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„Platelet TLR/PI3K/Akt signalling augments neutrophil mediated clearance of periodontal bacteria“

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1 Preface

This thesis is based upon studies conducted during December 2008 to January 2010 at the Institute of Clinical Microbiology and the Institute of Physiology at the Medical University of Vienna.

I would like to express my sincere gratitude to my supervisor Ivo Volf at the Institute of Physiology (Department of Physiology and Pharmacology) at the Medical University of Vienna. Without his advice and unique support this thesis would never have become reality.

I also would like to thank my co-supervisor Thomas Decker from the Institute of Genetics at the University of Vienna for admitting and supervising this project.

Further, I would like to express my gratitude to Markus Laky at the Bernhard Gottlieb Clinic for Dental Research and Alexander Hirschl at the Institute of Clinical Microbiology at the Medical University of Vienna for their advice and support.

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Alice Assinger, June 2010

2 Contents

Introduction

2.1 Periodontitis and periodontitis associated bacteria

Periodontitis associated diseases are highly prevalent and can affect up to 90 % of the worldwide population. Gingivitis, the mildest form of periodontitis associated disease, is caused by the bacterial biofilm that accumulates on teeth adjacent to the gingiva. However, gingivitits does not affect the underlying supporting structures of the teeth and is reversible. Inflammation that extends deep into the tissues and causes loss of supporting connective tissue and alveolar bone is designated as periodontitis. Periodontitis is a chronic inflammation of the supportive dental tissues, which occurs in response to a complex interplay of subgingival infections by certain gram-negative bacteria and host response. This results in continued loss of periodontitis associated attachment and makes periodontitis one of the main causes of tooth loss in adults (Pihlstrom et al., 2005).

Moreover accumulating evidence suggests that periodontitis is also associated with an increased risk for cardiovascular diseases. Several studies have investigated the association between periodontitis associated disease and atherosclerosis or its major clinical complication, coronary heart disease. These studies suggest that poor dental health may be associated with coronary heart disease events, even after adjustment for established cardiovascular risk factors (Beck et al., 2001;Buhlin et al., 2003a). Several clinical trials reported a causal relationship between cardiovascular disease and periodontitis associated disease and meta analysis revealed that the increase in risk appears to be approximately 20% (Janket et al., 2003).

The causal connection between oral conditions and cardiovascular disease might underlie to transient bacteraemia.

The ulcerated pocket epithelium in active periodontitis associated disease may facilitate entrance of oral microorganisms into the circulation. Gram-negative

periodontitis associated pathogens often find their way into the bloodstream (bacteraemia) in patients with periodontitis associated disease as a result of oral hygiene procedures like scaling or root planing or even during everyday activities like chewing or teeth brushing (Forner et al., 2006).

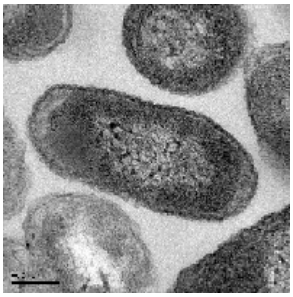

Figure 1 | Electron microscopic picture of A. actinomycetemcomitans Y; *kindly provided by Prof. Dr. Josef Neumüller (Medical University of Vienna)*

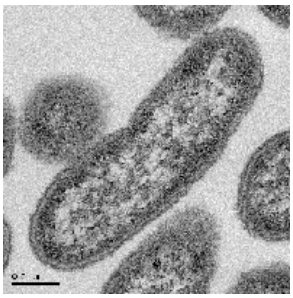

Figure 2 | Electron microscopic picture of P. gingivalis; *kindly provided by Prof. Dr. Josef Neumüller (Medical University of Vienna)*

There is a certain group of pathogens, among them Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis (depicted in Figure 1 and Figure 2), clearly associated with periodontitis associated diseases (Van Winkelhoff et al., 2002), proven to enter the blood flow (Lafaurie et al., 2007b) and also detected in various vascular lesions (Chiu, 1999).

Although these bacteraemia decline within an hour (Lafaurie et al., 2007a), it has long

been recognized that oral bacteria may cause distant site infections and systemic increases of various proinflammatory mediators, such as C reactive protein and interleukin 1b (IL-1b) and IL-6 and tumour-necrosis factor α (Buhlin et al., 2003b;Loos, 2005;Paraskevas et al., 2008).
In experimental models it could be shown that periodontitis associated pathogens promote the formation of foam-cells (Qi et al., 2003) and the development of atheroma (Li et al., 2002).
There is a growing body of evidence that platelets are involved in inflammatory processes and also have an impact on the fight against infection (Clark et al., 2007c;Yeaman, 2010). Moreover it could be shown that platelets from periodontitis patients have an increased activation status compared with platelets from healthy controls (Papapanagiotou et al., 2009).

2.2 Neutrophils

Neutrophils, the most abundant type of white blood cells, are the first line of defence against bacteria that invade tissues and blood.
They efficiently kill microbes by phagocytosis or exposing them to a variety of potent oxidizing agents.

Each phagocytic event results in the formation of a phagosome into which reactive oxygen species and bactericide enzymes are secreted.

Moreover neutrophils release these antibacterial substances and are thereby able to kill extracellular microbes.

Recently another mechanism on how neutrophils kill bacteria has been recognized. Neutrophils release of web-like structures of DNA, so called neutrophil extracellular traps (NETs) that trap and kill microbes extracellularly (Hickey and Kubes, 2009). These NETs kill microbes independent of phagocytic uptake and serve as a physical barrier that prevents spread of pathogens. It could be shown that platelets play an essential role in the formation of NETs (Clark et al., 2007b).

Besides defence against invading microbes neutrophil recruitment contributes to local inflammation and possibly tissue repair but may also encourage tissue damage, as recently reported in aggressive periodontitis (Kantarci et al., 2003).

2.3 Platelets

Platelets are produced in the bone marrow by megakaryocytes as anucleate cells lacking genomic DNA, but contain megakaryocyte-derived messenger RNA and the translational machinery for protein synthesis.

Upon injury of the blood vessel platelets build a barrier against blood loss by aggregation, a process called primary haemostasis.

Recently platelets have also been recognized as immunmodulatory cells. Since they secrete several antimicrobial peptides like “regulated upon activation normal T-cell expressed and secreted“ (RANTES), platelet factor 4 (PF-4), thymocin-β and its derivates, which can deaden microbes by forming pores in the microbial membrane (Peschel, 2002;Weber, 2005). RANTES and PF-4 also act as chemoattractants for monocytes and promote their differentiation into macrophages.

Apart from secreting factors important for immune response, platelets can also directly interact with cells of the immune system and platelet P-selectin binding to P-selectin glycoprotein ligand-1 (PSGP-1) on leukcytes enhances their adhesion to endothelial tissue.

Platelet-monocyte binding favours transmigration of monocytes to sites of inflammation (Diacovo et al., 1996a;Pitchford et al., 2005), which also leads to translocation of platelets into extravascular tissue, where they can further interact with leucocytes (Kehrel and Jurk, 2004).

Platelets also participate in inflammatory processes by the release of soluble agents, like Interleukin-1β (IL-1β) that induces endothelial cell gene expression that mediates adhesion of neutrophils and monocytes to the endothelium (Gawaz et al., 2005).

Another important inflammatory mediator released by activated platelets is the CD40 Ligand (CD40L, CD154), which is structurally related to cytokine tumour necrosis factor

α (TNFα). CD40L is stored in the cytoplasma of resting platelets and rapidly appears on the cell surface after platelet activation. In a period of minutes to hours (depending on the activator) CD40L undergoes cleavage from the platelet surface and a functional soluble fragment is generated (Hermann et al., 2001). CD40L can trigger specific immune response through dendritic cell maturation, T cell activation and isotype switching of immunoglobulin from IgM to IgG (Ni et al., 2000). Moreover, CD40L can induce inflammatory responses in the endothelium, such as the release of interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) (Henn et al., 1998), as well as secretion of chemokines and adhesion molecules for leukocyte recruitment (Langer and Gawaz, 2008).

Elevated serum levels of CD40L indicate an acute risk for coronary events (Heeschen et al., 2003;Liu et al., 2008). Since platelets are the main source of CD40L their crucial role in the pathogenesis of atherosclerosis is undoubted.

2.3.1 Platelet TLRs

Bacteria are commonly recognized by cellular components of the innate immune response via toll like receptors (TLRs), a class of proteins that bind conserved molecular structures of microorganism and represent evolutionarily conserved primary triggers of immune response.

Recent reports revealed that also platelets possess the majority of TLRs. TLR 1, 2, 4, 6 and 9 could be detected on the surface of murine and human platelets, whereat TLR4 and TLR9 are up-regulated activation dependently (Shiraki et al., 2004;Cognasse et al., 2005). Up to now TLR1/2 and TLR4 are proven to be functional and it could be shown that they trigger intracellular signalling which results in platelet activation (Andonegui et al., 2005b;Aslam et al., 2006a;Stahl et al., 2006a;Jayachandran et al., 2007;Blair et al., 2009g).

Platelet activation in response to TLR2 as well as TLR4 activation seems to be mediated by phosphoinositol-3-kinase (PI3K), a central mediator in platelet activation. Akt downstream of PI3K also appears to be essential in recent studies on TLR2 as well as

TLR4 mediated activation (Rex et al., 2009b;Zhang et al., 2009c;Blair et al., 2009f). Moreover it could be shown that platelet TLR4 is involved in leukocyte mediated clearance of activated neutrophils in response to LPS and therefore causes thrombocytopenia (Andonegui et al., 2005a;Aslam et al., 2006b). TLR4 also provides a threshold switch for neutrophil extracellular trapping of bacteria in severe sepsis (Clark et al., 2007a).

2.4 Aim of the study

Platelets play a pivotal role in the onset of atherosclerosis as well as in its major clinical complication, coronary heart diseases. Thus we were interested if platelets might play a role in linking periodontitis and cardiovascular diseases.

Since periodontitis goes along with a local chronic inflammation as well as transient bacteriaemia, we aimed to clarify which impact a single bacteriaemia has on platelet function by an in vitro model.

Moreover recent studies revealed that platelets bear immuno-modulatory functions, therefore we aimed to determine if platelets, in response to periodontitis associated bacteria, have an impact of the function of neutrophils.

We were further interested in the underlying processes of periodonititis associated bacteria induced platelet activation, the responsible surface receptors as well as signalling pathways induced upon stimulation with the two investigated bacteria A. actinomycetemcomitans and P. gingivalis.

Moreover we aimed to clarify if direct interactions of platelets and neutrophils are necessary for the observed effects of if platelet release products can mimic the effects.

3 Material and Methods

3.1 Material

3.1.1 Laboratory equipment

Centrifuge: Allegra X12R (Beckman Coulter)

Centrifuge: minispin 5415D (Eppendorf)

Flow cytometer: FACS Calibur analytic flow cytometer with a two laser system: Argon-488nm and 635nm Diode (Becton Dickinson)

Confocal microscope: LSM 510 Confocal Microscope (Zeiss)

3.1.2 Buffers

Phosphate buffered saline (PBS):

potassium phosphate (1.5mM)

potassium chloride (2.7mM)

sodium chloride (137mM)

sodium phosphate (8.3mM)

Full strength Hank´s buffered saline solution:

sodium chloride (0.137 M)

potassium chloride (5.4 mM)

disodium hydrogen phosphate (0.25 mM)

potassium dihydrogen phosphate (0.44 mM)

calcium chloride (1.3 mM)

magnesium sulphate (1.0 mM)

sodium hydrogen carbonate (4.2 mM)

Tyrode-HEPES buffer (with glucose and albumin):

sodium chloride (140mM)

potassium chloride (3mM)

magnesium chloride (1mM)

sodium hydrogen carbonate (16.62mM)

HEPES (10mM)

(D-glucose (5.5mM))

(human serum albumin (0.5%))

For platelet isolation by gel filtration and all functional platelet experiments buffer was set to a pH of 7.35.

3.1.3 Chemicals and solutions

Platelet agonists were always freshly prepared from ADP (Sigma-Aldrich) and TRAP-6 (Bachem) stock solutions.

ADP and TRAP-6 stock solutions were deeply frozen stored (-80°C) and never refrozen. Further dilutions were performed in phosphate buffered saline (PBS) and always freshly prepared immediately before use.

For cell fixation, 10% formaldehyde (37% stock solution; Sigma-Aldrich) or 10% paraformaldehyde (Sigma-Aldrich) stock solution in 0.9% sodium chloride were prepared. Paraformaldehyde was solved 1:10 (w/v), heated at 60°C for two hours and kept at -20°C.

For platelet gel filtration sepharose 4B (GE Healthcare) was used.

Lysis of erythrocytes for flow cytometric experiments was achieved by an ammonium chloride based whole blood lysis buffer (BD).

