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A impressão desta dissertação foi aprovada pelo Conselho Cientifico da Faculdade de Medicina de Lisboa em reunião de 18 de Dezembro de 2012.

Resumo

Os neutrófilos estão entre os primeiros leucócitos a migrar da corrente sanguínea para o local da inflamação. Um vez no local, estes possuem a capacidade de reconhecer e englobar os agentes causadores de infecções desenvolvendo estratégias anti-microbianas de forma a conseguir desativar e destruir esses mesmos agentes. Assim sendo, é importante salientar que um recrutamento de neutrófilos funcional e eficiente é fundamental para o sucesso da resposta inflamatória e para recuperação da saúde do hospedeiro.

Em situações inflamatórias, o recrutamento dos neutrófilos é feito a partir do sangue e através do endotélio vascular para os tecidos intersticiais, este recrutamento consiste num processo com várias etapas que requer interações sucessivas do neutrófilos com as células endoteliais. Entre as várias moléculas de adesão celular envolvidas neste processo, é a integrina-β2, Mac-1 (CD11b/CD18), que permite que neutrófilos se liguem á ICAM-1 endotelial, passo essencial para a adesão firme dos leucócitos e o seu consequente alongamento. Além disso, foi também demonstrado que o fibrinogénio, assim como a fibrina, são ligandos de alta afinidade para esta mesma integrina, para além de outros alvos moleculares.

O fibrinogénio é uma glicoproteina solúvel que está presente no plasma, sendo um dos elementos determinantes para o fluxo e viscosidade sanguínea, desempenhando ainda uma função fundamental na coagulação. Para além desta funções sobejamente conhecidas foi proposto por vários autores que fibrinogénio tem também uma função relevante no processo inflamatório tendo em conta a sua capacidade em modular a função do neutrófilo potenciando a sua desgranulação, fagocitose e atrasando a apoptose dos neutrófilos ativados. Uma das possíveis explicações para estes efeitos está diretamente relacionada com a ligação do fibrinogénio á Mac-1 quando esta se encontra em baixa afinidade, o fibrinogénio pode não só pre-ativar os neutrófilos mas

também facilitar a sua ativação. Tendo tudo isto em consideração, o objetivo deste trabalho consiste investigar se o fibrinogénio solúvel que se encontra em circulação pode ou não modular o recrutamento dos neutrófilos e desta forma modular a resposta inflamatória microvascular.

Na primeira abordagem a esta pergunta começou-se por investigar se o fibrinogénio solúvel ao ligar-se á Mac-1, poderia facilitar a interação entre as células endoteliais e os neutrófilos facilitando a sua adesão. Começou-se por tentar perceber se o fibrinogénio solúvel pode modular a ativação dos neutrófilos, tendo como medida de ativação a produção de radicais livres de oxigénio (OFR). Recorrendo á citometria de fluxo e á incubação dos neutrófilos com diidrorodamina 123, conseguiu-se provar que o fibrinogénio só por si induz a produção de OFR pelos neutrófilos isolados a partir de sangue periférico humano, ativando-os. Para além disso, demonstrou-se ainda que este efeito não é dependente da ligação do fibrinogénio á Mac-1, uma vez que, mesmo quando foi utilizado um anticorpo bloqueador da Mac-1 a produção de OFR não foi afectada. Para além disso, foi também verificado por microscopia confocal que não existe uma colocalização entre a marcação por Mac-1 e pelo fibrinogénio, o que sugere um receptor ainda não identificado para o fibrinogénio, na membrana do neutrófilo.

Seguidamente, utilizando uma abordagem in vitro, investigou-se se o fibrinogénio conseguia modular o efeito adesivo dos neutrófilos em circulação. Para tal recorreu-se ao uso de uma camara de fluxo laminar que permitiu a visualização do processo de adesão com “shear-stress constante. Os neutrófilos isolados a partir de sangue periférico humano foram colocados sobre uma monocamada de HUVECs com um fluxo constante de 0,1 dynes/cm2 na presença e na ausência de fibrinogénio solúvel. O comportamento adesivo dos neutrófilos foi avaliado contando o numero de neutrófilos em rolamento e aderidos e determinando a sua velocidade de rolamento. A uma concentração fisiológica (300mg/dL) de fibrinogénio, o numero de neutrófilos em rolamento não foi afectado significativamente quando comparado com o numero

encontrado na ausência de fibrinogénio. Mas surpreendentemente as velocidades de rolamento foram significativamente superiores na presença do fibrinogénio. Determinou-se ainda que o numero de neutrófilos aderidos na presença de fibrinogénio foi mais pequeno do que na sua ausência. O que conduziu á conclusão que o solúvel fibrinogénio em concentrações fisiológicas podem influenciar a adesão dos neutrófilos circulantes à parede endotelial ativada. Este efeito assume especial importância em situações não-inflamatórias, prevenindo a adesão excessiva de neutrófilos á parede vascular e também facilitando que células aderentes facilmente se libertem. Pode assim constituir um mecanismo importante para prevenir a acumulação não desejada de neutrófilos na vasculatura e impedir a formação e crescimento de trombos vasculares.

Estes resultados conduziram a que se propusesse que o fibrinogénio pode influenciar o recrutamento dos leucócitos in vivo, e particularmente, ter um importante impacto na normal progressão de uma inflamação aguda. De forma a explorar esta hipótese, o efeito do fibrinogénio solúvel no recrutamento dos leucócitos durante uma inflamação aguda, foi utilizada uma abordagem in vivo, recorrendo á microscopia intravital das vénulas pós-capilares do mesentério do murganho. Esta abordagem permitiu visualizar diretamente e contar o numero de leucócitos aderidos e em rolamento assim como determinar as sua velocidades de rolamento. O modelo animal utilizado nesta abordagem foi um murganho geneticamente alterado, sem a cadeia α do fibrinogénio o que faz com que os indivíduos homozigoticos não possuam fibrinogénio em circulação. Como grupo controlo foram utilizados murganhos “wild-type” e heterozigoticos que possuem um padrão de coagulação normal.

De forma a recriar uma situação de inflamação aguda, perfundiu-se o mesentério com PAF, um ativador de neutrófilos bem descrito na literatura. Nesta condições, foi encontrado um numero ligeiramente reduzido de leucócitos em rolamento nos murganhos homozigoticos(α-/-) quando comparado com o numero encontrado tanto

nos murganhos heterozigoticos como nos “wild-type”. No entanto, a velocidade de rolamento é superior nos murganhos homozigoticos(α-/-) quando comparada com os mesmos grupos controlo. Consistentemente com este resultado, também o numero de leucócitos aderentes foi inferior neste grupo quando comparado com o mesmo grupo controlo.

Estes resultados permitiram concluir que o recrutamento leucocitário ficou comprometido na ausência do fibrinogénio solúvel em circulação.

De uma forma global, este trabalho permitiu concluir que o fibrinogénio solúvel pode modular a ativação dos neutrófilos e ainda constituir um factor importante para o recrutamento leucocitário durante uma situação de inflamação aguda.

O fibrinogénio solúvel tem a capacidade de se ligar não só ao neutrófilo mas também ás células endoteliais, podendo assim servir de ponte de ligação entre estas duas identidades celulares e desta forma favorecer um recrutamento leucocitário mais eficaz e mais estável.

Estes resultados tem implicações importantes no processo inflamatório. Sendo os neutrófilos células determinantes neste processo, precisam de conseguir atravessar rapidamente a parede vascular e chegar ao local onde ocorre a inflamação. Se este processo se encontra comprometido devido a uma concentração reduzida ou mesmo á ausência de fibrinogénio solúvel em circulação, a resposta imunitária fica comprometida e suscetível á invasão por agentes patogénicos. O que compromete a saúde do organismo aumentando o risco de um possível choque séptico entre outras consequências patológicas.

Abstract

Neutrophils are among the first leukocytes to migrate from the blood to sites of inflammation. Here, they are able to recognize and engulf inflammatory agents, such as infectious agents and activate various anti-microbial strategies to inactivate and destroy them. Importantly, an efficient neutrophil recruitment and function will be pivotal for the success of the inflammatory response in recovering the host's health.

In inflammatory conditions, neutrophil recruitment from the bloodstream through the vascular endothelium into interstitial tissues requires successive interactions of neutrophils with the endothelial cells throughout this multistep process. Among diverse cellular adhesion molecules involved is the β2-integrin Mac-1 (CD11b/CD18), that by binding the endothelial ICAM-1 is specifically required during firm adhesion and leukocyte crawling. Additionally, this integrin has been shown to bind fibrinogen with a strong affinity as well as to its clotting product fibrin, among other molecular targets.

Fibrinogen is a soluble glycoprotein present in the plasma that is importantly required for coagulation and the major determinant of blood viscosity and flow. It has been also proposed as a relevant player in inflammation in view of its ability to modulate the neutrophil function by potentiating degranulation, phagocytosis and antibody-dependent cellular cytotoxicity and by providing survival cues that delay apoptosis of activated neutrophils. One possible explanation for these effects could be that by binding Mac-1 in a low-avidity state, fibrinogen could function as a primer for resting neutrophils and thus decrease the threshold for cell activation. Taken all this, our goal in the present work was to address whether circulating fibrinogen could also modulate neutrophil recruitment and thus understand its role in the mechanism of microvascular inflammatory response.

In this respect, we hypothesized that upon binding Mac-1, plasma fibrinogen could potentiate neutrophil-endothelial cell interaction by priming the neutrophil to a more

adhesive phenotype. To test this, we first investigated whether soluble fibrinogen could modulate neutrophil activation, more precisely concerning the production of oxygen free radicals (OFR). By combining flow cytometry with dihydrorhodamine 123, we showed that by itself, soluble fibrinogen could induce OFR production by neutrophils isolated from human peripheral blood, arguing it could modulate neutrophil activation. Furthermore, we demonstrated this effect not to be Mac-1 dependent as: (i) fibrinogen did not interfere with the neutrophil Mac-1 activation and (2) an anti-total Mac-1 antibody known to block this integrin function did not impair the induced OFR production. Finally, binding of fibrinogen to the neutrophil's membrane was observed independently of its co-localization with Mac-1 staining, arguing for the fibrinogen interaction with another unidentified neutrophil membrane receptor.

Next, we addressed *in vitro* whether fibrinogen could modulate the adhesive behaviour of circulating neutrophils. For such, we used flow chamber assays that allowed the visualization and study of cell adhesion processes under well-defined wall shear stress. Human neutrophils were flown over monolayers of HUVECs at a constant wall shear stress of 0,1 dynes/cm^2 in the presence or absence of soluble fibrinogen. Neutrophil's adhesive behaviour was evaluated by determining the numbers of rolling and adherent neutrophils and the velocity of rolling. Under a physiological concentration (300mg/dL) of fibrinogen, the number of rolling neutrophils was not significantly affected in comparison to that measured in its absence. Surprisingly, the rolling velocities measured for the first were significantly higher than those exhibited by the latter. Additionally, the number of adherent fibrinogen-treated neutrophils was smaller than that determined for non-treated ones. This showed that at physiological concentrations, soluble fibrinogen could modulate the adhesive behaviour of circulating neutrophils towards activated endothelium. This effect may be important in a non-inflammatory condition by preventing excessive adhesion of neutrophils to the

vascular wall and enabling adherent cells to easily detach from it. It may constitute an important mechanism to prevent unwanted accumulation of neutrophils in the vasculature and to avoid thrombus formation and growth.

These results led us to propose that fibrinogen could further influence leukocyte recruitment *in vivo* and particularly, impact on the normal progression of acute inflammation. To explore this, the effect of soluble fibrinogen on leukocyte recruitment in acute inflammation was next evaluated *in vivo* by intravital microscopy of post-capillary venules of mouse mesentery. This approach allowed us to address specifically its contribution for leukocyte rolling and adhesion by determining the number of rolling leukocytes, their rolling velocities and the number of adherent leukocytes. To enable the *in vivo* study of leukocyte recruitment in the absence of soluble fibrinogen in the bloodstream, we made use of a mouse model bearing a disrupted fibrinogen α chain gene for which, homozygous individuals had been shown not to express any experimentally detected plasmatic soluble fibrinogen. As control groups, wild-type and heterozygous mice, known to possess normal coagulation patterns, were used.

Acute inflammation was mimicked by superfusing the mesentery with PAF, a well-known neutrophil activator. Under these conditions, a slightly reduced number of rolling leukocytes was observed in homozygous ($\alpha^{-/-}$) mice when compared to both control groups. Despite this, rolling leukocytes migrated with increased velocities in homozygous ($\alpha^{-/-}$) mice when compared to those from control animals. Consistently, homozygous mice further displayed a diminished number of adherent leukocytes than the other groups. Overall, these observations led us to conclude that leukocyte recruitment is compromised in the absence of soluble fibrinogen in the blood circulation.

Altogether our studies led us to conclude that soluble fibrinogen can modulate neutrophil activation and importantly, that it should constitute an important factor for

leukocyte recruitment in acute inflammation. As soluble fibrinogen is able to bind either to the neutrophil or to the endothelial cell, one could envisage that it could also be able to bridge these two cellular identities and possibly favour a more stable and effective leukocyte recruitment.

These results have profound implications in inflammation. As central players in the process, leukocytes need rapidly to cross the vascular wall and reach the inflamed areas. If this is affected due to absent or reduced levels of plasmatic soluble fibrinogen, ineffective immune responses will be elicited to invading pathogens. This will compromise the organism's health by enhancing the risk of sepsis development among other possible pathological consequences.

Agradecimentos

Em cada passo que dou aprendo!

Aprendo com o caminhar, com o caminho que percorro, com o meu estado de espírito durante a caminhada mas acima de tudo aprendo com os que me acompanham durante a caminhada.

Se começar um projecto cientifico é sinónimo de iniciar uma busca de conhecimento numa área especifica, para mim este projecto de doutoramento consistiu não só num aprofundar dos meus conhecimentos científicos mas essencialmente num profundo conhecimento de mim mesma.

Este percurso de alguns anos permitiu-me experimentar quase todas as fazes que definem o ser humano, os limites, os momentos altos e os baixos, a tristeza e a alegria, o sucesso e o insucesso , a frustração e o sentimento de tarefa cumprida, o principio e o fim e o saber que contribui para a mudança.

Os que me acompanharam , conseguem com certeza atribuir a cada um desses momentos um ou vários episódios e, é por isso, que mais uma vez escrevo que acima de tudo aprendi com os que comigo fizeram este percurso.

Agradeço em primeiro lugar a quem me permitiu viver esta experiência, Professora Carlota Saldanha, que como minha orientadora me aceitou nesta equipa e que soube ajudar-me ao ritmo da minha compreensão.

A todos os meus colegas: Ana, Sofia , Sónia, Henrique, Teresa Raquel e Cláudia que me ajudaram e aturaram, no sentido mais lato da palavra, durante todo este tempo. O que para aqueles me conhecem, é fácil de entender a dimensão hercúlea da tarefa...

Quero agradecer especialmente ao Ângelo, que me deu a mão quando eu mais precisei, e sem o qual a escrita desta tese era practicamente impossível.

A todos aqueles que cruzaram o meu caminho aqui no IMM e que por muitas vezes com uma pequena ajuda ou palavra fizeram toda a diferença nesse dia.

Agradeço aos meus pais, os meus modelos de vida, ao meu irmão, a minha melga assistente,

e ao meu marido, pelo meu passado, o meu presente e o meu futuro.

"Aqueles que passam por nós, não vão sós, não nos deixam sós; deixam um pouco de si, levam um pouco de nós".

Antoine de Saint-Exupery

Abbreviations

Acronym	
DAMPs	Damage-associated Molecular Patterns
EC	Endothelial Cell
ESL-1	E-selectin ligand 1
Fib	Fibrinogen
fMLP	formyl-Met-Leu-Phe
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balance salt solution
HP1	Heterochromatin Protein 1
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule-1
IL-1b	Interleukin-1b
i.p.	Intraperitoneal
LBR	Lamin B receptor
LF	Lactoferrin
LFA-1	Leukocyte Function-Associated Molecule 1 / $\alpha_L \beta_2$
LTB4	Leukotriene B4
HBP	Heparin Binding Protein
MAC-1	CD11b/CD18 / $\alpha_M \beta_2$

MPO	Myeloperoxidase
NETs	Neutrophils Extracellular Traps
PAF	Platelet Activating Factor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PMN	Neutrophils / Polymorphonuclear neutrophilic granulocytes
PRR	Pattern Recognition Receptors
PSGL-1	P-selectin ligand 1
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SPF	Specific Pathogen Free
STSS	Streptococcal Toxic Shock Syndrome
TLRs	Toll-Like Microbial Pattern Recognition Receptors
TNF-α	Tumor Necrosis Factor-alpha

Contents

Objectives

The main goal of this thesis was to understand if soluble fibrinogen was able to modulate neutrophil recruitment in the mechanism of microvascular inflammatory response.

This work was divided in three different goals:

- Understand if fibrinogen was able to modulate the pattern of neutrophil activation and thus contribute to neutrophil recruitment.
- Understand if soluble fibrinogen as able to modulate the neutrophil adhesive behavior, *in vitro*.
- Understand if fibrinogen modulates leukocyte recruitment *in vivo* during the acute inflammatory response.

PART ONE

Chapter I – Introduction

1. Inflammation

Inflammation is a protective tissue response towards infection or injury. It comprises a series of vascular and cellular events set into motion so as to destroy, dilute, or isolate the causative agent and further to reconstitute the damaged tissue and regain tissue homeostasis. An inflammatory process can be classified into two distinct patterns: acute and chronic inflammation.

The acute process constitutes a rapidly elicited response (seconds or minutes) with a relatively short duration, lasting from minutes to several hours or a few days. Its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils and monocytes, towards the affected tissues. On the other hand, chronic inflammation consists of a response of longer duration that normally arises when an acute inflammatory response is not completely turned off or extinguished. It acts like a slow-burning fire, continuing to stimulate pro-inflammatory immune cells when they may not be needed [1]. In the next sections, these two processes will be briefly outlined.

1.1. Acute inflammation

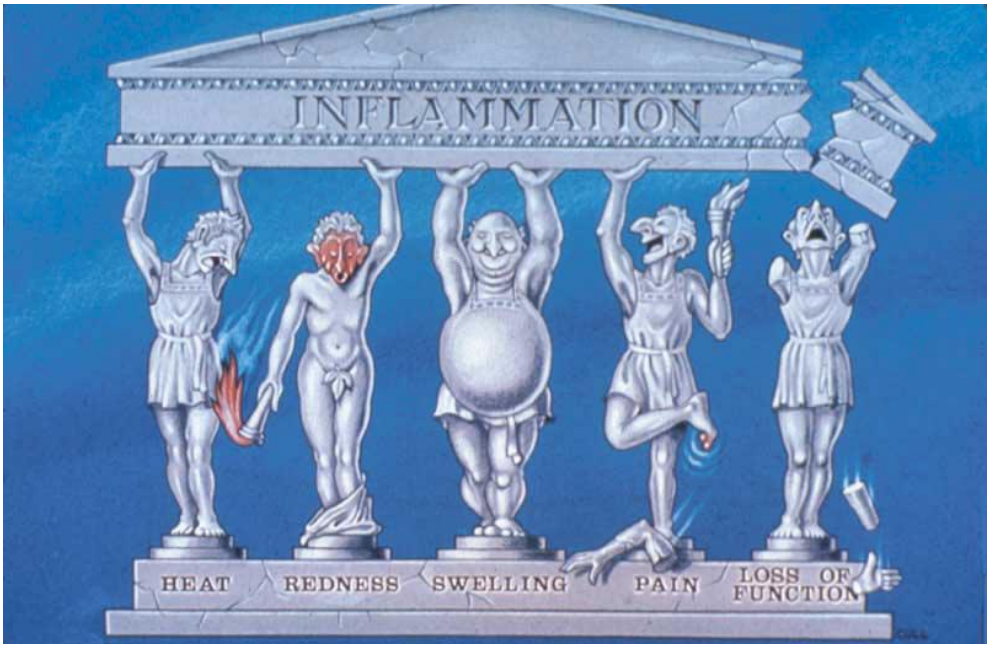

Fig.1: adapted from the [2]. Cardinal signs of inflammation. This cartoon depicts five Greeks representing the cardinal signs of inflammation — heat, redness, swelling, pain and loss of function — which are as appropriate today as they were when first described by Celsus more than 2000 years ago. This figure was commissioned by D.A.W. and drawn by P. Cull for the Medical Illustration Department at St Bartholomew's Medical College.

