animals are anesthetised and the cremaster muscle exteriorised. Anethesia is maintained by iv. infusion into a jugular vein.The preparation can be observed directly through the microscope or via camera on the screen. A velocimeter measures RBC flow rate.

Fig 4. Leukocyte recruitment in a murine cremasteric postcapillary venule observed by intravital microscopy. Clearly visible are 2 emigrated cells (upper right quadrant, solid arrows) and multiple rolling and adherent cells. Slow rolling and adherent cells are deformed due to opposing forces from blood flow and adhesion molecules.

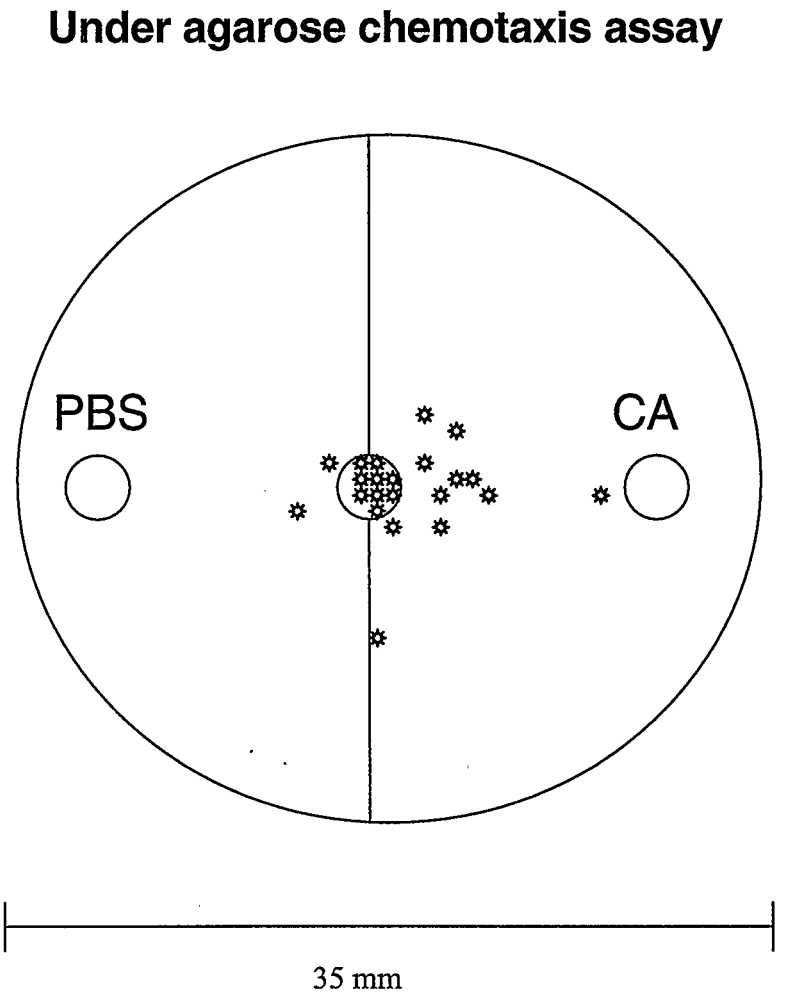

Fig 5. Schematic representation of the under agarose chemotaxis assay (not to scale). 10^7 neutrophils are inserted in the center well. After 30 min. incubation at 37 °C, the number of cells that has migrated towards the chemoattractant (CA) source is divided by the total number of migrated cells. This ratio determines the degree of directed migration (chemotaxis). Phosphate buffered saline (PBS) is used as control solution.

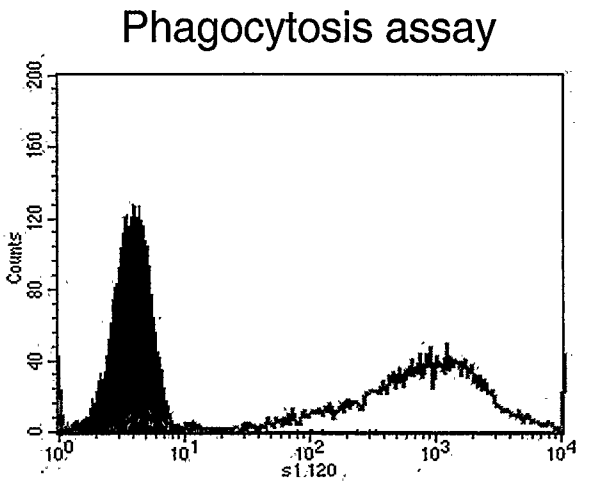

Fig 6. Representative sample indicating the phagocytic capcity of neutrophils by flow cytometry. At t=0, all cells have a low fluorescence (black curve). At t=30, most cells have phagocytosed FITC labeled bioparticle(s) and are fluorescent (white curve with gray boundary).

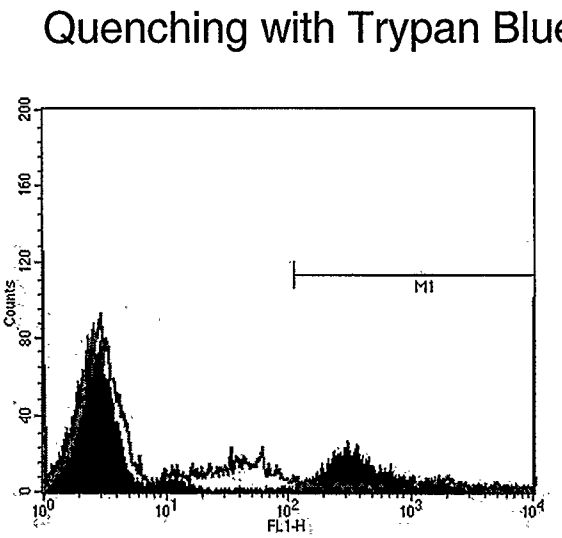

Fig 7. Representative sample illustrating the use of trypan blue to differentiate between truly phagocytosed or merely extracellularly adherent fluorescent bioparticles. The sample was incubated at 4 °C to allow binding but not phagocytosis. A sample of the cells was then analyzed in normal buffer (black curve) while the remainder was analyzed in buffer containing trypan blue (white curve with gray border). Bioparticles that are adherent but not phagocytosed are sufficiently quenched by trypan blue to make exclusion (marker M1) possible.

Chapter 3

CD43 deficiency in ischemia-reperfusion injury

3. CD43 deficiency in I/R injury

The IR model is a modification of previously published reports regarding the effect of CD43 on leukocyte recruitment in murine cremaster tissue (18). In that model, the tissue was superfused with the inflammatory mediators PAF or fMLP. CD43 KO animals showed increased adherence in comparison to WT animals although their total recruitment was actually decreased, demonstrating impaired leukocyte emigration in CD43 KO animals.

The goal of these experiments was to confirm these observations on leukocyte kinetics under physiologically more relevant conditions. The model of IR injury utilized here is well characterized, does not require superfusion and induces a comprehensive inflammatory response (90, 229, 230)

Preparation of murine cremaster tissue was performed as described in Materials & Methods. After a control measurement, bloodflow to the cremaster was halted for 30 min. to induce ischemia. A weak surgical clamp was used as not to damage the vessels. Subsequent recordings were made 5, 20, 30 and 60 min after the clamp had been released. We compared 7 WT animals to 7 CD43 KO animals.

3.1. Results

Figure 8 shows the effect of reperfusion on leukocyte kinetics. The increase in leukocyte rolling flux for both mouse strains was less than what is typically reported in the literature. Generally, reperfusion induces up to three times as many rolling cells compared to control (90). The reason for this difference is unknown. Still, the effect of reperfusion is clear; the number of rolling leukocytes following reperfusion increased significantly, while the average rolling velocity was strongly reduced. These results are indicative of an inflammatory response and have typically been attributed to upregulation of adhesion molecules on the reperfused endothelium. In the murine cremaster tissue, leukocyte rolling was shown to be exclusively P-selectin mediated for up to 2 hrs. post-ischemia (90). In our experiments, leukocyte rolling flux and velocity returned to baseline values 30 min. following the start of reperfusion.

