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**Institution för medicin, solna**

# PLATELET-LYMPHOCYTE CROSS-TALK IN ATHEROGENESIS

## Experimental Studies on Platelet-Regulated Lymphocyte Adhesion and $CD4^+$ T Cell Activation

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# PLATELET-LYMPHOCYTE CROSS-TALK

# IN ATHEROGENESIS

Experimental Studies on Platelet-Regulated
Lymphocyte Adhesion and $CD4^+$ T Cell Activation

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# ABSTRACT

Atherosclerosis is an inflammatory and thrombotic disease. Platelets and lymphocytes, especially $CD4^+$ T cells, are indispensable players during the initiation and progression of atherosclerotic lesion formation. During the early stage of atherogenesis, lymphocytes are generally thought to be recruited from arterial blood flow. It is, however, not clear how they are recruited. Platelets can regulate various functional aspects of $CD4^+$ T cells, but the mechanisms of platelet-$CD4^+$ T cell interactions have not be well defined. Therefore, aims of the present thesis work are to investigate how platelets influence lymphocyte adhesion under arterial flow conditions, and how platelets regulate $CD4^+$ T cell functions.

Platelet-supported lymphocyte adhesion was first investigated using reconstituted human blood and a collagen-coated parallel-plate flow chamber. Adhered platelets supported lymphocyte adhesion under arterial flow conditions (e.g., 500 $s^{-1}$), which involved multiple adhesion molecules (e.g., P-selectin, CD40L, and GPIIb/IIIa). Platelet-dependent lymphocyte adhesion was selective among larger $CD4^+$ and $CD8^+$ T cells, and small B cells. In vivo model of arterial thrombosis confirmed that arterial thrombi supported lymphocyte recruitment, and that platelet GPIIb/IIIa blockade prevented thrombus formation and subsequently abolished lymphocyte recruitment.

We continued to study how platelets enhance lymphocyte adhesion on subendothelial matrix protein (SEMP)-coated surface under arterial flow conditions by using Cone and Plate(let) analyser (CPA). Collagen markedly, fibrinogen and VWF mildly provoked platelet deposition that proportionally enhanced lymphocyte adhesion. The data confirmed that platelets selectively enhance adhesion of large $CD4^+$ and $CD8^+$ T cells and NK cells, and of small B cells. The lymphocyte adhesion positively correlates with their platelet conjugating potential and expression of PSGL-1, Mac-1, and CD40L.

Platelet-regulated $CD4^+$ T cell function was investigated in autologous human platelet-$CD4^+$ T cell co-cultures. Platelets attenuated $CD4^+$ T cell proliferation, but enhanced activation of Th1/Th17/Treg cells. The enhancements were exerted by both direct cell-cell contact and platelet-derived soluble mediators PF4, RANTES, and TGFβ. The enhancements were shown as increased $CD4^+$ T effector cell phenotypes and corresponding cytokine production.

Dynamics of platelet-regulated $CD4^+$ T cell activation were also monitored. Platelets constantly promoted Treg cell activation, but exerted a biphasic regulation on Th1/Th17 activation, namely a transient enhancement followed by a secondary suppression. The distinct regulations were achieved by a selective, TGFβ-mediated inhibition of $FoxP3^-$ T cell proliferation.

Together, the thesis work has elucidated novel mechanisms of platelet-regulated $CD4^+$ T effector cell responses. Platelets selectively enhance lymphocyte adhesion under arterial flow conditions, and regulate distinct dynamics of Th1/Th17/Treg cell activation.

# LIST OF PUBLICATIONS

**I.** Hu H, **Zhu L**, Huang Z, Ji Q, Chatterjee M, Zhang W, Li N.

Platelets enhance lymphocyte adhesion and infiltration into arterial thrombus.

*Thromb Haemost. 2010; 104(6):1184-92.*

**II.** Gerdes N*, **Zhu L***, Ersoy M, Hermansson A, Hjemdahl P, Hu H, Hansson GK, Li N.

Platelets regulate $CD4^+$ T-cell differentiation via multiple chemokines in humans.

*Thromb Haemost. 2011; 106(2):353-362.*

**III.** Spectre G*, **Zhu L***, Ersoy M, Hjemdahl P, Savion N, Varon D, Li N.
Platelets selectively enhance lymphocyte adhesion on subendothelial matrix under arterial flow conditions.

*Thromb Haemost. 2012;108(2):328-337*

**IV.** **Zhu L**, Huang Z, Stålesen R, Hansson GK, Li N.
Platelets provoke distinct dynamics of immune responses of different $CD4^+$ T cell subsets via selective regulations of cell proliferation
*Manuscript*

*These authors contributed equally to the study.