Anyone who has experienced injuries, knows from common observation that an injured site rapidly becomes inflamed presenting the four cardinal signs of inflammation that Celsus described in the first century. For example, a burn rapidly becomes red (*rubor*), hot (*calor*), swollen (*tumor*) and painful (*dolor*) [2].

Such acute inflammatory response is triggered by the recognition of the inflammatory agent by specialized cells, such as tissue resident macrophages or even by neighbouring host cells. In the specific case of an infectious process, this initial event involves more precisely the recognition of specific molecular patterns expressed by the

invading pathogens, collectively referred as Pathogen-associated molecular patterns (PAMPs) [3]. These are a diverse set of microbial molecules which share a number of different recognizable biochemical features (entire molecules or, more often, part of molecules or polymeric assemblages) that alert the organism for the presence of intruding pathogens. Such exogenous PAMPs are recognized by specific receptors, the pattern recognition receptors (PRRs), expressed in the diverse immune and non-immune cells. Among the diverse groups of PRRs so far identified, Toll-like receptors (TLRs) stand as the best-known and more extensively studied [4]. The recognition of PAMPs by specific PRRs further triggers an immunological response that comprehends: (i) initially an acute inflammatory process by which specific innate immune cells are activated so as to mediate the destruction or the confinement of the pathogen and/or of pathogen-infected cells, and (ii) that if required, is then followed by an adaptive immune response that more specifically will fight the infectious agent as well as trigger the generation of an immunological memory so as to more rapidly handle a future encounter with the pathogen [5]. Besides pathogens, trauma is the other main reason for tissue and cell damage that can elicit an acute inflammatory response. It is triggered by the recognition of intracellular proteins released by the dead cells, the damage-associated molecular patterns (DAMPs) also known as alarmins, by some of the previously referred PRRs [4].

In response to the detection of PAMPs and DAMPs, pro-inflammatory cues are expressed, such as the cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1b (IL-1b), which further signal for endothelium activation. Among other functions, activated endothelium controls the recruitment of specific leukocyte populations from the blood circulation towards the inflamed area. This leukocyte recruitment process normally takes place at a particular microcirculatory area, more precisely at the level of post-capillary venules. Although distinct leukocytes are recruited in response to different stimuli through the use of specific factors, these recruitment processes

generally comprise a cascade of events, which will be described in section 1.4 in further detail. Leukocyte recruitment is mediated by molecular interactions sequentially established between the leukocyte and the endothelium and involving cell surface proteins, such as cell adhesion molecules (like the selectins) and receptors (such as the integrins) as well as, small secreted chemotactic cytokines, the chemokines [6].

Importantly, acute inflammatory processes are classically characterized by a differential recruitment of leukocytes towards the inflamed area. Neutrophils dominate the initial influx to the inflamed area where once activated, they make use of a myriad of anti-microbial processes, such as phagocytosis, oxidative burst and the action of specific anti-microbial peptides or neutrophils-derived proteases, so as to eliminate the inflammatory agent. Some of these processes are not solely specific for these agents but can also damage surrounding host tissues. Ultimately, neutrophil activation leads to their apoptosis [7].

At later stages of acute inflammation, monocyte extravasation is favoured. Once in the inflamed area, monocytes differentiate into macrophages with either: (i) a pro-inflammatory functional profile, termed as inflammatory dendritic cells or classically-activated macrophages or (ii) an anti-inflammatory and pro-resolving function, the alternatively-activated macrophages that ensure the clearance of apoptotic neutrophils and of cellular debris and that further mediate resolution of the inflammatory process and the subsequent tissue regeneration. As apoptotic cell clearance is complete, most macrophages are believed to leave the inflamed area by lymphatic drainage. Macrophages can also function as antigen-presenting cells and thus bridge innate and acquired immunity responses [8] [9].

1.2. Chronic Inflammation

Chronic inflammation is commonly described as an inflammation of prolonged duration in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously. Although it may follow acute inflammation, chronic inflammation frequently begins insidiously, as a low-grade, often asymptomatic response. Distinct pathological conditions can lead to this scenario, such as, persistent infection by low toxicity organisms, prolonged exposure to potentially toxic agents or autoimmune diseases [8].

Histologically, it is characterized by: (i) the infiltration with mononuclear cells such as macrophages and lymphocytes, a reflection of persistent reaction to injury; (ii) tissue destruction and necrosis, largely induced by the inflammatory cells and (iii) in attempting to heal the inflamed area, by the replacement of the damaged tissue by connective tissue which often requires the proliferation of small blood vessels (angiogenesis). As an example of this later histological marker, the original tissue can be replaced particularly by fibrotic tissue and thus evolve into fibrosis [1].

1.3. Neutrophils

Neutrophils (PMNs) constitute the majority of the blood leukocytes circulating in the bloodstream: there are about $4x10^6$ neutrophils per millilitre of blood under normal healthy conditions. In view of their limited lifespan of only 48-72h in circulation, the maintenance of these neutrophil counts requires the daily release of about 10^{11} PMNs from a healthy adult bone marrow. These leukocytes play a significant role in the innate immune response as they are responsible for eliminating invading pathogens through a process requiring sequentially neutrophil recruitment, chemotaxis and the

engulfment and destruction of the offending microbe [10]. These several topics will be briefly reviewed in subsequent sections.

1.3.1. Morphological aspects

The neutrophil has a diameter of about 10-12 µm and typically, possesses a multi-lobed nucleus. Usually 3-4 lobes attached to each other by very fine filaments can be observed [10]. In its cytoplasm, this leukocyte importantly contains four granule compartments that are normally classified into: (i) primary (or azurophilic) granules, (ii) secondary (or specific) granules, (iii) tertiary (or gelatinase) granules and (iv) secretory vesicles. These granules are defined by their high content in specific protein factors, namely and respectively, myeloperoxidase (MPO), lactoferrin (LF), gelatinase, and latent alkaline phosphatase. Importantly, this classification does not only reflect differences in their protein content but as well, differences in their mobilization with the later granules to be formed being the first to be released. As outlined in the following section, these cytoplasmic compartments are formed sequentially during granulocytic differentiation in the bone marrow [11] [10].

1.3.2. Neutrophil differentiation

Much of the activity of the bone marrow is dedicated to the production of neutrophils, where approximately two-thirds of the blood-cell-forming activity (hematopoiesis) is devoted to myelopoiesis, the term used for the collective production of monocytes and granulocytes. The first step in myeloid differentiation is the commitment of the pluripotent stem cell to become a multipotent stem cell. Afterwards, the first morphologically identified myeloid cell is the myeloblast, which is sequentially differentiated into four morphologically different maturation forms before originating

the mature neutrophil. Namely, these forms are in the sequence of their appearance: the promyelocyte, the myelocyte, the metamyelocyte and the band.

The myeloblast is a very large cell of up to 20 μm in size that is mostly occupied by its nucleus (more than 85% of the cell size) which presents very smooth chromatin and usually possesses 1 to 3 nucleoli. Its cytoplasm does not present granules or vacuoles of any type. In the next differentiation step, the promyelocyte presents the first cytoplasmic granules, the non-specific primary granules, known as well as the azurophilic granules.

In the myelocyte stage, cells cover a wider range of sizes (16-24 μ). The nucleus of the myelocyte contains no nucleoli and although usually round, it can also adopt an oval shape and present slightly condensed chromatin. Its cytoplasm presents secondary granules (also known as specific granules) that contain alkaline phosphatase, hydrolases, lysozymes, and other proteins. The myelocyte is the last stage capable of mitosis, i.e., cells in the following stages cannot proliferate but only mature (differentiate). In the metamyelocyte, the nucleus takes a kidney shape and in the band, the nucleus further starts to lobulate despite the fact that the filaments that will attach the different lobes, have not yet developed.

1.4. Neutrophil recruitment

The recruitment of neutrophils from the blood is a central event in the innate immune responses to invading pathogens and tissue damage. In fact, before reaching the inflamed tissues, these leukocytes need to cross the vascular wall in a presently well studied process.

As referred above, neutrophil recruitment takes place largely at postcapillary venules. Here, the vessel wall is rather thin and the diameter of the vessel is adequate to sustain

the recruitment process. In fact, the diameter of these venules is sufficiently small so as to enable neutrophils to establish contact with the vessel wall. On the other hand, it is also sufficiently large not to be occluded by the circulating neutrophils when arresting and making firm contact with the endothelium. The slow blood flow associated with the presence of erythrocytes in greater numbers promotes neutrophil margination. This can be stimulated under conditions of blood hyperviscosity or by hemodynamic changes or pro-inflammatory stimuli.

Overall, this recruitment process comprises a series of sequential steps, namely "tethering", rolling, slow rolling, arrest, post-adhesion strengthening, crawling, and paracellular or transcellular transmigration. In the following sections, the most relevant steps of this process will be herein briefly presented.

1.4.1. Neutrophil rolling

The initial attachment of neutrophils to the vascular wall is determined by the activation state of the endothelial cells that compose this tissue. Endothelial cell activation is elicited in response to their exposure to several pro-inflammatory stimuli such as, TNF-α, IL-1β and IL-17 that are generated upon initiation of the inflammatory processes. Such stimulation results in the expression on their luminal surface of specific cell adhesion molecules of relevance in the leukocyte recruitment process. Among these, are selectins, such as, P-selectin and E-selectin as well as, members of the Immunoglobulin superfamily, such as ICAMs (intercellular adhesion molecules) and VCAMs (vascular cell adhesion molecules).

The endothelial selectins bind specific factors expressed on the neutrophil membrane, namely PSGL-1 (P-selectin ligand 1) and L-selectin. The binding of PSGL-1 to P-selectin and E-selectin mediates the initial contact between the circulating neutrophils and the

activated endothelial cells. E-selectin and E-selectin ligand 1 (ESL-1) interaction further mediates a slower rolling of the neutrophil along the endothelium, while the binding of E-selectin to CD44 induces a redistribution of PSGL-1 and L-selectin on the leukocyte membrane that typically promotes their clustering and is further associated with a subsequent reduction in the speed of leukocyte rolling [12].

1.4.2. Neutrophil adhesion

Neutrophil will roll along the venular endothelium until they finally arrest. In order to resist detachment by disruptive shear flow, neutrophils must subsequently strengthen their adhesion [13]. The key molecules mediating the termination of rolling and the firm adhesion of neutrophils to the activated endothelium are the β2-integrins, leukocyte function-associated molecule 1 (LFA-1 , αLβ2) and Mac-1 (αMβ2, CD11b/CD18) present on neutrophils, and their ligands, members of the immunoglobulin superfamily, ICAM-1 and ICAM-2, expressed on the endothelial cell surface. The regulation of cell adhesion by the β2-integrins is crucial for neutrophil recruitment. In fact, LFA-1 mediates the termination of the rolling of the neutrophil along the endothelium and in conjunction with Mac-1, the subsequent firm adhesion of this leukocyte to endothelial cells.

1.4.3. Transendothelial migration

Once firm adhesion is established, two roads can be taken for transendothelial migration: (i) the transcellular road, whereby neutrophils penetrate the individual endothelial cell, or (ii) the paracellular road, whereby neutrophils squeeze in between endothelial cells. The key players involved in guidance toward paracellular or

transcellular migration are again the major neutrophil β2 integrins LFA-1 and Mac-1 and their ligands ICAM-1 and ICAM-2.

1.4.4. Integrins

Integrins are a large family of heterodimeric transmembrane glycoproteins that are normally enrolled in processes involving cellular adhesion. For such, these receptors interact with specific extracellular matrix proteins of the basement membrane or with specific ligands expressed on the surface of other cells. They contain large (α) and small (β) subunits of sizes of 120-170 kDa and 90-100 kDa, respectively. Integrins contain binding sites for divalent cations Mg^{2+} and Ca^{2+}, which are necessary for their adhesive function. Mammalian integrins can be grouped into several subfamilies, such that members of a given subfamily share a common β subunit and can associate with different α subunits.

Among these subfamilies stand the β2-integrins which are exclusively expressed on leukocytes and that undergo a conformational change encompassing the phosphorylation of the β subunit upon their activation. This leukocyte-specific integrins are required for neutrophil adhesion and transmigration across the activated endothelium. Concerning their interaction with their ligands, these integrins can adopt three distinct states of activation: (i) a bent form incapable of ligand binding, (ii) an extended, but not fully opened form with intermediate ligand binding capacity and (iii) an extended and opened form with full avidity for ligand binding.

Circulating neutrophils maintain their integrins in a low affinity and non-adhesive state. In response to local inflammatory stimuli, these protein factors are rapidly activated to bind ligands in a process known as “inside-out” activation. Neutrophil integrin affinity

and/or avidity may be stimulated by endogenous and bacterial chemotactic factors, including interleukin 8 (IL-8), leukotriene B4 (LTB4), formyl-Met-Leu-Phe (fMLP) and anaphylatoxin C5a. In the vasculature, endothelial-derived chemokines (e.g. CXCL8) may stimulate the neutrophil to arrest and transmigrate, and tissue-derived chemotactic factors (e.g. bacterial-released fMLP or C5a) induce the neutrophils to migrate through the extracellular matrix toward the source of infection. The N-formyl peptide receptor on human neutrophils binds to products of bacterial protein synthesis (N-formyl peptides), resulting in G protein activation and the subsequent G protein-dependent cellular responses including, chemotaxis and as well as, oxidative burst [14].

Among neutrophil β2-integrins stands Mac-1 that is importantly involved in the neutrophil recruitment as presented above. This integrin is normally stored in specific granules which are shuttled to the granulocyte surface. Under normal conditions, the unstimulated Mac-1 remains in a resting, non-adhesive state. This integrin constitutes a chemoattractant activation-dependent receptor that undergoes a conformational change upon stimulation that is known to expose new epitopes on its surface, which can be detected by specific reporter monoclonal antibodies. Via its interaction with endothelial ICAM-1, active Mac-1 mediates neutrophil adhesion to and transmigration across the endothelium [15].

The interaction of neutrophil integrins with extracellular matrix proteins does not only allow cell adhesion but also initiates signal transduction cascades that influence chemotaxis, cell proliferation, survival as well as the change of nuclear shape [16]. In what concerns this later change, it is believed that a malleable interphase neutrophil nucleus, as evidenced by its unusually lobulated or annulated shape (lobulate in humans; annulate in rodents), is crucial for the activated neutrophil to squeeze through endothelial walls and migrate through tight tissue spaces. Among other components of the nuclear envelope, adequate levels of the lamin B receptor (LBR) are critically necessary for the differentiation of the lobulated nucleus as well as for

determining the nuclear shape and peripheral heterochromatin distribution within mature blood granulocytes [17] [18].

1.5. Neutrophil function in inflammation

Having reached the site of inflammation, the activated neutrophil makes use of an array of anti-microbial mechanisms so as to achieve its fundamental goal: the local elimination of the inflammatory agent (Fig.2). Among diverse mechanisms, the neutrophils kill pathogens locally by disposing of the contents of their intracellular granules and through more complex mechanisms, such as phagocytosis, oxidative burst and NETosis. These latter mechanisms will be briefly outlined in subsequent sections.

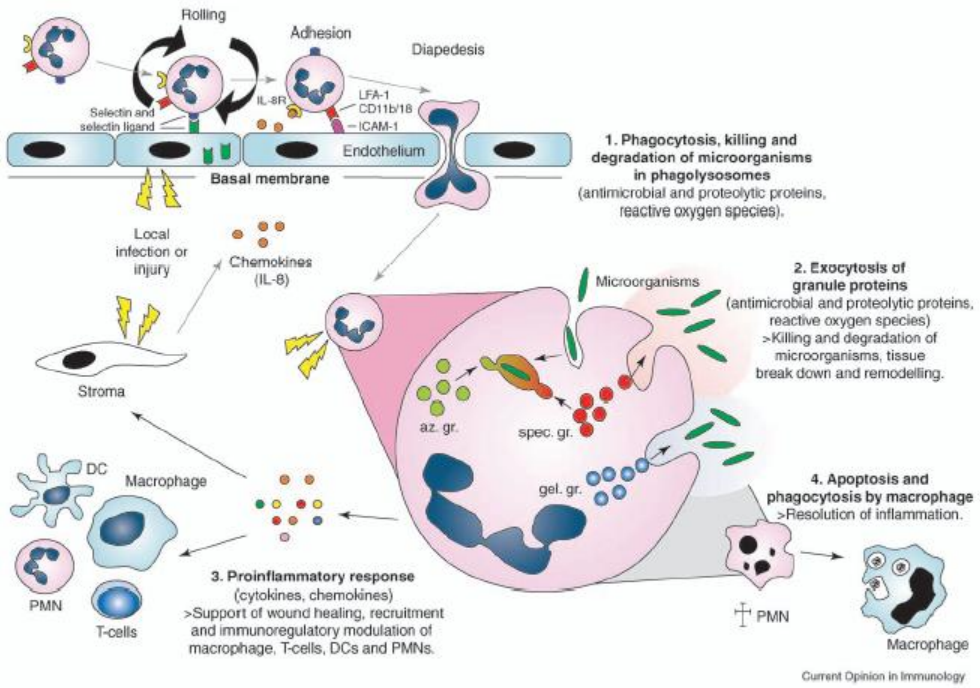

Fig.2: The life of the neutrophil during the inflammatory process. In response to local infection or injury, neutrophils attach to the endothelium via a series of interactions

among adhesion molecules and their corresponding receptors. Attachment in combination with locally secreted chemokines direct neutrophil migration to the site of infection or injury, where they become activated and execute a cascade of defence mechanisms. Finally, neutrophils commit apoptosis, resulting in phagocytosis by macrophages and subsequent resolution of inflammation.This figure has been adapted from reference [11].

1.5.1. Phagocytosis

Along with macrophages, neutrophils are professional phagocytes and are thus endowed with a unique capacity to engulf and thereby eliminate pathogens and cell debris, termed as phagocytosis. For such, phagocytes are equipped with specialized receptors to recognize their targets. Upon their recognition, a complex machinery mediates its internalization and initiates an assortment of degradative mechanisms that culminate in killing and disposal of the engulfed particles (Fig. 2). Neutrophils can internalize both opsonized and non-opsonized particles. The principal opsonin receptors of neutrophils, the Fcγ receptors and a subgroup of β2-integrins, bind to immunoglobulin or complement-coated particles, respectively. The main Fcγ receptors present in human resting neutrophils are FcγRIIA (CD32) and FcγRIIIb (CD16), while the high-affinity FcγRI (CD64) functions predominantly after neutrophils have been primed with interferon. On the other hand, particles coated with complement fragment C3bi are specifically recognized by the activated β2 integrin Mac-1.

Complement-opsonized particles are internalized by gently “sinking” into the cell, whereas the usage of Fcγ receptors initiates a vigorous extension of pseudopods that surround and ultimately, entrap the particle. As a result of this process, the particle is engulfed in a vacuole, specifically named as the phagosome, whose initial composition is not antimicrobial. This phagossome undergoes then a dynamic maturation process that involves multiple fusion events with components of the endocytic pathway, as

well as removal of components by vesicular fission. This process results in radical changes in the phagosomal contents and in its membrane composition, including the acquisition of microbicidal enzymes, vacuolar ATPases and the NADPH oxidase complex [19]. Importantly, these novel components provide the armoury required for the matured compartment to kill pathogens and dispose of debris (Fig. 2). For instance, phagocytosis ultimately results in the activation of the oxidative burst and subsequently, in the generation of reactive oxygen species (ROS). Among other anti-microbial functions thus activated, the action of microbicidal enzymes derived from cytoplasmic granules is as well of notice in this killing activity.

1.5.2. Oxidative burst

Oxidative burst comprises an oxygen-dependent antimicrobial process that is essential for bacterial killing. It involves the generation of reactive oxygen species (ROS) that are thought to function by oxidizing and thus damaging important bacterial components such as proteins, nucleic acids and other molecules. (Fig.3) This killing mechanism starts with the assembly of a multimeric NADPH oxidase enzyme complex at the phagosomal membrane. By oxidizing NADPH, this complex transfers two electrons to O_2 so as to generate superoxide (O_2^-). This oxygen radical further dismutes into hydrogen peroxide (H_2O_2), either spontaneously or by a superoxide dismutase (SOD)-mediated process. In turn, H_2O_2 is then converted by myeloperoxidase (MPO) into hypochlorite (HOCl) that is further enrolled in the generation of secondary ROS.