Due to the clamping procedure, blood flow rate (V_{RBC}) during reperfusion may sometimes be slightly lower than before ischemia. To demonstrate that the reduced leukocyte rolling velocity (V_{WBC}) is not caused by this, the V_{WBC} was normalized through division with the V_{RBC}. Clearly, the decrease in V_{WBC} is disproportionate to the decrease in V_{RBC}, indicating an inflammatory response. Similar to previously reported data, no difference in V_{WBC} between WT and CD43 KO animals was observed.

The adhesive behavior of leukocytes revealed the expected difference between the two animal strains: CD43 KO animals had approximately twice as many adherent cells directly after reperfusion. This difference stayed significant for up to 30 min. This confirms the anti-adhesive effect of CD43 under physiologically relevant conditions *in vivo*. Unexpectedly however, the reduction in total cellular recruitment in CD43 KO animals was not observed in this model.

3.2.Discussion

These experiments confirm the anti-adhesive role of CD43 under physiologically relevant circumstances *in vivo*. The observations of increased adhesion but normal recruitment in the CD43 KO animals suggest a reduced ability of adherent CD43-deficient leukocytes to migrate out of the vasculature. This is consistent with results generated in the PAF superfusion model. It is important to realize however, that the number of recruited cells in this model is very low in comparison to PAF superfusion in which as many as 30-50 cells would extravasate. Because of the low recruitment, small percentage differences in recruitment between WT and CD43 KO animals could be extremely difficult to demonstrate statistically. Furthermore, since the surgical procedure alone, without any further proinflammatory agonist, also invariably results in some emigration (not shown), it can be argued that the number of recruited cells observed in this model is in fact background. In retrospect, a model resulting in more recruitment could have confirmed reduced emigration in CD43 KO animals. (Much) longer ischemic periods (up to 6 hrs.) have been used to induce substantial recruitment, both in murine as well as other animal models (258, 259).

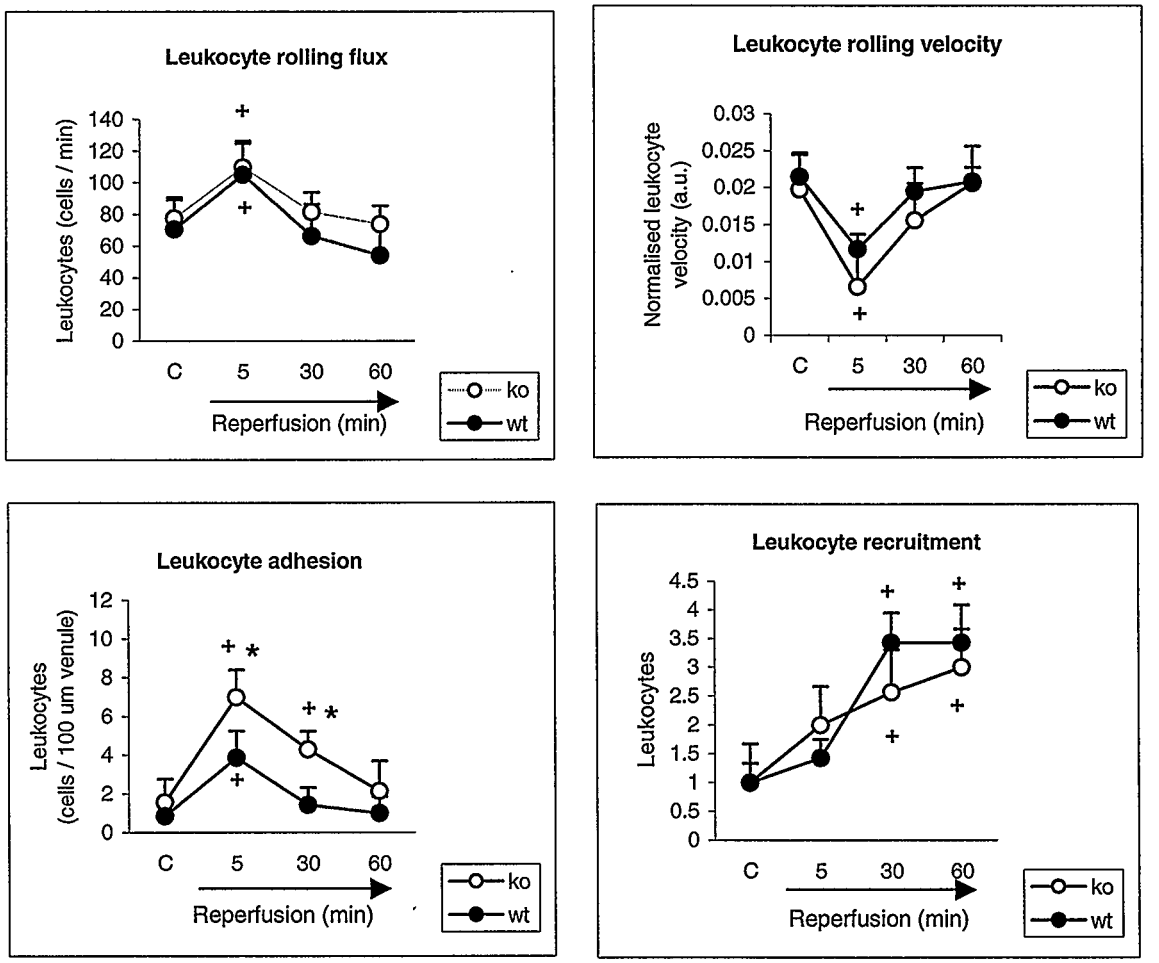

Figure 8. Leukocyte kinetics in the murine cremaster. After a control measurement (C), ischemia was induced for 30 min. by clamping the tissue off. Blood flow was then restored and measurements done at the indicated time points. CD43 KO animals showed significantly more adhering cells although total emigration was similar between the two strains.CD43 KO n=7, WT n=7. +p <0.05 compared to control, *p<0.05 compared between CD43 KO and WT.

Chapter 4

CD43 deficiency in bacterial peritonitis

Chapter 4. CD43 deficiency in bacterial peritonitis

The experiments described in the previous chapter (IR injury) did not reveal the expected decrease in leukocyte recruitment associated with CD43 deficiency. We had previously demonstrated that leukocyte recruitment into the peritoneum of CD43 KO animals was impaired in response to a single inflammatory stimulus (oyster glycogen)(18). A similar result was obtained by others using a CD43 mAb (57). We hypothesized that a similar impairment in recruitment would occur in response to bacterial infection and that the reduced effector cell recruitment would have negative consequences for host defense.

To test this hypothesis, we examined a murine model of bacteria-induced peritonitis. *E.coli* was chosen as the infecting pathogen since it is commonly isolated in clinical cases of spontaneous peritonitis and its use in experimental models is well accepted (60, 71). The use of this particular strain in a similar experimental model has previously been published (129).

Paired groups of CD43 KO and WT animals were injected with 10^7 CFUs *E.coli* bacteria (Examination of paired groups compensated for differences in bacterial load inherent to procedures involving bacteria). After 4 or 24 hrs. the animals were sacrificed, their peripheral blood harvested and leukocytes and bacteria extracted from the peritoneum as described in Material & Methods.

4.1. Results and discussion

A striking increase in bacterial load was observed in the CD43 KO animals compared to WT. The trend visible after 4 hrs. became statistically significant at 24 hrs. (figure 9). Whereas the CD43 KO animals showed a ~3000 fold increase, the WT animals cleared the pathogen almost completely, reducing it to ~ 20% of the initial injected load (KO 2.9 E10; WT 2.2 E6; $p<0.01$; $n=14$).