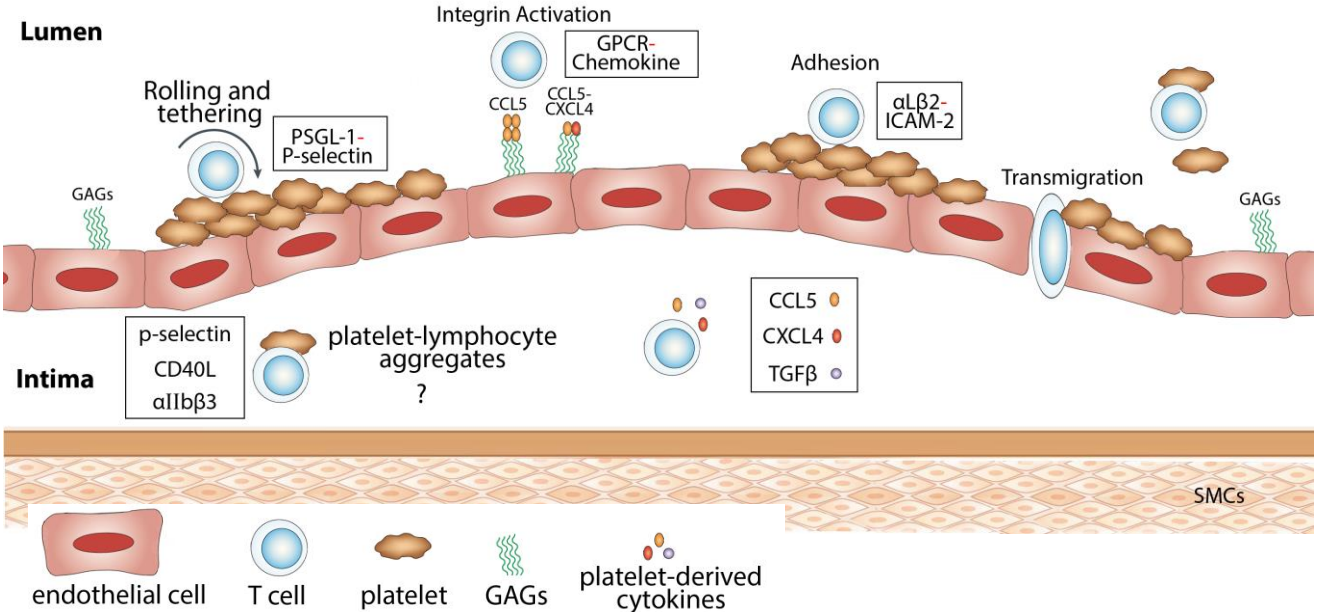
# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| APC | antigen presenting cell |
| cAMP | cyclic adenosine monophosphate |
| CCR | CC chemokine receptor |
| cGMP | cyclic guanosine monophosphate |
| CPA | cone-and-plate(let) analyzer |
| CXCR | CXC chemokin receptor |
| DAG | diacylglycerol |
| EC | endothelial cell |
| GAG | glycosaminoglycan |
| GEFs | guanine-nucleotide-exchange factors |
| GlyCAM-1 | Glycosylation-dependent cell adhesion molecule-1 |
| GPCR | G protein-coupled receptors |
| GTPases | guanosine triphosphate hydrolase enzymes |
| HEV | high endothelial venule |
| ICAM-2 | intercellular adhesion molecule 2 |
| IFNγ | Interferon γ |
| IL | interleukin |
| IL-12R | Interleukin 12 receptor |
| Insp3 | inositol trisphosphate |
| MadCAM-1 | mucosal vascular addressin cell adhesion molecule 1 |
| MAP kinase | mitogen-activated protein kinases |
| MHC | major histocompatibility complex |
| OCS | open canalicular system |
| PAF | platelet-activating factor |
| PDGF | platelet derived growth factor |
| PF4 | platelet factor 4 |
| $PGI_2$ | prostacyclin |
| PLC | phospholipase C |
| PS | phosphatidylserine |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| PtdIns(4,5)P2 | Phosphatidylinositol,4,5-bisphosphate |
| RIAM | Rap1-GTP-interacting adaptor molecule |
| sGC | soluble guanylyl cyclase |
| TCR | T cell receptor |
| TGFβ | transforming growth factor β |
| $TxA_2$ | thromboxane $A_2$ |
| VCAM-1 | vascular cell adhesion molecule-1 |
| vWF | von Willebrand factor |

# 1 INTRODUCTION

Atherosclerosis is a thrombotic and inflammatory disease (1, 2), and is the principle pathological cause of ischemic cardiovascular diseases (3). Atherogenesis involves dysfunction and interaction of blood cells and vascular cells, such as platelets and leukocytes in the circulation and endothelial cell and smooth muscle cells of the vessel wall.




**Figue1. Platelet-lymphocyte interaction in atherosclerosis.** During atherogenic process, lymphocytes roll and tether on the platelet-covered endothelial surface, which is mediated by PSGL-1 (lymphocyte) and P-selectin (platelet). Platelet-derived chemokines can bind to the GAGs on endothelial cells, attract lymphocytes, and induce integrin activation and firm adhesion of lymphocytes. The firm adhesion between platelets and lymphocytes is mainly mediated by ICAM-2 and αLβ2. Platelets facilitate lymphocyte transmigration into the plaque, and cytokines and chemokines released from platelets can influence lymphocyte activation, differentiation and proliferation. Abbreviations: GAGs: glycosaminoglycans; GPCR: G protein-coupled receptors; PSGL-1: P-selectin glycoprotein ligand-1; ICAM-2: intercellular adhesion molecule 2; CCL5: RANTES; CXCL4: platelet factor 4; TGFβ: transforming growth factor β.

Atherosclerotic lesions contain a large number of blood-born immune and inflammatory cells, including macrophages and lymphocytes, which are the driving force of inflammatory processes in atherosclerotic lesion development. Platelets are known to be present in atherosclerotic lesions (4), and are closely involved throughout the whole process of atherosclerosis, from the initiation of lesion formation, to plaque development, and to plaque rupture (5). Platelets and leukocytes, particularly concerned in the present thesis work, $CD4^+$ T cells, do not simply co-exist in the lesions. There is increasing evidence showing that interactions between platelets and lymphocytes

play pivotal roles in atherogenesis. Hence, the present thesis work has studied the regulatory roles of platelets on lymphocyte, especially $CD4^+$ T cells, adhesion and activation in experimental settings mimicking arterial flow conditions and lesion development.

## 1.1 PLATELET PHYSIOLOGY

Platelets are annucleic cells, and are shed cytoplasmic fragments of megakaryocytes in the bone marrow. Platelets are the smallest blood cells, 1-4 μm in size, but circulate in large numbers, 200–$300\times10^{-9}$/L. The life span of platelets in the circulation is around 10 days. Aged and apoptotic platelets, as well as activated platelets are mainly cleaned by macrophages in the liver and the spleen.