3.1.4 Antibodies and fluorescence marker

The following antibodies were used:

monoclonal anti PAC-1 (BD, cat # 340507, FITC conjugated)

monoclonal anti CD11b (BD, cat # 557397, PE conjugated)

monoclonal anti CD14 (R & D, cat # BAF383)

monoclonal anti CD32, AT10 antibody (Serotec, cat # MCA1075XZ)

monoclonal anti CD40L (BD, cat # 555699, FITC conjugated)

monoclonal anti CD45 (Acris, cat # AM01077FC-N, FITC conjugated)

monoclonal anti CD62L (Coulter, cat# IM1231U FITC conjugated)

monoclonal anti CD62P (BD, cat # 348107, PE conjugated)

monoclonal anti CD62P (Santa Cruz, cat # sc-19672)

polyclonal anti CD62P (R & D ,cat # AF137)

monoclonal anti CD63 (BD, cat # 556020, FITC conjugated)

The following fluorescence markers were used:

Annexin V/ FITC Kit (Bender MedSystems, cat# 30622-C1F1)

Cell Tracker Green CMFDA (Invitrogen, cat# C7025)

pHrodo succinimidyl ester (Invitrogen, cat# P36600)

3.2 Blocking of surface receptors and signalling

If indicated, platelets were inhibited with Akt inhibitor VIII (2µM), U73122 (2µM) or wortmannin (2µM) for 10 minutes and the gel filtered (as precedingly described).
In some cases platelet surface receptors were blocked, by incubating platelets with anti P-selectin Abs (10µg/ml) or anti CD40L mAb (10µg/ml) before adding neutrophils. Or neutrophils were incubated with P-selectin (10µg/ml) before addition of platelets. Moreover TLR2 on platelets and/or leukocytes was blocked with anti TLR2 mAb (25µg/ml) and TLR4 was blocked with anti TLR4 mAb (25µg/ml).
To avoid any effects by preservatives like azide, antibodies were re-buffered in PBS by a mini dialysis kit with a 1kDa cut off (Amersham Bioscience, United Kingdom) before use.
All reagents were tested for their effects on platelets and/or neutrophil activation by determination of surface expression of CD62P and CD11b.

3.2.1 Methods

3.2.2 Bacteria: strains and culture conditions

Porphyromonas gingivalis (CCUG 27724) and Aggregatibacter actinomycetemcomitans (CCUG 13227) were obtained from the Culture Collection of the University of Göteborg.
P. gingivalis was cultured on Schaedler blood agar plates at 37° C. Anaerobic conditions were generated by a sealed chamber containing GENbag anaer (bioMerieux, France). A. actinomycetemcomitans was also cultured on Schaedler blood agar in 5% CO_2 atmosphere at 37°C.

3.2.3 Blood donors

Venous blood from healthy donors, who declared to be free of any medication for at least one week, was drawn by a 20 G needle and collected into anticoagulation tubes filled with 3.8% sodium citrate (used in a 1:9 volume ratio). All volunteers gave informed written consent and the study was approved by the Human Ethics Committee of the Medical University of Vienna (EK-Number 321/2007).

Platelet-rich plasma (PRP) was obtained by centrifugation immediately after taking the sample at 125g for 20 minutes.

3.2.4 Platelet isolation

To avoid contaminations with other cell types only the upper two thirds of the PRP fraction were used to isolate platelets. To obtain gel filtered platelets, a sepharose 4B filled column was prepared, by cutting off the tip of a serological, plastic pipette (10ml) with hot wire and stuffing the cone end with a piece of nylon to hold back liquid

sepharose. Thereafter the pipette was filled with 10ml sepharose and washed with Tyrode-HEPES buffer containing glucose and albumin (pH: 7.35). Then 1ml of PRP was loaded and 500µl fractions collected in test tubes. The fractions change from clear fractions (buffer) to a milky clouding (platelets), followed by a yellowish tint (plasma). Only the platelet fractions were used for platelet studies.

3.2.5 Isolation of neutrophils

After platelet rich plasma has been taken of, the tubes were filled up with 15% dextran in isotonic NaCl solution. After 30 minutes most of the erythrocytes sedimented and the supernatant was taken off, centrifuged at 350g for 5 minutes and resuspended in Hank´s buffered salt solution. Thereafter a Ficoll Hypaque density gradient centrifugation was performed to separate mononuclear and polynuclear leukocytes (as depicted in Figure 3).

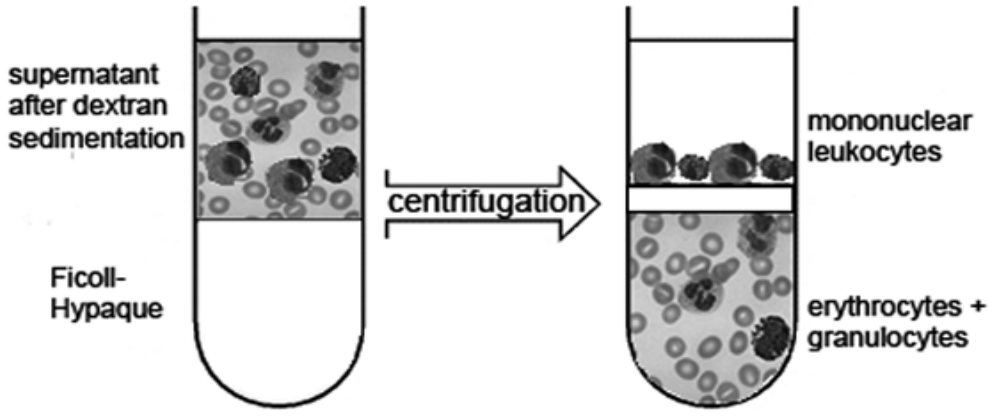

Figure 3 | Ficoll Hypague centrifugation: Ficoll Hypaque has a specific density of 1.078, therefore mononuclear leukocytes (monocytes, lymphocytes) are unable to sediment at the bottom. The heavier erythrocytes and granulocytes are found at the bottom at the tube.

400µl Ficoll was pipetted in an eppendorf tube and overlaid with the blood cells and centrifuged at 350g for 30 minutes. The granulocytes in the pellet were then depleted of contaminating erythrocytes by lysis in NH_4Cl for 5 minutes on ice. Thereafter the

cells were centrifuged at 350g for 5 minutes, the supernatant discarded and the pellet washed in PBS and resuspended at of 1 x 10^6cells/ml.

3.2.6 Plasma isolation

Platelet poor plasma (PPP) was obtained by centrifugation of anticoagulated whole blood at 1300g for 20 minutes at 4°C. PPP and PRP was routinely assessed by flow cytometry using anti CD61 or anti CD45 antibody to exclude potential contaminations. Flow cytometric studies were conducted using a FACSCalibur flow cytometer with CellQuest Pro software.

3.2.7 Phagocytosis assay

Neutrophils were stimulated with pHrodo stained bacteria A. actinomycetemcomitans and P. gingivalis. After 20 minutes incubation time cells were fixed in 1% paraformaldehyde and analysed flow cytometrically. The dye is non-fluorescent at neutral pH and fluorescences bright red in acidic environments. A bright red fluorescence signal in FL2 indicates that the bacteria have reached the acidic vacuoles of the neutrophils.

3.2.8 Confocal microscopy

Freshly isolated neutrophils were stained with 50µM Cell Tracker Green CMFDA for 30 minutes. Then neutrophils were washed twice in PBS and finally 30% PPP or PRP were added. The cells were then stimulated with either pHrodo stained A. actinomycetemcomitans or P. gingivalis for 20 minutes. Then cells were fixed in 1% paraformaldehyde for 10 minutes, washed in PBS and analysed by confocal microscopy.

3.2.9 Determination of bacterial killing and phagocytosis

To determine A. actinomycetemcomitans killing and phagocytosis by human neutrophils with or without platelets, bacteria (1 x 10^8/ml) were incubated with neutrophils (5 x 10^5/ml) with or without platelets (1 x 10^7/ml) in 30% plasma for 20 minutes. Thereafter the samples were centrifuged at 350g for 5 minutes and the supernatant was diluted up to 10^{-10} times and 3 x 20µl of each dilution plated on Schaedler blood agar plates and incubated at 37°C for 24 hours. Then the colonies were counted and the colony forming units (CFU) per ml calculated. This experiment could not be successfully performed with P. gingivalis since large amounts of the anaerobic species died during sample preparation.

3.2.10 Bacteria binding assay

A. actinomycetemcomitans and P. gingivalis were stained with Alexa Fluor 633 succinimidyl ester according to the instructions of the manufacturer. After repeated washing in PBS, Alexa 633 labelled bacteria were added to freshly isolated neutrophils in the presence of 30% PPP or PRP. After 5, 10, 20 and 30 minutes probes were fixed in 1% formaldehyde for 10 minutes. Thereafter neutrophils were stained with anti CD45-FITC for 30 minutes and bacteria positive neutrophils determined by double positive (FL1 and FL4) events.

3.2.11 Determination of platelet leukocyte aggregates

Platelet leukocyte aggregates (PLA) were measured in whole blood and analysed by two-colour flow cytometry, according to a protocol by (Li et al., 1997). Whole blood was stimulated with live A. actinomycetemcomitans, live P. gingivalis in the concentrations indicated. For control experiments blood cells were also actuated with classical platelet agonists ADP or TRAP-6, LPS or Pam_3CSK_4. FITC anti CD45 mAb

(leukocyte specific) and PerCP anti CD61 mAb (platelet specific) were added and erythrocytes lysed by an ammonium chloride-based lyses buffer according to the instructions of the manufacturer.
For platelet neutrophil aggregates PRP or isolated platelets were added to freshly isolated neutrophils (5 x 10^5/ml) from the same donor. Platelets and neutrophils were always added in a physiological ratio of 20:1. Samples were treated as described for PLA.
Leukocytes/neutrophils were gated by light scattering and FITC-CD45 fluorescence and further gated into PerCP-CD61-negative and PerCP-CD61-positive populations. A minimum of 5000 leukocytes/neutrophils were acquired, and the percentage of platelet-bound leukocytes/neutrophils (CD45-positive/CD61-positive) was quantified.

3.2.12 Analysis of neutrophil activation

Neutrophil activation was determined as increase of surface expression of CD11b and a decrease of CD62L.
Freshly isolated neutrophils were incubated in PPP, PRP or with isolated platelets in buffer, whereat platelets and neutrophils were always added in a physiological ratio of 20:1 and the final plasma concentration was always 30%. Cells were then stimulated with the indicated amounts of A. actinomycetemcomitans or P. gingivalis for 20 minutes. Then the antibodies anti CD11b-PE or anti CD62L-FITC were added (1:20) and after 30 minutes of incubation surface expression of CD11b and CD62L determined by flow cytometry. Only an increase in CD11b and shedding of CD62L was regarded as a parameter for neutrophil activation.

3.2.13 Flow cytometric analysis of surface- and intracellular markers of platelet activation

3.2.13.1 Surface expression of P-selectin

Surface exposure of P-selectin (CD62P) was determined by flow cytometry. 30µl of PRP or gel filtered platelets were stimulated with ADP (50µM), A. actinomycetemcomitans or P. gingivalis (3 x 10^9bacteria/ml) for 20 minutes. Platelets were then fixed by adding 4µl of 10% formaldehyde and incubated for 15 minutes. Thereafter the samples were centrifuged at 1000g for 2 minutes. The supernatant was discarded, the pellet resuspended in 30µl PBS and 2µl of antibody directed against CD62P-PE was added. Incubation was performed for one hour at room temperature and probes were kept in a dark place. Thereafter probes were diluted in 470µl PBS and analysed immediately. PE marked CD62 was detected in FL2. 10 000 events, gated for platelets according their size, were measured and analysed using BD CellQuest Pro and Microsoft Excel.

3.2.13.2 Surface expression of CD63

PRP or isolated platelets with and without CD14 (5µg/ml, R&D Systems, United States) were stimulated with ADP (50µM), E. coli LPS (1µg/ml), Pam_3CSK_4 (1µg/ml) or the indicated amount of A. actinomycetemcomitans or P. gingivalis (3 x 10^5-10^9bacteria/ml). If not indicated otherwise incubation time was 20 minutes. For some experiments platelets were preincubated with antibodies blocking TLR2 or TLR4 or inhibitors of signalling pathways as described in chapter 3.2 prior to stimulation with agonists. Thereafter cells were fixed in 1% formaldehyde and after washing in PBS incubated with anti CD63-FITC for 60 minutes. Thereafter the samples were diluted in BPS and analysed flow cytometrically in FL1.