In conjunction with the increased bacterial load, decreased overall health (observation) and survival rate (figure 10) was observed in the CD43 KO animals as a result of these experiments. The death of some of the experimental animals was unintentional. Although the survival data cannot be analyzed statistically, it clearly agrees with the increase in bacterial load. Since animals that died could not be sampled for other parameters, lower survival of CD43 KO animals actually biased against our hypothesis. This means that the differences in bacterial load between the two mouse strains may in fact still be an underestimation.

Associated with the increase in bacterial load, decreased cellular recruitment was also observed in the CD43 KO animals compared to WT (figure 11). The trend seen at 4 hrs. was statistically significant at 24 hrs. after injection of the bacteria. At 4 hrs. the leukocyte infiltrate in the peritoneum consisted predominantly (>60%) of neutrophils and at 24 hrs. mostly (>70%) of monocytes. Differences in circulating leukocyte counts

between (untreated) WT and CD43 KO mice do not explain this result since total and differential leukocyte counts were similar in both animal groups as measured by us and reported by others (18, 45).

After 4 and 24 hrs. leukocyte depletion of the peripheral blood occurred in both murine strains (Figure 12). Interestingly, peripheral leukocyte counts fell significantly more in the CD43 KO animals compared to WT. This suggests that CD43-deficient cells are removed from the circulation more avidly than WT leukocytes (but do not reach the target site). This agrees with the increased adhesion and decreased emigration associated with CD43 deficiency. As an alternative explanation we considered the possibility that CD43-deficient cells were accumulating in different tissues. The lung is an important organ to consider in this respect because of its rheological properties of small vessel diameter and low flow rates (236). It has been previously demonstrated that i.p. injection of a pro-inflammatory stimulus and its subsequent appearance in the blood stream has profound systemic effects. This can lead to augmented neutrophil recruitment (up to 350 %) into the lungs of experimental animals (91).

To investigate this possibility, assays for MPO were performed as described in Materials & Methods. Although generally used as an indicator of neutrophil numbers, monocytes/macrophages also carry this enzyme (237). We utilized MPO levels as a suitable indicator for inflammatory cell influx during acute inflammation.

Figure 13 shows the results of an experiment in which inflammation was induced in CD43 KO and WT animals following i.p. injection of oyster glycogen. After 4 h. the animals were killed and their lungs analyzed for MPO activity. As discussed in the background section and demonstrated in figure 13, normal, healthy lungs always contain some (sequestered) neutrophils. The background MPO values were similar for both animal strains. After 4 hrs. however, a significant difference was found: Lungs of CD43 KO animals showed higher MPO activity than those of WT animals. This suggested that at least part of the reduced recruitment into the peritoneum in CD43 KO animals following bacterial peritonitis is a result of augmented neutrophil accumulation within the lungs, excessively depleting the peripheral blood. This accumulation does not necessarily involve recruitment into the pulmonary tissue but may simply reflect increased adhesion to, and trapping in, pulmonary blood vessels. Abnormally high increases in adhesion or recruitment into the lung and perhaps throughout the whole CD43-deficient animal, would lead to fewer neutrophils being available for recruitment into the peritoneum.

In this model, recruitment was reduced in the CD43 KO animals as was seen in the PAF superfusion model (18). The accelerated peripheral blood depletion and augmented leukocyte accumulation in CD43 KO animals in other than target tissue, suggests that the mechanisms responsible for reduced leukocyte recruitment between the two models may differ. Although reduced emigration of CD43-deficient cells may contribute to the reduced recruitment into the peritoneum, it appears that when the inflammatory response is systemic (peritonitis) rather than local (cremasteric PAF superfusion), depletion of the circulating leukocyte pool becomes an additional factor. Future experiments could

address if the accumulation of leukocytes occurs only in lungs or if it occurs in other organs as well. It would also be of interest to determine if CD43-deficient cells are truly recruited into the tissue or that they simply become immobilized intravascularly through increased adherence.

This data demonstrates for the first time a significant impairment in host defense against a bacterial pathogen associated with CD43 deficiency.

4.2. Limitations

We demonstrated the phenotype of reduced recruitment and impaired host defense under provocative conditions. Note that CD43 KO animals do not normally develop any form of disease spontaneously.

The MPO data was generated in animals injected with oyster glycogen. It seems highly likely that similar accumulation in the lungs would also occur in *E.coli*-induced peritonitis but this needs to be confirmed.

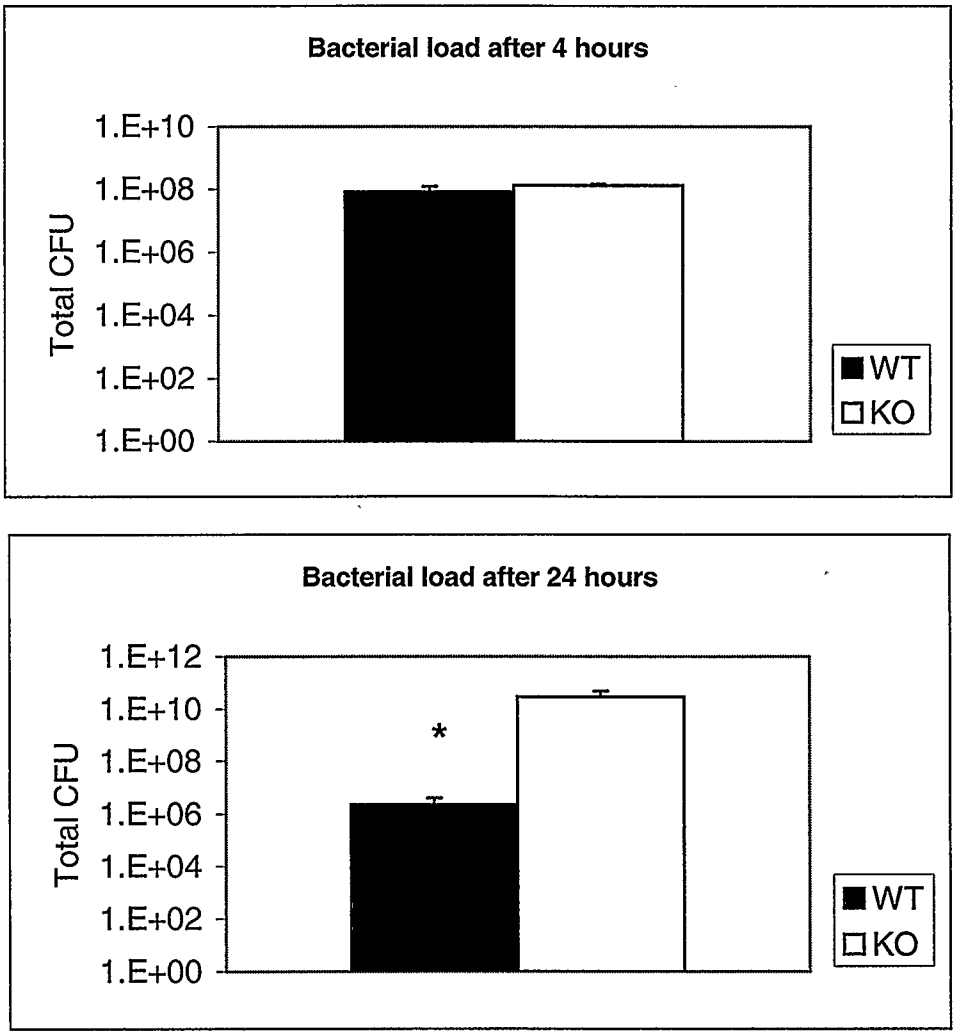

Figure 9. Total bacterial load 4 or 24 hrs. after i.p injection of 10^7 *E.coli* bacteria. While WT animals clear the pathogen almost completely, CD43 KO animals dramatically increase their load. Mean + SEM; n=8 and 14 for 4 hrs.and 24 hrs. respectively, for both animal strains. *$p<0.05$.