Except nucleus, platelets are equipped with all other organelles seen in ordinary cells. Some of the organelles are specialized in platelets, e.g., the two types of secretory granules in cytoplasm, namely α granules and dense granules. The α granules are the storage of high molecular weight proteins and peptides, such as von Willebrand factor (vWF), fibrinogen, platelet factor 4 (PF4), and coagulation factor V. The dense granules contain low molecular weight compounds, including ADP, ATP, and calcium. Platelets have two specialized tubular systems. The open canalicular system (OCS) is a reservoir of platelet membrane, and provides a transportation highway for the release of platelet granule contents. The dense tubular system is a calcium storage pool, and is the site for thromboxane $A_2$ and prostaglandin syntheses (6).

Platelet membrane is embedded with a large number of proteins. Some of them are adhesion molecules, are they may be platelet-specific, such as glycoprotein (GP) Ib/IX/V complex and GPIIb/IIIa, which are the principle receptors for vWF and fibrinogen, respectively. Some other proteins serve as receptors for various platelet agonists. For example, platelet membrane expresses the thrombin receptor PAR1 and PAR4 and the ADP receptor $P2Y_{12}$ and $P2Y_1$ (7).

### 1.1.1 Platelet activation

Platelets are sensitive cells, and can be activated by a number of stimuli. Table 1 lists a panel of common agonists and their corresponding receptors of platelets. The potencies of platelet agonists differ considerably. Thrombin, the most potent physiological agonist of platelets, induces full platelet activation, including platelet shape change, adhesion and aggregation, secretion, as well as vesiculation (microparticle generation). Some platelet agonists, such as ATP and epinephrine, are so-called weak agonists, which only induce platelet shape change and/or reversible platelet aggregation.

**Table 1. Common agonists and their receptors of platelets**

| Agonist | Receptor | Receptor type |
|---|---|---|
| Thrombin | PAR1, PAR4 | Seven transmembrane receptors |
| Thromboxane $A_2$ | TPa | Seven transmembrane receptor |
| ADP | $P2Y_1$, $P2Y_{12}$ | Seven transmembrane receptors |
| Serotonin (5HT) | $5HT_{2A}$ | Seven transmembrane receptor |
| Epinephrine | Adrenergic receptor α2 | Seven transmembrane receptor |
| collagen | GPⅥ<br>$\alpha_2\beta_1$ | Immunoglobulin superfamily<br>integrin |
| ATP | $P2X_1$ | ion channel |

Platelet activation increases surface expression and affinity of platelet adhesion molecules, and enables platelets to roll, to tether, and to adhere on the vessel wall and/or adjacent cells. P-selectin expressed on activated platelets primarily mediates platelet rolling on inflamed endothelial surface, mainly via P-selectin glycoprotein ligand-1 (PSGL-1). Platelet GPIb-IX-V ligation with VWF also contributes importantly in platelet rolling and tethering on endothelial surface and subendothelial matrices, especially under the conditions with high shear rates, and is critical in initiating and building up arterial thrombus. Under arterial blood flow conditions, i.e., in the presence of high level shear stress, VWF immobilizes on the subendothelial matrix. The immobilized VWF changes to an active conformation that allows VWF binding to GPIb with a high affinity. This interaction subsequently activates integrin GPIIb/IIIa and other integrins. GPIIb/III ligation can sufficiently support firm adhesion of platelets under the conditions with a shear rate up to 900 $s^{-1}$. When the shear rate raises to >1500 $s^{-1}$, GPIb-VWF binding becomes essential to slow down the velocity of platelet rolling, and can mediate platelet rolling on the matrix at the shear rate up to 6000 $s^{-1}$ (8).

Collagen is another important subendothothelial matrix protein for platelet activation. Platelets tether on collagen via GPVI. The ligation further increases affinity and clustering of GPVI, and also induces inside-out signaling leading to the activation of α2β1, another collagen receptor of platelets, and GPIIb/IIIa. These complex adhesion molecule cross-talks coordinate their adhesion affinity, and enhance stability of platelet adhesion (9).

GPIIb/IIIa (αIIbβ3) is the most abundant membrane protein on platelets, and mediates aggregation and firm adhesion of platelets. GPIIb/IIIa can bind to many ligands, including fibrinogen, fibronection, VWF, thrombospondin-1, and CD40L (10, 11). GPIIb/IIIa binds to its primary ligand fibrinogen that bridges adjacent platelets, and leads to platelet aggregation and eventually thrombus formation. Moreover, GPIIb/IIIa-ligand binding induces outside-in signalling, which leads to and/or enhances further platelet activation, such as cytoskeletal change, platelet spreading, and granule secretion (10, 12).

Alongside platelet adhesion and aggregation, activated platelets undergo granule secretion. Platelet secretion releases granule contents through granule fusion with cell membrane or via OCSs. Platelets release a wide range of substances. Releasates of α granules include adhesion molecules (e.g., fibrinogen and von Willebrand factor), growth factors (platelet derived growth factor <PDGF> and transforming growth factor β <TGFβ>), chemokines (platelet factor 4 <PF4> and RANTES <regulated on activation, normal T cell expressed and secreted>) and so on. Platelet dense granules release low molecular weight substences, such as ADP, ATP and serotonin. Platelet-released substances exert various actions on platelet themselves and/or on adjacent/distant platelets and other type of cells, to amplify platelet activation and to regulate the functions of other cells, e.g., T cell activation and differentiation.

Platelet activation also triggers the metabolisms of platelet membrane phospholipids, leading to the synthesis and release of thromboxane $A_2$ ($TxA_2$) and platelet-activating factor (PAF). Platelet activation is also a dispensable component of coagulation cascade. Most remarkedly, membrane phosphatidylserine (PS) exposure of activated platelets provides a docking site for FXa and FVa to form prothrombinase complex, which converts prothrombin to thrombin (13).