3.2.13.3 CD40L surface expression

CD40L surface expression was detected according to a protocol by (Inwald et al., 2000). In detail, 10µl PRP were incubated with periodontitis associated bacteria (3 x 10^9bacteria/ml) or TRAP (10µM) for 20 minutes. Then 1µl of anti CD40L was added and the samples incubated for 20 minutes at room temperature at dark. Thereafter platelets were fixed with 2µl of 10% formaldehyde and analyzed flow cytometrically within 15 minutes.
To identify if TLRs are involved in bacterial recognition by platelets, platelets were incubated with anti TLR2 and ant TLR4 (see chapter 3.2). To compare platelet signalling by TLR agonists and periodontitis associated bacteria, platelets were preincubated with the respective inhibitors (see also chapter 3.2).

3.2.13.4 Detection of activated glycoprotein IIb/IIIa

Activated glycoprotein IIb/IIIa (GPIIb/IIIa) was determined by PAC-1 antibody. 10µl of PRP was incubated with 2µl ADP (f.c.:50µM), A. actinomycetemcomitans or P. gingivalis (3 x 10^7-10^9bacteria/ml). Incubations were performed in a volume of 20µl and 1µl of PAC-1 antibody was added to each probe. Incubation was performed at dark for 30 minutes. Thereafter probes were fixed with 2µl of 10% formaldehyde and analysed flow cytometrically.

3.2.14 Statistical evaluation and graphics

Results are presented as mean values ± standard deviation. Each measurement was taken in duplicate. All parameters were subjected to KS test for confirming normal distribution and to subsequent t-tests for unpaired samples. For binding studies results were compared using repeated-measures analysis of variance (ANOVA). In case of the existence of an overall effect, differences were analyzed using an ANOVA followed by

Bonferroni correction. Statistical analysis was performed using SPSS 16.0 and Microsoft Excel software. An error probability of less than 0.05 was considered as statistically significant and less than 0.01 as statistically extremely significant. Graphics of the calculated data were drawn with Sigma Plot 10.0.

4 Results

4.1 Periodontitis associated bacteria induce platelet activation

To determine the impact of periodontitis associated bacteria on platelet function, platelet rich plasma (PRP) was incubated with different concentrations of periodontitis associated bacteria A. actinomycetemcomitans and P. gingivalis and the effects on platelet function were assessed by flow cytometrical analysis of *GPIIb/IIIa activation and surface expression of* CD40L, CD62P and CD63.

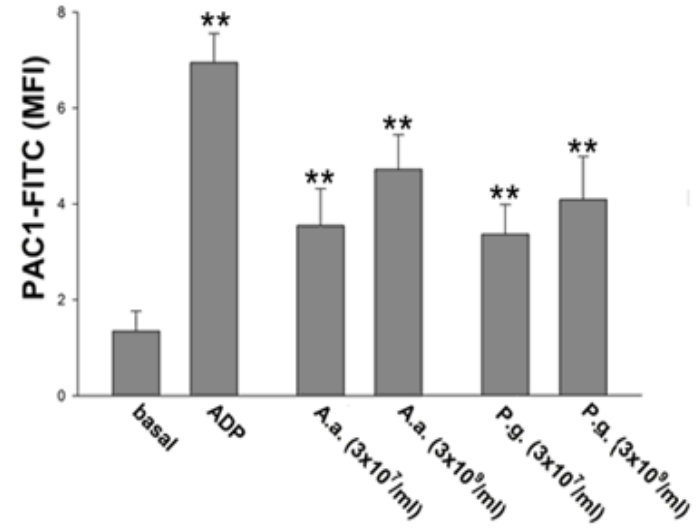

Figure 4| Influence of periodontitis associated bacteria A. actinomycetemcomitans and P. gingivalis on GPIIb/IIIa activation: *PRP ($3x10^8$ platelets/ml) was incubated for 20 minutes with ADP (50µM), A. actinomycetemcomitans or P. gingivalis ($3 x 10^7$-10^9 bacteria/ml) and activation of GPIIb/IIIa receptor measured and compared to unstimulated control; means and standard deviations of 6 independent experiments with PRP from 6 different donors; **indicates statistically very significant difference to basal GPIIb/IIIa activation (P<0.01)*

In Figure 4 the influence of periodontitis associated bacteria on activation of GPIIb/IIIa is depicted. Activation of GPIIb/IIIa (integrin αIIbβ3) is an important marker for platelet activation and a prerequisite for platelet aggregation. Platelet activation induces a conformational change in GPIIb/IIIa, which leads to the exposure of a binding site for fibrinogen, fibronectin, vitronectin and von Willebrand factor (vWf). Antibody PAC-1

recognizes an epitope near the platelet fibrinogen receptor and therefore binds only activated GPIIb/IIIa.

As depicted in Figure 4 both types of bacteria, A. actinomycetemcomitans and P. gingivalis induce GPIIb/IIIa activation. A ratio of one bacterium per 10 platelets is sufficient to induce a conformal change in GPIIb/IIIa.

Similar results can be shown for surface expression of CD40L, as depicted in Figure 5. In resting platelets CD40 ligand is located in cytoplasma of platelets. Upon activation CD40L is exocytosed and exposed on the platelet surface. Therefore CD40L represents another important marker of platelet activation, which is exposed independently of GPIIb/IIIa. Incubation of A. actinomycetemcomitans and P. gingivalis lead to a dose-dependent expression of CD40L. Whereat, similar to results of GPIIb/IIIa activation, a ratio of one bacterium per 10 platelets seems to be sufficient for activation. But even at a concentration of 10 bacteria per platelet the observed extent of activation significantly differs from activation induced by 50µM ADP.

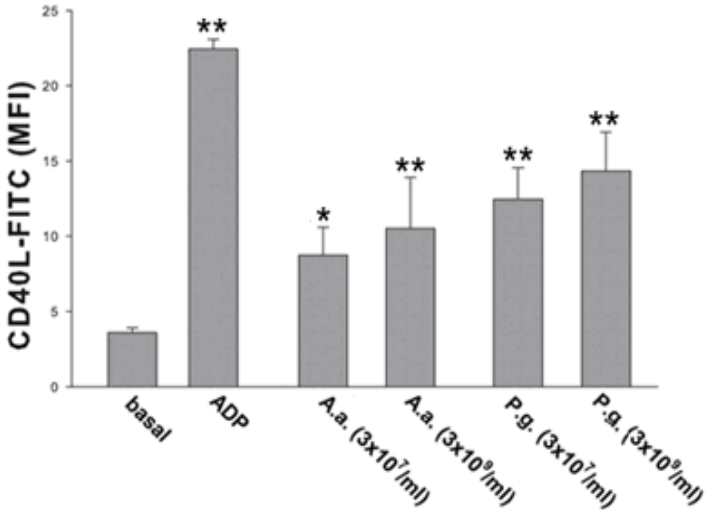

Figure 5| Influence of periodontitis associated bacteria A. actinomycetemcomitans and P. gingivalis surface expression of CD40L: *PRP ($3x10^8$ platelets/ml) was incubated for 20 minutes with ADP (50µM), A. actinomycetemcomitans or P. gingivalis (10^7-10^9 bacteria/ml) and surface expression of CD40L measured and compared to unstimulated control; means and standard deviations of 6 independent experiments with PRP from 6 different donors; *indicates statistically significant difference to basal surface expression of CD40L (P<0.05); **indicates statistically very significant difference to basal surface expression of CD40L (P<0.01)*

To determine the effects of periodontitis associated bacteria on platelet degranulation two other surface marker, CD62P and CD63, were investigated.

CD62P (P-selectin, PADGEM) is stored in the membrane of platelet α-granules and upon granule exocytosis it becomes part of the platelet surface membrane. P-selectin is thought to mediate adhesion of activated platelets to neutrophils and monocytes.

CD63 (granulophysin, LAMP-1, MLA 1) is stored in the membrane of dense bodies and becomes exocytosed on the platelet surface upon dense body degranulation.

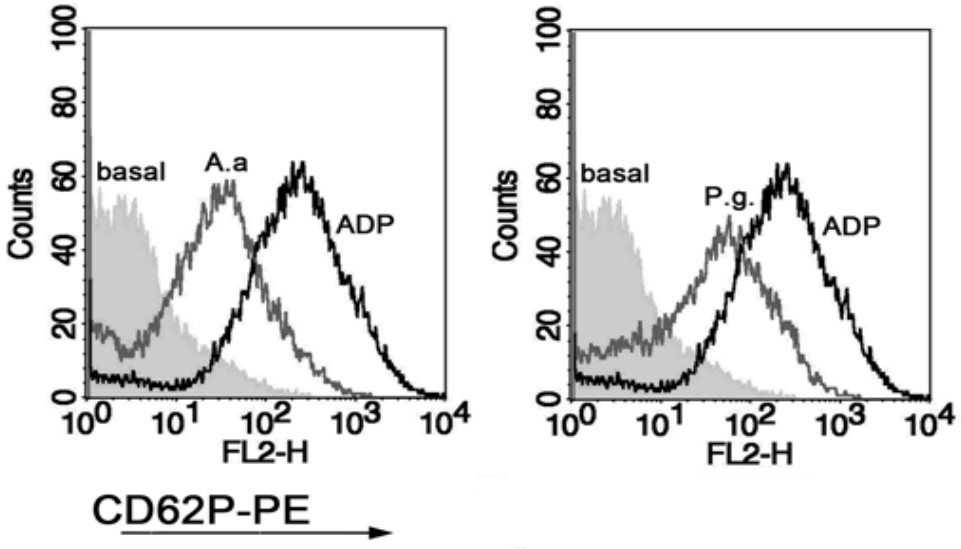

Figure 6| Influence of periodontitis associated bacteria A. actinomycetemcomitans and P. gingivalis surface expression of CD62P: PRP ($3x10^8$ platelets/ml) was incubated for 20 minutes with ADP (50µM), A. actinomycetemcomitans or P. gingivalis (10^9 bacteria/ml) and surface expression of CD62P measured; FACS histogram overlay of a typical experiment out of 6 independent experiments with PRP from 6 different donors;

As shown in

Figure **6**, A. actinomycetemcomitans or P. gingivalis incubation with platelets leads to an increase of surface expression of CD62P on platelets.

Also in terms of CD62P surface expression, platelet activation is rather low compared to activation induced with a classical platelet activator like ADP (50µM).

As shown in Figure 7, periodontitis associated bacteria time- and dose-dependently induce surface expression of CD63 on the platelet surface. While platelet activation by classical agonists occurs within 5 minutes (data not shown), significant platelet

activation by periodontitis associated bacteria takes up to 15 minutes in lower bacteria concentrations and up to 10 minutes in higher bacteria concentrations.

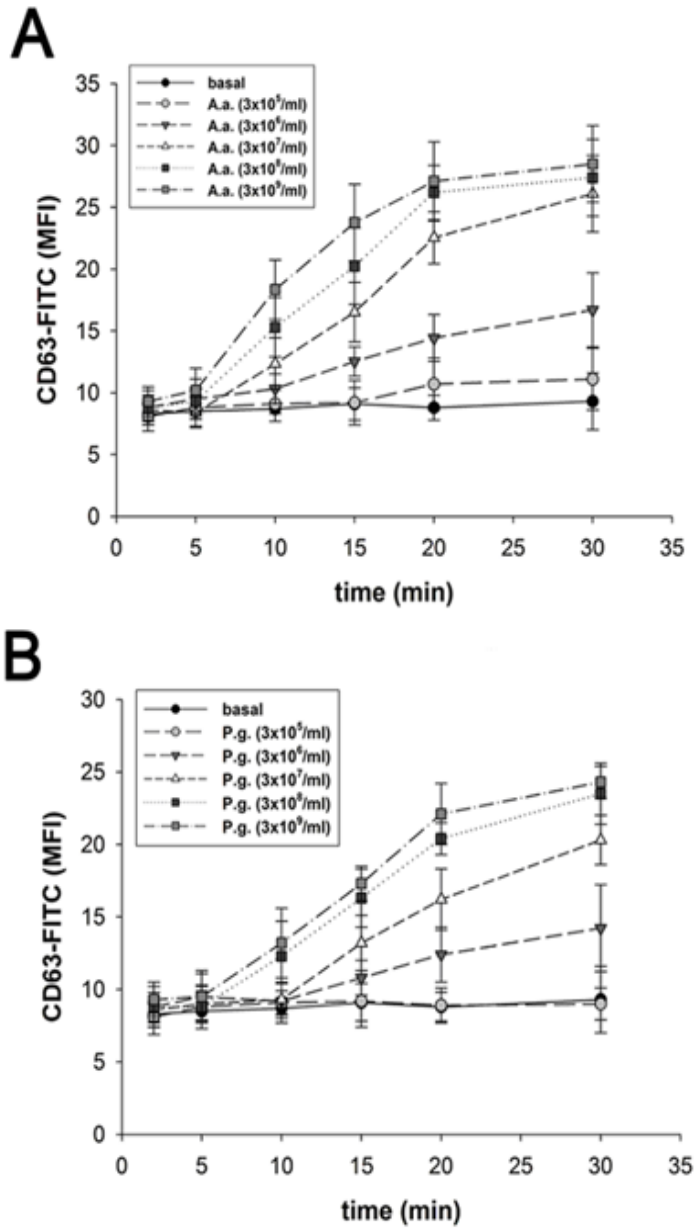

Figure 7| Time and dose dependency of periodontitis associated bacteria induced surface expression of CD63: *PRP (3x10^8 platelets/ml) was incubated with different concentrations of A. actinomycetemcomitans (A) or P. gingivalis (B) (10^5-10^9 bacteria/ml) and surface expression of CD63 was measured at different time points (2-30 minutes) and compared to unstimulated control; means and standard deviations of 6 independent experiments with PRP from 6 different donors;*

Moreover Figure 7 shows that the critical bacteria concentration for induction of significant platelet degranulation is between 3 x 10^6-10^7bacteria/ml for both periodontitis associated bacteria, which corresponds to a bacterium to platelet ratio of 1 bacterium to 10-100 platelets.