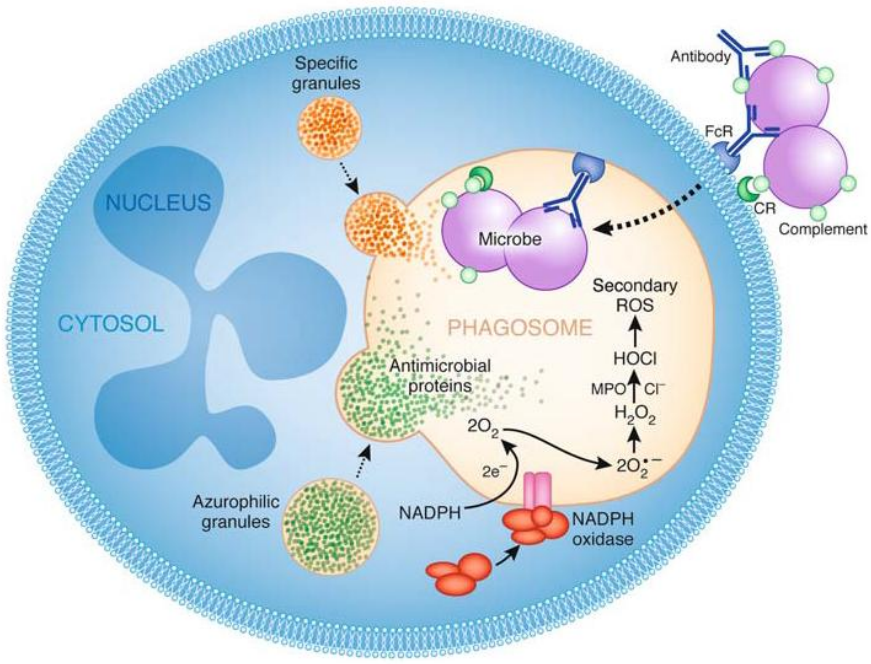

Fig.3 Neutrophil phagocytosis and microbicidal activity. Following phagocytosis, microbes are destroyed by ROS and antimicrobial proteins released from granules. This figure has been adapted from reference [7].

1.5.3. Terminating their function: apoptosis and clearance

Activated neutrophils are terminally differentiated cells that have a circulating half-life of around 7h, upon which undergo constitutive apoptosis.

In inflammation, the net accumulation of neutrophils in the inflamed tissues results from a balance between the rate of their recruitment and that of their removal. After performing their function at the site of inflammation, neutrophils undergo as well apoptosis. As discussed above, these apoptotic neutrophils are subsequently phagocytosed by resident macrophages, providing the means to resolve the

inflammatory response without releasing cytotoxic molecules that would otherwise damage host tissues [7] [20].

Neutrophil apoptosis can be initiated by stimuli of: (i) either the extrinsic pathway, such as TNF-alpha, or (ii) the intrinsic pathway, like ROS. Regardless of the inducing pathway, the apoptotic process is characterized by loss of cytoplasmic granules, rounding of the nucleus and condensation of nuclear heterochromatin. Besides these mechanisms, the neutrophils can also undergo apoptosis after phagocytosis by a process known as phagocytosis-induced cell death.

In addition, an alternative form of neutrophil death has been described more recently and termed as NETosis. In the next section, we will be briefly outlining this particular mechanism.

1.5.4. NETosis

NETosis consists on the generation of neutrophils extracellular traps (NETs) upon explosion of the neutrophil. This mechanism is not only a way for neutrophils to die but most importantly, it provides the organism with an additional tool to fight pathogens. NETs interact with a variety of different pathogens. They capture both Gram-positive (*Staphylococcus aureus, Streptococcus pneumoniae* and Group A streptococci [GAS]) and Gram-negative bacteria (*Salmonella enterica* serovar Typhimurium and *Shigella flexneri*) as well as fungi (*Candida albicans*). By providing a high local concentration of antimicrobial proteins, NETs could disarm and kill bacteria.

NETs are complex extracellular structures composed of threads of approximately 15nm in diameter, which are likely to constitute remnant chains of nucleosomes from unfolded chromatin associated with specific proteins from attached neutrophilic granules [21].

NETosis takes advantage of the unique properties of the neutrophil nuclear envelope as briefly described above (section 1.4.4). This process comprises three major steps which are here briefly described. A first step takes place in the post-mitotic granulopoietic phase, when lamins decline in amount while LBR increases in cellular content. As a consequence, the more malleable nuclear envelope is tied tightly to the underlying peripheral heterochromatin, while the nuclear envelope is distorted by forces associated with the microtubules (MT)-centrosomal system. In the next step, LBR decreases in amount and lamins, other nuclear integral membrane proteins and Heterochromatin Protein 1 (HP1) continue to disappear. The peripheral heterochromatin, stabilized by modifications in the chromatin and particularly in associated histone proteins maintains the fragile lobulated nuclear shape. The third and final step occurs in the mature granulocyte. NET formation is thought to occur when the structural constraints on chromatin are relaxed, leading to a sudden explosion of the chromatin through the fragile nuclear envelope [18].

1.5.5. Neutrophil transdifferentiation

During inflammation, neutrophils leave the circulation and enter new habitats, the inflamed tissues in which they are exposed for extended periods to multiple factors such as cytokines, endogenous growth factors, bacterial products as well as multiple other local products.

According to the traditional definition view on the blood circulating neutrophil, its lifespan is measured in hours. In clear contradiction, several observations now suggest that neutrophil lifespan can be considerably prolonged in response to stimuli such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-a (TNF-a), interleukins (ILs), interferons and bacterial products. In certain inflammatory conditions, it has been further shown that exposure to several of these cytokines,

induces a change in postmitotic neutrophils towards a resident neutrophil phenotype with different and novel features, among which some ressemble those of macrophages. In fact, these long-lived neutrophils were described to exhibit a reduced migration to specific neutrophil chemotactic stimuli, to possess an increased phagocytic capacity and to release a reduced amount of destructive enzymes [22] [23].

2. Fibrinogen

2.1. Molecular details on the fibrinogen

Human fibrinogen is a circulating 340 kDa glycoprotein, primarily synthesised by hepatocytes. It is comprised of two symmetric half molecules, each consisting of one set of three different polypeptide chains termed α, β and γ. This protein is highly heterogenous due to alternative splicing, extensive post-translational modifications as well as proteolytic degradation and given the naturally-observed polymorphic variation within the α and β chains loci (Fig.4). Taken this, it has been suggested that each individual may actually carry over one million of nonidentical fibrinogen molecules in their blood.

Each of the three polypeptide chains of the fibrinogen molecule is encoded by a separate gene located on chromosome four. The α and γ fibrinogen chain genes are orientated in tandem and are transcribed in the opposite direction to the β fibrinogen chain gene. Alternative splicing may occur in the α and γ fibrinogen chain leading to the production of peptide variants. The predominant α chain of circulating fibrinogen contains around 610 amino acid residues (70 kDa) whereas the β chain consists of 461 amino acids residues (56 kDa). The γ chain is rather heterogenous with respect to both charge and size. However, the most abundant form, denoted as γ_{-} or γ_{A} , consists of 411 residues (48kDa).

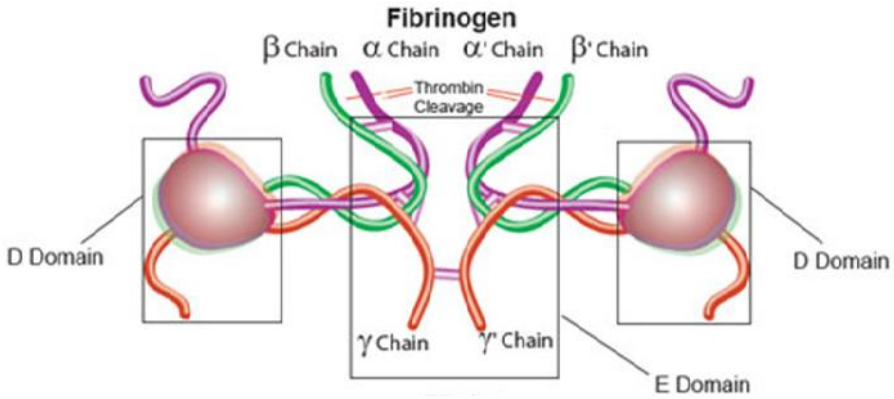

Fig.4: Fibrinogen structure. This figure has been adapted from the following website: http://www.sigmaaldrich.com/img/assets/27040/fibrinogen-cleave.jpg.

Structurally, the fibrinogen molecule has three distinct domains: two terminal D domains (67kDa), connected to a single central E domain (33kDa) by a triple-stranded array of polypeptide chains in the form of α helical coiled coils. The three constitutive chains and the two halves of fibrinogen molecule are held together by a series of 29 disulphide bonds with all 58 cysteine residues of fibrinogen participating in these interactions [24] [25].

2.2. Fibrinogen functions

Fibrinogen circulates as a component of blood at a concentration of approximately 9μM with a half-life of around 100 hours and its main functions overlap with those of fibrin in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia. These functions are regulated by interactive sites on fibrin(ogen). Some are masked or otherwise not available on fibrinogen and

they commonly become exposed as a consequence of fibrin formation or fibrinogen–surface interactions [25].

During inflammation the synthesis of fibrinogen in the liver is drastically enhanced. It has been also suggested that extra-hepatic epithelial cells, in particular lung alveolar epithelium, are also able to synthesise and secrete fibrinogen locally in response to proinflammatory mediators [26]. At sites of tissue injury, fibrinogen is converted to fibrin by α-thrombin with the release of fibrinopeptides A and B from the amino-terminal ends of the α and β chains, respectively. The fibrin monomers further polymerise spontaneously and undergo intermolecular crosslinking to form a stable fibrin clot in the presence of transglutaminase (factor XIIIa).

Following vascular injury, there are two principal mechanism by which fibrinogen participates in controlling blood loss. It acts as an adhesive protein essential for platelet aggregation as well as forming an insoluble fibrin clot in the final stage of blood coagulation cascade. The fibrin clot also provides a scaffold for cell adhesion, spreading, migration and proliferation.

2.3. Fibrinogen and Mac-1

Fibrinogen has a high affinity for the integrin receptor Mac-1 (α_M β_2) on neutrophils and monocytes. Importantly, it is through binding to this integrin that it has been proposed to play a role in inflammatory cell function (Fig.5).

The Mac-1-binding site within the fibrinogen D domain contains two peptide sequences, γ190-202 (P1) and γ377-395 (P2), which form adjacent antiparallel β-strands [27]. Consistently, the two peptides inhibit Mac-1-mediated cell adhesion, directly support adhesion and are able to promote cell migration. The ‘‘I-domain’’

within the α_M subunit, a 200–amino acid ‘‘insert’’ within the β-propeller structure, is central to integrin binding to fibrinogen [28].

Along the last decade, it has been shown that immobilized fibrin(ogen) importantly bridges hemostatic factors and inflammatory systems [29]. In view of this notion, fibrinogen became a biologically significant ligand for the Mac-1 (α_M β_2) integrin. Several *in vitro* studies have further shown that leukocyte engagement of fibrinogen can profoundly alter leukocyte function, leading to changes in cell migration, phagocytosis, NF-κB-mediated transcription, production of chemokines and cytokines, degranulation, and other processes [30] [31] [20] [32]. However, the contribution of soluble fibrinogen to those changes is nowadays still not clear. As such, these findings altogether enhance the need to better define the molecular details and the *in vivo* consequences of the interaction between soluble fibrinogen and the Mac-1 (α_M β_2) integrin.

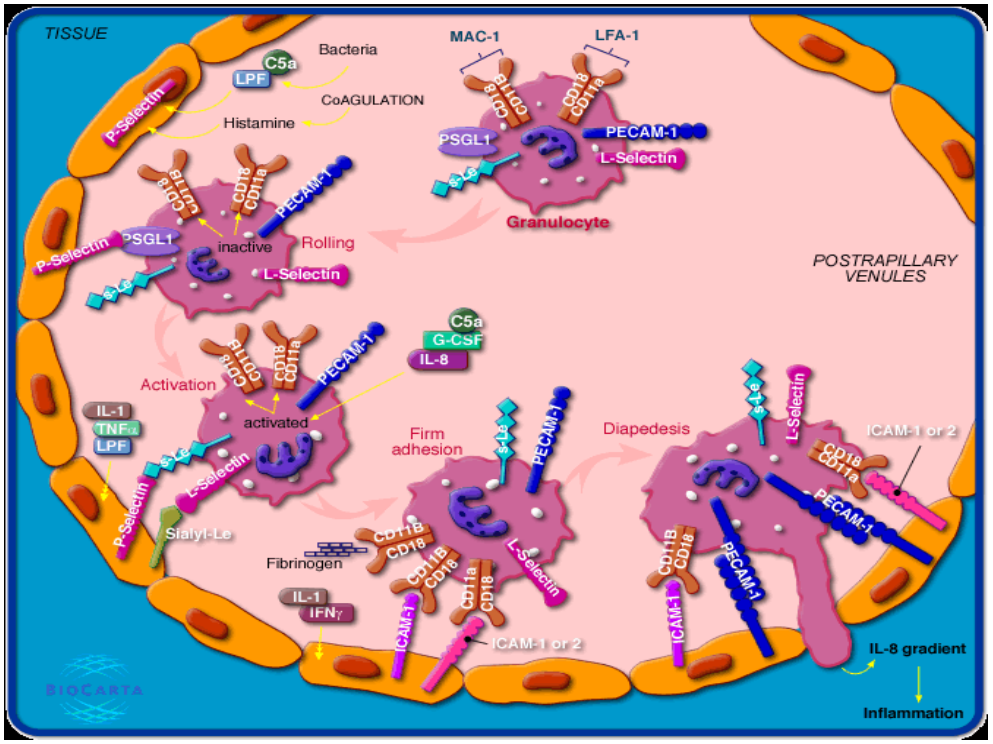

Fig.5: The present figure illustrates a summary of neutrophil recruitment. It has been adapted from Biocarta (http://www.biocarta.com/).

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PART TWO

Chapter II - Differential effect of soluble fibrinogen as a neutrophil activator

Abstract

A fundamental paradigm involved in acute inflammatory responses to invading pathogens and tissue damage is the migration of specific leukocyte populations to the affected tissues to mount there an initial innate response to the aggression. The recruitment of polymorphonuclear neutrophils (PMNs) from the blood is a central event in this respect.

The aim of this study was to understand whether fibrinogen is able to modulate the pattern of neutrophil activation and thus contribute to neutrophil recruitment. We demonstrated that fibrinogen induces free radical production by neutrophils without modifying the activation status of Mac-1 ($\alpha M\beta 2$, CD11b/CD18), the previously identified neutrophil receptor for fibrinogen. This data indicates that fibrinogen must have an additional different binding site in the neutrophil membrane. Importantly, we propose that as Mac-1 activation was not affected by the binding of fibrinogen, activated neutrophils can further maintain their ability to marginate, roll and adhere to the endothelial walls.

Keywords: Fibrinogen; Neutrophils; Mac-1; Inflammation

Published as:

V. Vitorino de Almeida, A. Calado, H. S. Rosário and C. Saldanha; *"Differential effect of soluble fibrinogen as a neutrophil activator."*; **Microvascular Research**.; 83(3):332-6 **(2012)**

1. Introduction

Neutrophil recruitment is crucial for a successful inflammatory response. In this process, neutrophils initially establish several contacts with the vascular endothelium leading to rolling and firm adhesion to endothelium. This initial step is determinant for the migration of neutrophils to the inflammation site. This process is mediated by several integrins, including the β_2 integrin family, such as Mac-1 ($\alpha_M\beta_{2,}$ CD11b/CD18). Circulating neutrophils maintain their integrin receptors in a low affinity, non-adhesive state and, in response to local inflammatory stimuli, are rapidly activated to bind specific ligands [1,2].

Mac-1 is a multiligand receptor that mediates leukocyte adhesion during the inflammatory response. One of its ligands is the plasma protein, fibrinogen, a large multidomain protein consisting of two pairs of α, β and γ polypeptide chains organized into three major structural regions: a central E and two peripheral D regions held together by coiled-coil connectors. This multifunctional glycoprotein participates in haemostasis and has adhesive and inflammatory functions through specific interactions with cells. Fibrinogen is synthesized by hepatocytes, and then secreted into the circulating bloodstream and it is the main protein component of blood clots. However fibrinogen can mediate diverse biological responses due to its particular molecular structure that comprises binding sites for receptors expressed on cells that are central mediators of the inflammatory process [3].

As a ligand for the neutrophil Mac-1 integrin, it has been reported to play a role in neutrophil signalling by modulating the generation of second messengers, production of oxygen free radicals and cell adhesion in inflammatory conditions.

The concentration of this glycoprotein increases in inflammatory conditions, and for this reason, it is considered a risk factor for diverse pathological conditions such as cardiovascular disease and stroke [4].

2. Material and methods

2.1. Reagents

HBSS*: Hank's Balance salt solution with $NaHCO_3$ without phenol red, calcium chloride and magnesium sulphate, Sigma*; red blood cell lysing buffer: *155mM ammonium chloride (NH_4Cl), 10 mm potassium hydrogen carbonate* ($KHCO_3$)*, 0,1mM EDTA*; Ficoll-Paque Plus, *Amersham Biosciences*; DextranT500 a 6% (p/v), Pharmacosmos; Human Fibrinogen, *Sigma;* Fibrinogen Alexa 488; anti- total Mac-1 antibody labelled with Phycoerythrin (PE) referred in this manuscript as anti-Mac-1(total antibody, eBioscience; anti-activated Mac-1 antibody labelled with Allophycocyanin (APC) referred in this manuscript as anti-Mac-1(activated) antibody, eBiosience; fMLP (N-formyl-methionine -leucine-phenylalanine), Calbiochem.

2.2. Methods

2.2.1. Isolation of human neutrophils

Human neutrophils were isolated from heparinised peripheral venous blood from healthy donors. After erythrocyte sedimentation in the presence of Dextran T500, the leukocyte-rich plasma was layered onto a Ficoll-Paque Plus gradient and centrifuged at 400g for 30 min at 4ºC. The PMN pellet was collected and further washed with HBSS buffer.

The viability and activation state of the isolated cells were verified, by flow cytometry analysis for the presence of specific PMN markers such as: CD69, CD66b, CD11b, CD16, and CD62L.

Neutrophils were activated with fMLP (10 6 M) for 15 min at 37ºC, as needed.

2.2.2. Oxygen free radical production

To detect oxygen free radical production, isolated neutrophils at 1,0x 10^6 cell/mL, were loaded with the fluorescent probe dihydrorhodamine 123 (DHR123) (1µM) [5], and then incubated with a range of concentrations of fibrinogen (0-400mg/dL) for 15 minutes immediately before flow cytometry analysis.

2.2.3. Activation of neutrophil Mac-1

The evaluation of the Mac-1 activation was performed both by flow cytometry and confocal microscopy by making use of antibodies specific for the non-activated and activated conformations of this integrin, respectively the anti-Mac-1(total) and the anti-Mac-1(activated) antibodies.

2.2.4. Flow cytometry and confocal microscopy analysis

All the flow cytometry data were acquired in a FACSCalibur Cytometer and for each experiment 10000 events were counted. The confocal immunofluorescence microscopy was performed using a Zeiss LSM 510 META Microscope.

3. Results

The purity, viability and cell activation status were checked before further experimentation by flow cytometry (fig.1) by making use of the following specific markers: CD69 which labels activated PMN; CD11b, CD66b and CD16 which are positive

for PMN; CD62L which is positive for non-activated PMN and CD64 which does not label PMN. A small percentage of CD69-labelled neutrophils was observed indicating that some neutrophils were primed most likely as a result of the isolation procedure. However, these were excluded from our studies. Despite this, the use of these specific markers showed that the isolation procedure employed yielded non-activated neutrophils.

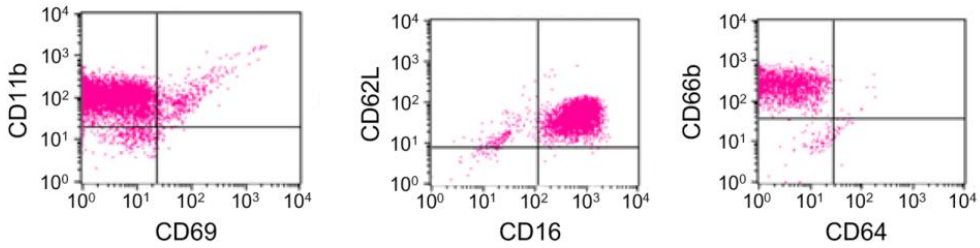

Fig.1: The used neutrophil isolation protocol yielded non-activated cells as demonstrated by flow cytometry analysis. The dot plots presented here illustrate FACS analysis performed by labelling the obtained cellular isolates with specific cellular markers, namely CD11b, CD69, CD62L, CD16, CD66b and CD69.