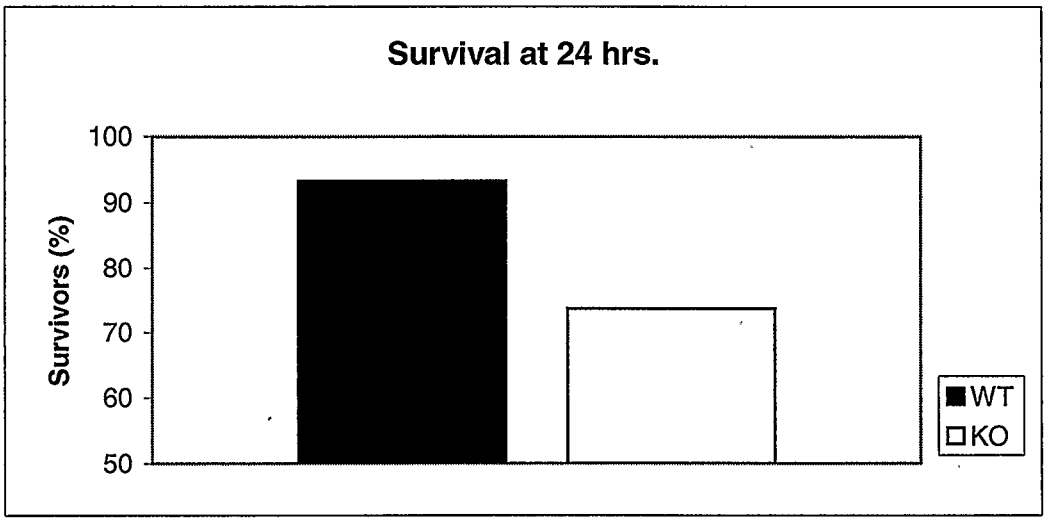

Figure 10. Survival of WT and CD43 KO animals 24 h. after i.p. injection of 10^7 *E.coli* bacteria.WT, n=15; CD43 KO, n=19.

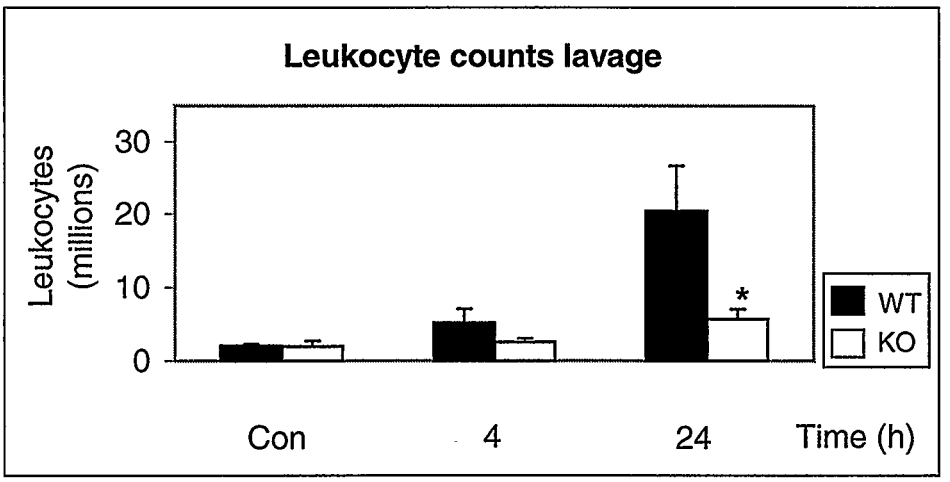

Figure 11. Leukocyte recruitment in the bacterial peritonitis model 4 or 24 h.after i.p. injection of 10^7 *E.coli* bacteria (Con = untreated animals). CD43 KO animals show a significant reduction in recruitment compared to WT animals. Mean + SEM; n=3, 8 and 14 for control, 4 hrs. and 24 hrs. respectively, for both animal strains. *p<0.05.

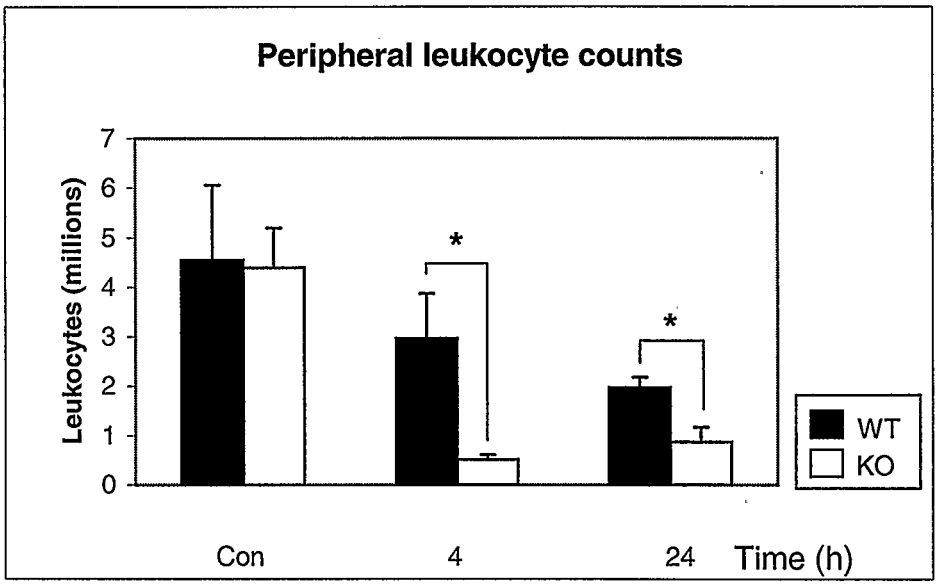

Figure 12. Circulating leukocyte counts in the bacterial peritonitis model 4 or 24 h. after i.p. injection of 10^7 *E.coli* bacteria (Con = untreated animals). Values of untreated WT and KO animals (Con) are similar. At 4. and 24h. KO animals have lower counts than the WT animals. Within strains, both animal types show significant depletion between 0 and 4, and between 4 and 24 h. Mean + SEM; n=3, 8 and 14 for control, 4 hrs. and 24 hrs. respectively, for both animal strains. *p<0.05.

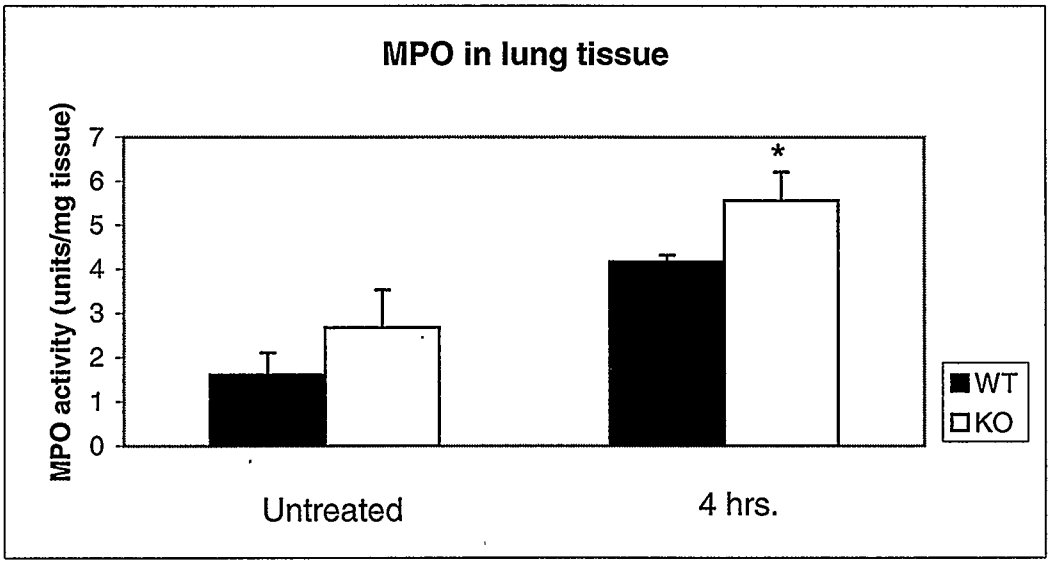

Figure 13. Lung MPO activity as a measure of neutrophil and moncoyte/macrophage recruitment. Lungs from CD43 KO and WT animals were harvested 4 hrs. after an i.p.injection of 1 ml 1% oyster glycogen. Values were compared to untreated animals. After 4 hrs. both strains showed significantly higher MPO activity indicating increased accumulation. The difference between the two strains is significant at 4 hrs. Mean + SEM; WT, n=3 and 3 for untreated and 4 hrs. respectively; CD43 KO; n=3 and 5 for untreated and 4 hrs. respectively; *$p<0.05$.