Furthermore, besides classical platelet functions in thrombosis and haemostasis, an accumulating body of evidence indicates that platelets are not a simple thrombocyte, but are versatile cells closely involved in other physiological and pathophysiological processes, such as inflammation, immunity, tissue regeneration, and angiogenesis. Indeed, platelets have been recognized as an important link between thrombosis and inflammation during atherogenesis (14).

## 1.2 ADHESION CASCADE OF LYMPHOCYTES

Similar to adhesion and recruitment of platelets and other leukocytes, lymphocyte adhesion on the vessel wall follows the sequential processes of rolling, tethering, adhesion, and migration. The adhesion capacity of lymphocytes is, however, considerably weaker as compared to

monocytes and granulocytes, due to the several reasons that will be discussed later on.

When coming in contact with inflamed endothelial cells, lymphocytes may roll on endothelial surface and slow down their trafficking. Lymphocyte rolling is mainly mediated by selectins and their ligands. Lymphocytes express L-selectin (CD62L). The selectin is important for lymphocyte recruitment at the secondary lymphoid tissues, and is therefore also called "homing receptor". L-selectin mediates lymphocyte rolling via binding at their endothelial ligands of sialylated carbohydrates. The latter includes GlyCAM-1, which is highly expressed on ECs of high endothelial venules, and MadCAM-1, which is expressed on ECs of gut-associated lymphoid tissues (15, 16). L-selectin also binds to PSGL-1, but with a low affinity (17). Lymphocyte activation may lead to L-selectin shedding, which reduces L-selectin expression intensity and has a major impact on lymphocyte homing, for example, selective recruitment of naïve T cells (L-selectin$^{+++}$) but not T effector cells (L-selectin$^{+/-}$) (18).

Inflamed ECs express E-selectin and P-selectin, and both selectins primarily bind to PSGL-1 on lymphocytes. E-selectin also binds other ligands on lymphocytes, such as CD44 and E-selectin ligand 1 (19), while P-selectin also binds to CD44 variant isoforms (20). Moreover, PSGL-1 and CD44 have been shown to cooperate at mediating the rolling of inflammatory T cells on ECs (21). Integrins, even in their low affinity form, also take part in lymphocyte rolling and contribute importantly to slow down the rolling velocity, which subsequently lead to tethering of lymphocytes. It should be noted that lymphocyte-expressed PSGL-1 may be responsible for 90% P-selectin binding (22). However, unlike the constitutive expression of functional PSGL-1 on monocytes and granulocytes, quiescent lymphocytes do not constitutively express functional PSGL-1 (23). Transformation of PSGL-1 from its inactive to functional form requires modifications catalysed by, e.g., fucosyltransferases IV and VII. The expression of these PSGL-1 "activating" enzymes is, however, regulated by cytokines and transcription factors (24, 25). The regulation is lymphocyte activation- and subset-dependent. For example, it has been shown that fucosyltransferases VII is absent in naive T cells, but is highly expressed in Th1 and Treg cells (26, 27). Hence, T cell activation and differentiation may influence their adhesion behaviours.

Lymphocyte rolling and tethering are important steps in initiating the adhesion process, but are weak interactions between lymphocytes and the vessel wall. The interactions do not resist the blood flow well, especially in the blood flow with a high shear stress. Thus, rolling lymphocytes need to engage activated integrins that eventually lead to firm adhesion of lymphocytes. Integrins are transmembrane heterodimers, which contain α and β subunits. Four leukocyte-

specific β2 integrins (αLβ2, αMβ2, αXβ2, and αDβ2), two β7 integrins (α4β7 and αEβ7) and the extracellular matrix (ECM)-binding β1 integrins (α1–α6β1) are found on T cells. Among them, αLβ2 (leukocyte function-associated antigen-1; LFA-1) is the most abundant integrin, αLβ2-ICAM-1 ligation is the primary support of lymphocyte adhesion (28), and α4β7 and α4β1 are also widely distributed among T helper cells.

Mechanisms of integrin activation are complex, and some remains controversial. In general, integrin activation may be achieved by two ways, inside-out and outside-in signaling pathways. The inside-out signaling pathway refers to the processes: a) lymphocyte stimulating events, such as selectin ligation, TCR binding, and/or chemokine-receptor interaction, trigger diverse intracellular signalings; b) all signals eventually lead to activation/mobilization of the common signaling protein Talin; c) Talin binds to β cytoplasmic tail, separates the connection between α and β subunit cytoplasmic tails, and unfolds the packing of α and β transmembrane domains; and d) Talin's mechanical "intervention" leads a conformation change and thus an increase of affinity (activation) of integrin (29-32). Outside-in signaling pathway refers to the sequencial procedures that an integrin binds to its ligand, induces a conformational change of its molecule, and thus increases its affinity (33). It is believed that outside-in pathway of lymphocytes likely works as an amplifier or co-stimulus to the inside-out signaling (34).

The inside-out signaling pathway of integrin activation can be exampled by the data of P-selectin ligation induced integrin activation, albeit the data were generated from studies using neutrophils and monocytes. Thus, the interaction between P-selectin and PSGL-1 triggers neutrophil αMβ2 and monocyte α4β1 activation, promotes leukocyte adhesion to VCAM-1, and enhances platelet-leukocyte aggregate formation (35, 36). An earlier study also found that P-selectin and PSGL-1 binding induces a moderate activation and clustering of αMβ2 (37). Moreover, there is evidence indicating that PSGL-1 ligation causes Src kinase–MAP kinase pathway activation and the cytoskeletal rearrangement (38), and that P-selectin–PSGL-1 ligation leads to αMβ2 and αLβ2 activation via Src kinase and PI3 kinase signalling (39).

Together, inside-out and outside-in signalling cooperate to enhance ligand-binding affinity of integrins, and to induce clustering of integrins (40), both of which result in stronger integrin ligation and thus firmer adhesion of lymphocytes (41, 42). However, current knowledge of lymphocyte, as well as monocyte and granulocyte, adhesion is mainly gained from studies of cell adhesion under venous flow conditions. There is a paucity of information concerning if and how lymphocytes can adhere under arterial flow conditions. Studies on this regard are thus

warranted.