3.1. The effects of fibrinogen on neutrophil activation

In order to characterize how fibrinogen modulates the pattern of neutrophil activation, we first addressed the production of oxygen free radicals (OFR) in the presence of this protein. For such, isolated neutrophils, previously loaded with DHR123, were incubated with increasing concentrations of fibrinogen and OFR production was evaluated by flow cytometry (fig.2). In this figure, the columns of histograms displayed on the left and on the right panels are representative of the two different patterns of fibrinogen-induced activation that were detected in our experiments for the various neutrophil isolates. Obtained data indicate that a sub-population of neutrophils became activated at higher

concentrations of fibrinogen (300-400 mg/dL). In fact on the left panel, the number of PMNs with high fluorescence intensity increased with the increase of fibrinogen concentration showing a gradient of responsive neutrophils. On the other hand on the right panel, the OFR-producing neutrophils were distinctly sub-divided in two sub-populations with the increase of fibrinogen concentration: a low-responsive one and a high-responsive subpopulation. Overall, this experiment led us to suggest that fibrinogen by itself can activate the production of OFR and thus induce neutrophil activation. Based on these data, the following studies in this report were performed with fibrinogen at concentration of 300 mg/dL.

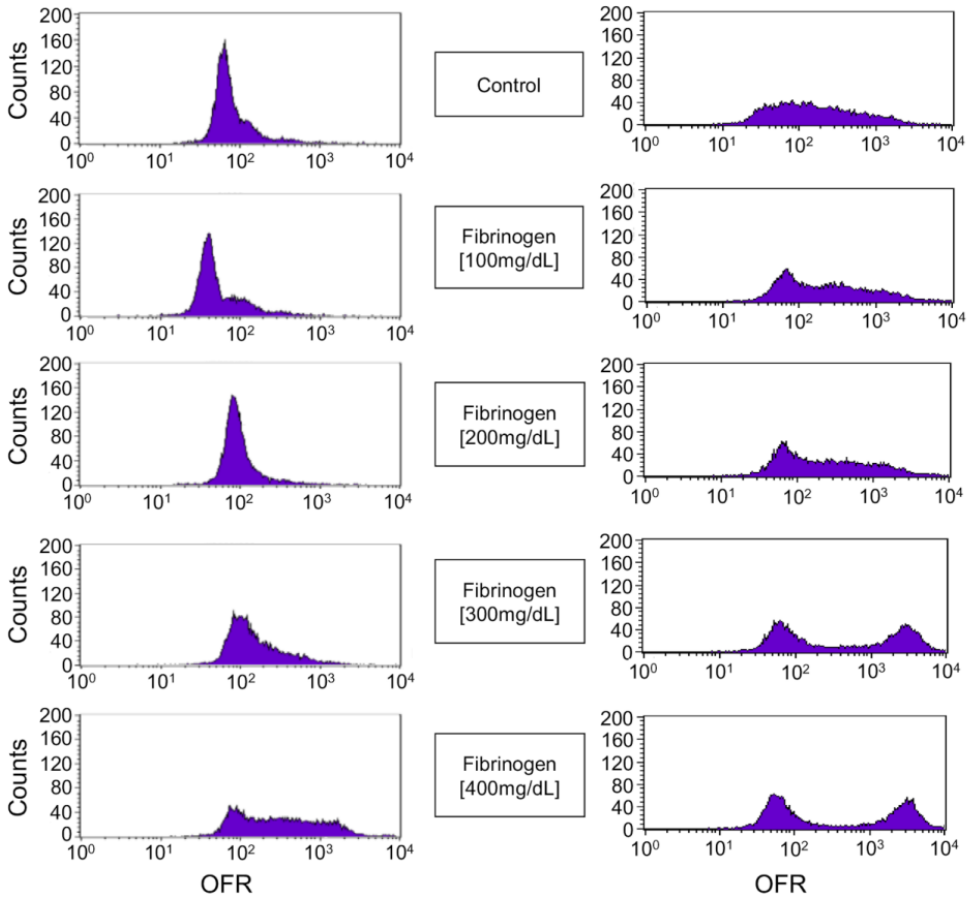

Fig.2: Increasing fibrinogen concentrations induced production of OFR by isolated human neutrophils probed with DHR123. Neutrophils were first loaded with DHR123, incubated with four increasing doses of fibrinogen and then analysed by flow cytometry for determining OFR production.

The histograms presented here are representative of the two distinct patterns of neutrophil activation encountered for the different neutrophil isolates collected for this experiment: an example of one is displayed on the left panel and one of the other on the right panel of the figure. For each experiment 10000 events were counted. Representative of N=6.

As in the previous experiment, neutrophils were solely incubated with fibrinogen in the presence of DHR123, we argued that the effects observed on neutrophil activation could be mediated by the interaction of fibrinogen with the neutrophil membrane. To investigate this, neutrophils were first incubated with Alexa488-labeled fibrinogen in the absence or presence of a known neutrophil activator, fMLP and the interaction of fibrinogen with these cells was then evaluated by flow cytometry (fig.3). As depicted in the presented histograms, fibrinogen was able to bind to neutrophils in the absence of fMLP thus showing its ability to bind to resting neutrophils.

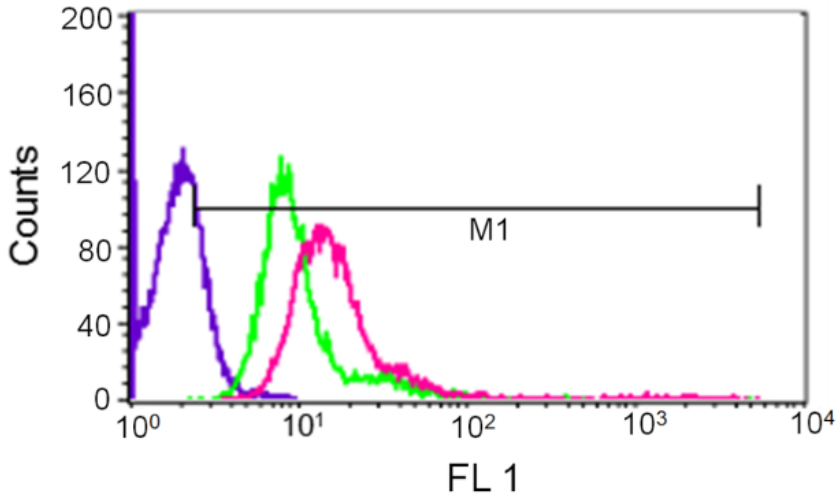

Fig. 3: Fibrinogen binding to resting neutrophils. Neutrophils were incubated with fibrinogen in the presence or absence of fMLP [10^{-6} M] and fibrinogen-neutrophil binding was evaluated by flow cytometry. The histogram depicted in blue shows the autofluorescence of neutrophils. The histogram displayed in green indicates the binding of fluorescently labelled fibrinogen (Alexa488-labelled fibrinogen) to resting cells. The histogram in pink refers to neutrophils activated with fMLP and further incubated with Alexa488-labelled fibrinogen. These data suggest the ability of fibrinogen to bind to resting neutrophils which was further potentiated after neutrophil activation with fMLP.

As described in the Introduction, the main binding site for fibrinogen in neutrophils is the Mac-1 integrin. In this respect, we then aimed at understanding whether the binding of fibrinogen to resting neutrophils was mediated by this integrin. To address this issue, we made use of an antibody targeting the total Mac-1 integrin (anti-Mac-1(total)) that has been described to block this integrin. First, we confirmed by flow cytometry that all the neutrophils present in our isolates were positive for this marker (fig.4). Next, we evaluated the effect of the anti-Mac-1(total) antibody on neutrophil-fibrinogen binding by sequentially incubating isolated neutrophils first with the blocking antibody and then with Alexa488-labelled fibrinogen. Upon flow cytometry analysis, we were able to observe that 100% of the neutrophils are both positive for anti-Mac-1(total) antibody labelling and for fibrinogen binding. Thus despite the blocking effect of this antibody, the fluorescently-labelled fibrinogen still retained its ability to bind to neutrophils. This result led us to propose that fibrinogen can use a binding site distinct of Mac-1 to interact with the neutrophil membrane.

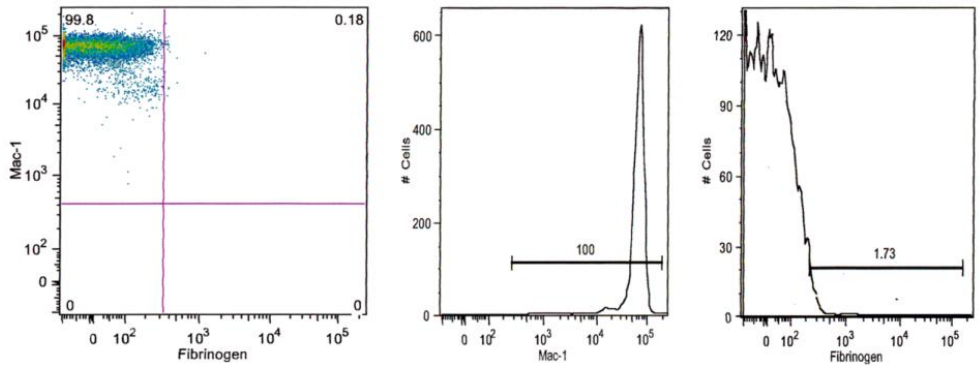

Fig.4: Neutrophil isolates were 100% positive for anti-Mac-1(total), a blocking antibody for total Mac-1 integrins. The flow cytometry plots are representative of neutrophils labelled with anti-Mac-1(total) in the absence of fibrinogen.

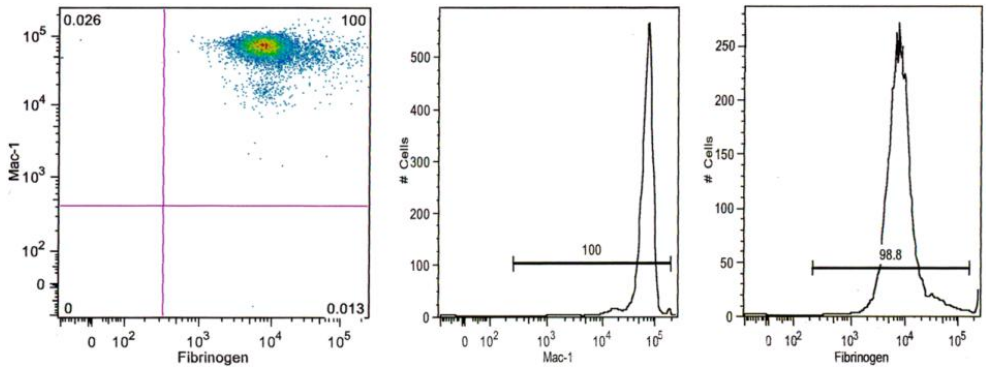

Fig. 5: By blocking Mac-1 with the anti-Mac-1 (total) antibody, neutrophils still retained their ability of binding to fibrinogen. Flow cytometry plots represent neutrophils labelled first with anti-Mac-1(total) and then with Alexa488-labelled fibrinogen. Here, we were able to observe that 100% of neutrophils were positive for anti-Mac-1(total) as well as for fibrinogen binding, suggesting that fibrinogen was able to bind neutrophils despite the blocking effect of the anti-Mac-1 (total) antibody.

To reinforce our proposal, we next addressed whether the binding of fibrinogen to the neutrophil membrane was able or not to affect Mac-1 expression and activation. For such, we made use of the previous anti-Mac-1(total) antibody as well as of an antibody directed to the activated fraction of Mac-1 (anti-Mac-1 (activated)) present in the neutrophil membrane. In this set of experiments, isolated neutrophils were first incubated in the presence or absence of either fibrinogen or fMLP, subsequently probed with either anti-Mac-1(total) antibody or Mac-1 (anti-Mac-1 (activated)) antibodies and analysed by flow cytometry. In line with the data presented before, both antibodies were able to label positively the entire neutrophil population (left panels in fig. 6). Additionally, the pre-incubation with unlabelled fibrinogen in the absence or presence of DHR123 did not change the binding pattern of total or activated Mac-1. These results indicate that despite the fact that fibrinogen was able to

potentiate neutrophil activation in relation to the production of OFR, Mac-1 activation status was not altered in the presence of this protein factor.

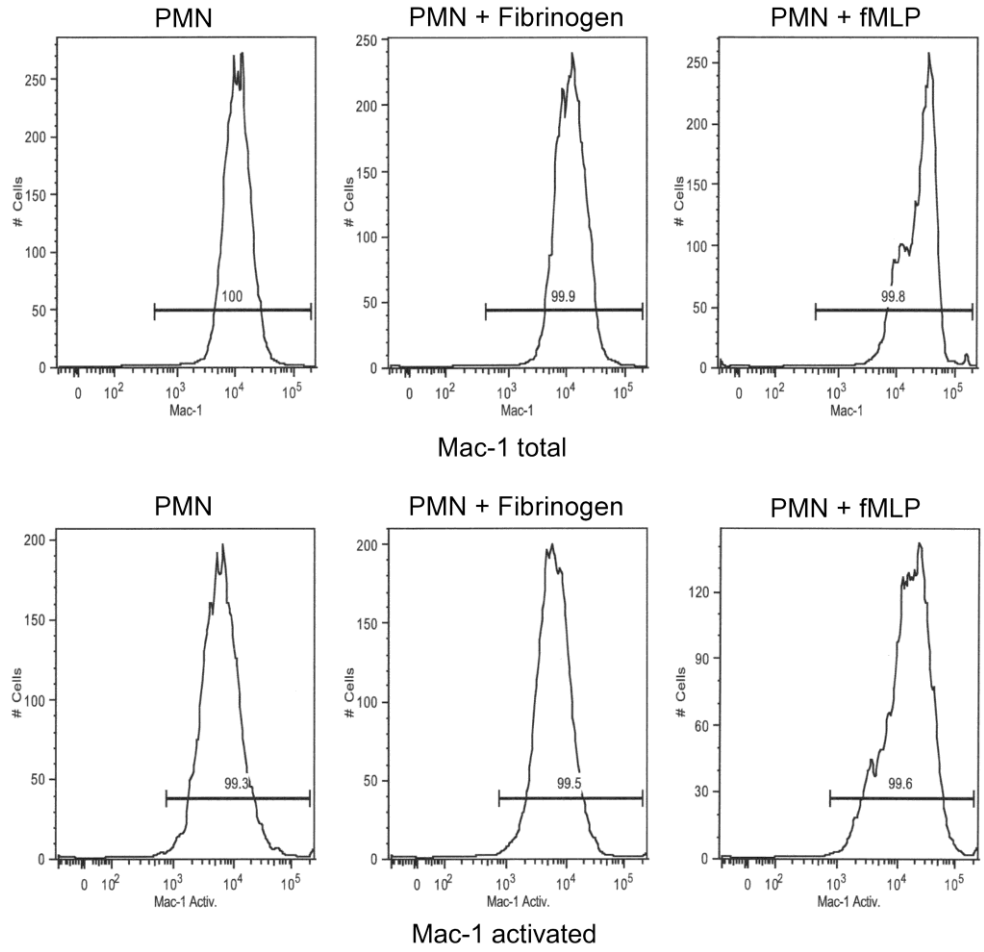

Fig.6: In the presence of fibrinogen, no difference in expression of total and active Mac-1 was detected. The first row is a representation of flow cytometry histograms of isolated neutrophils incubated first with DHR123 in the presence of either fibrinogen (designated here as fib) or fMLP and then labelled with the anti-Mac-1 (total) antibody. The second row is a representation of flow cytometry histograms of isolated neutrophils subjected to a similar incubation procedure but where the final antibody labelling was performed with the anti-Mac-1(active) antibody.

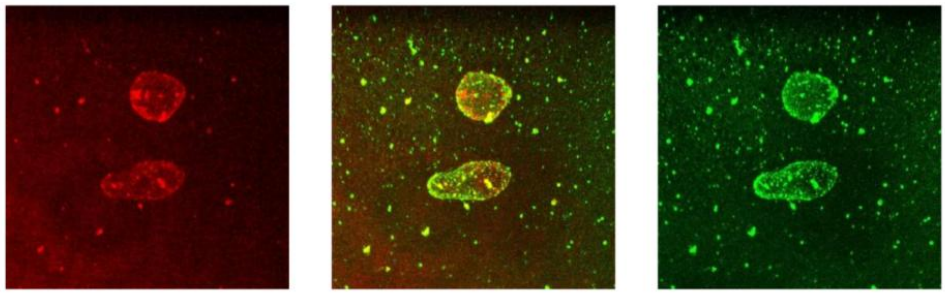

Fig.7: Fibrinogen labelling does not co-localize completely with the active Mac-1 labelling. Confocal analyses of isolated PMNs labelled with: the anti-Mac-1(active) antibody (red) (a) and fibrinogen Alexa488 (green) (c). The merged image in the middle indicates partial co-localization of fibrinogen and Mac-1 (yellow) (b).

The data so far collected supported our proposal that the Mac-1 integrin may not be the sole fibrinogen binding site in the neutrophil membrane. A prediction from such working hypothesis would be that the binding of fibrinogen to the neutrophil should not be restricted to membrane areas expressing activated Mac-1. To address this issue, isolated neutrophils labelled with anti-Mac-1(activated) antibody and Alexa488-labelled fibrinogen were analysed by confocal fluorescence microscopy (fig.7). As expected from the published data [6] we observed here that fibrinogen labelling partly co-localized with the activated Mac-1 labelling. However, this co-localization was not complete as the fibrinogen was also able to decorate areas of the neutrophil membrane completely devoid of detectable activated Mac-1 labelling.

Altogether, our results showed that fibrinogen is able by itself to induce neutrophil activation as measured by free radical production. This effect was evident at

borderline-high concentrations (300-400mg/dL) of fibrinogen. Particularly in part of the neutrophil samples used, it was possible to differentiate two subpopulations of neutrophils with distinct responses of OFR production upon activation by fibrinogen. As high concentrations of fibrinogen have been identified as a risk factor for cardiovascular diseases, we hypothesize that in this regard, this protein might promote the setting of an inflammatory microenvironment in the circulation and thus facilitate the progression and development of these diseases.

Moreover, we produced experimental evidences that strongly indicate that this effect involves the binding of fibrinogen to the neutrophil membrane and that this interaction is not dependent of Mac-1, the receptor previously identified for this ligand in the neutrophil. Importantly, the membrane expression and activation of this integrin was not modified in the presence of fibrinogen. Overall, this observation suggests to us that the activation of neutrophils by fibrinogen may not interfere in the rolling and adhesion steps during recruitment towards the endothelium.

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Chapter III - An *in vitro* study on the modulation of the neutrophil adhesive behavior by soluble fibrinogen

Abstract

Fibrinogen constitutes an important plasma glycoprotein involved in hemostasis and in inflammation. Previously, we have shown that at physiological concentrations, soluble fibrinogen is able to modulate the pattern of neutrophil activation. This led us to propose that under these conditions, fibrinogen could as well interfere with the adhesive behaviour of circulating neutrophils which is of utmost importance in their recruitment to the vascular wall during inflammatory processes.

To address our working hypothesis, *in vitro* adhesion assays were here performed in a flow chamber by using primary cultures of human umbilical vein endothelial cells (HUVEC) and neutrophils isolated from peripheral venous blood of healthy human donors. In the presence of a physiological concentration of soluble fibrinogen (300mg/dL), we observed that despite the number of neutrophils rolling on an activated endothelium was not affected, their rolling velocity was increased in comparison to that of non-activated neutrophils. Consequently as expected, the number of fibrinogen-treated neutrophils adhering to activated HUVEC monolayers was significantly diminished. Overall, we have here demonstrated that at least *in vitro*, soluble fibrinogen under physiological concentrations is able to modulate the interaction of neutrophils with the vascular endothelium. *In vivo* studies will enable us in the future to study the physiological relevance of these findings and further to understand the mechanisms underlying this function.

Keywords: Neutrophil recruitment; Fibrinogen; Flow chamber

Published as:

V. Vitorino de Almeida, A. Calado , A.S.S. Herdade, H. S. Rosário and C. Saldanha*; "An in vitro study on the modulation of the neutrophil adhesive behavior by soluble fibrinogen"*; accepted for publication at **Clin Hemorheol Microcirc. (2012)**

1. Introduction:

Inflammation triggers an extensive variety of physiological and pathological processes as a protective response to insult or injury. Acute inflammation is characterized by the recruitment of specific leukocyte populations, namely neutrophils and monocytes, from the blood circulation towards the affected area. Initially, neutrophils are predominantly recruited so as to eradicate the inflammatory agent. These leukocytes are later followed by monocytes that by maturing into macrophages further set the stage for inflammation resolution and subsequent tissue repair [1].