Chapter 5

In vitro effector function of CD43-deficient neutrophils

Chapter 5 *In vitro* effector function of CD43-deficient neutrophils

Although there is a strong correlation between reduced recruitment and increased bacterial load in the peritonitis model, the observed reduction in bacterial clearance in CD43 KO animals could additionally have been caused by other defects in CD43-deficient leukocytes. As previously discussed, effector cells have to be able to detect gradients of inflammatory signals and migrate towards the site of infection (chemotaxis). Upon arrival, the bacterial pathogens have to be recognized and eliminated. The latter involves mechanisms that are phagocytosis-dependent and -independent.
As discussed in chapter 1, *in vitro* reports suggest CD43 may affect such functions:
- CD43 is involved in the proliferation and activation of leukocytes (31, 45, 59, 70, 74);
- CD43 may facilitate neutrophil chemotaxis by localization to the uropod (72, 76, 234);
- CD43 crosslinking may induce respiratory burst activity in neutrophils (96)
- CD43 may be involved in the binding of mycobacteria to phagocytic cells (92)
- CD43 crosslinking may induce neutrophil locomotion (1)

We hypothesized therefore, that part of the impairment in host defense in the bacterial peritonitis model may, in addition to recruitment problems, have been related to co-existing defects in bactericidal function of the recruited leukocytes. To investigate the CD43 dependency of these processes, we assessed the ability of peritoneally recruited neutrophils to migrate towards a chemotactic stimulus, bind and phagocytose a target and kill *E.coli* bacteria *in vitro*.

5.1. Results and discussion

For most of the in vitro assays described in this chapter, murine neutrophils were harvested from the peritoneum as described in Materials & Methods. This allowed us to confirm decreased recruitment in response to oyster glycogen in CD43 KO animals (figure 14).

5.1.1. CD43 and chemotaxis

To investigate if chemotactic responses were affected in CD43 KO neutrophils, their migratory response to fMLP, a chemotactic bacterial peptide, was determined. fMLP was considered a relevant microbial stimulus since analogs are found in many bacterial species (including *E.coli*) as the N-terminus amino acids in proteins (81, 208). Since CD43-deficient leukocytes showed reduced recruitment, Boyden chamber approaches would most likely have an inherent bias. Instead, an under agarose assay was used in which the directional behavior of the cells can be determined without the use of a physical barrier (see M&M). Neutrophils isolated from the peritoneum were exposed to a distant source of fMLP (0.2, 2 and 20 mM). Such concentrations are high compared to those used for human cells that may respond to concentrations as low as 0.1 pmolar in this system (19). Higher concentrations are necessary to induce chemokinesis or chemotaxis in murine cells probably because these express a low-affinity fMLP receptor (93).

Both CD43 KO and WT neutrophils elicited from the peritoneum showed similar chemotaxis and total chemokinetic responses to fMLP (Figure 15). This result does not entirely exclude the possibility of chemotactic defects related to CD43-deficiency. In a complex *in vivo* micro-environment where cells interact with various substrata and receive multiple directional stimuli, impairments may exist.

5.1.2. CD43 and phagocytosis

Phaogocytic assays were performed as described in Materials & Methods. Trypan blue was used to distinguish between cell adherent and truly phagocytosed *E.coli* particles. WT and CD43-deficient neutrophils isolated from the peritoneum were equally efficient in binding the FITC labeled *E.coli* particles (fig 16). Interestingly, additional experiments performed with neutrophils isolated from peripheral blood did reveal a difference: WT neutrophils showed a greater tendency to associate with bioparticles, a difference that disappeared with the addition of trypan blue. This suggests it is not phagocytosis but rather surface binding that is reduced in CD43-deficient neutrophils. This is consistent with data demonstrating that the extracellular domain of CD43 is critical in the binding of certain M*ycobacterium* species (92).

The difference in surface binding between peripheral CD43 KO and WT cells was not observed for recruited cells. The reason for this is unclear but may be related to changes in activation state resulting from recruitment or from CD43 shedding. Due to shedding,

emigrated WT neutrophils in the peritoneal space may resemble CD43 KO cells to a large degree (This matter is addressed in chapter 6).

It is unclear why the reduced binding in CD43-deficient peripheral neutrophils does not result in reduced phagocytosis as well. It may be that CD43-mediated binding is not required for phagocytosis. It is also possible that with the ratio of 10 bioparticles to every neutrophil, it is not the rate of binding but the rate of phagocytosis that is limiting. In retrospect, additional experiments with different ratios and/or time points could have addressed this.

5.1.3. CD43 and bacterial killing

To specifically examine bactericidal function, murine neutrophils elicited from the peritoneum were tested *in vitro* for their ability to kill *E.coli* as described in M&M. WT and KO cells demonstrated a similar bactericidal capacity, killing approximately 70% of the bacteria within 2 h. (Figure 17).

These experiments suggest that bactericidal function of recruited CD43-deficient neutrophils is not impaired. Although it remains possible that *in vivo* cell function impairments exist, *in vitro*, CD43 deficient neutrophils appear capable of proper homing to a chemotactic stimulus, phagocytosis and bacterial killing. This suggests that reduced recruitment and not phagocytic or bactericidal defects in recruited cells were primarily if not solely responsible for the decreased host defense observed in our peritonitis model.

5.2. Limitations

It is important to realize that besides the binding and phagocytosis assay, experiments were only performed with recruited and not with circulating neutrophils. As discussed, emigrated neutrophils in the peritoneal space may resemble KO cells to a large degree due to shedding. This may partly explain why all tested variables showed independence of CD43. This is however, the physiological phenotype that effector cells acquire when migrating to a site of infection and is therefore relevant to the assessment of the role of CD43 under physiological conditions, including our model of peritonitis.

There are several important limitations concerning the experiments leading to the conclusion that reduced recruitment is responsible for the impaired host defense:

1. Although the neutrophil is a crucial effector, it is not the only phagocytic cell involved. Resident and recruited macrophages can perform similar functions. It may be that CD43 deficiency affects their function and subsequently host defense. Additional experiments on this cell type would be important to consider.
2. Cell viability counts were performed before, but not after the phagocytosis assays. It cannot be excluded that differences in the degree of cell lysis or apoptosis after phagocytosis between WT and CD43-deficient neutrophils skewed the data.
4. Chemotaxis assays were performed *in vitro* in response to only 1 stimulus (fMLP). This is hardly representative of the *in vivo* situation where multiple signals are present. Examples are LTB_4, C3a and C5a.

5. Shiota et al. (84) have demonstrated that CD43 shedding continues at the effector site even after emigration. This means that CD43 fragments are available *in vivo* at the site of infection. For the *in vitro* experiments, cells were harvested by lavage and washed, eliminating such fragments and any potential function they may have in the microbicidal process. It cannot be excluded that these fragments play an important role *in vivo*, for example as opsonins.