## 1.3 $CD4^+$ T CELLS IN ATHEROGENESIS AND THEIR REGULATION BY PLATELETS

$CD4^+$ T cells are a key T cell population in adoptive immune system. Their main function is to help and regulate activation, differentiation and proliferation of other immune cells by releasing cytokines. $CD4^+$ T cells are thus termed T helper (Th) cells. They are engaged in various immune responses, such as antibody class switching of B cells, activation of cytotoxic T cells, and phagocytosis of macrophages. These actions also gain Th cells a nickname "conductors" (43).

### 1.3.1 CD4 T cell activation and differentiation

Activation of naïve $CD4^+$ T cells is achieved by two signals. Signal 1 is the recognition between T cell receptor (TCR) of $CD4^+$ T cells and peptide-MHC complex of antigen presenting cells (APCs). A TCR is associated with CD3, a transmembrane protein that transduces signal 1 into the cells. However, signal 1 may also signal cell apoptosis or cell tolerance. Signal 2 is called as costimulation, which is mediated by CD28 expressed on T cells and B7 protein on APCs. Signal 2 can not only amplify and verify TCR/CD3-mediated signal 1, but also inhibit apoptosis of the cells. After stimulation of signal 1 and 2, T cells undergo proliferation and differentiation into T effector cells (44). Hence, according to their characteristics of cytokine production and biological functions, $CD4^+$ T effector cells are generally divided into type 1 T helper (Th1; pro-inflammatory and producing pro-inflammatory cytokines, e.g., interferon γ <IFNγ> and IL-2), Th2 (anti-inflammatory; IL-4 and IL-5), Th17 (pro-inflammatory; IL-17A), and regulatory T (Treg) cells (anti-inflammatory; TGFβ and IL-10) (45).

### 1.3.2 $CD4^+$ T cells are an active player in atherosclerosis

$CD4^+$ T cells are present in atherosclerotic lesions (1). Studies using animal models showed that $CD4^+$ T cells are present already in the fatty streak stage and throughout all stages of lesion development, and that $CD4^+$ T cells are the most abundant T cell subpopulation in atherosclerotic lesions (46, 47). Those findings indicate that $CD4^+$ T cells may infiltrate into the atherogenic sites at the very early stage, and play important roles throughout the whole process of atherogenesis.

The effects of $CD4^+$ T cells in atherogenesis are complex. Many studies showed that $CD4^+$ T

cells aggravate atherosclerotic lesion development (47-49). Some studies showed, however, a more complex story that $CD4^+$ T cells appeared to have an athero-protective effect at the lower part of the aorta (50). The complexity of $CD4^+$ T cell effects may owe to the diverse effects of different $CD4^+$ T cell subpopulations, of which some subpopulations may play exactly opposite roles in atherogenesis. Th1 cells are generally regarded as a pro-atherogentic subset, and Th1-cytokines, such as IFNγ and IL-2, are the most expressed cytokines in plaques (51). Inhibition of Th1 responses or Th1 cell deficiencies markedly reduced atherosclerotic lesion formation in $ApoE^{-/-}$ mice (52-54). Treg cells, which produce TGFβ and IL-10, are anti-inflammatory, and have been proved to be anti-atherosclerotic during the atherogenesis (55). There is evidence showing that increased presence of Treg cells reduces lesion formation (56). Impacts of Th2 and Th17 cells in atherogenesis are vague and controversial. Some studies showed that Th2 switch reduced atherosclerotic lesion formation (54), whilst some other investigations demonstrated that Th2 deficiency had no effect on atherosclerotic lesion formation (57), or even attenuated lesion formation (58). Th17 cells are regarded as a highly pro-inflammatory subset, and have been shown to promote atherosclerotic lesion development (59). However, Th17 intervention may also present an athero-protective effect (60).

### 1.3.3 Platelet-regulated $CD4^+$ T cell function in atherosclerosis

Atherosclerosis is an inflammatory disease (61), and is also a thrombotic disease (62). The latter is evidenced by that repeated infusion of activated platelets markedly enhanced atherosclerotic lesion formation in a pro-atherosclerotic $ApoE^{-/-}$ mouse model (63, 64), and that pro-atherosclerotic effects of activated platelets are associated with platelet-leukocyte aggregation and platelet-enhanced leukocyte adhesion at the sites of atherosclerotic lesions (63). Moreover, deficiency or interventions of platelet adhesion molecules attenuated lesion formation in pro-atherosclerotic murine models (65). Hence, it is highly possible that platelets and lymphocytes, especially $CD4^+$ T cells that are rich in the lesions, interact closely during atherogenesis, albeit research on this regard has not been intensive.

### 1.3.4 Platelets facilitate lymphocyte recruitment

Platelets facilities adhesion and recruitment of leukocytes, including lymphocytes, under flow conditions (35, 66). Our current knowledge on this regard is, however, mainly generated from studies performed under venous (i.e., low shear stress or rate) flow conditions (67). Our current understanding with regard to if and how platelets support lymphocyte adhesion under arterial flow conditions is limited.

Platelets assist lymphocyte adhesion mainly in two ways. Platelet adhesion on inflamed endothelial cells and subendothelial matrices provide lymphocytes with a highly adhesive surface and/or an additional adhesion support. On the other hand, platelets may bind to lymphocytes to form platelet-lymphocyte aggregates, and thus enhance lymphocyte adhesion.

Platelets have been reported to mediate T cell delivery to high endothelial venules (HEVs) via P-selectin bridging (68). In addition, ligation between platelet P-selectin and T cell PSGL-1 is stronger than the ligation between T cell L-selectin and endothelial addressin, and can thus support T cell recruitment at high endothelial venules more efficiently (69). Platelet-mediated lymphocyte rolling also induces expression and activation of α4β1 and αLβ2 on lymphocytes, which subsequently enhance firm adhesion of lymphocytes (70, 71).