Targeting of leukocytes to sites of inflammation is a complex process that relies initially in a multistep recruitment cascade that enables leukocyte migration through an activated endothelium of postcapillary venules via cell-cell interactions [2]. In brief, this recruitment process comprises the following steps: “tethering”, rolling, slow rolling, arrest, post-adhesion strengthening, crawling, and paracellular or transcellular transmigration [3] [4].

Distinct *in vitro* and *in vivo* methodologies have been pivotal in the study of leukocyte recruitment through the past decades. Among *in vitro* approaches, flow assays performed in specially devised chambers as that depicted in Fig. 1 have shown a potential application in this respect by more closely mimicking an *in vivo* situation than static assays. In fact, *in vitro* experimental systems have proven capable of generating controlled mechanical forces similar to those induced by hemodynamic forces *in vivo*. Among these, the most relevant mechanical force in leukocyte–endothelial cell adhesion is shear stress and normal time-averaged levels of venous shear stresses usually range *in vivo* between 1–5 dyn/cm^2. Assuming a unidirectional and laminar flow, the shear rate, γ, at the bottom surface of the flow chamber is given by $\gamma = \frac{6Q}{wh^2}$ (where Q is the volumetric flow rate; w, the width of the flow field; and h, the height of the flow field). As the shear stress, τ, is related to the shear rate by the equation

$\tau = \gamma\mu$ (where μ is the viscosity of the fluid used in the experiments, typically 0.007 poise for a diluted saline solution at 37°C), it can be represented by the expression, $\tau = \frac{6Q\mu}{wh^2}$. Ideally, any of these parameters could be changed to modulate shear stress. However as the geometry of the flow chamber is held constant, only the volumetric flow rate can be normally altered for such purpose. With this experimental design the shear stress exerted on the cells is assumed to be approximately equal to the chamber wall shear stress. Of utmost importance, flow chamber assays thus enable the study and visualization of the leukocyte recruitment cascade under well-defined wall shear stress conditions. In these assays, steps as rolling and cell adhesion can be quantified by selective image acquisition and subsequent image processing. In particular, these assays are especially appropriate for addressing adhesive events which occur very rapidly in a short time scale. In addition, they also enable the study of initial events such as cell stabilization and spreading giving insight into the kinetics of particular cell-cell or cell-substrate adhesive behaviour [5] [6].

The different steps of the leukocyte recruitment cascade involve sequentially an array of specific factors among which stand distinct adhesion molecules expressed on the cell surface of leukocytes and endothelial cells, such as integrins. In steady state conditions, circulating leukocytes maintain their integrin receptors in a low affinity and non-adhesive state. But in response to local inflammatory stimuli, integrins are rapidly activated to bind specific ligands [7] [8]. Members of the β2 integrin family, such as Mac-1 (αMβ2, CD11b/CD18), play crucial roles in the particular case of neutrophil recruitment [7].

Among other ligands, Mac-1 serves as the receptor for fibrinogen, a large multidomain plasma glycoprotein consisting of two pairs of α, β and γ polypeptide chains organized into three major structural regions: a central E and two peripheral D regions held

together by coiled-coil connectors [9]. Fibrinogen is synthesized by hepatocytes in the liver and further secreted into the circulating bloodstream. This protein is a crucial player in the coagulation cascade through which it polymerizes into fibrin, the major protein component of blood clots [10] [11] [12]. Moreover, fibrinogen is also known to constitute an acute phase protein involved in other biological responses, such as in inflammation [10]. This is due to its particular molecular structure which comprises binding sites for several receptors expressed on cells that act as central mediators of the inflammatory process. As a ligand for Mac-1, fibrinogen has been shown to play a role in neutrophil signalling by modulating the generation of second messengers, production of oxygen free radicals and cell adhesion in inflammatory conditions [8].

Previously, we have shown that at physiological concentrations, fibrinogen is able to modulate the pattern of neutrophil activation without interfering with the expression and activation of the Mac-1 integrin [13]. In fact, an increased free radical production was observed in neutrophils freshly isolated from peripheral blood of human healthy donors upon their incubation with physiological concentrations of fibrinogen. Interestingly however, both the membrane expression and the activation status of the Mac-1 integrin were not modified under these conditions. Overall, these results led us to suggest that fibrinogen could as well play a role in modulating the adhesive behaviour of the neutrophil towards the vascular endothelium under normal physiological conditions. In the present report, we aimed to address this issue by assaying neutrophil recruitment *in vitro* in flow assays performed under a physiological concentration of soluble fibrinogen (300mg/dL). For such, we employed flow chambers and analysed the recruitment of neutrophils isolated from peripheral venous blood of healthy human donors onto monolayer of human umbilical vein endothelial cells (HUVEC).

2. Materials and methods:

2.1. Human Umbilical Vein Endothelial Cells (HUVECs) Monolayer Preparation

Human umbilical vein endothelial cells (purchased from Invitrogen) were cultured in tissue culture dishes in Medium 200 supplemented according to manufacturer's instructions and maintained at 37^{o} C in an atmosphere of 5% CO_2. HUVECs were used between passages 3 and 6. All supplements and media were purchased from Invitrogen.

For the flow assays, HUVECs were grown on 40mm sterile round glass coverslip up to a cell confluence of around 80%. When required, HUVECs were activated in the presence of TNF-α (10ng/mL) for 2 hour prior to the flow assay.

2.2. Isolation of human neutrophils

Human neutrophils were isolated from heparinised peripheral venous blood from healthy donors. After erythrocyte sedimentation in the presence of 6% of Dextran T500 (*Pharmacosmos*), the leukocyte-rich plasma was layered onto a Ficoll-Paque Plus (*Amersham Biosciences*) gradient and centrifuged at 400g during 30 min at 4^{o}C. The pellet of neutrophils was collected and further washed in HBSS buffer *(*Hank's Balance salt solution, *Sigma*).

When required, neutrophils were incubated in 10 μM fMLP (N-formyl-methionine-leucine-phenylalanine, *Calbiochem*) or in 300 mg/dL fibrinogen (Sigma-Aldrich) for 15 min at 37^{o}C prior to being used in the flow assays.

2.3. Flow assay

The flow chamber and all the required components were purchased from Warner Instruments. This chamber was assembled according to the scheme presented in Fig. 1 and to the manufacturer's instructions. Briefly, the bottom round coverslip of 40 mm diameter (designated as component #2 in Fig. 1), over which a HUVECs monolayer was previously grown, was inserted into the base of the chamber (component #1). The chamber forming gasket (component #3) was further placed on the top of the culture. The 30mm round coverslip (component #4) was inserted in the top cover slip (component #5) of the chamber and the pressure plate (component #6) tight.

The flow chamber was further assembled onto to the flow assay scheme presented in Fig. 2. In brief, a syringe filled with the neutrophil suspension at a concentration of 10^6 cells/mL in HBSS (purchased from Invitrogen) was placed onto a syringe pump and further connected to the outlet flow chamber at a shear stress of 0,1dynes/cm^2. This flow chamber was then coupled onto the heated stage of a Fluorescence Inverted Microscope (Leitz Fluovert FU, objective 10x / 0,30).

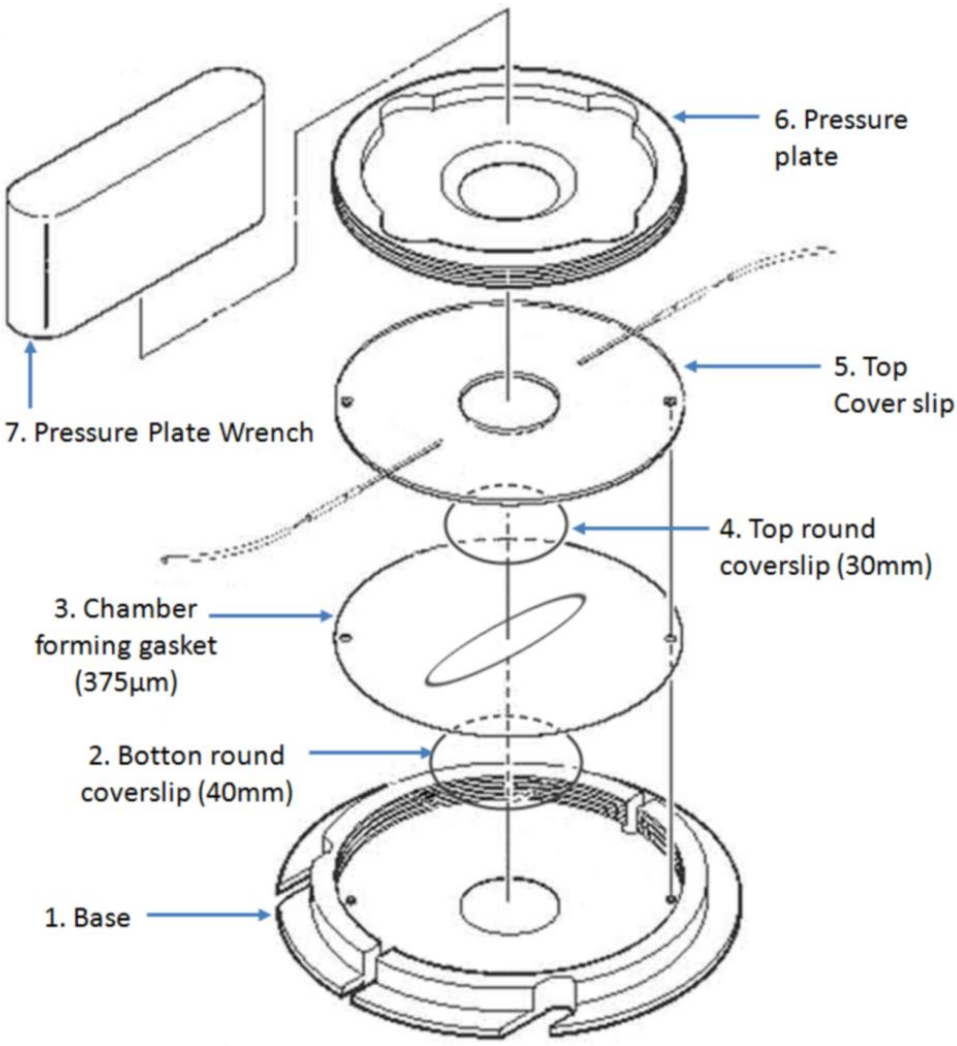

Fig.1: Scheme illustrating the assembly of the flow chamber with the indication of the required components (herein numbered).

For the flow assay, neutrophils were initially allowed to flow for 5 minutes so as to ensure for flow stabilization and to attain an adequate number of cells interacting with the HUVECs monolayer. Image acquisition was then performed for up 10 to 25 min by employing a CCD-Iris color video camera (Sony). For each assay, images were collected

at several locations on the dish but always concentrating in the middle of the flow chamber and avoiding its edges.

Acquired video sequences were further analysed with ImagePro-Plus software to quantify: (i) the number of rolling leukocytes (defined as the number of leukocytes moving at a low-velocity with a high-variance translation of 100μm) as well as their rolling velocities (calculated by v = d/t, where d stands for the displacement of the leukocyte over a period of time, t) and (ii) the number of adherent leukocytes (defined as the total number of leukocytes adhering to the HUVEC monolayer per unit area for at least 30 sec). For this analysis, neutrophils were manually tagged and their movements on the monolayer monitored.

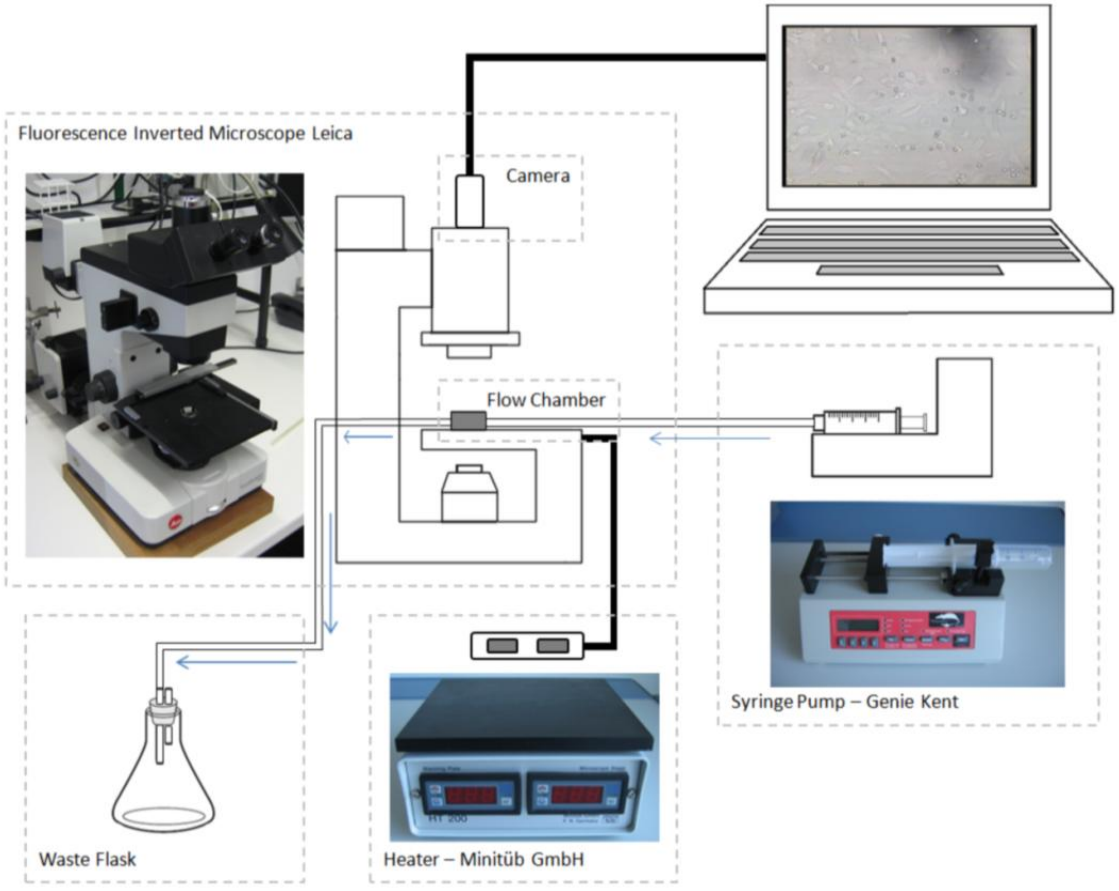

Fig.2: Assembly of the flow chamber assays. Blue arrows depict the flow orientation.

2.4. Statistical analysis

Data were obtained from six independent experiments and expressed as mean values ± standard error deviation (SD). Student´s t test was used to determine the significance of differences between sample means. Statistical analysis was performed using Origin Pro8.

3. Results

In this report, we made use of *in vitro* flow assays to understand whether soluble fibrinogen is able to interfere with the adhesive behaviour of the neutrophil in relation to an endothelial cell layer under physiological flow conditions. For such, we employed a flow chamber assay schematically depicted in Fig. 1 in which neutrophils isolated from peripheral blood of human healthy donors were flown over monolayers of HUVECs. In order to mimic more closely the *in vivo* situation, we used a constant wall shear stress of 0,1 dynes/cm^2, a value comparable to that found in postcapillary venules and that has been shown not to allow the direct adhesion of neutrophils to endothelial cells in the absence of an increased expression of selectins [5]. The neutrophil adhesive behaviour was further analysed by determining the following parameters: number of rolling leukocytes; velocity of rolling and number of adherent leukocytes.

To ensure that the obtained neutrophils were not activated by our isolation procedure, fresh neutrophil isolates were first assayed on HUVEC monolayers previously activated or not. HUVEC activation was performed by incubating the cell monolayer in the presence of a known inflammatory activator of endothelial cells, the tumour necrosis factor-alpha, TNF-α, at a concentration of 10 ng/mL for a period of 2 hours prior to use in the flow assay. In these assays, we observed a significantly smaller number of

neutrophils either rolling on or adhering to a non-activated HUVEC monolayer in comparison to the numbers obtained when using a TNF-α-activated endothelial monolayer (Fig. 3, Fig. 4 and Table 1). These results demonstrated that the isolated neutrophils were not able to bind spontaneously to the non-activated HUVECs and were not therefore activated during the isolation procedure used.

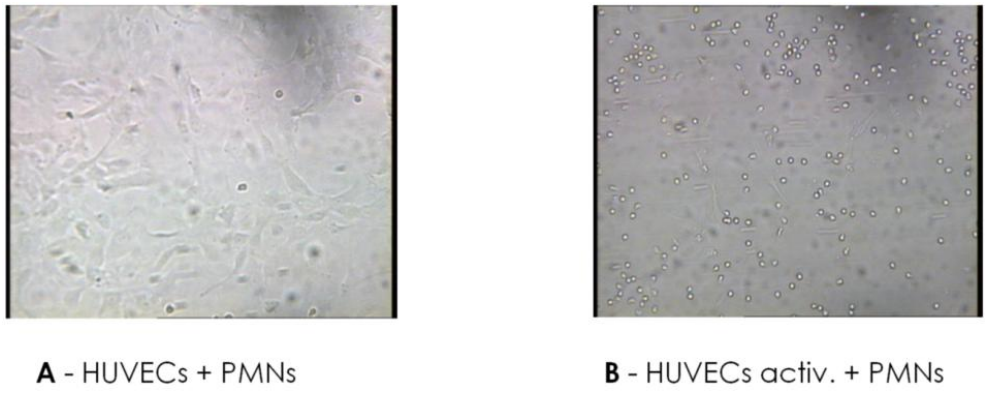

Fig.3: Images acquired from the flow assays performed with non-treated neutrophils (here referred as PMNs) and HUVECs (panel **A**) or TNF-α-activated HUVECs, referred here as HUVECs activ (panel **B**). For each condition six assays were performed (N=6). As depicted in the micrographs presented, an increased number of neutrophils were observed either rolling or adhering to the HUVEC monolayers in the assays shown in panel **B**.

As a proof-of-concept of our flow assays, we further assayed the recruitment of neutrophils pre-treated with fMLP (a well-known neutrophil activator [14] onto TNF-α-activated HUVECs. In comparison with assays using non-treated neutrophils and activated HUVEC, we observed here a decreased number of rolling leukocytes (Fig. 4 and Table 1) with lower rolling velocities (Fig. 5 and Table 1) and an increased number

of adherent neutrophils (Fig. 4 and Table 1). As discussed below, this scenario is consistent with that expected for an inflammatory situation [15].

Finally to address for a role for fibrinogen on the adhesive behaviour of neutrophils under normal conditions, neutrophils were pre-incubated with soluble fibrinogen at a concentration of 300 mg/dL for 15 min at room temperature prior to the flow assay. By probing these neutrophils on flow assays performed on activated HUVECs, we were able to observe that both neutrophil rolling as well as adhesion were differently affected when comparing to results obtained from flow assays using non-treated neutrophils and TNF-α-activated HUVEC (Fig. 4 and 5; Table 1). In fact, despite the number of rolling neutrophils was not affected in the presence of a physiological concentration of fibrinogen (Fig. 4 and Table 1), the rolling velocities of fibrinogen-treated neutrophils were significantly augmented (Fig. 5 and Table 1). Moreover, the number of adherent neutrophils was substantially diminished when these cells were pre-incubated with fibrinogen (Fig. 4 and Table 1).