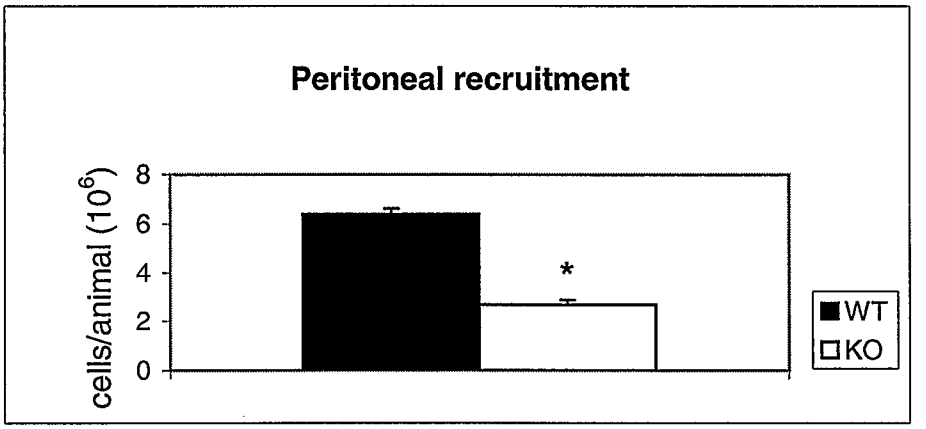

Figure 14. Neutrophils were harvested from the peritoneum 2 hrs. after i.p.injection of 1% oyster glycogen.CD43 KO animals recruited significantly less than WT. Mean + SEM; CD43 KO, n=10; WT, n=10 *$p<0.01$.

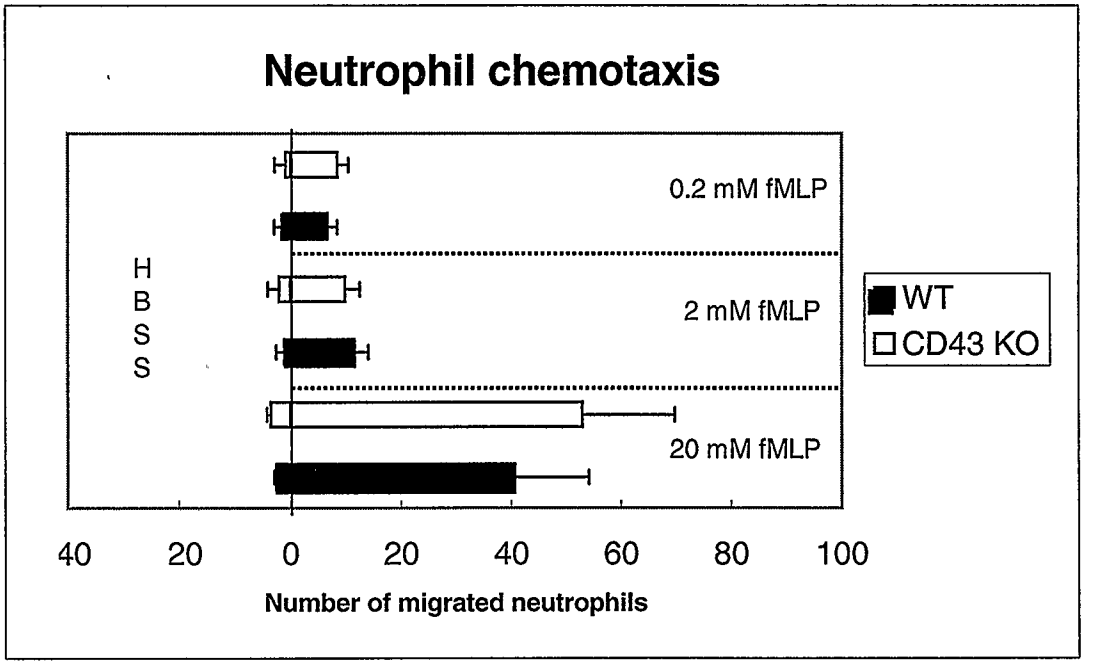

Figure 15. Chemotaxis towards fMLP in the under agarose assay at the indicated concentrations. WT and CD43 KO cells show similar chemotactic responses. Mean + SEM; WT, n=3, CD43 KO, n=3.

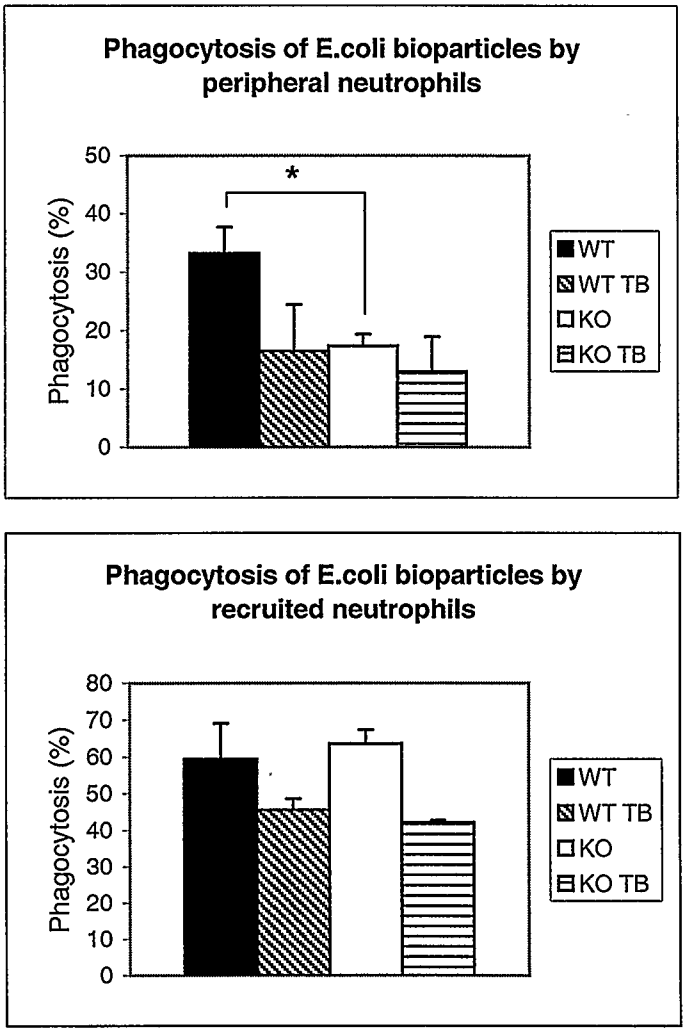

Figure 16. Binding and phagocytosis of FITC-labeled *E.coli* bioparticles by peripheral and recruited (isolated from peritoneum) neutrophils. Samples treated with trypan blue (TB) indicate true phagocytosis. CD43 deficient neutrophils (KO) show reduced binding while phagocytosis is unaffected. Recruited neutrophils show no differences in binding or phagocytosis. Mean + SEM; n=3 for both cell types. *$p<0.05$.

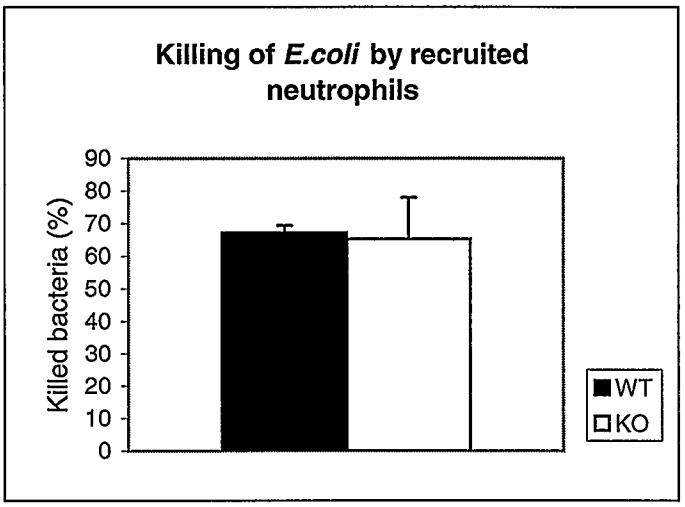

Figure 17. Bacterial killing assay. CD43-deficient and WT neutrophils were equally efficient in killing *E.coli* . Mean + SEM; n=4 for both cell types.