Platelets can adhere to leukocytes to form platelet-leukocyte aggregates, and platelet-leukocyte aggregates have been shown to enhance leukocyte recruitment at the sites of inflammation (72). All leukocyte subpopulations can form heterotypic conjugates, with higher conjugating potentials among neutrophils and monocytes and a much weaker potential in lymphocytes. For example, platelet activation increased platelet conjugation in neutrophils and monocytes up to 80%, but only to <10% in lymphocytes (73). However, activated platelets tend to bind the monocyte-size, i.e., activated lymphocytes. When $CD4^+$ T cells were activated with PHA, platelet-large $CD4^+$ T cell aggregation could increase to 20%, while platelet-small $CD4^+$ T cell aggregation remained below 10% (74). Elevated platelet-$CD4^+$ T cell aggregation has indeed been shown to enhance $CD4^+$ T cell adhesion under venous flow conditions, and the enhancement involved multiple platelet adhesion molecules, including P-selectin and CD40L (75).

### 1.3.5 Platelet derived chemokines regulate CD4 T cell function

Platelets store a number of chemokines in α-granules, and can secrete them upon activation. Platelet derived chemokines may regulated multiple functions of $CD4^+$ T cells. Platelet-released chemokines can exert their effects in distance. For example, the platelet-specific chemokine platelet factor 4 (PF4) is abundant in circulation, and can act on $CD4^+$ T cells and other target cells in distance. On the other hand, both platelets and $CD4^+$ T cells have been found in atherosclerotic lesions (4, 76, 77), suggesting that lesional platelets may also influence $CD4^+$ T cells in vicinity.

#### *1.3.5.1 Chemokine receptors on CD4$^+$ T cells*

Platelet-derived chemokines regulate CD4$^+$ T cell function through their corresponding receptors. It is interesting that profiles of chemokine receptor expression vary considerably among different CD4$^+$ T cell subsets. Some receptors are more widely expressed. For instance, the chemokine receptors CCR2 and CXCR4 are present in all T helper cells. Some chemokine receptors are restricted to certain subsets. For example, CXCR5 is restricted to follicular B helper T cells (78). In addition, some receptors are constitutively expressed on naive CD4$^+$ T cells (e.g., IL-12Rβ1) (79), while the expression of CCR1 and CCR2 is only seen after stimulation by IL-2 (80). Therefore, the expression profile of chemokine receptors may play an important role in selectively recruitment of T helper cell subsets.

#### *1.3.5.2 CD4$^+$ T cell chemotaxis and adhesion regulated by platelet-derived chemokines*

Platelet-derived chemokines are important chemoattractants for CD4$^+$ T cells during their adhesion on and infiltration into the vessel wall. Importantly, platelet-derived chemokines can immobilize themselves on the endothelial cell surface. Thus, the chemokines bind to the glycosaminoglycans (GAGs) on endothelial surface after secretion, in order to reach a certain concentration locally and to avoid being flushed away by blood flow. Binding to GAGs also protects chemokines from cleavage, and ensures the high affinity interactions of the chemokines to their corresponding receptors. Moreover, chemokine binding to the receptors on rolling and tethering lymphocytes results in integrin activation. The interaction is very efficient and can lead to integrin activation within milliseconds (33). Several platelelt-derived chemokines, such as RANTES (CCL5) and SDF-1α (CXCL12), are known to activate β1, β2 and β7 integrins and to enhance integrin-dependent adhesion (81). Therefore, platelelt-derived chemokines contribute importantly to firm adhesion of CD4$^+$ T cells, and the effects should be more important for T cell adhesion in an arterial flow where high shear stresses are present.

#### *1.3.5.3 Key platelet-derived regulators*

##### 1.3.5.3.1 RANTES

CCL5 (RANTES) is one of chemokines that can be most efficiently immobilized on endothelial surface, and can efficiently support T cell arrest (82). RANTES can rapidly increase integrin avidity through its ligation on CCR1 receptor (83), while RANTES interaction with its CCR5 receptor contributes importantly to the lymphocyte spreading in a shear flow (84). RANTES also contributes to T cell-subendothelial matrix protein interaction. Thus, it has been shown that

RANTES enhanced the activation of T cells exposed to immobilized fibronectin, and promoted T cell adhesion to fibronectin-coated surfaces in a β1 integrin-dependent manner (85). It is also known that both CCR1 and CCR5 support transendothelial chemotaxis toward RANTES (86), and that RANTES seems to recruit Th1 cells selectively (87).

Besides T cell chemotaxis and adhesion, RANTES may also enhance cell proliferation of $CD4^+$ T cells stimulated by antiCD3/CD28 MAbs (88), in which RANTES exerts the effect by sustainably elevated intracellular calcium levels of $CD4^+$ T cells (89). RANTES also enhances Th1 differentiation of CD3/CD28 MAb-stimulated $CD4^+$ T cells, which is accompanied by elevations of Th1 cytokine, such as IL-2, production (88). However, our knowledge on if and how RANTES can influence differentiation and cytokine production of other $CD4^+$ T effector cells is limited.

1.3.5.3.2 PF4

PF4 (CXCL4) is the most abundant chemokines secreted by activated platelets. PF4 has been detected in human atherosclerotic plaque (4), and has been shown to be closely involved in atherosclerosis. PF4 may facilitate leukocyte adhesion by increasing endothelial E-selectin expression (90). It can also aggravate atherogenesis by inhibiting LDL receptor degradation, therefore retaining lipoproteins in the plaque (91). Moreover, PF4 may form a hetero-dimer with RANTES, which increases binding affinity of both PF4 and RANTES to glycosaminoglycans (GAGs) (92). The hetero-ligation can subsequently promote their function, such as a higher efficiency in supporting leukocyte arrest on endothelial surface (92).