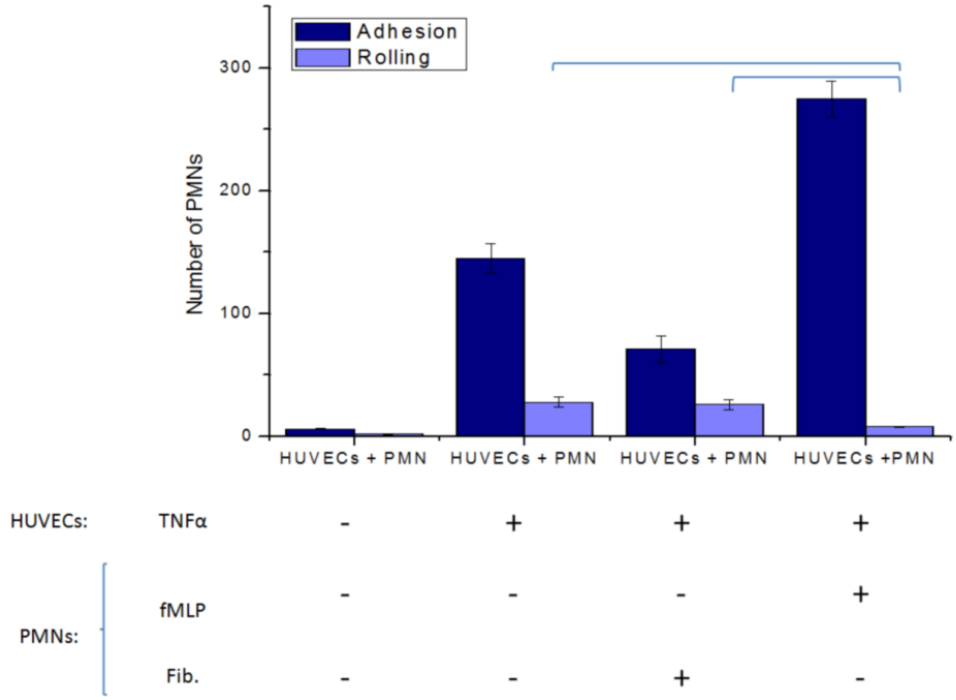

Fig.4: Graphic representation of the mean values of the numbers of neutrophils (referred here as PMNs) rolling on HUVECs (labelled as Rolling in the Figure legend) or adhering to this endothelial cell layer (labelled as Adhesion in the Figure legend) determined in the distinct experimental conditions employed in the present manuscript. In the lower part of the Figure, the treatments inflicted to HUVECs or neutrophils are indicated for each of the experimental conditions tested (Fib stands for fibrinogen). Error bars represent the standard error deviation obtained in a total of six experiments performed per experimental condition. In the presence of a physiological concentration of fibrinogen 300mg/dL, the number of neutrophils rolling on an activated endothelium is not affected whereas the number of adherent neutrophils is substantially decreased ($p < 0,01$) when comparing to the recruitment data collected when using non-activated neutrophils and TNF-α-activated HUVECs.

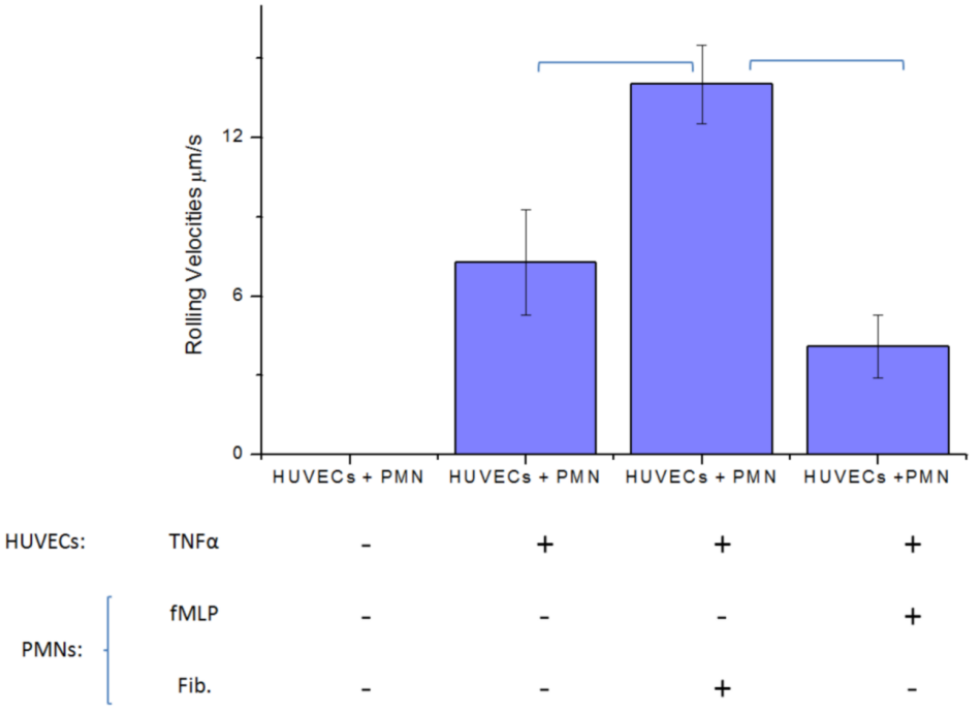

Fig.5: Graphic representation of the mean values of the rolling velocities of neutrophils (referred here as PMNs) on HUVECs monolayers determined for the different experimental conditions employed in the present manuscript. In the lower part of the Figure, the treatments inflicted to HUVECs or neutrophils prior to the flow assays are indicated for each of the experimental conditions tested. Here (-) stands for absence of treatment and (+) for the incubation with the mentioned product (Fib stands for fibrinogen). Error bars represent the standard error deviation obtained in a total of six experiments performed per experimental condition. The rolling velocities of neutrophils increased significantly when the neutrophils were pre-incubated with soluble fibrinogen at 300mg/dL in comparison to those measured for non-activated or fMLP-activated neutrophils ($p < 0,01$).

Table 1: Summary of the results obtained for the neutrophil recruitment parameters determined in the flow assays performed in this study. For each condition six assays were performed (N=6). Results are presented as mean values and the associated standard error deviations. PMN (abbreviated from polymorphonuclear cells) refers to the neutrophils; HUVECs activ stands for TNF-α-activated HUVECs; PMN Fib stands for neutrophils pre-incubated with fibrinogen (Fib) prior to the flow assay; PMN active stands for neutrophils pre-incubated with fMLP prior to the flow assay.

	Number of adherent neutrophils	**Number of rolling neutrophils**	**Rolling Velocities (µm/s)**
HUVECs + PMN	6 ± 0,35	2 ± 0,35	0 ± 0
HUVECs activ + PMN	145 ± 12,21	28 ± 4,25	7,27 ± 2
HUVECs activ + PMN Fib.	71 ± 10,79	26 ± 4,03	14 ± 1,48
HUVECs activ + PMN activ	275 ± 14,70	8 ± 0,50	4,1 ± 1,18

4. Discussion

In a previous report, we demonstrated that soluble fibrinogen is able to modulate neutrophil activation when present at physiological concentrations [13]. This led us to propose that this plasma glycoprotein could as well modulate the adhesive behaviour of circulating neutrophils and therefore could modulate their recruitment towards the vascular endothelium.

To address this working hypothesis, we made here use of *in vitro* flow assays by using (i) HUVEC monolayers and (ii) neutrophils freshly isolated from peripheral blood of human healthy donors. For the isolation of human neutrophils, we made use of a protocol shown previously to yield non-activated cells. Consistently, our neutrophil isolates did not bind spontaneously to non-activated endothelium under flow.

In vitro flow assays closely reproduce leukocyte recruitment under inflammatory conditions. In fact in these assays, activated neutrophils are expected to be significantly recruited towards an activated endothelium under flow. Accordingly, we were able to observe that neutrophils pre-incubated with fMLP, a known neutrophil activator [16] [17], adhered more extensively to the TNF-α-activated HUVEC monolayer in comparison to non-activated neutrophils. Additionally, rolling velocities for the former were also lower than those exhibited by non-activated cells. Altogether, these results are in agreement with the current knowledge on how leukocyte recruitment towards the vascular endothelial wall occurs in inflammation.

During the recruitment cascade, leukocytes are able to roll along the wall of inflamed post-capillary venules until they halt, adhere and then transmigrate. It is assumed that the recognition of endothelial-bound chemokines triggers the activation of particular leukocyte integrins which are responsible for the transition from rolling to adhesion. Depending on their conformational state, binding of an activated integrin to their specific ligands on the endothelium will lead either to a reduction of the rolling velocity or to the arrest of the leukocyte. Subsequent firm adhesion further will require additional activation of neutrophil integrins mediated via their binding to ligands expressed on the surface endothelial cell. Along this process, a slow rolling step will also contribute to the firm adhesion of the leukocyte to the vascular wall [4]. In fact at higher rolling velocities, strong leukocyte adhesion is not favoured and therefore a reduction in the rolling velocity must take place for a rolling leukocyte to adhere more firmly.

Having proven that our neutrophil isolates exhibited the expected adhesive behaviour in the used flow assays, we further assessed whether fibrinogen in a physiological concentration (300mg/dL) could play a role in this respect. Under these conditions, we observed that the numbers of rolling neutrophils were not significantly affected when compared to those measured for non-activated neutrophils. Surprisingly however,

rolling velocities measured for the former situation were significantly higher than those exhibited by the latter. As higher rolling velocities would predictably preclude neutrophil adhesion to the activated HUVECs, the number of adherent fibrinogen-treated neutrophils was consistently smaller than that determined for non-activated leukocytes.

Altogether, our current analysis of neutrophil recruitment under flow enabled us to conclude that at least *in vitro*, soluble fibrinogen at physiological concentrations is able to accelerate neutrophil rolling and consequently, to reduce their adhesion towards the vascular wall. Such reduction will necessarily translate into a decreased ability of the neutrophil to transmigrate towards an affected area and therefore into a diminished inflammatory response.

By combining these results with the published data [15] [18] where the authors suggested that leukocytes that engage fibrinogen molecules loosely bound to the surface of fibrin(ogen) matrix, we propose here that soluble fibrinogen at physiological concentrations is normally able to modulate the adhesive behaviour of the circulating neutrophil to activated endothelium. This action may reflect an ability of fibrinogen to shield the neutrophil from excessive adhesion towards the vascular wall. In other words, binding of soluble fibrinogen to the neutrophil membrane under normal conditions may loosen putative neutrophil-endothelium interactions so that the neutrophil may then be able to easily detach from the endothelial wall. Such a putative role for soluble fibrinogen could for instance be instrumental by preventing unwanted accumulation of neutrophils in the vasculature and subsequently, avoiding thrombus formation and growth.

Our future studies will address the *in vivo* functional relevance of such activity and importantly, the molecular mechanisms underlying this proposed shielding effect of soluble fibrinogen.

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Chapter IV - Fibrinogen modulates leukocyte recruitment *in vivo* during the acute inflammatory response

Abstract:

Besides playing an important role in blood hemostases, fibrinogen also regulates leukocyte function in inflammation. Our previous *in vitro* studies showed that the adhesive behaviour of the neutrophil is modulated by soluble fibrinogen when present at a physiological concentration. This led us to propose that this plasma glycoprotein might further influence leukocyte recruitment in vivo and thus contribute to the inflammatory response.

To address this in vivo, leukocyte recruitment was here investigated under acute inflammatory conditions in the absence of soluble fibrinogen in the blood circulation. For such, intravital microscopy on mesentery post-capillary venules was performed on homozygous fibrinogen α chain-deficient mice (($\alpha^{-/-}$) mice). Acute inflammatory states were induced by perfusing platelet activating factor (PAF) over the exposed tissue. As control animals, two groups of mice expressing soluble fibrinogen in circulation were used, namely, C57BL/6 wild type animals and heterozygous fibrinogen α chain-deficient mice (($\alpha^{+/-}$) mice).

Under acute inflammatory conditions, an abnormal pattern of recruitment was observed for leukocytes in homozygous ($\alpha^{-/-}$) mice in comparison to both control groups. In fact, the former exhibited a significantly decreased number of rolling leukocytes that nevertheless, migrated with increased rolling velocities when compared to leukocytes from control animals. Consistently, homozygous mice further

displayed a diminished number of adherent leukocytes than the other groups. Altogether our observations led us to conclude that leukocyte recruitment in homozygous ($\alpha^{-/-}$) mice is compromised what strongly suggests a role for soluble fibrinogen in leukocyte recruitment in inflammation.

Keywords: Neutrophil recruitment; Fibrinogen; Intravital microscopy; Inflammation

Published as:

V. Vitorino de Almeida, A.S.S. Herdade, A. Calado, H. S. Rosário and C. Saldanha; *"Fibrinogen modulates leukocyte recruitment in vivo during the acute inflammatory response"*; accepted for publication at **Clin. Hemorheol. Microcirc. (2012)**

1. Introduction:

Acute inflammation constitutes an essential part of the host response to eradicate the agent of lesion or infection. This response importantly requires the migration of specific leukocyte populations from the blood circulation towards the inflamed area. Leukocyte recruitment constitutes a complex cellular process by which leukocytes are first recruited to the endothelial vascular wall of postcapillary venules across which they further extravasate into the interstitial tissue. It is mediated via cell-cell interactions between the leukocyte and the endothelium and occurs through a cascade of multiple steps, namely: "tethering", rolling, slow rolling, arrest, post-adhesion strengthening, crawling, and paracellular or transcellular transmigration [1] [2].

Throughout these distinct steps, a myriad of specific factors is sequentially involved. Among these, several adhesion molecules expressed on the cell surface of leukocytes and endothelial cells, such as integrins and selectins, have been shown to be central for leukocyte recruitment. This is the case of members of the β2 integrin family, such as $\alpha_L\beta_2$ and $\alpha_M\beta_2$ (also known as Mac-1 or CD11b/CD18), that have been shown to cooperatively contribute for the firm adhesion of leukocytes to the vessel wall [3]. For such, β2 integrins are required to bind to members of the immunoglobulin superfamily expressed on the surface of endothelial cells, such as endothelial intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [4]. In order to control for their function in leukocyte recruitment, circulating leukocytes maintain their integrin receptors in a low affinity and non-adhesive state under steady state conditions. But in response to local inflammatory stimuli, integrins are rapidly activated to bind specific ligands [5] [6] [7] [8] [9]. Besides ICAM-1, the $\alpha_M\beta_2$ integrin is further capable of binding a remarkable assortment of seemingly unrelated ligands, including the complement C3 derivative, platelet membrane glycoprotein GP1bα and importantly, immobilized fibrinogen [10].

Fibrinogen is a large multidomain plasma glycoprotein that is normally expressed as a dimer of α, β and γ polypeptide chains. This protein is a major player in the coagulation process by polymerizing into the major protein component of blood clots, fibrin [11]. As an acute phase protein, fibrinogen is also involved in other biological responses, such as in inflammation [12]. For this, it requires its recognition by a variety of integrin and non-integrin receptors on multiple cell types. Via these interactions, it can even serve as an intercellular bridging molecule. In inflammation, fibrinogen has been shown to target cellular mediators central to this response, such as neutrophils and endothelial cells [10]. In particular and as a ligand for neutrophil Mac-1, fibrinogen has been shown to play a role in neutrophil signalling by modulating: the generation of second messengers, the production of oxygen free radicals and cell adhesion under inflammatory conditions. Overall, these findings clearly illustrate that leukocyte-fibrinogen interaction can alter leukocyte function and thus can lead to changes in cell migration [3].

Accordingly, our previous studies showed that soluble fibrinogen when present at a physiological concentration (300 mg/dL) modulated the adhesive behaviour of neutrophils *in vitro* [13]. In flow chamber assays, fibrinogen significantly increased the velocity of neutrophils rolling on activated endothelial monolayers without interfering with their total number. Consistently, the capacity of fibrinogen-treated neutrophils to adhere to this endothelial bedding was significantly diminished. These data importantly suggest to us that soluble fibrinogen may modulate leukocyte recruitment towards the vascular wall and thus further influence the inflammatory response [14]. In this respect, fibrinogen may actually act as a non-diffusible cue for leukocyte targeting, for instance through its deposition in the form of a provisional fibrin matrix, an event due to occur at virtually any site of overt tissue damage.

To address the role of fibrinogen in leukocyte recruitment, we decided here to investigate what effects may be imposed onto the recruitment of leukocytes in

inflammation by the absolute absence of fibrinogen in the blood circulation. For such, we employed a mice model bearing a disrupted fibrinogen α chain gene. In both neonatal and adult homozygous fibrinogen α chain-deficient mice (referred throughout this study as homozygous ($\alpha^{-/-}$) mice), all the three distinct fibrinogen chain components (α, β and γ) are immunologically undetectable in the circulation. Accordingly, their blood samples fail either to clot or support platelet aggregation in vitro [15]. With these mice, we made here use of an intravital microscopy approach to inspect the mesentery microcirculation. Such approach has been used extensively in the past to evaluate leukocyte trafficking (rolling velocities, leukocyte flux, adhesion, and transmigration) after exposure to an inflammatory stimuli, as it provides a direct visual observation of leukocytes moving, tether to and roll along the vessel wall before adhering at the site of inflammation [16]. In this study, we were able to show that the lack of circulating soluble fibrinogen interferes with the pattern of leukocyte recruitment in inflammation.

2. Materials and Methods:

2.1. Mice and reagents

Fibrinogen α chain-deficient mice were kindly provided by Stephen Smiley (Trudeau Institute). In order to screen for homozygous and heterozygous fibrinogen α chain-deficient mice, all animals were genotyped by PCR prior to their use in this study. For PCR genotyping, we made use of the following oligonucleotide primers presented from the 5′ to the 3’ ends: mFGAx1F: GCTTCAGCTCCAGTTCTCCTCATGAGCCAT; mFGAiIR: TGCTGGATCAATCCCCAGCAACCGTGAGAG and HPRTx1R: TATTACCAGTGAATCTTTGTCAGCAG. The experimental control group of wild-type (WT) mice referred below were age-matched C57BL/6 mice purchased from Charles River.

All animal were housed in specific pathogen-free (SPF) animal facilities, according to the FELASA recommendation, at Instituto de Medicina Molecular (IMM). They were maintained on a low-fat chow pellet diet with tap water ad libitum using a 12h light/dark cycle. All animals used were euthanized with an intraperitoneal injection of an overdose of sodium pentobarbital (Eutasil 20%, Ceva, Sante Animale) at the end of the experiment or when any signs of pain or distress were observed. The experimental protocol was previously analysed and approved by the competent governmental institution (DGV- Direcção Geral de Veterinaria).

Where not referred, chemicals were purchased from Sigma-Aldrich.

2.2. Animal preparation and surgery

Mice were anesthetized with an intraperitoneal (i.p.) injection of a cocktail containing ketamine hydrochloride 50mg/mL (Ketalar, Pfizer), Xylazine hydrochloride 20mg/mL (Rompun 2%, Bayer) and a saline solution of 0,9% sodium chloride. In order to maintain body temperature, mice were laid on a heating pad kept at 37°C during surgery. This consisted in performing an abdominal midline incision and subsequently exposing the mesentery into the observation platform.

2.3. Intravital microscopy

After surgery, the mesentery was prepared for intravital microscopy for which a schematic representation is depicted in Fig. 1. Throughout tissue exteriorization and the remainder of the experiment, the mesentery was superfused with a thermocontrolled (37°C) Krebs-Henseleit buffered saline solution (132,0mM NaCl ; 2,2mM $NaHCO_3$; 4,7mM KCl ; 2,0 mM $CaCl_2$ and 1,2 mM $MgCl_2$) equilibrated with 5% CO_2 in N_2 (Air Liquide). Upon exteriorization of the mesentery, the mouse was transferred to the stage of a Zeiss LSM 5 Live *confocal line-scanning microscope* (Carl

Zeiss MicroImaging). Microscopic observations were performed with a saline immersion objective (Objective W Plan-Apochromat 20x/1,0) [17].

Under normal conditions, tissue observation immediately following exteriorization of the mesentery revealed an initial increase of the numbers of rolling and adherent leukocytes that declined to new steady state levels after 10 minutes of stabilization. These levels were further considered as our experimental baselines as thereafter, leukocyte adhesion, rolling and rolling velocities in each individual were constant through all the time of acquisition under normal conditions. As such, video acquisition was routinely initiated 10 minutes after exteriorization of the mesentery and performed for a total time of acquisition of 30 minutes for all animals assayed.

Three distinct experimental groups of mice were employed: wild-type C57BL/6 mice (thereafter referred as WT or wild-type); heterozygous fibrinogen α chain-deficient mice (referred throughout this study as heterozygous ($\alpha^{+/-}$) mice) and homozygous fibrinogen α chain-deficient mice (homozygous ($\alpha^{-/-}$) mice). On the other hand, for each group two experimental conditions were assayed: a normal condition and acute inflammatory condition, locally elicited by supplemeting the superfusion saline solution with 10^{-2}M PAF (CalBiochem) 10 min after exteriorization. In each group in each experimental condition, a total of six mice were assayed (n=6).

Acquired video sequences were further analysed with ImagePro-Plus software (Media Cybernetics) to quantify: (i) the number of rolling leukocytes (defined as the number of leukocytes moving at a low-velocity with a high-variance translation of 100μm) as well as their rolling velocities (calculated by v = d/t, where d stands for the displacement of the leukocyte over a period of time, t) and (ii) the number of adherent leukocytes (defined as the total number of leukocytes bound to the vascular wall per unit area for at least 30 sec) [18] [19]. These leukocyte recruitment parameters were determined at 15 min after image acquisition started. For this analysis, leukocytes were manually tagged and their movements on the post-capillary venules monitored.