Chapter 6

Downregulation of CD43

Chapter 6. Downregulation of murine CD43

As discussed, there is considerable in *vitro* and *in vivo* data to suggest that surface CD43 of phagocytic cells (neutrophils, monocytes/macrophages) is partially downregulated during recruitment. In order to interpret our data correctly, it was imperative to determine if this downregulation occurred in the murine system and to what degree.
For this we used flowcytometric analysis to compare CD43 expression on peripheral and recruited neutrophils. Because of the difficulty in obtaining sufficient numbers of unactivated peripheral neutrophils, a whole blood procedure was adopted as described in Materials & Methods (Figure 21 shows a representative sample of the flow profiles obtained in these experiments).

6.1. Results and discussion

To demonstrate our ability to measure CD43 downregulation, WT neutrophils isolated from the peritoneum were either kept at 4 °C or incubated at 37 °C with or without 10^{-5} M PAF. Both conditions induced significant (up to 39.5 %) shedding of the 115 kDa glycoform compared to the 4 °C control (figure 18). Because this high concentration of PAF caused clotting in our whole blood samples, 10^{-5} M fMLP was chosen as the agonist for CD43 shedding from peripheral neutrophils. Both glycoforms were significantly downregulated (figure 19) and to a similar degree (115 kDa 17 % ; 130 kDa 13 %). This data is consistent with reports on human neutrophils and demonstrates that CD43 can also be downregulated even more on cells that have already undergone recruitment. Although

a shedding-resistant subpopulation of CD43 molecules may exist on neutrophils (49), this data suggests this is not the reason why a large proportion of CD43 persists on the cell surface. Shedding from neutrophils isolated from the peritoneum (resuspended in PBS) confirms that shedding can occur independent of plasma proteases.

Next, we compared CD43 expression on peripheral and recruited neutrophils (Figure 20). The physiological downregulation was small compared to the *in vitro* results: as little as 16 % total downregulation was demonstrated (115 kDa 9 % ; 130 kDa 7 %). Downregulation of CD43 in the murine system has not previously been published. The total (115 and 130 kDa) extent of shedding we observed is consistent with the discussed clinical data (84).

We presume the mechanism of CD43 downregulation in these experiments to be shedding, although this was not directly confirmed. There is however no evidence for total CD43 downregulation through internalization as alternative to shedding in the literature. In fact, the one *in vitro* study that specifically addressed this matter, argues against internalization of CD43 as a mechanism of downregulation on granulocytes (279). It is believed however that (on T cells at least) one glycoform of CD43 can be internalized and replaced by the other:

"Like many other surface molecules, CD43 is constantly internalized, degraded, synthesized and re-expressed on the cell surface. The half-life of a CD43 molecule on the cell surface is approximately 20 min. (unpublished results generated by A.T. Jones). It is believed that when a cell changes surface expression from one glycoform to another (e.g.

downregulation of 130 kDa CD43 on double positive $CD4^+CD8^+$ thymocytes to 115 kDa CD43 on a naïve single-positive $CD4^+$ or $CD8^+$ cell), the cell will downregulate that glycoform through internalization and replace it with newly synthesized CD43 molecules of the other glycoform. Loss of both glycoforms of CD43 can be seen as a good indication of downregulation through shedding (Dr. Hermann Ziltener, University of British Columbia, abstract of personal communication).

The simultaneous decrease of both glycoforms in these experiments clearly suggests that CD43 downregulation and not a shift in glycoform expression was responsible for the reduction in CD43 expression of either glycoform. It is important to note that without direct evidendence for shedding (e.g. the detection of shed CD43 fragments), downregulation of both glycoforms of CD43 through internalization cannot be excluded.

We are uncertain how to interpret the extent of downregulation with respect to the data on the binding of bioparticles (figure 16) described in the previous chapter. Downregulation of CD43 during recruitment to a KO-like phenotype does not occur since 85 % remains on the surface. It seems unlikely (but not impossible) that a loss of only 15 % of surface CD43 could explain the disappearance of the difference in bioparticle affinity between WT and CD43-deficient cells once these are recruited.

6.2. Limitations

We used oyster glycogen to recruit neutrophils into the peritoneum. This was relevant in relation to the *in vitro* assays described in chapter 5. This stimulus is not physiological and may have over- or underestimated physiological shedding. Cells recruited in response

to *E.coli* would have provided an alternative, although concentration would again be an issue.

Both glycoforms of CD43 showed a similar MFI. This does not mean they are expressed in the same amount on the cell surface. The antibodies used may differ in specificity or the ratio of antibody to epitope may be different.

Since we and others have shown that CD43 shedding may continue at the target site we cannot exclude the possibility that the observed reduction in CD43 expression occurred after recruitment.

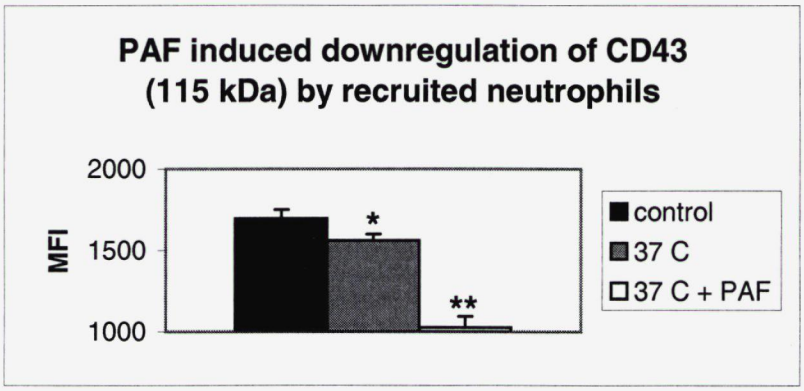

Figure 18. Incubation at 37 °C with and without 10^{-5} M PAF induced significant shedding of the CD43 115 kDa glycoform compared to control cells kept at 4 °C. Mean + SEM; n=3. *p<0.05; **p<0.01.

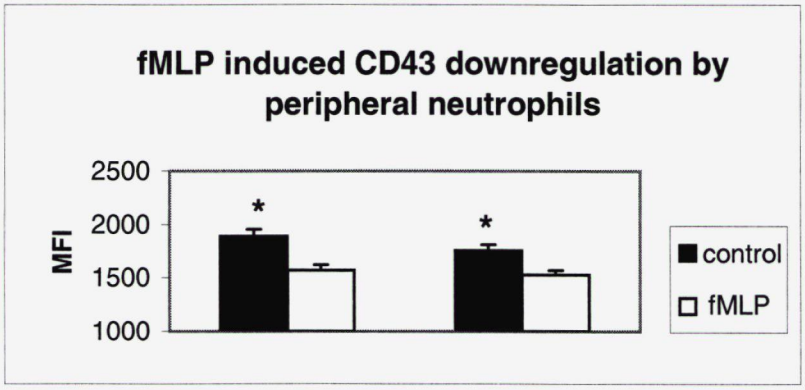

Figure 19. Incubation at 37 °C for 30 min. with 10^{-5} M fMLP induced significant shedding in murine neutrophils. Both CD43 glycoforms showed a similar reduction. Mean + SEM; n=3. *p<0.05

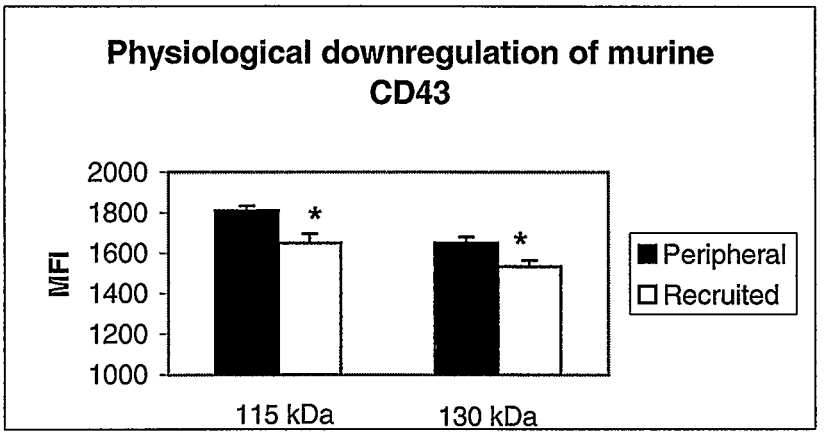

Figure 20. Expression of the 115 and 130 kDa glycoforms of CD43 on neutrophils isolated from peripheral blood or from the peritoneum. Mean + SEM; n=3; *$p<0.05$.