PF4 inhibits cell proliferation of cultured total $CD4^+$ T cells, and may inhibit Th1 differentiation and cytokine production (93). Further studies revealed, however, that PF4 effects are more complex. Thus, it has been shown that PF4 enhances Th1 response (e.g., IL-2 and INFγ production) in $CD4^+CD25^+$ T cells, but inhibits Th1 response in $CD4^+CD25^-$ cells (94). Moreover, it has also been shown that PF4 can enhance Treg cell responses through selectively inhibiting $CD4^+CD25^-$ cell proliferation but promoting $CD4^+CD25^+$ proliferation (94).

1.3.5.3.3 CD40L

As a costimulatory molecule, CD40L, through ligation with its receptor CD40, is well known for its roles of bridging between cellular immunity and humoral immunity, as well as regulating antigen-presenting cell (APC) and endothelial cell activation (95, 96). Thus, CD40L of activated $CD4^+$ T cells, which can also be substituted by CD40L expressed on activated platelets, binds to

B cell CD40, and thus induces B cell proliferation and isotope switching (95, 97). CD40L can also stimulate adhesion molecule, including CD40, expression on macrophages, and promote their antigen presenting activities (95). Moreover, CD40L binding with endothelial CD40 increases expression of E-selectin, VCAM-1 and ICAM-1, and also enhances endothelial release of IL-8 and MCP-1 (14).

CD40L also serves $CD4^+$ T cells themselves. Hence, CD40-CD40L ligation enhances platelet-T cell aggregation (74), which may in turn enhance T cell adhesion under flow conditions. Indeed, a recent study using a pro-atherosclerotic murine model showed that $CD40L^{-/-}$ deficiency hampers platelet-leukocyte aggregation, reduces leukocytes adhesion on endothelium, and therefore decreases atherosclerotic lesion development (98). Because infusion of wild-type, i.e., CD40L-expressing platelets temporarily reduced circulating Treg cells, it has also been proposed that platelet CD40L could aggravate atherogenesis through reducing Treg recruitment at the lesions (98). Moreover, platelet-derived soluble CD40L has been shown to enhance Th1 differentiation (99). Recombinant CD40L can also enhance activation, proliferation, Th1 differentiation and cytokine production of TCR-stimulated $CD4^+$ T cells (100).

1.3.5.3.4 TGFβ

Platelets store and release a large amount of TGFβ upon activation. Platelet-derived TGFβ is known to be the principle source of TGFβ in circulation (101). Animal studies using pro-atherosclerotic mouse models demonstrated that disruption of TGFβ signalling enhanced atherosclerotic lesion formation (102), and that increased leukocyte production of TGFβ reduced lesion development (103). It is generally accepted that TGFβ has a protective role against atherogenesis (104).

Athero-protective effects of TGFβ is partially attributed to the effects that TGFβ inhibited cell proliferation and differentiation of $CD4^+$ T cells (105), especially Th1 differentiation and cytokine production (106, 107). However, TGFβ has also been shown to inhibit Th2 cell development and cytokine production (106, 107). As a common factor required for both Th17 and Treg cell polarization, TGFβ promotes Th17 development at low concentrations, but enhances Treg cell development at high contractions (108).

# 2 AIMS OF THE STUDY

The overall aim of the thesis work is to investigate if platelets can facilitate the recruitment of lymphocytes at the sites of atherogenesis, and if and how platelet regulation $CD4^+$ T cell function. Specifically, we aimed to:

- Study if platelets facilitate lymphocyte adhesion under arterial flow conditions (**Paper I and II**)
- Elucidate how platelets enhance lymphocyte adhesion on subendothelial matrix protein-coated surface under different shear rates (**Paper I and II**)
- Investigate if and how platelets influence proliferation, activation, and cytokine production of $CD4^+$ T cells (**Paper III**)
- Study how platelets regulate the activation dynamics and interactions of $CD4^+$ T cell subsets (**Paper IV**)

# 3 METHODOLOGY

## 3.1 PLATELET AND LYMPHOCYTE ADHESION ASSAYS

### 3.1.1 In vitro adhesion assays

#### *3.1.1.1 Parallel-plate flow chamber*

The parallel-plate flow chamber (PPFC) is a widely used device to observe cell-cell or cell-protein interaction under certain flow conditions. For different applications, PPFC has been designed and modified into several types. The PPFC used in the present work is set up based on the design of Frangos et al (109).

The complete flow circuit includes a peristaltic pump, which can generate different fluid velocities; one or two containers to hold the perfusion-in and/or perfusion-out samples; PPFC; a vacuum syringe to take away the bubbles; and a microscope as an observation platform. (Figure 2)