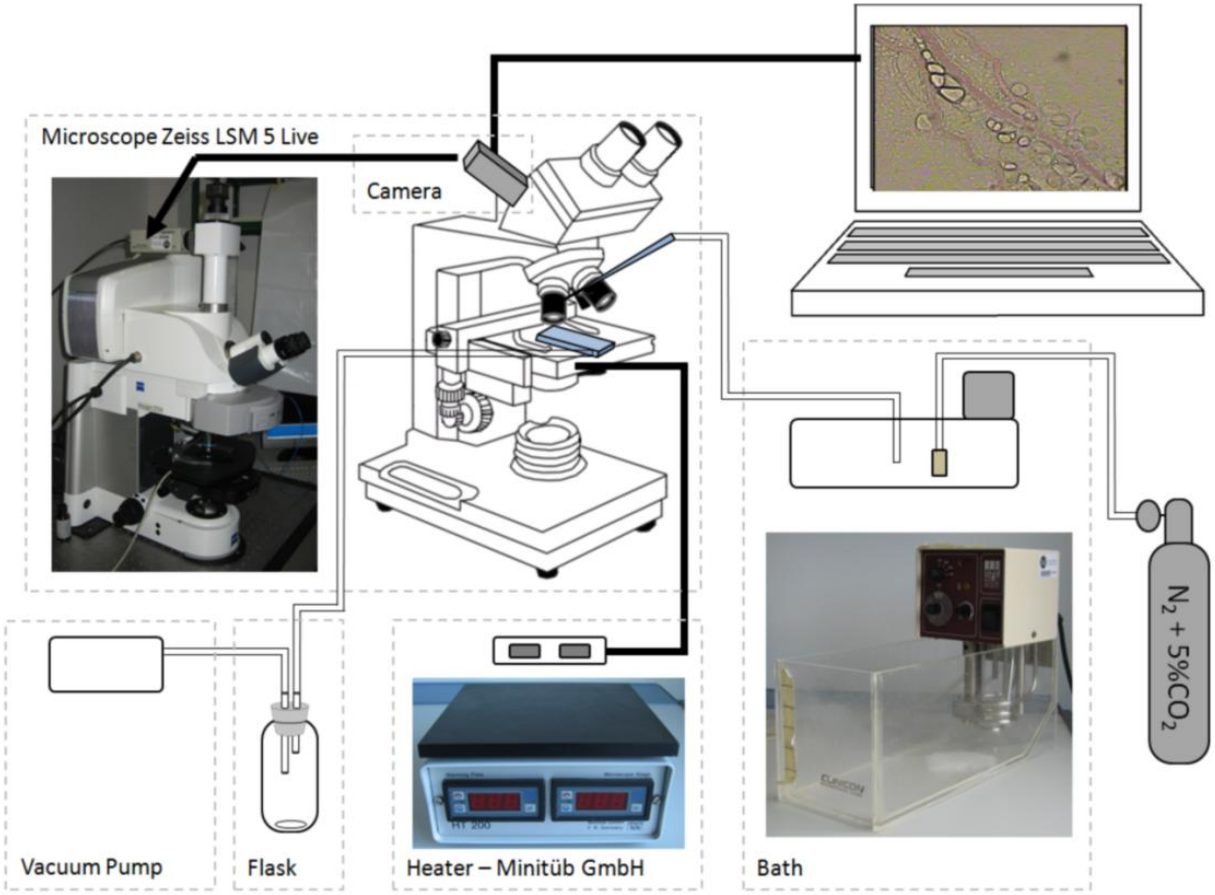

Fig.1: Schematic representation of the assembly of the intravital microscopy experiment.

2.4. Statistical analysis

For each experimental group in a given experimental condition, data were obtained from six independent experiments and expressed as mean values ± standard error deviation (SD). Student´s t test was used to determine the significance of differences between samples means. Statistical analysis was performed using Origin Pro8.

3. Results:

To further investigate this in vivo, we addressed here whether the absence of soluble fibrinogen in the blood circulation could interfere with the normal leukocyte recruitment pattern observed under acute inflammatory processes.

For such, leukocyte recruitment was analysed by using an intravital microscopy approach for the visualization of the mesenteric microcirculation. Here, we made use of a mouse model harbouring a disrupted fibrinogen α chain gene, for which homozygous animals have been shown to express no fibrinogen in the blood circulation [15]. Homozygous mice ($\alpha^{-/-}$) were here assayed in parallel with two groups of animals possessing soluble fibrinogen in their blood circulation, namely wild-type animals as well as in heterozygous ($\alpha^{+/-}$) mice. All these three experimental groups were studied under both normal and acute inflammatory conditions. In order to simulate locally an acute inflammatory environment, the mesentery tissue perfusion was performed in the presence of the platelet activating factor (PAF), a known promoter of neutrophil activation and adhesion [20]. Image acquisition was initiated 10 minutes after the exteriorization of the mesentery from the animal body and performed for a total time of 30 minutes for all animals assayed. The acquired data was further analysed so as to determine three distinct recruitment parameters, namely: the number of leukocytes rolling on the vascular endothelium for which results are

presented in Table 1 and Fig.2; their rolling velocities presented in Table 1 and graphically depicted in Fig.3 and the number of leukocytes adhering to vascular wall, for which results are shown in table 1 and Fig.4. These leukocyte recruitment parameters were determined at 15 min after image acquisition started and 25min after mesentery exteriorization.

Our first aim in this study was to establish whether heterozygous ($\alpha^{+/-}$) mice would show a similar leukocyte recruitment pattern to that observed in wild type animals. The obtained results depicted in Figs 2 to 4 showed that under both experimental conditions, there was no significant difference for the several leukocyte recruitment parameters determined between these two experimental groups. In both groups by comparing acute inflammatory conditions to normal conditions, we observed as expected, an increase in the number of rolling leukocytes and in the number of adherent leukocytes and a reduction of leukocyte rolling. This led us to conclude that leukocytes from both animal groups displayed identical recruitment behaviour.

Leukocyte recruitment was further addressed in the absence of any circulating fibrinogen in the homozygous ($\alpha^{-/-}$) mice. Under inflammatory conditions, these mice displayed a slightly reduced number of rolling leukocytes when compared to normal as depicted in Fig.2 and Table 1. Despite the fact that the observed difference is not significant, this result is unexpected taking into account the data collected for the other two experimental groups. In comparison to these control groups, we further observed that homozygous ($\alpha^{-/-}$) mice exhibited increased and decreased counts of rolling leukocytes, respectively at normal and acute inflammatory conditions.

As obtained for the control groups, we also observed in homozygous ($\alpha^{-/-}$) mice that leukocyte rolling velocities were decreased in the presence of PAF when comparing to the normal conditions, as depicted in Fig.3 and Table 1. Additionally, these results were

higher than those determined for the other two experimental groups both under normal conditions and acute inflammatory.

Finally in what concerns specifically to leukocyte adhesion, our results showed that in homozygous ($\alpha^{-/-}$) mice as for the other animal groups, the induction of an acute inflammatory process resulted in an increased count of adherent leukocytes in comparison to normal conditions as depicted in Fig.4 and Table 1. When comparing these results to those obtained for both control groups, we further observed that similarly as for leukocyte rolling, homozygous ($\alpha^{-/-}$) mice exhibited increased and decreased counts of adherent leukocytes, respectively at normal and acute inflammatory conditions.

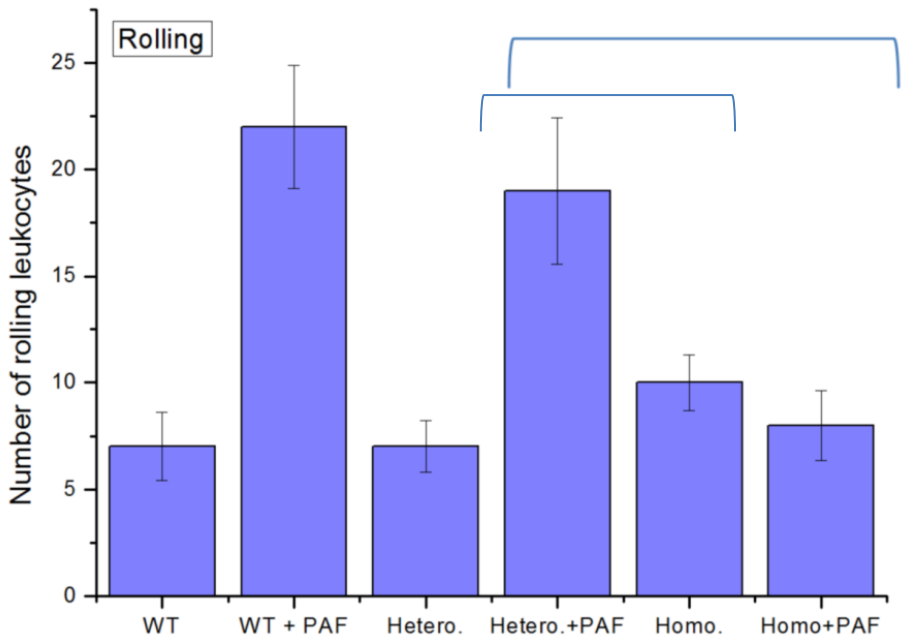

Fig.2: These leukocyte recruitment parameters were determined at 15 min after image acquisition started. Homozygous ($\alpha^{-/-}$) mice displayed a reduced number of rolling leukocytes under acute inflammatory conditions when compared to either wild-type

animals and heterozygous (α $^{+/-}$) mice. This differences were statistically significant ($p < 0,05$). In both control groups, acute inflammatory conditions induced an increase of the number of rolling leukocytes when compared to normal conditions. Paradoxically, an inverse behaviour was observed for homozygous (α $^{-/-}$) mice. This figure displays graphically the number of rolling leukocytes determined for each of experimental groups under each experimental condition. Three experimental groups were assayed: wild-type mice (referred here as WT); heterozygous (α $^{+/-}$) mice (referred here as Hetero) and homozygous(α $^{-/-}$) mice (referred here as Homo). On the other hand, two distinct experimental conditions were used here: normal conditions (used when no treatment is stated) and acute inflammatory conditions stimulated by tissue perfusion with PAF (this condition is here designated as PAF). Represented results are mean values of the data obtained from a total of six mice (N=6) per experimental group per experimental condition. Error bars depict the associated standard error deviations.

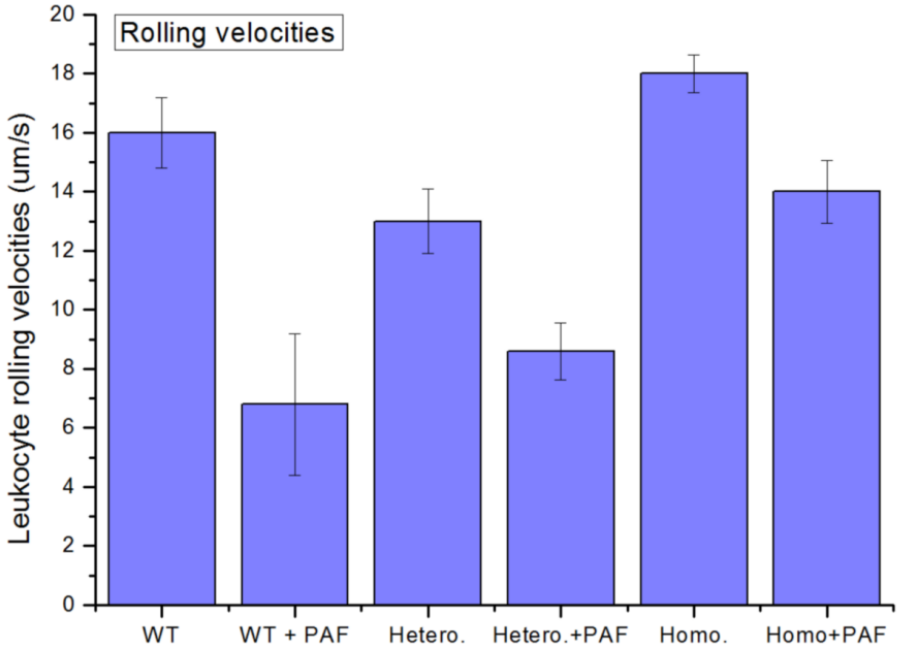

Fig.3: These leukocyte recruitment parameters were determined at 15 min after image acquisition started. The leukocyte rolling velocity in homozygous (α $^{-/-}$) mice is

significantly ($p<0.05$) higher than that determined for heterozygous ($\alpha^{+/-}$) mice as well as for wild-type mice both in the basal state and in an inflammatory environment induced by PAF tissue perfusion. In all experimental groups, leukocyte rolling velocities measured under PAF treatment were lower in comparison to those determined for the normal conditions. This figure displays graphically the leukocyte rolling velocities determined for each of experimental groups under each experimental condition. Three experimental groups were assayed: wild-type mice (referred here as WT); heterozygous ($\alpha^{+/-}$) mice (referred here as Hetero) and homozygous($\alpha^{-/-}$) mice (referred here as Homo). On the other hand, two distinct experimental conditions were used here: normal conditions (used when no treatment is stated) and acute inflammatory conditions stimulated by tissue perfusion with PAF (this condition is here designated as PAF). Results are here represented as mean values of the data obtained from a total of six mice (N=6) per experimental group per experimental condition. Error bars depict the associated standard error deviations.

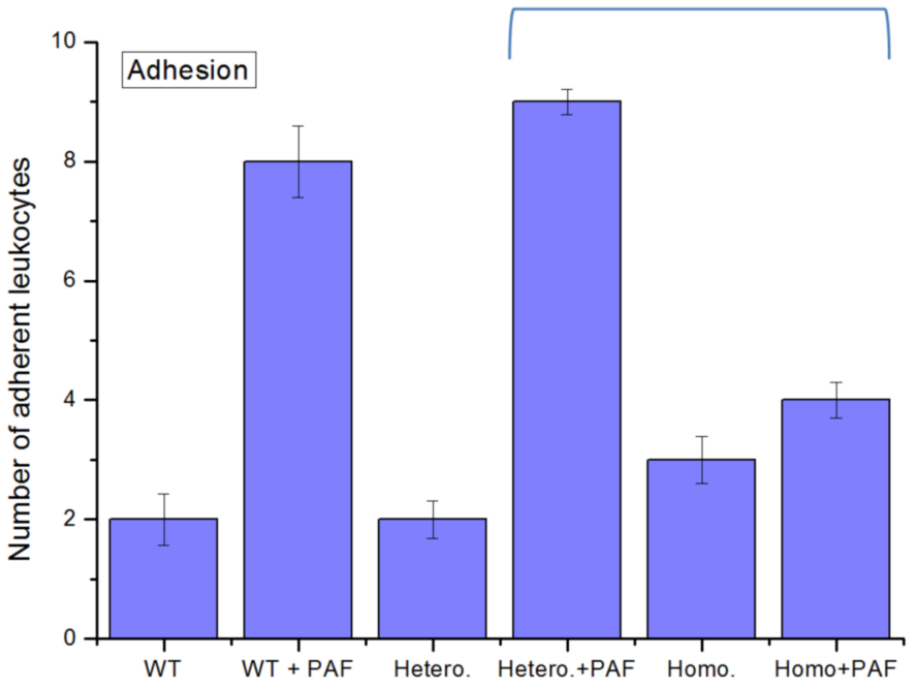

Fig.4: These leukocyte recruitment parameters were determined at 15 min after image acquisition started. The number of adherent leukocytes observed in homozygous ($\alpha^{-/-}$) mice was significantly lower than that measured in either wild-type animals or heterozygous ($\alpha^{+/-}$) mice under acute inflammatory conditions ($p < 0,01$). Wild type animals and heterozygous ($\alpha^{+/-}$) mice displayed a similar number of adherent leukocytes in presence of PAF. This figure displays graphically the number of adherent leukocytes determined for each of experimental groups under each experimental condition. Three experimental groups were assayed: wild-type mice (referred here as WT); heterozygous ($\alpha^{+/-}$) mice (referred here as Hetero) and homozygous($\alpha^{-/-}$) mice (referred here as Homo). On the other hand, two distinct experimental conditions were used here: normal conditions (used when no treatment is stated) and acute inflammatory conditions stimulated by tissue perfusion with PAF (this condition is here designated as PAF). Represented results are mean values of the data obtained from a total of six mice (N=6) per experimental group per experimental condition. Error bars depict the associated standard error deviations.

Table 1: Leukocyte recruitment parameters determined under normal conditions as well as under acute inflammatory conditions. Three experimental groups were used namely, wild-type mice (referred here as wild-type); heterozygous $\alpha^{+/-}$ mice (referred here as Heterozygous) and homozygous $\alpha^{-/-}$ animals (referred here as Homozygous). Acute inflammatory conditions were elicited via mesentery perfusion with a saline solution supplemented with PAF (this conditions is here designated as PAF) and under normal conditions for which tissue perfusion was performed in the absence of PAF (condition used when no PAF treatment is stated). For each condition in each experimental group, six assays were performed (N=6). Results are presented as mean values with the associated standard error deviations.

	Number of rolling neutrophils	Number of adherent neutrophils	Rolling Velocities (µm/s)
Wild-type	7 ± 1,6	2 ± 0,4	16 ± 1,2
Wild-type + PAF	22 ± 2,8	8 ± 0,6	6,8 ± 2,4
Heterozygous	7 ± 1,2	2 ± 0,3	13 ± 1,1
Heterozygous + PAF	19 ± 3,4	9 ± 0,2	8,6 ± 0,9
Homozygous	10 ± 1,3	3 ± 0,4	18 ± 0,6
Homozygous + PAF	8± 1,6	4 ± 0,3	14 ± 1,1

4. Discussion:

Previously, the use of flow chamber assays have enabled us to demonstrate *in vitro* that the presence of soluble fibrinogen at a physiological concentration modulated the adhesive behaviour of human neutrophils [14]. The evaluation of these data led us to suggest that apart from other contributions to the inflammatory process, fibrinogen could as well modulate specifically the recruitment of leukocytes towards the vascular wall in inflammation. This was precisely the main goal of the present study.

To address this, leukocyte recruitment was assayed by intravital microscopy of the post-capillary venules of mouse mesentery under acute inflammatory conditions in the absence or presence of soluble fibrinogen in the blood circulation. For such, we made use of a mouse model bearing a disrupted fibrinogen α chain gene for which homozygous mice do not express any experimentally detected plasmatic soluble fibrinogen. As control mice, we made use of two groups of animals namely, wild-type mice and heterozygous mice, which express soluble fibrinogen in the blood circulation.

Leukocyte recruitment is initiated with the capture of free-flowing leukocytes to the vessel wall which is then followed by the rolling of leukocytes along the endothelial surface layer. This movement is further slowed down so as to favour the subsequent adhesion of leukocytes to and their transmigration through the vascular wall. Our experimental setting allowed us here to observe directly the contribution of this plasmatic protein for the two major steps of this recruitment process namely, leukocyte rolling and leukocyte adhesion. For such, the detailed analysis of the acquired videos enabled us to determine three important leukocyte recruitment parameters: the number of rolling leukocytes; the rolling velocities of these leukocytes and finally, the number of leukocytes adhering to the vascular wall of the imaged post-capillary venules.

Through this approach, we were initially able to understand that although possessing different levels of plasmatic soluble fibrinogen, leukocytes from the two control groups in use exhibited a normal and identical recruitment behaviour. These results suggest that the levels of soluble fibrinogen expressed in heterozygous ($\alpha^{+/-}$) mice should be enough to support its putative contribution to leukocyte recruitment.

In addition, we were able to further conclude that leukocyte recruitment is negatively affected by the absence of soluble fibrinogen in the blood circulation. In fact, we observed that in the mesentery of homozygous ($\alpha^{-/-}$) mice subjected locally to PAF,

leukocytes in the microcirculation presented an abnormal pattern of rolling and adhesion along the endothelial wall of postcapillary venules. These in vivo results came in support to our previous in vitro observations obtained in a flow chamber model [14]. In this first study, we have shown that neutrophil rolling velocities were substantially diminished when these cells were pre-incubated in the presence of a physiological concentration of soluble fibrinogen in comparison to an untreated condition. Consistently, the number of neutrophils adhering to the endothelial monolayer was augmented upon pre-treatment with soluble fibrinogen. In the present study that information was further complemented with the fact that in comparison to the control animals, displayed consistently under inflammatory conditions: a substantially reduced number of rolling leukocytes that were actually migrating at higher rolling velocities and consistently, a diminished number of adherent leukocytes.