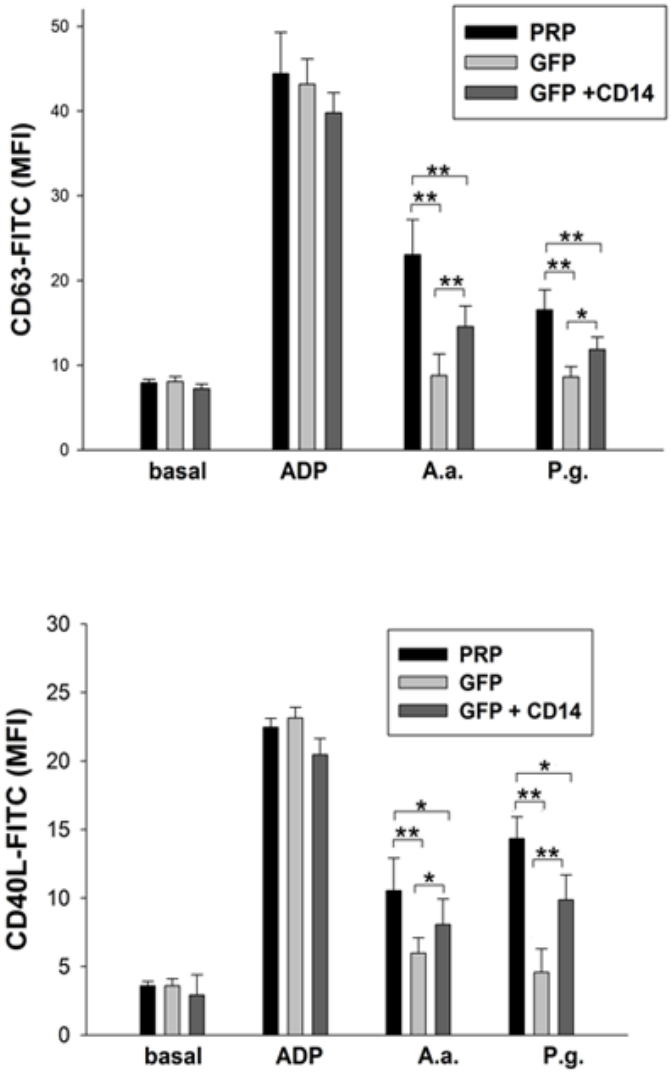

Figure 8| Influence of plasma factors on periodontitis associated bacteria induced surface expression of CD63 and CD40L: *PRP (3x10^8 platelets/ml) or GFP (3x10^8 platelets/ml) with or without CD14 (5µg/ml) was incubated for 20 minutes with ADP (50µM), A. actinomycetemcomitans or P. gingivalis (10^9 bacteria/ml) and surface expression of CD63 (upper figure) and CD40L (lower figure) measured and compared to unstimulated control; means and standard deviations of 6 independent experiments with PRP from 6 different donors; **indicates statistically very significant difference to PRP (P<0.01)*

4.2 Mechanisms underlying the interaction of periodontitis associated bacteria and human platelets

Platelet activation depicted in the last chapter was determined in platelet rich plasma. To identify if plasma factors are necessary in platelet interaction with periodontitis associated bacteria, platelets were purified from plasma by gel filtration, as described in Material and Methods.

As shown in Figure 8 for CD63 and CD40L, plasma factors appear to be essential for platelet activation by A. actinomycetemcomitans and P. gingivalis, since gel filtered platelets show a significantly reduced response to periodontitis associated bacteria compared to platelet rich plasma.

Upon addition of recombinant CD14, a protein which is not expressed by platelets, bacteria induced platelet activation can be (at least partly) restored.

The importance of CD14 in bacteria mediated platelet activation indicates an involvement of toll like receptors for the interaction, since CD14 is part of the LPS-binding complex and also crucial in TLR2 recognition.

It has been shown that the interaction of A. actinomycetemcomitans with other human cell types is mediated via TLR2 and TLR4, while P. gingivalis is bound mainly via TLR2(Kikkert et al., 2007b).

To proof if platelet bacteria binding is mediated via the same receptor(s), platelets were treated with antibodies against TLR2 and TLR4 or both before they were stimulated with bacteria.

Since bacteria platelet interactions have been discussed to depend on immunoglobulin opsonisation of bacteria (and therefore might be mediated via FcγRII), platelet rich plasma was also treated with AT10, an antibody that inhibits binding of IgG to FcγRII.

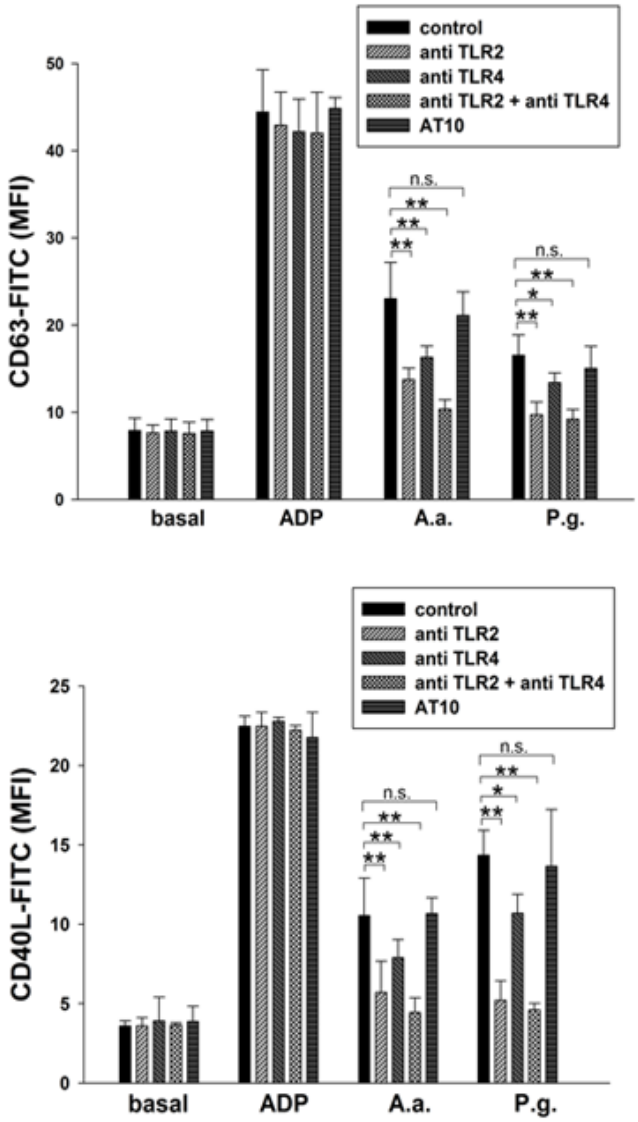

Figure 9 | Surface receptors involved in periodontitis associated bacteria induced surface expression of CD63 and CD40L: *PRP (3x10^8 platelets/ml) was pre-incubated with either antiTLR2, antiTLR4 or both, or AT10 (to block binding to FcγRII). Thereafter PRP was stimulated for 20 minutes with ADP (50μM), A. actinomycetemcomitans or P. gingivalis (10^9 bacteria/ml) and surface expression of CD63 and CD40L measured and compared to unstimulated control; means and standard deviations of 6 independent experiments with PRP from 6 different donors; *indicates statistically significant difference to control (P<0.05) **indicates statistically very significant difference to control (P<0.01); n.s. indicates statistically not significant;*

As depicted in Figure 9 for surface expression of CD63 and CD40L, platelet activation by the two tested periodontitis associated bacteria does not seem to be IgG dependent or mediated by FcγRII.

However, upon blocking TLR2 or TLR4 on human platelets bacteria induced activation is significantly reduced. As demonstrated in Figure 9, TLR2 seems to be more important in this process. Blocking of TLR4 has more impact on A. actinomycetemcomitans than P. gingivalis induced platelet activation, although also in P. gingivalis platelet interaction TLR4 seems to be involved.

4.3 Influence of periodontitis associated bacteria on formation of platelet leukocyte aggregates

As shown in chapter 4.1, platelet activation by periodontitis associated bacteria takes longer than platelet activation by classic agonists. Moreover platelet activation is rather weak even after prolonged incubation times and high concentrations of bacteria, though this platelet activation pattern is typical for TLR signalling.
Incubation of platelets with LPS, the classical activator of TLR4, or PAM_3CSK_4, a synthetic activator of TLR2, shows a slow and weak platelet response in terms of platelet activation (surface expression of CD62P or GPIIb/IIIa activation), but a strong increase in platelet leukocyte interactions (Andonegui et al., 2005c;Blair et al., 2009e).
Platelet leukocyte aggregates were measured flow cytometrically, by double staining with platelet specific anti CD61-perCP and leukocyte specific anti CD45-FITC.
As shown in Figure 10, leukocytes were first determined by their size (FSC-H), shown as R1, then the R1 population was separated by determination of CD45-FITC positive events (FL-1), and thereby further divided in subpopulation by their granularity (SSC-H).
The populations, leukocytes (A), lymphocytes (B), monocytes (C) and neutrophils (D) were then separately analysed for the percentage of CD61-perCP (FL-3) positive cells.

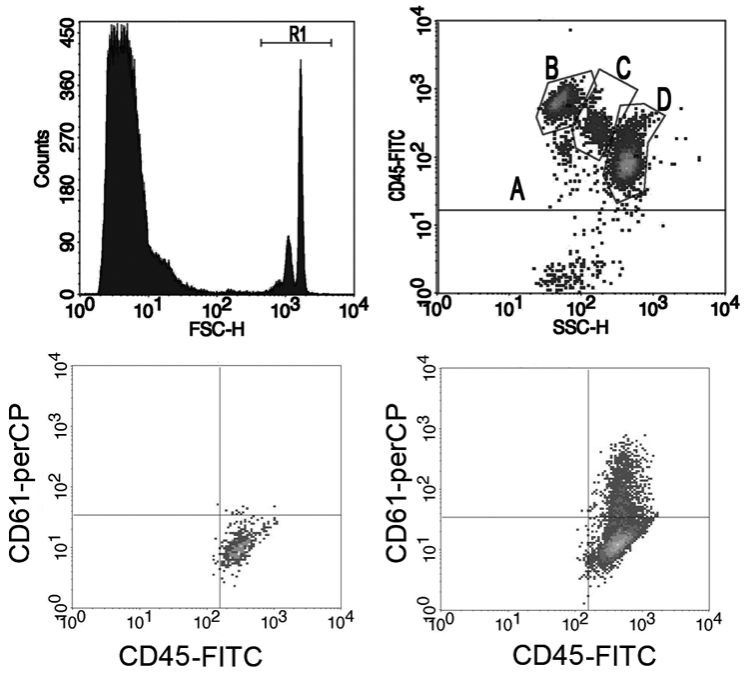

Figure 10| Analysis of platelet leukocyte aggregates by flow cytometry: *whole blood was stimulated for 20 minutes with TRAP-6 (10µM); upper left figure shows forward scatter (FSC-H) of whole blood cells after lysis of erythrocytes, the population with the biggest size was then gated (R1) and discriminated by their FL-1 fluorescence intensity (CD45-FITC) and their inner complexity (SSC-H), as shown in the upper right; The population was then divided into leukocytes (A) lymphocytes (B), monocytes (C) and neutrophils (D), which were then separately analyzed for their FL-3 fluorescence intensity (CD61-perCP), as shown in the lower left for untreated neutrophils and in the lower right figure for TRAP-6 treated neutrophils.*

As depicted in Figure 11, classical platelet activators are not able to induce strong formation of platelet leukocyte aggregates but upon incubation with LPS or PAM_3CSK_4 more platelets stick to leukocytes. Similar results were obtained when analysing platelet-monocyte and platelet-neutrophil aggregates (data not shown). But in no case platelets adhere to lymphocytes what is in line with previous reports (Blair et al., 2009d).