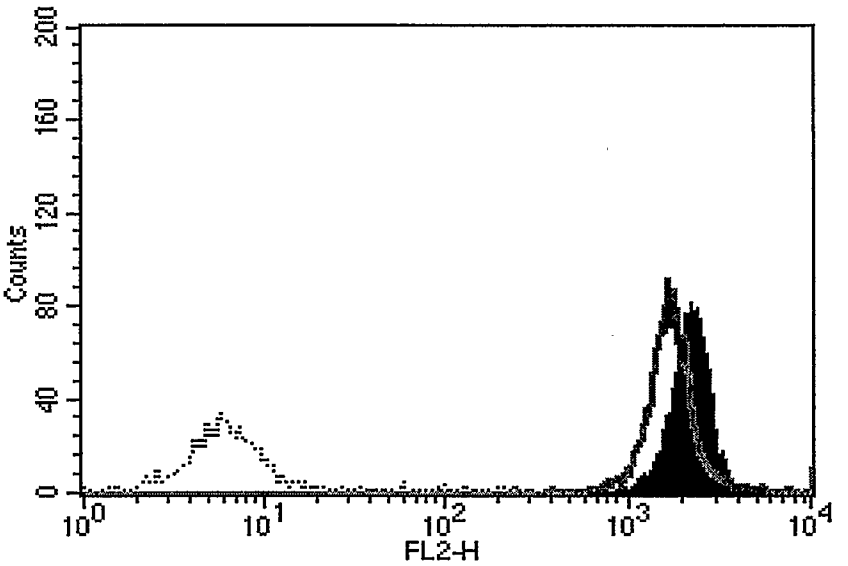

Fig 21. Representative (overlay) flow profile of PAF-mediated CD43 shedding. Black curve is untreated lavage sample at 37 °C.
Gray curve is a lavage sample at 37 °C treated with 10^{-5} M PAF.
Also shown is the isotype control (dotted curve to the left).

Chapter 7

Conclusions and future directions

7. Conclusions and future directions

It is becoming increasingly clear that CD43 is an important regulator of leukocyte recruitment. Its properties appear to make it uniquely suited to combine both inhibition and promotion of cell-cell interactions within one molecule, depending on the (inflammatory) conditions. To me, the data on CD43 generated in the CD43 KO animal vs. investigations in which antibodies were used suggest a subtle modifiable role for CD43 in leukocyte recruitment. In this scenario the antiadhesive properties of the molecule can be overcome under inflammatory circumstances by removal of (part of) the molecule (although this does not occur in lymphocytes) and/or by engaging an active domain, ultimately resulting in a direct or indirect proadhesive role for CD43. It stands to reason that engagement of an active domain on CD43 involves an upregulated inflammatory mediator the identity of which is currently unknown.

Demonstration that an antibody against the EC domain of CD43 can inhibit leukocyte recruitment as early as the rolling phase, not only suggests this domain is involved in promoting recruitment but also that CD43 may be engaged early in the recruitment cascade.

The most obvious mechanism by which CD43 could promote recruitment is interaction with a ligand during emigration to directly increase adhesive forces. As discussed in the Background section, potential candidates include selectins, integrin ligands and ECM components (156, 157, 160, 162). Some of these meet the requirement of being upregulated under inflammatory conditions.

It is important to realize that CD43 does not necessarily have to be adhesive itself (beyond the interaction with its unknown, perhaps even soluble, ligand) to promote recruitment. Activation of CD43 and subsequent signaling through its IC domain may enable CD43 to promote recruitment by contributing to the activation of rolling or adherent cells and the upregulation of adhesion molecules on the leukocyte. Numerous reports have shown CD43-mediated activation of intracellular signaling pathways. In neutrophils, CD43 crosslinking can induce respiratory burst initiation, integrin activation and locomotion (76, 83, 96, 233, 234, 238, 247, 248). CD43 may therefore aid in the proper activation of leukocytes, which is essential for their movement out of the vasculature (232).

Finally, CD43 may decrease adhesive forces between the adherent cell and the endothelium, or perhaps between emigrating leukocytes and leukocytes still in the blood, thereby facilitating transmigration (76, 234).

This thesis and other studies show that neutrophil recruitment is associated with CD43 downregulation and that complete absence of CD43 leads to a strong increase in adhesive interactions *in vivo*. These characteristics potentially make CD43 an interesting target for anti-inflammatory therapies. It is possible that if CD43 shedding were prevented or minimized, adhesive interactions could be reduced.

As discussed in the background section, CD43 shedding appears to be mainly mediated by serine proteases (chymotrypsin, elastase and trypsin). Serpins (serine protease inhibitors) are naturally occurring inhibitors of these enzymes. An *in vitro* study has

shown that serpins are effective in preventing neutrophil CD43 shedding but do not affect L-selectin, CD18 or CD29 expression. Serpins significantly inhibited neutrophil adhesion to ECM components, most notably fibronectin (241).

More specific inhibitors of CD43 shedding are feasible. Two proteolytic cleavage sites have been identified on the EC domain of CD43 (246). Theoretically, specific antibodies could protect such sites. Besides difficulties in developing such antibodies, a potential obstacle could be the abundance of CD43 on leukocytes, which may create the need for high dosages to provide sufficient coverage.

It is also important to consider that although *in vitro* studies reported as much as 80% CD43 shedding in some cases (61), the extent of CD43 shedding in clinical studies and measured by us in the murine system is relatively small (14, 84). This raises obvious questions on the therapeutic effectiveness of preventing CD43 shedding. If a total reduction of CD43 expression of 15 % is sufficient for recruitment to occur, inhibition of shedding would need to be highly effective to prevent this. This is likely to be very difficult to achieve clinically.

Lastly, and potentially the most promising approach, is the blocking of sites that are crucial to the ability of CD43 to promote leukocyte recruitment under inflammatory conditions. As discussed in the Background, numerous studies have demonstrated that CD43 crosslinking may induce IC signaling, resulting in leukocyte activation and adhesion, possibly through regulation of integrins (76, 247, 248). There is *in vivo* evidence to suggest that certain antibodies may prevent leukocyte adhesion and

recruitment by interfering with such processes. An anti-CD43 mAb showed the capacity to prevent murine neutrophil and monocyte recruitment (238). The mechanism responsible for this did not appear to involve the inhibition of shedding since the same antibody also reduced *lymphocyte* extravasation in non-obese diabetic mice, preventing disease. The authors suggest this mAb may prevent CD43-mediated signaling (43). It remains unclear however what physiological mediator(s) initiate(s) CD43 signaling.

Although valuable data has been generated on CD43 since its initial characterization over two decades ago, many questions remain. The molecular tools now available to us may answer many of these. Possible approaches include the generation of cells expressing only the IC or the EC domain of CD43 or truncations thereof. This would permit experiments addressing the differential roles of the EC and IC domain in recruitment, if these domains affect each other and whether CD43-mediated signaling is dependent on external signals (for example by a ligand) or that it is generated by intracellular mediators (for example due to cell activation through other receptors).

Investigation into these aspects of CD43 function could provide further insights into the regulation of leukocyte activation and recruitment and may ultimately provide important clues for additional therapeutic approaches aimed at modulating the intensity of the inflammatory response.

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