Overall these results are consistent as higher rolling velocities can logically be converted in a reduced number of slow rolling events and consequently into a reduced number of adherent leukocytes. In fact, for a successful leukocyte adhesion, the rolling velocities must gradually decrease as a function of their contact time with the inflamed endothelium.

Altogether these studies further lead us to propose that soluble fibrinogen constitutes an important factor for the capture of the leukocytes by the activated endothelium. The mechanisms behind this effect are still not fully understood. Notwithstanding, they most probably, relate to the fact that soluble fibrinogen is not only able of individually binding either to the neutrophil membrane or to the endothelial cell, but is moreover able to bridge these two cellular entities thus leading to a more stable and effective recruitment process.

Importantly, these results have profound implications for the inflammatory response. Leukocyte recruitment from the blood is a central event in the innate immune

responses to invading pathogens. In these pathological situations, leukocytes need to cross rapidly the vascular wall to reach the site where the inflammatory is taking place. If this ability is somehow compromised as observed here in homozygous ($\alpha^{-/-}$) mice due to the absence of proper levels of soluble fibrinogen in the blood circulation, the normal progression of the elicited inflammatory process will be as well severely affected and a deficient inflammatory response will be mounted. Such a situation may ultimately evolve towards extreme consequences such as sepsis among others that will further put the organism's health at risk.

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PART THREE

Chapter V –Discussion and Conclusion

Fibrinogen is a multifunctional glycoprotein that besides being critically enrolled in haemostasis plays adhesive and inflammatory functions through specific interactions with specific cellular types. In inflammation, it has been importantly reported to impact on the function of a cellular player that stands as one of the first lines of defence against invading pathogens, the neutrophil. More precisely, it has been shown to modulate neutrophil signalling so as to potentiate the generation of second messengers, production of oxygen free radicals and cell adhesion under inflammatory conditions.

Among several molecular targets, fibrinogen constitutes a high-affinity ligand for the Mac-1 integrin. This protein receptor is a member of the family of the β2-integrins and is expressed in a low-affinity state on the membrane surface of resting neutrophils. During neutrophil recruitment in inflammation, G protein–coupled receptors on rolling cells encounter and bind to chemokines sequestered on the apical endothelium. These binding events lead to 'inside-out' signalling which induces conformational changes in β2 integrins, such as Mac-1, and that increase their avidity and affinity. As a result, rolling neutrophils arrest and subsequently, crawl intravascularly to transmigration sites.

Taking all this data into account, one possible scenario would be that fibrinogen could modulate the neutrophil function via binding to Mac-1. More precisely, one could envisage that the fibrinogen could induce those effects by targeting this integrin in a low-avidity state. This plasmatic glycoprotein could then function as a primer for resting neutrophils and thus decrease the threshold for cell activation. Moreover, this line of thinking led us to hypothesize that upon binding Mac-1, plasma fibrinogen might potentiate neutrophil-endothelial cell interaction by priming the neutrophil to a more

adhesive phenotype. Taken this, our goal in the present work was to address whether circulating fibrinogen could also modulate neutrophil recruitment and thus understand its role in the mechanism of the microvascular inflammatory response.

To address our working hypothesis, we first investigated whether soluble fibrinogen could modulate neutrophil activation independently of any occurring inflammatory process. This was evaluated in vitro in the presence of this glycoprotein, by analysing the production of oxygen free radicals (OFR) of human neutrophils isolated from peripheral blood. We could observe here, that at physiological concentrations, fibrinogen could in fact induce by itself OFR production. Such result strongly supports the idea that under physiological conditions, this plasmatic glycoprotein can already modulate the neutrophil activation status. In view of the above mentioned data, the neutrophil Mac-1 could come as a logical mediator of such effect. Despite this, we presented here evidence to support that this is not actually the case: the fibrinogen-mediated OFR production occurred independently of Mac-1 activation status and most importantly, of the binding of fibrinogen to this integrin. As such, these results suggest that fibrinogen should exert this effect by targeting an unravelled receptor on the neutrophil surface. On the other hand, these results led us further to propose that soluble fibrinogen may as well modulate the adhesive behaviour of the neutrophil, i.e. it may interfere with the ability of the neutrophil to adhere to the endothelium under normal conditions.

In order to investigate this possibility, we first made use of an in vitro approach based on the employment of a flow chamber assay in which neutrophils were flown over endothelial monolayers at wall shear stress conditions closely mimicking the in vivo microcirculatory situation. Our results showed that at least in vitro, the number of rolling neutrophils is not affected by the presence of physiological concentrations of soluble fibrinogen. However, the velocity of these rolling leukocytes was significantly increased and the number of adherent neutrophils significantly diminished under flow

in the presence of a physiological concentration of fibrinogen. On the one hand, these latter results are intrinsically consistent, as leukocyte adhesion is a direct consequence of the leukocyte rolling velocity. In fact, it has been extensively documented that the speed of a leukocyte rolling along the endothelium decreases gradually until its successful arrest [1] [2]. On the other hand, these results obtained *in vitro* strongly suggested to us that fibrinogen should importantly act as a regulator of the microcirculation homeostasis by modulating the interaction between neutrophils and the endothelium wall at normal physiological concentrations. In this respect, fibrinogen can be part of a vascular mechanism devised to prevent undesirable accumulation of neutrophils in the vasculature and moreover, to refrain thrombus growth.

If playing such a homeostatic role, we reasoned that the absence of soluble fibrinogen in the blood circulation could interfere with the normal leukocyte recruitment pattern observed under acute inflammatory processes. To address this issue, we employed a mouse model bearing a disrupted fibrinogen α chain gene that consequently, does not express any experimentally detectable fibrinogen in the blood circulation in homozygous animals. By studying leukocyte recruitment by an intravital microscopy approach, we were able to observe *in vivo* that leukocyte recruitment is negatively affected by the absence of soluble fibrinogen in the blood circulation. In fact, leukocytes in the microcirculation of the mesentery of homozygous ($\alpha^{-/-}$) mice subjected locally to PAF presented an abnormal pattern of rolling and adhesion along the endothelial wall of postcapillary venules in comparison to what was observed in animal control groups. More precisely, leukocytes in fibrinogen deficient mice were observed to roll along the vascular endothelium with higher rolling velocities and the number of slow rolling events and that of adherent leukocytes were significantly reduced. As such, this study highlighted the requirement of soluble fibrinogen for leukocytes to be efficiently recruited to the endothelium in acute inflammatory

conditions. Accordingly, a delay in the inflammatory response has been described previously for this fibrinogen-deficient animal model upon exposure to endotoxin [3].

Altogether, the results obtained in this work support the role of fibrinogen as an important factor in the capture of leukocytes by the activated endothelium. As described in the Introduction, this plasmatic glycoprotein is possibly able to interact with several receptors on multiple cell types for what its symmetrical dimer structure of αβγ heterotrimers greatly contributes. In view of this notion, one can envisage that this soluble plasmatic protein should be able to interact simultaneously with neutrophils and endothelial cells. This way, fibrinogen would function as a bridge between these leukocytes and the vascular wall and would thus stabilize this inter-cellular interaction and actively favour a more efficient leukocyte recruitment.

Such bridging activity has been actually proposed to be of utmost relevance in the infection by the leading invasive strain of group A Streptococcus [4]. As a major virulence factor, this strain expresses the M1 protein, an α-helical coiled-coil surface protein that upon release from the streptococcal surface, induces toxic shock-like vascular leakage and tissue injury. The net result of such activity is a massive inflammatory response with profound pathophysiological consequences that lead to a streptococcal toxic shock syndrome (STSS), one of the most acute and severe forms of septic shock. These M1-mediated pathological properties require the interaction of this bacterial protein with the host's fibrinogen. In fact, these events are triggered by the formation of a complex between M1 and fibrinogen that, unlike M1 alone, leads to neutrophil activation. For such, the M1-fibrinogen complex has been shown to bind to β2 integrins on neutrophils and thus to trigger the release of heparin binding protein (HBP), a potent vasodilator, which induces vascular leakage and intravascular coagulation with the consequent end-organ damage. Such complex has been also shown to activate platelets in an integrin dependent manner, leading to further activation of neutrophils as well as of monocytes. Importantly, it has been further

demonstrated that disruption of the M1-fibrinogen network into fibers or sparse networks resulted in loss of neutrophil activation strongly suggesting that the density of fibrinogen in the network is the critical factor in neutrophil activation [4] .

Besides standing as a paradigmatic example of how fibrinogen may bridge distinct molecular and cellular players, this streptococcal strain represents as well one of several menaces that currently challenge our knowledge on human immunity. Among these, one can also encounter antibiotic-resistant bacteria, such as *Staphylococcus aureus* and multidrug-resistant Gram-negative bacilli, and viruses (such as the H5N1 and H1N1 influenza viruses and the SARS corona virus). Upon infection with these pathogenic agents, the host is rendered more susceptible to secondary infections.

Tweaking host immunity may be a way to fight these emerging pathogens. This will necessarily imply a deeper insight into the mechanisms that govern the action of the several cellular and molecular players of the immune response in response to such threats. In this respect, the neutrophil plays a central role by patrolling the blood in a continuous search of “prey”, which can present itself primarily in the form of bacteria or dead and dying host cells and by further acting so as to eliminate and eradicate the infectious agents. However, the task of preventing pathogen invasion is not necessarily a trivial one. A major contribution of the present work was to stress that besides contributing to the neutrophil function in inflammation, soluble fibrinogen plays as well an important role in modulating the activation and recruitment of the neutrophil under physiological conditions. Importantly, this work came in support of the notion that there is still a lot to know about the partnership between the fibrinogen and neutrophils in the battle towards invading pathogens.

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Neutrophil Polarization and Migration," *Molecular Biology of the Cell,* vol. 16, p. 5773–5783, 2005.

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PART FOUR

Curriculum Vitae

Personal information	
Name	**Vanda Lúcia de Carvalho Vitorino de Almeida**
Address	Avenida Paulo VI nº2, 1950-229 Lisboa, Portugal
Mobile	00351 962309996
Email	vandaalmeida@fm.ul.pt / vlcvalmeida@gmail.com
Nationality	Portuguese
Date of Birth	18th of June, 1979
Gender	Female
Research Interests	**Inflammation, Microcirculation, Intravital microscopy, Biology and Function of Neutrophils, Teaching**
Current Status	**PhD student** granted by Fundação para a Ciência e Tecnologia (FCT) at Unit of Microvascular Biology and Inflammation, Institute of Molecular Medicine, Lisbon –Portugal since January 2007
Work Experience	
Dates	21st - 29th of June 2010
Employer name	Institute of Molecular Medicine – Faculty of Medicine form University of Lisbon - Unit of Microvascular Biology and Inflammation. Av. Professor Egas Moniz 1649-028, Lisboa – Portugal
Position	**Invited teacher**
Main activity	Preparing and lecturing the "14º initiation of investigation course" for medical students
Dates	March 2010 – until June 2010
Employer name	Institute of Molecular Medicine – Faculty of Medicine form University of Lisbon - Unit of Microvascular Biology and Inflammation. Av. Professor Egas Moniz 1649-028, Lisboa – Portugal

Position	**Co-orientation of Laboratory Training course for medical students**
Main activity	Primary Culture of Hepatocytes and Microsurgical techniques for Intravital Microscopy and collecting the liver.
Dates	March 2008 – until June 2008
Employer name	Institute of Molecular Medicine – Faculty of Medicine form University of Lisbon - Unit of Microvascular Biology and Inflammation. Av. Professor Egas Moniz 1649-028, Lisboa – Portugal
Position	**Co-orientation of Laboratory Training course for medical students**
Main activity	Studying Leukocyte adhesion in hypertensive Rats with Microsurgical techniques for Intravital Microscopy of the mesentery and cremaster muscle.
Dates	January 2007-Until Now
Employer name	Institute of Molecular Medicine – Faculty of Medicine form University of Lisbon - Unit of Microvascular Biology and Inflammation. Av. Professor Egas Moniz 1649-028, Lisboa – Portugal
Position	**PhD student** (project reference: SFRH / BD / 30145 / 2006)
Main activity	Research work concerning the role of fibrinogen in the mechanism of microvascular inflammatory response. The following experimental techniques have been used: fluorescence confocal microscopy, Intravital microscopy, flow cytometry, cell culture techniques, and microcirculation studies with in vitro and in vivo models.
Dates	February 2006 – January 2007
Employer name	Institute of Molecular Medicine – Faculty of Medicine form University of Lisbon – Unit of Vascular Biopathology. Av. Professor Egas Moniz 1649-028, Lisboa – Portugal
Position	**Researcher**
Main activity	Research work concerning the biology of neutrophils, using techniques as flow cytometry, confocal microscopy and fluorescence spectroscopy.
Dates	June 2005 - February 2006
Employer name	Project “CHLASTS- Chemical Laboraty Safety Training System”

	from the program Leonardo Da Vinci. CiCTSUL Interdisciplinary Centre of Science Technology and Society of University of Lisbon
Position	**Researcher**
Main activity	Developing new materials and techniques to teach chemical laboratory safety, to students, teachers and laboratory technicians.
Dates	June 2004 – June 2005
Employer name	Project "For a History of the Teaching of Chemistry, in the XIXth and XXth Centuries (until 1974 Revolution)". From CiCTSUL Interdisciplinary Centre of Science Technology and Society of University of Lisbon. Financed by Fundação para a Ciência e Tecnologia (FCT) (project reference: POCTI / CED / 41990 / 2001)
Position	**Researcher**
Main activity	Investigated teaching techniques, laboratory facilities, and documents from the XIXth and XXth Centuries until 1974 Revolution.

Education and training

Dates	10th – 12th of May, 2010
Institution name	Institute of Molecular Medicine
Course name	"**Cell Migration in Health and Disease**"
Principal subjects/ occupational skills covered and achieved	Fundamental mechanisms in cell behaviour during adhesion and migration both during normal development and in diseases.
Dates	04th – 06th of May, 2009
Institution name	Institute of Molecular Medicine
Course name	"**Nanomedicine**"
Principal subjects/ occupational skills covered and achieved	Nanomedicine, or nanotechnology applied to medical problems, artificial nanostructures, such as nanoparticles and nanodevices, nanotechnology-based imaging, targeted drug delivery and release, regenerative medicine and practical sessions on atomic force microscopy (AFM)

Dates	16th – 20th of February, 2009
Institution name	ICVS – Life and Health Sciences Research Institute, University of Minho- Portugal
Course name	**Animal Cell and Tissue Culture: From Basic Principles to Advanced Techniques**
Principal subjects/ occupational skills covered and achieved	Cell and tissue culture techniques. Topics covered: cell differentiation, cloning and selection, cell separation, characterization, transformation and molecular techniques.
Dates	20th – 22nd of January, 2009
Institution name	Institute of Molecular Medicine in collaboration with Zeiss International
Course name	**Advanced Optical Sectioning Microscopy Course**
Principal subjects/ occupational skills covered and achieved	Spatial resolution, optical sectioning methods, single molecule resolution in microscopy and techniques to separate multiple fluorescence dyes with laser scanning microscopy.
Dates	31st March – 3rd April, 2008
Institution name	Institute of Molecular Medicine
Course name	**Flow Cytometry**
Principal subjects/ occupational skills covered and achieved	Fundamentals of Flow Cytometry, Calibration of FACSCalibur/FACSCanto, Potential Flow Cytometry Applications, Fluorescence-Activated Cell Sorting, FACSAria principles, Three-color calibration / acquisition – FACSCalibur, Analysis – CELLQUEST / FLOWJO, Intro to Diva. Six-color calibration / acquisition - FACSCanto
Dates	14th – 16th January, 2008
Institution name	Institute of Molecular Medicine
Course name	**Nano Course on Immunology**
Principal subjects	Immunology research, imaging in immunology, dendritic cells, NKT cells, T cells, tumour immunology and transplantation immunology.
Dates	12th – 19th November, 2007

Institution name	Institute of Molecular Medicine
Course name	**BioImaging Nano Courses**
Principal subjects/ occupational skills covered and achieved	Light Microscopy, Fluorescent Microscopy, Confocal Microscopy, 3D Microscopy and Live Imaging.
Dates	19th – 30th March, 2007
Institution name	CIISA / Faculty of Veterinary Medicine of Technical University of Lisbon in collaboration with the Department of Laboratory Animal Science, Utrecht University
Course name	**VI Course on Laboratory Animal Science**
Principal subjects/ occupational skills covered and achieved	Ethics of animal experimentations, legislation, housing and caging, welfare and experimental procedures, practical handling and restraining techniques, collection of body fluids, administration of substances, microsurgical techniques, peri-operative care, analgesia, anaesthesia, euthanasia and necropsy
Dates	1999 – 2005
Institution name	Faculty of Science of the University of Lisbon-Portugal
Degree achieved	**Graduation on Biochemistry**
Final grade	15 (0 – 20)

Personal skills

Mother tongue(s) **Portuguese**

Other language(s)

Self-assesssment	Understanding		Speaking		Writing
	Listening	Reading	Spoken interaction	Spoken production	
English	Excellent	Excellent	Excellent	Excellent	Excellent
Spanish	Good	Good	Fair	Fair	Fair
French	Fair	Fair	Fair	Fair	Fair

Computer skills and Windows, Microsoft Office, Statistical analysis (Graph prism,

competences (as a user)	OriginPro8, Mathematica6), Image Analysis (Image J, Photo Shop, Image Pro Plus).
Attended Conferences	
2010	Participation in the: "Joint FEBS/EFIS Workshop: Inflammatory Diseases and Immune Response: Basic Aspects, Novel Approaches and Experimental Models" with a poster entitle: **"Modulation of neutrophil recruitment by fibrinogen"**.
2009	Participation in the "Tri-Society Conference of the International Cytokine Society, International Society of Interferon and Cytokine Research and Society for Leukocyte Biology" with a poster entitle **"Fibrinogen and Neutrophil Recruitment"**.
2008	Participation in the "25th Conference of the European Society for Microcirculation" with a poster entitle: **"Differential effect of soluble fibrinogen as a neutrophil activator".**
2007	Participation in the: 8th World Congress on Inflammation" with a poster entitle: **"Neutrophil Activation: fibrinogen dependency"**.
2007	Participation in the XXVIII Congresso Português de Cardiologia: **"The role of fibrinogen in the mechanism of microvascular inflammatory response"** with an oral communication.
2006	Participation in the: V Congresso do Investigação em Medicina em Pedagogia e Ciência with a poster entitle: **"Fibrinogen as a neutrophil activator"**.
2005	Participation in the: 4º Encontro Nacional da Divisão de Ensino e Divulgação da Química" with one poster entitle: **"Química Viva! – Química Segura!"**.
2005	Participation in the: "5th International Conference on the History of Chemistry" with one poster entitle: **"Retorts – Mythic pieces of chemical equipment"**.

2002	Participation in the: XIII Congresso Nacional de Bioquímica, with two posters entitle: **"TBT Effects on Cholesterol and Lipids Metabolism"** and **"Mammals evaluation of TBT effects as an endocrine disruptors".**

Publications

V. Vitorino de Almeida, A. Calado, H. S. Rosário and C. Saldanha; **"*Differential effect of soluble fibrinogen as a neutrophil activator.*"**; (2012) Microvascular Research.; 83(3):332-6.

S. De Oliveira, V. Vitorino de Almeida, A.Calado, H. S. Rosário, and C. Saldanha; ***"Integrin-associated protein (CD47) is a putative mediator for soluble fibrinogen interaction with human red blood cells membrane"***; (2011) Biochim Biophys Acta.; 1818(3):481-490

P. de Oliveira, A.Q. Gomes, T.R. Pacheco, V. Vitorino de Almeida, C.Saldanha and A. Calado; **"*Cell-specific regulation of acetylcholinesterase expression under inflammatory conditions*"**; (2012) Clin Hemorheol Microcirc.

V. Vitorino de Almeida, A. Calado , A.S.S. Herdade, H. S. Rosário and C. Saldanha; **"*An in vitro study on the modulation of the neutrophil adhesive behavior by soluble fibrinogen*"**; (2012) accepted for publication at Clin Hemorheol Microcirc.

V. Vitorino de Almeida, A.S.S. Herdade, A. Calado, H. S. Rosário and C. Saldanha; **"*Fibrinogen modulates leukocyte recruitment in vivo during the acute inflammatory response*"**; (2012) accepted for publication at Clin Hemorheol Microcirc

Additional information

Driving license	Since October 1999