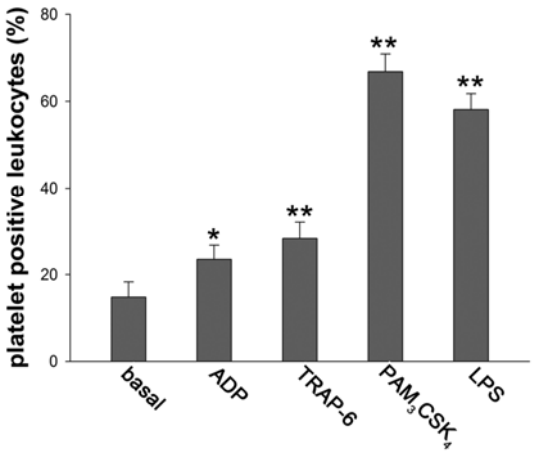

Figure 11| Platelet leukocyte aggregates induced by classical platelet activators and TLR ligands: *whole blood was stimulated for 20 minutes with ADP (50µM), TRAP-6 (10µM),* LPS (*1µg/ml*) PAM_3CSK_4 *(1µg/ml); means and standard deviations of 6 independent experiments with blood from 6 different donors; **indicates statistically very significant difference to basal, ADP and TRAP-6 treatment (P<0.01)*

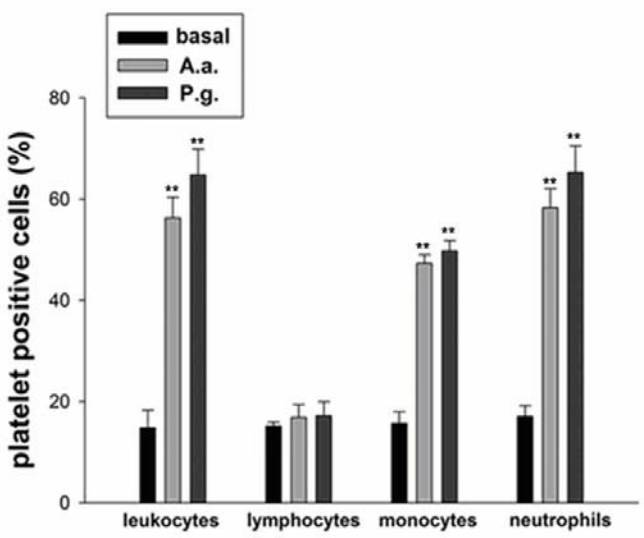

Figure 12| Platelet leukocyte aggregates induced by classical platelet activators and TLR ligands: whole blood was stimulated for 20 minutes A. actinomycetemcomitans or P. gingivalis (10^9 bacteria/ml); means and standard deviations of 6 independent experiments with blood from 6 different donors; **indicates statistically very significant difference to basal, ADP and TRAP-6 treatment (P<0.01)

Therefore the effects of periodontitis associated bacteria on the formation of platelet leukocyte aggregates were investigated. As shown in Figure 12, A.

actinomycetemcomitans and P. gingivalis appear to be strong inducers of platelet-leukocyte, and thereby especially platelet-monocyte and platelet-neutrophil aggregates.

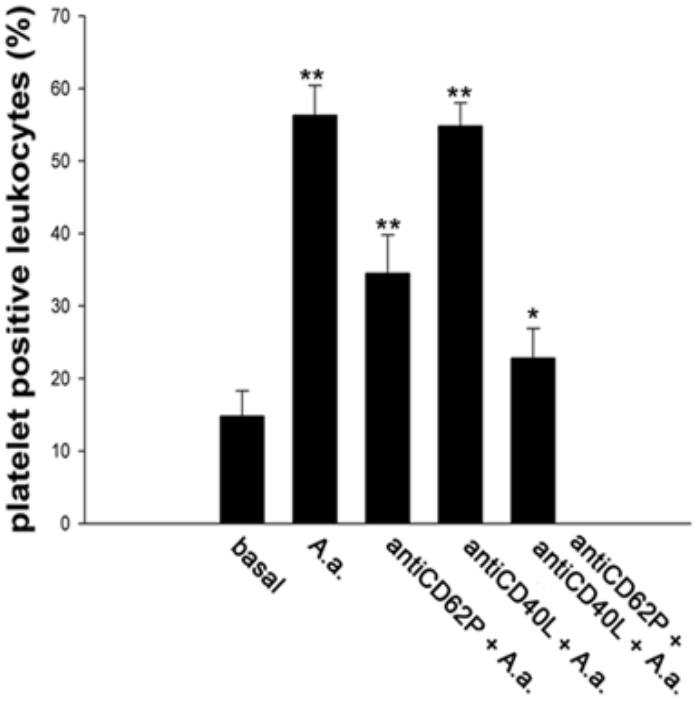

Figure 13 | Identification of the responsible receptors on platelets involved in A. actinomycetemcomitans induced adhesion to leukocytes: *PRP ($3x10^8$/ml) was preincubated with anti CD62P and/or anti CD40L, then isolated neutrophils ($5x10^5$/ml) and A. actinomycetemcomitans ($3x10^9$ bacteria/ml) were added and after 20 minutes the formation of platelet leukocyte aggregates was determined; means and standard deviations of 6 independent experiments with blood from 6 different donors; *indicates statistically significant difference to control (P<0.05) **indicates statistically very significant difference to control (P<0.01)*

To determine if these effects were platelet specific or just a result of indirect binding by leukocyte-bacteria, bacteria-platelet interactions, platelets were treated with azide free CD62P antibody and/or CD40L antibody. CD62P and CD40L are regarded as the two most common leukocyte receptors on platelets.

As shown in Figure 13 for A. actinomycetemcomitans and in Figure 14 for P. gingivalis, the effects of platelet leukocyte binding seem to be specific and mediated mainly via CD62P, and only if CD62P is blocked the CD40L antibody has additional effects.

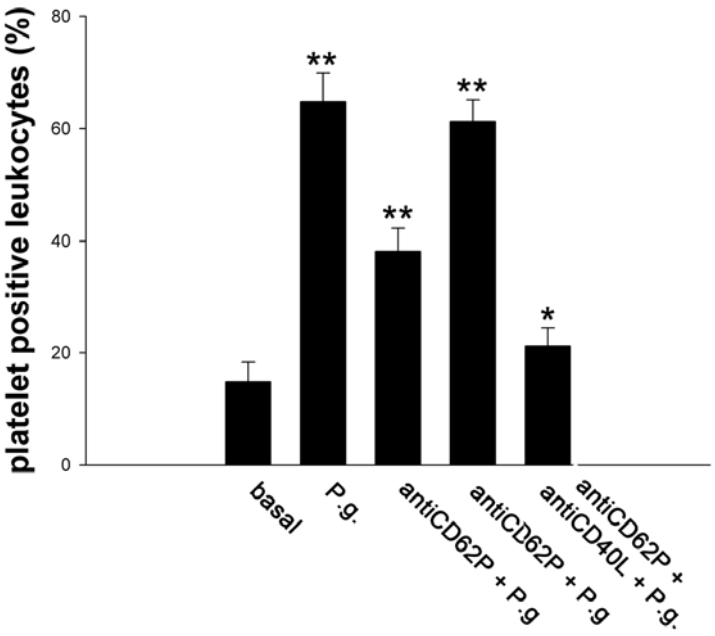

Figure 14 | Identification of the responsible receptors on platelets involved in P. gingivalis induced adhesion to leukocytes: *PRP ($3x10^8$/ml) was preincubated with anti CD62P and/or anti CD40L, then isolated neutrophils ($5x10^5$/ml) and P. gingivalis ($3x10^9$ bacteria/ml) were added and after 20 minutes the formation of platelet leukocyte aggregates was determined; means and standard deviations of 6 independent experiments with blood from 6 different donors; *indicates statistically significant difference to control (P<0.05) **indicates statistically very significant difference to control (P<0.01)*

We then investigated if TLRs are involved in the bacteria induced formation of platelet leukocyte aggregates. Therefore, whole blood was incubated either with anti TLR2, anti TLR4 or both antibodies and the influence on A. actinomycetemcomitans (Figure 15) and P. gingivalis (Figure 16) in platelet leukocyte aggregation formation was determined.

As can be seen an antibody against TLR2 significantly reduced platelet neutrophil aggregates (PNA) formation by ~50% in the case of A. actinomycetemcomitans and ~25% in the case of P. gingivalis. A blocking antibody against TLR4 had no effect on P. gingivalis induced PNA formation and was less effective compared to TLR2 blocking antibody in the case of A. actinomycetemcomitans induced PNA formation. However, when PNA formation was analysed in the presence of both blocking antibodies a synergistic effect was observed in A. actinomycetemcomitans and P. gingivalis dependent PNA formation.

From these data we conclude that TLR2 and TLR4 are involved in periodontitis associated bacteria induced platelet activation and PLA formation.

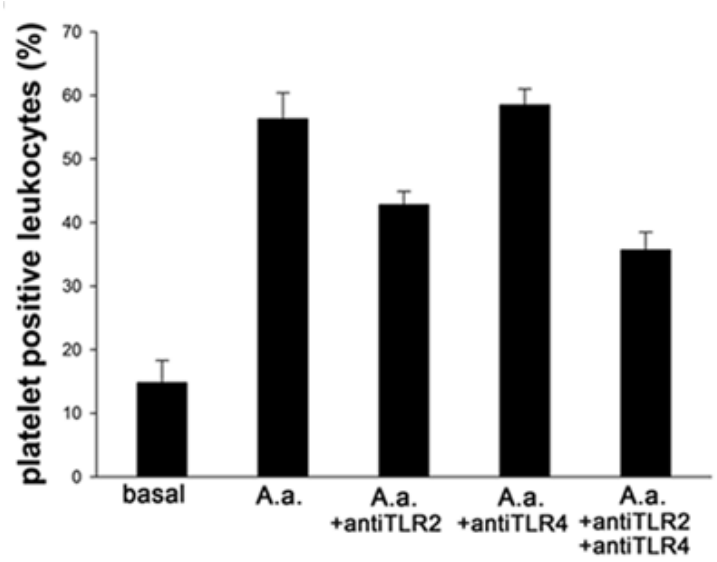

Figure 15 | Influence of TLR receptors on formation of platelet leukocyte aggregates induced by A. actinomycetemcomitans: *PRP (3x10^8/ml) was preincubated with anti TLR2 and/or anti TLR4, then isolated neutrophils (5x10^5/ml) and A. actinomycetemcomitans (3x10^9 bacteria/ml) were added and after 20 minutes the formation of platelet leukocyte aggregates was determined; means and standard deviations of 6 independent experiments with blood from 6 different donors;*

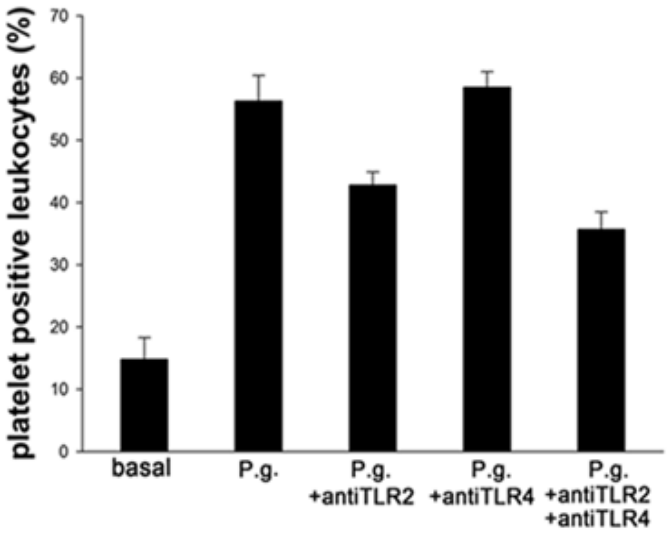

Figure 16 | Influence of TLR receptors on formation of platelet leukocyte aggregates induced by P. gingivalis: *PRP (3x10^8/ml) was preincubated with anti TLR2 and/or anti TLR4, then isolated neutrophils (5x10^5/ml) and P. gingivalis (3x10^9 bacteria/ml) were added and after 20 minutes the formation of platelet leukocyte aggregates was determined; means and standard deviations of 6 independent experiments with blood from 6 different donors;*

4.4 Platelet signalling induced by periodontitis associated bacteria

It has been shown by other groups that PI3K and Akt are involved in TLR signalling in human platelets (Zhang et al., 2009b;Blair et al., 2009c;Kalvegren et al., 2010a). Therefore, we blocked PI3K, PLC or Akt and determined the effects on ADP, Pam2CSK4 (TLR2/6 agonist), Pam3CSK4 (TLR1/2 agonist), LPS from E. coli (TLR4 agonist) and S. typhi (TLR4 agonist) on induced surface expression of CD40L (depicted in Figure 17). Moreover we investigated the effects of the same inhibitors on periodontitis associated bacteria induced surface expression of CD40L (shown in Figure 18).

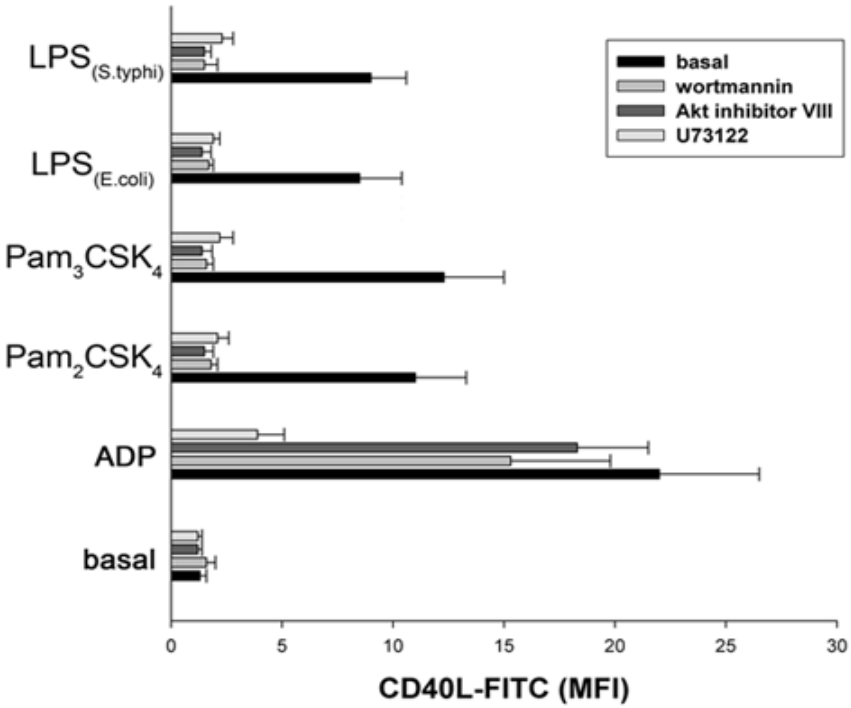

Figure 17 | Underlying mechanisms of surface expression of CD40L by ADP and TLR agonists: *PRP ($3x10^8$ platelets/ml) was preincubated with wortmannin (2µM), U73122 (2µM) or Akt inhibitor VIII (2µM) for 10 minutes or left untreated, thereafter platelets were incubated with ADP (50µM), Pam_2CSK_4 (1µg/ml), Pam_3CSK_4 (1µg/ml), LPS from E. coli or S. thyphi (1µg/ml) for 20 minutes. Samples were analyzed by flow cytometry. Platelet activation was determined by surface expression of CD40L. Means and standard deviations of 6 independent experiments;*

Blocking of PI3K, PLC or Akt led to a total inhibition of TLR agonist and bacteria induced surface CD40L (Figure 17 and Figure 18), but PI3K and Akt inhibition had no significant impact on activation induced by maximal effective doses of ADP (Figure 17). These results indicate that platelet activation by periodontitis associated bacteria is mediated via the PI3K/Akt pathway.

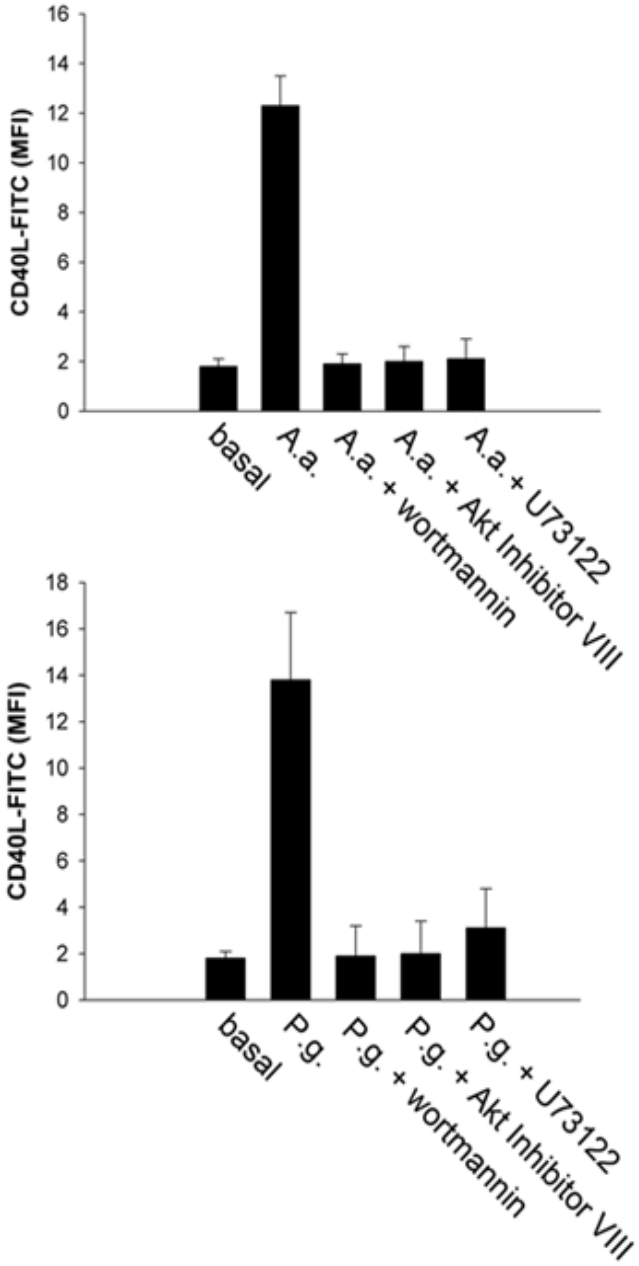

Figure 18 | Underlying mechanisms of bacteria induced surface expression of CD40L: *PRP ($3x10^8$ platelets/ml) was preincubated with wortmannin (2µM), U73122 (2µM) or Akt inhibitor VIII (2µM) for 10 minutes or left untreated, thereafter platelets were incubated with A. actinomycetemcomitans or P. gingivalis ($3x10^9$ bacteria/ml) for 20 minutes. Samples were analyzed by flow cytometry. Means and standard deviations of 6 independent experiments;*

4.5 Effects of platelets on neutrophil mediated phagocytosis of periodontitis associated bacteria

To determine if platelets have an impact on the rate of phagocytosis of periodontitis associated bacteria by neutrophils, uptake of pHrodo stained A. actinomycetemcomitans or P. gingivalis by freshly isolated neutrophils in the presence and absence of platelets was quantified (Figure 19).

In the absence of plasma addition of platelets had no effect on phagocytosis by neutrophils. In the presence of plasma phagocytosis significantly enhanced for both bacteria tested (Figure 19) and the presence of platelets even further increased the rate of phagocytosis.

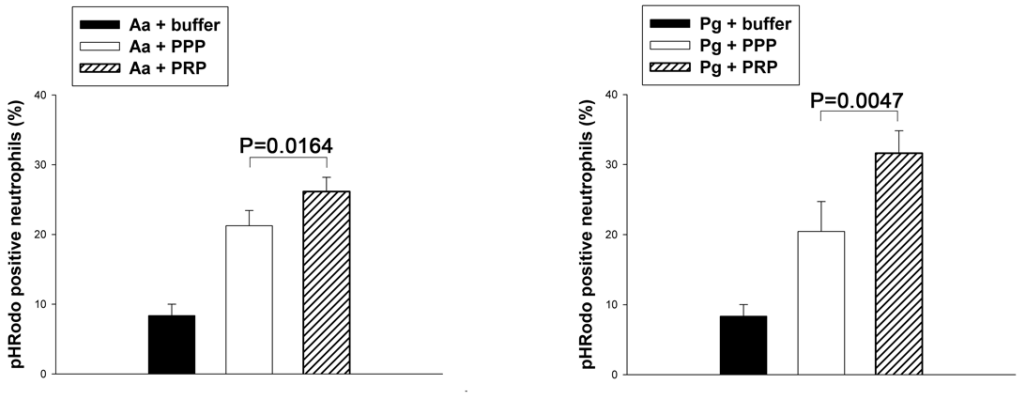

Figure 19| Influence of platelets on neutrophil mediated phagocytosis of periodontitis associated bacteria: *Isolated neutrophils were either incubated with buffer, PPP or PRP ($3x10^8$/ml) and the effects on pHrodo stained A. actinomycetemcomitans (left) and P. gingivalis (right) were determined; In the presence of platelets neutrophils phagocytose significantly more bacteria (P=0.0164 for A. actinomycetemcomitans and P=0.0047 for P. gingivalis); means and standard deviations of 5 independent experiments with blood from 5 different donors*

That bacteria are actually phagocytosed in that assay was determined and confirmed by confocal microscopy (Figure 20), revealing that pHrodo stained bacteria are found in the acidic environment of phagosomes (intensive red stain).

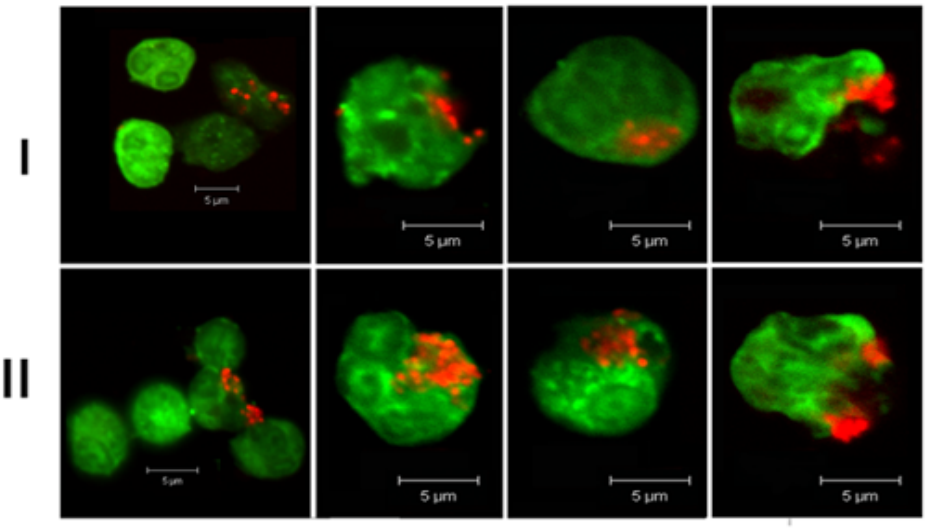

Figure 20| Isolated neutrophils undergoing phagocytosis: *merged z-stack confocal image of bacterial uptake; green: Cell Tracker Green CMFDA stained neutrophils; red: pHrodo labelled A. actinomycetemcomitans;* ***I****: in the presence of PPP;* ***II****: in the presence of PRP (3x10^8/ml)*

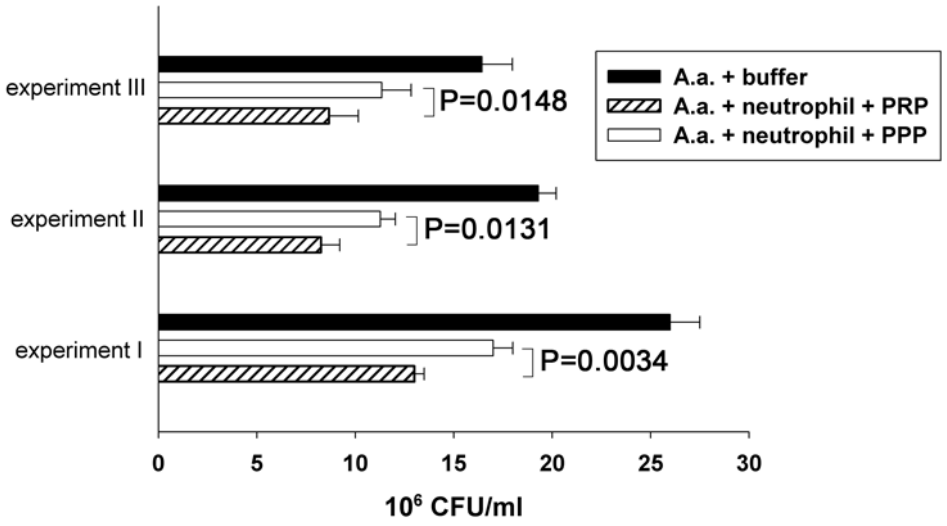

Figure 21| The effects platelets on bacteria survival: *neutrophils were incubated for 20minutes with A. actinomycetemcomitans and P. gingivalis (10^{9}bacteria/ml) in the presence and absence of platelets; neutrophils were removed by centrifugation and the surviving bacteria in the supernatant determined as described in Material and Methods by colony forming units (CFU) after 24 hour incubation on blood agar; 3 independent experiments;*

To proof that platelet induced increase in the rate of phagocytosis by neutrophils also has a biological consequence, the percentage of surviving bacteria was evaluated in a plasma containing phagocytosis assay in the absence (PPP) and presence (PRP) of platelets. In fact the increased rate of phagocytosis (*Figure 19*, PPP versus PRP) is reflected by a decreased percentage of viable bacteria in the presence of platelets (*Figure 21*, PRP versus PRP).
From these data we conclude that the rate of phagocytosis of periodontitis associated bacteria by neutrophils is increased in the presence of platelets.

4.6 Mechanisms of bacteria induced platelet leukocyte interaction

Next we determined if platelet TLR/PI3K/Akt signalling is also necessary for the supporting effects on neutrophil mediated clearance of periodontitis associated bacteria. Moreover we investigated if a direct interaction between platelets and neutrophils is required for increased bacterial clearance by blocking P-selectin/PSGL-1 and CD40L/CD40 interactions.
Since P-selectin and TLR4 co-localize on human platelets (Stahl et al., 2006b), blocking of P-selectin might lead to a interference with bacteria binding to TLR4. Therefore we blocked platelet P-selectin by two different antibodies and preincubated neutrophils with soluble P-selectin to saturate P-selectin glycoprotein ligand-1 (PSGL-1).
Blocking of either PSGL-1 or CD62P leads to a highly significant decrease in formation of platelet neutrophil aggregates. In contrast, blocking of CD40L had no significant impact on platelet adhesion to neutrophils. Moreover, blocking either Akt or PI3K activation in platelets also leads to a highly significant decrease in platelet neutrophil aggregates induced by both types of bacteria (Figure 22).
Similar effects could be observed in terms of surface expression of CD11b (Figure 23) where the increased surface expression of bacteria stimulated neutrophils in the presence of platelets is completely reversed if platelet PI3K or Akt signalling is interrupted. Also blocking of platelet P-selectin by relevant antibodies leads to a significant decrease of CD11b when compared to fully functional platelets.

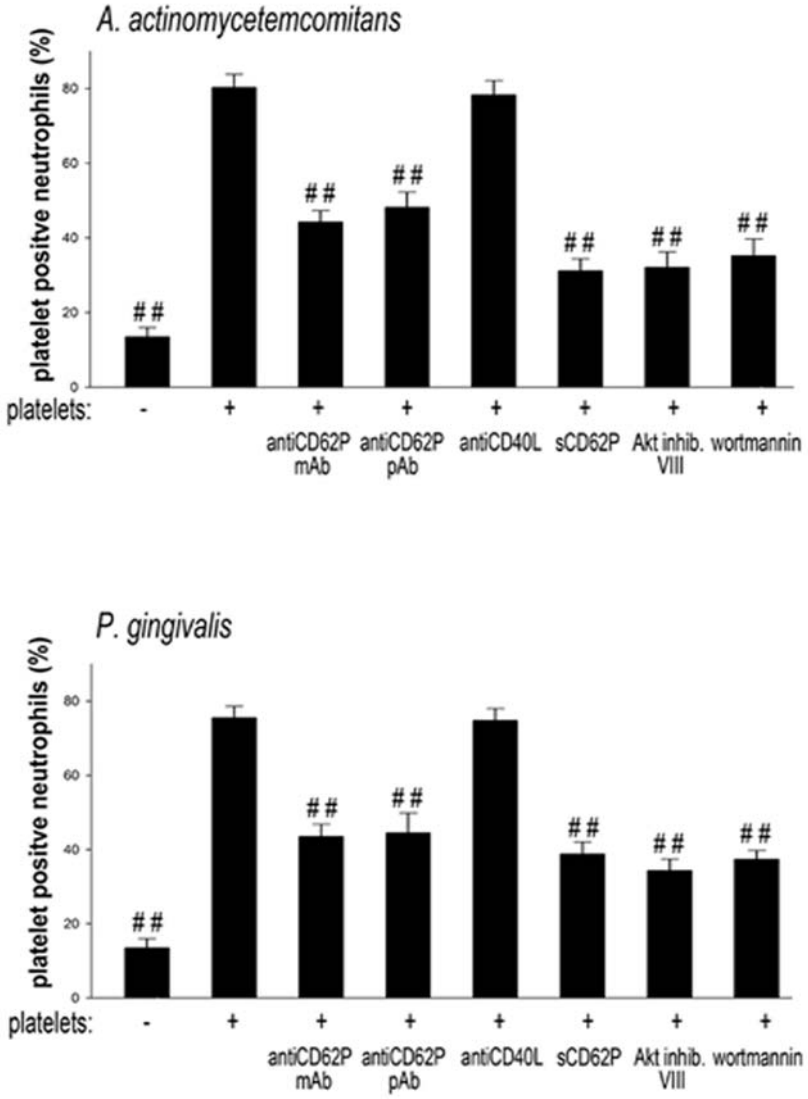

Figure 22| Influence of platelet PI3K, Akt, P-selectin and CD40L on bacteria induced formation of platelet leukocyte aggregates: *GFP (1x10^7 platelets/ml) were incubated with the respective inhibitors and P-selectin/PSGL-1 interaction was inhibited as described in Material and Methods and incubated with freshly isolated neutrophils (5x10^5/ml) in 30% plasma from the same donor. PLA were measured after 20 minutes of stimulation with A. actinomycetemcomitans or P. gingivalis. Samples were analyzed by flow cytometry; mean and standard deviations of 3 independent experiments; significances are either reported as change in mean fluorescence ± standard deviation relative to samples lacking platelets (*) or relative to control ($^{\#,\#\#}$); *,$^{\#}$ indicate P<0.01; $^{\#\#}$ indicates P<0.01;*

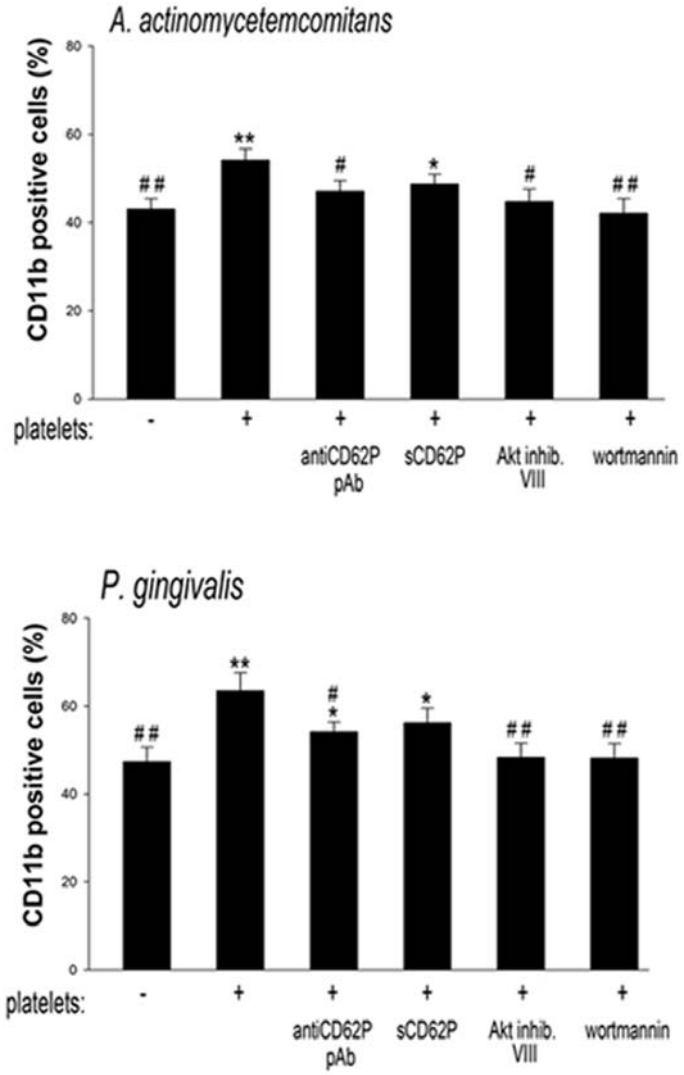

Figure 23| Influence of platelet PI3K, Akt, P-selectin and CD40L on activation of neutrophils: *GFP ($1x10^7$ platelets/ml) under conditions of P-selectin/PSGL-1 interaction inhibition as described in Material and Methods and incubated with freshly isolated neutrophils ($5x10^5$/ml) in 30% plasma from the same donor. After 20 minutes of stimulation with A. actinomycetemcomitans or P. gingivalis CD11b on the neutrophil surface was determined; final concentration of bacteria was always $1x10^8$ bacteria/ml; Samples were analyzed by flow cytometry; mean and standard deviations of 3 independent experiments; significances are either reported as change in mean fluorescence ± standard deviation relative to samples lacking platelets (*) or relative to control ($^{\#,\#\#}$); $*,^{\#}$ indicate P<0.01; **, $^{\#\#}$ indicates P<0.01*

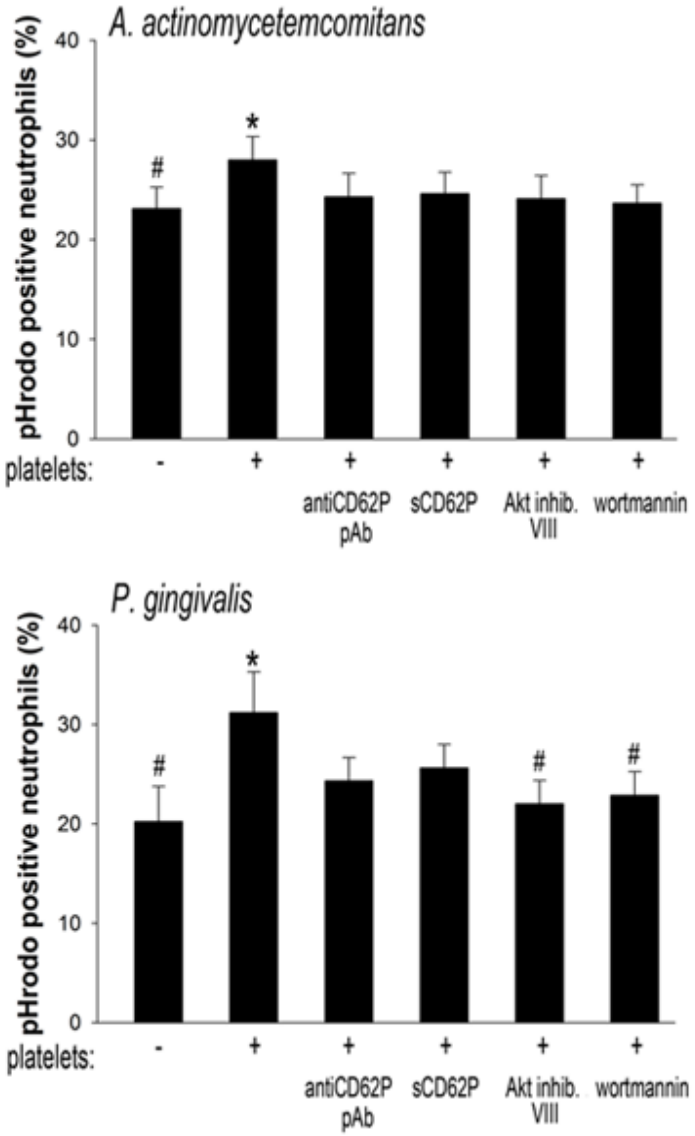

Figure 24| Influence of platelet PI3K, Akt, P-selectin/PSGL-1 interaction on the supportive role of platelets on the phagocytotic activity of neutrophils:
*GFP ($1x10^7$ platelets/ml) were incubated with the respective inhibitors and inhibition of P-selectin/PSGL-1 interaction as described in Material and Methods and incubated with freshly isolated neutrophils ($5x10^5$/ml) in 30% plasma from the same donor. PLA were measured after 20 minutes of stimulation with A. actinomycetemcomitans or P. gingivalis; Phagocytosis by neutrophils was measured after 20 minutes of stimulation with pHrodo stained A. actinomycetemcomitans or P. gingivalis; final concentration of bacteria was always $1x10^8$ bacteria/ml; Samples were analyzed by flow cytometry; mean and standard deviations of 3 independent experiments; significances are either reported as change in mean fluorescence ± standard deviation relative to samples lacking platelets (*)or relative to control ($^{\#,\#\#}$); *,$^{\#}$ indicate P<0.01; $^{\#\#}$ indicates P<0.01;*

Interestingly even high amounts of soluble P-selectin can only partially reduce the supportive effects of platelets on bacterial induced surface expression of CD11b by neutrophils, indicating that soluble P-selectin can to some extent mimic the effects of platelet P-selectin/PSGL-1 binding.
Furthermore, the increased phagocytotic activity of neutrophils in the presence of platelets is undone when platelets are preincubated with Akt or PI3K inhibitor or upon blocking P-selectin-PSGL-1 interaction (

Figure 24). These data indicate that a direct interaction between platelets and neutrophils mediated via P-selectin/PSGL-1 is essential for the supportive role of platelets in bacterial clearance. Moreover platelet activation via TLR/PI3K/Akt is essential for the formation of platelet neutrophil aggregates and the supportive effects on bacterial clearance.

5 Discussion

Although platelets are able to take up bacteria, as previously shown for P. gingivalis (Li et al., 2008), they are unable to form a functional killing chamber and therefore fail to directly kill bacteria (White, 2006b). Therefore, it has been speculated that platelet enclosure of microorganisms results in a protection of pathogens from antibiotics or clearance by leukocytes (Clawson and White, 1971;White, 2006a). Nevertheless, the functional significance of platelet mediated uptake of bacteria is currently unclear as a rat model revealed that thrombocytopenia leads to a delay of periodontal healing (Spolidorio et al., 2009), which argues for a supportive role of platelets in immune response.

Within this work we demonstrate that in the presence of platelets significantly more periodontitis associated bacteria (A. actinomycetemcomitans and P. gingivalis) are phagocytosed and killed by neutrophils. Moreover, upon incubation with the investigated bacteria platelets do not only enhance phagocytosis but also neutrophil surface expression of CD11b.

Since these effects of platelets on neutrophil function might be mediated by direct interaction of platelets with neutrophils or indirectly via platelet release products, we investigated the effects of the two periodontitis associated bacteria on the formation of platelet neutrophil aggregates as well as on platelet activation. Incubation of blood cells with A. actinomycetemcomitans and P. gingivalis results in an enormous increase in the number of platelet leukocyte aggregates, thereby especially in platelet neutrophil aggregates and platelet monocyte aggregates.

TLRs seem to be involved in the formation of platelet leukocyte aggregates induced by the two periodontitis associated bacteria, as the extensive formation of platelet leukocyte aggregates can be undone by blocking of TLR2 and in the case of A. actinomycetemcomitans also (though to a lesser extent) by blocking of TLR4. Recognition of A. actinomycetemcomitans by TLR2 and TLR4 and recognition of P. gingivalis by TLR2 is in line with results on periodontitis associated bacteria induced cytokine production in whole blood, which indicates that gram-negative bacteria predominantly stimulate TLR2 (Kikkert et al., 2007a).

Furthermore we demonstrate that plasma is a mandatory cofactor for the supportive effect of platelets on bacteria phagocytosis by neutrophils and also necessary for platelet activation by the two investigated bacteria itself, which suggests that platelet activation by periodontitis associated bacteria might be essential for the observed effects.

Detailed analyses on platelet activation in response to periodontitis associated bacteria reveals that GPIIb/IIIa activation, as well as surface expression of CD63, CD40L and P-selectin occur in a dose and time dependent way. These results are in line with previous studies on murine platelets which revealed that P. gingivalis induces platelet activation in vitro and in vivo (Sharma et al., 2000;Blair et al., 2009b).

Nevertheless, we find that even at high concentrations of bacteria and prolonged incubation platelets do not become fully activated. The effects of A. actinomycetemcomitans and P. gingivalis on platelet activation after 20 minutes of incubation are significantly below those induced by maximal effective concentrations of ADP, whereat the formation of platelet leukocyte aggregates increases enormously compared to ADP activation.

Accordingly, platelet leukocyte formation seems to be the most apparent outcome of stimulation by the two periodontitis associated bacteria. Such extensive interactions are characteristic for TLR2 induced platelet activation (Blair et al., 2009a) and increased cross-talk of platelets with monocytes and neutrophils as a consequence of platelet activation has also been found in patients with periodontal diseases (Nicu et al., 2009).

While the PI3K/Akt signalling pathway downstream of TLRs acts as a negative feedback in leukocyte activation (Schabbauer et al., 2004), in platelets PI3K/Akt is essential for TLR2 and TLR4 mediated activation (Rex et al., 2009a;Zhang et al., 2009a;Blair et al., 2009h;Kalvegren et al., 2010b) and our results indicate that signalling pathways in platelets are identical after stimulation with either periodontitis associated bacteria or TLR agonists. Moreover we demonstrate the importance of PLC, responsible for maintenance of Akt phosphorylation (Resendiz et al., 2007a) in platelet activation induced by periodontitis associated bacteria.

Our data further indicate an exclusive role of TLR signalling by ruling out an involvement of FcγRII in periodontitis associated bacteria mediated platelet activation, which has been shown to be involved in P. gingivalis Hgp44 induced platelet aggregation (Naito et al., 2006). An involvement of protease activated receptor 1 (PAR-1) and PAR-4 (previously reported for platelet interaction with P. gingivalis toxins (Lourbakos et al., 2001)) can be ruled out as CD14 was found to be essential for the observed effects. Even more, Akt inhibition would only lead to delayed platelet activation in PAR stimulated platelets but to no inhibition of activation after 20 minutes (Resendiz et al., 2007b).

Our data indicate that P-selectin/PSGL-1 engagement is required for platelet adhesion to neutrophils and also necessary for increased CD11b activation and enhanced phagocytosis, which is in line with previous reports that binding of P-selectin to PSGL-1 triggers tyrosine kinase-dependent mechanisms that lead to CD11b activation in neutrophils(Evangelista et al., 1999;Evangelista et al., 2007) and that CD11b is important for phagocytosis by neutrophils (Schymeinsky et al., 2009).

It is known that soluble P-selectin can induce surface expression of CD11b on human neutrophils (Woollard et al., 2006) - nevertheless we found that soluble P-selectin can mimic the effects of platelets on neutrophil CD11b expression only to a limited extent and does not have the ability to fully replace the effects of functional platelets, since only a tendency but no statistically significant influence of soluble P-selectin on the phagocytotic activity of neutrophils could be detected. This indicates that neutrophil interaction with activated platelets boosts activation of both cells mutually.

Taken together, our data suggest that in response to periodontitis associated bacteria platelets rather support immune response by increasing the phagocytotic activity of neutrophils in a TLR/PI3K/Akt dependent mechanism which results in P-selectin/PSGL-1 mediated direct interaction with neutrophils. Thereby our findings add functional relevance to previous observations that platelet leukocyte aggregates bind bacteria more rapidly than unbound leukocytes in whole blood (Peters et al., 1999b;Nicu et al., 2009).

P-selectin dependent interaction of neutrophils and platelets amplifies neutrophil

activation - an intended process when dying or severely damaged endothelial cells fail to sustain leukocyte rolling and adhesion and platelet P-selectin facilitates leukocyte migration to perivascular tissues (Diacovo et al., 1996b;Zwaginga et al., 1999). Nevertheless, the interaction between neutrophils and activated platelets also occurs in circulating blood, yielding in activated platelet leukocyte aggregates, which is a hallmark of acute myocardial infarction, sepsis, and inflammatory disorders (Maugeri et al., 2006;Linden et al., 2007). Since platelet neutrophil complexes have a more activated adhesion molecule profile and a greater capacity for phagocytosis and toxic oxygen metabolite production (Peters et al., 1999a) they also release more destructive enzymes. This results in exacerbating tissue injury and might contribute to the clinical signs of periodontal diseases and subsequent systemic complications. Therefore platelets appear to be double edged players in periodontal diseases, which augment bacterial clearance but might thereby also encourage the development of atherosclerosis.

6 Summary

Periodontitis is a chronic infection of supportive dental tissues caused by distinct gram negative bacteria. Interaction with host immune system leads to a chronic inflammation and since transient bacteraemia frequently occurs also distant site infections are associated with periodontitis. Moreover periodontitis has been associated with an increased risk for cardiovascular diseases.

The present work aimed to clarify the role of platelets in immune response to periodontitis associated bacteria. Furthermore we aimed to determine if platelets are involved in linking periodontitis with cardiovascular complications.

Although platelets are unable to directly kill bacteria our data reveal that platelets enhance neutrophil mediated clearance of A. actinomycetemcomitans and P. gingivalis by increasing the phagocytotic activity of neutrophils as well as neutrophil bacteria binding and upregulation of neutrophil surface expression of CD11b (Mac-1).

The supportive role of platelets on neutrophil function mainly depends on direct interactions of platelets with neutrophils, mediated by P-selectin/PSGL-1 binding. Platelet activation, which is required for interaction with neutrophils, by both bacterial strains occurs via toll like receptor (TLR) 2 and to a lesser extent TLR4 and the observed effects can be totally reversed by inhibition of platelet phosphoinositol-3-kinase (PI3K) or Akt.

This platelet-mediated increase in neutrophil activation enhances bacterial clearance but may also exacerbate tissue injury. Therefore platelets might be double edged players in periodontitis which augment bacterial clearance but thereby also encourage the development of cardiovascular disease.

7 Zusammenfassung

Parodontose ist eine chronische Infektion des Zahnhalteapparates durch bestimmte Gruppen gram-negativer Bakterien. Durch die Immunantwort des befallenden Organismus führt die bakterielle Infektion zu einer chronischen lokalen Entzündung.
Da es häufig zu Bakteriämien kommt können auch an anderen Stellen des Körpers zu Parodontose assoziierten Infektionen kommen. Zudem konnte nachgewiesen werden, dass Parodontose einen Risikofaktor für kardiovaskuläre Erkrankungen darstellt.
Ziel der hier vorliegenden Arbeit war es nachzuweisen welche Rolle der Thrombozyt in der Immunantwort gegen Parodontose assoziierten Keime hat. Sowie festzustellen ob Thrombozyten in Parodontose assoziierten kardiovaskulären Komplikationen eine Rolle spielen.
Wenngleich Thrombozyten Bakterien nicht direkt abtöten können, konnte in dieser Arbeit nachgewiesen werden, dass Thrombozyten die neutrophilen Granulozyten bei der Vernichtung von A. actinomycetemcomitans and P. gingivalis unterstützt. Da sie die phagozytotische Aktivität der Neutrophilen, wie auch die Bakterienbindung an Neutrophile verstärken und die Oberflächenexpression von CD11b (Mac-1) hochregulieren.
Die verstärkende Wirkung der Thrombozyten auf die Neutrophilenfunktion ist hauptsächlich auf eine direkte Interaktion zwischen Thrombozyten und Neutrophile zurückzuführen. Diese Interaktion wird durch die P-Selektin am Thrombozyten und PSGL-1 am Neutrophilen vermittelt. Thrombozytenaktivierung, die die Voraussetzung für deren Interaktion mit Neutrophilen darstellt, wird bei beiden Bakterienstämmen durch Toll Like Rezeptor (TLR) 2 und zu einem geringeren Ausmaß durch TLR4 vermittelt und kann durch Hemmung von thrombozytärer Phosphoinositol-3-Kinase (PI3K) oder Akt komplett verhindert werden.
Dieser Thrombozyten vermittelte Anstieg der Neutrophilenaktivierung verstärkt die Beseitigung der Bakterien kann aber auch zu unerwünschten Gewebeschäden führen. Daher scheinen Thrombozyten eine zweischneidige Rolle in der Parodontose zu haben, da sie einerseits zur Beseitigung der Bakterien beitragen aber andererseits auch zur Entstehung von kardiovaskulären Erkrankungen beitragen.

8 Table of figures

9 References

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10 Curriculum Vitae

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EDUCATION

1988-1992	Elementary school Spittal/Drau, Austria
1992-2000	Grammar school “Porcia“, Spittal/Drau, Austria
Jan.-Jun.1999	Duringthon Sixthform College, Worthing, Great Britain
Jun 2000	final examinations: grammar school “Porcia”, Spittal/Drau, Austria
2000-2006	Diploma in nutritional sciences, University of Vienna, Austria
2006-2009	PhD programme nutritional sciences, University of Vienna, Austria

WORK EXPERIENCE

September 2002	Internship: bacteriological laboratory, Kärntnermilch; Austria
September 2004	Internship: bacteriological laboratory, Kärntnermilch, Austria
April 2006- Jan. 2009	Scientific assistant (post graduate) at the Institute of Physiology; Medical University of Vienna, Austria
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THESIS

Master of Science thesis (Nutritional Sciences): Nutritional status of children in rural and urban Ecuador (University of Vienna; 2005)

Doctoral thesis (Life Sciences): Platelet function under systemic oxidative stress –the role of native and modified lipoproteins (University of Vienna; 2008)

PUBLICATIONS

Assinger,A., Koller, F., Schmid,D., Zellner, M., Babeluk,R., Koller,E. & Volf,I. (2010) Oxidized HDL trigger CD40L expression in human blood platelets by a CD36-dependent mechanism. Atherosclerosis *in press*

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CONGRESS CONTRIBUTIONS

Assinger, A., Koller, F, Schmid,W., Koller,E., Volf,I. (2010) Hypochlorite-oxidized LDL induce the release of CD40L in human platelets, 1. Joint Meeting GTH & NVTH, February 2010, Nürnberg

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