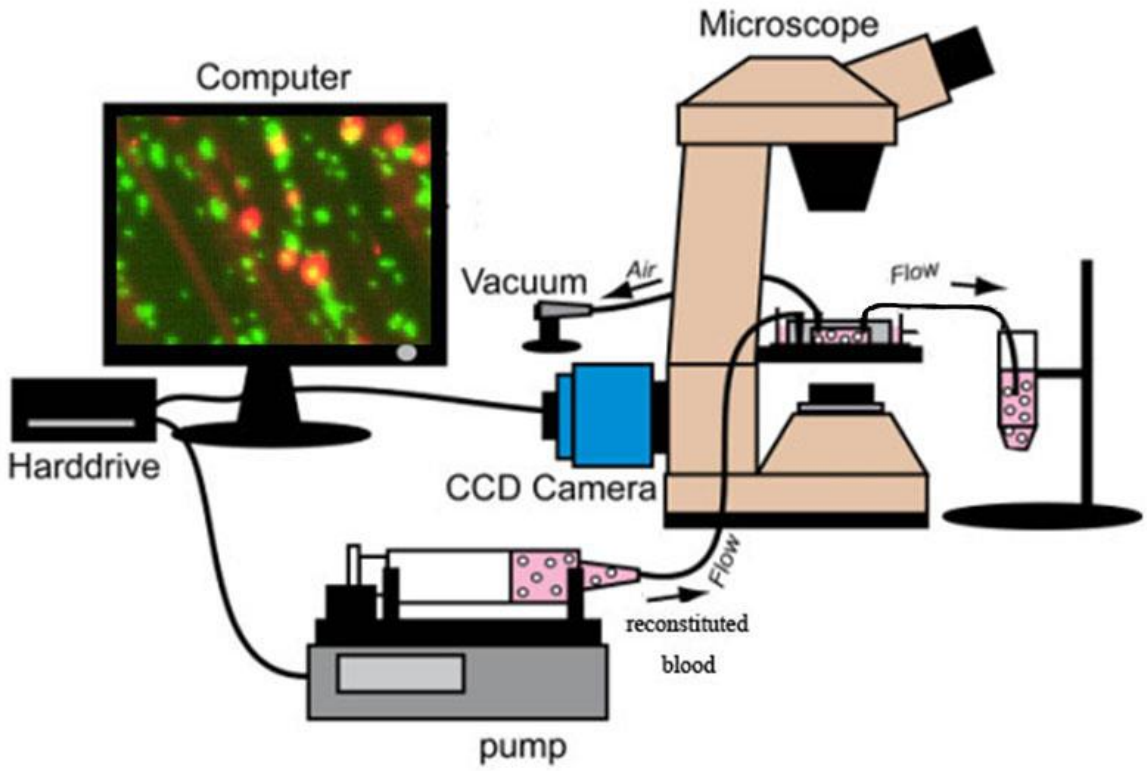



**Figure 2. Schematic illustration of parallel plate flow chamber system.**

Details of the flow chamber are shown in figure 3. From the top to bottom, the chamber contains upper layer with entrance and exit slots, as well as inlet and outlet manifolds; a

silicone gasket with an internal rectangle, which is used to create a space for perfusion; a glass slide, which can be coated with cells or proteins; and the lower layer. All these parts are held firmly by screws.

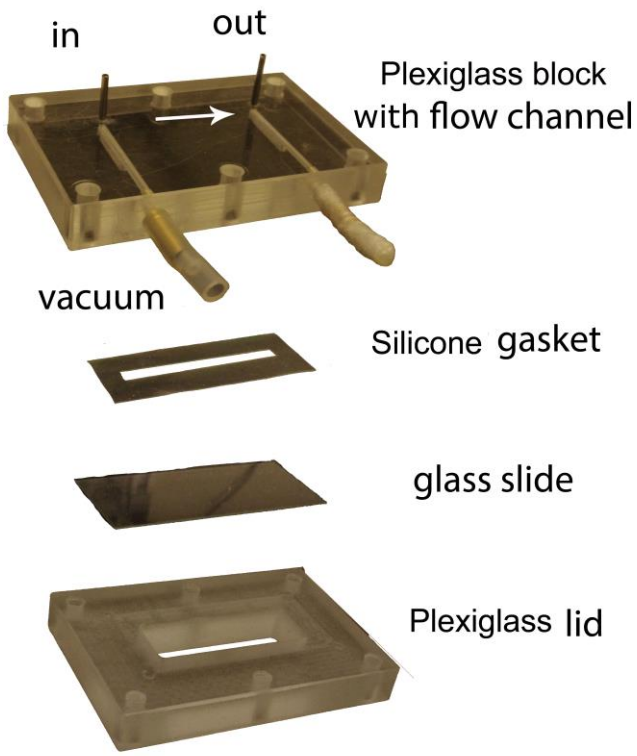



**Figure 3. Parallel plate flow chamber set-up.**

When setting up a certain viscosity by using the peristaltic pump, the fluid flowing between the parallel plates will create a corresponding shear stress. The relation can be described as a mathematical formula, $\tau=6Q\mu/(wh^2)$, with $\tau$ is shear stress, Q is the flow rate, $\mu$ is the medium viscosity, *w* is the width of the flow channel, and *h* is the height of the flow channel (110). The unit for shear stress $\tau$ is dynes/cm$^2$ (1 dynes/cm$^2$ = 26.3 s$^{-1}$, when expressed as shear rates).

When the cells in the perfusion fluid are subjected to the hydrodynamic stress, the cells tether or adhere to the protein coated on the glass. The shear stress has two counter-acting actions on the cells. On the one hand, it pushes cells to contact the coated proteins. On the other hand, it also drives cells away from the binding sites (111, 112). According to the ranges of shear rates in different blood vessels (113), the shear rates chosen in this thesis work were 200, 500 and 1,000 s$^{-1}$, corresponding to the shear rates found in the veins, arteries, and narrowed arteries,

respectively.

In our set up of the experiment, the PPFC was used to observe the behaviors of platelets and lymphocytes tethering and adhesion. The glass coverslip set in the PPFC was coated with 1 mg/ml collagen or 1% bovine serum albumin (BSA). We labeled the platelets and lymphocytes with florescent dyes Calcein-green AM and Calcein red-orange, respectively. The flow chamber was mounted on the object platform of a Nikon TE2000-U intravital fluorescence microscope equipped with a Nikon DS-2MBWc-U1 CCD video camera (Nikon Corp., Tokyo, Japan). Images of platelet deposition and lymphocyte adhesion were elucidated with 450-490 nm and 510-560 nm excitation lights, respectively. Images of platelet and lymphocyte tethering and adhesion were recorded during or after perfusion. Image analysis was performed off-line using Image-Pro Plus version 5.3 (MediaCybernetics Inc., Bethesda, MD, USA).

#### *3.1.1.2 Cone-and-Plate (let) Analyser (CPA) procedure*

The Cone-and-Plate(let) Analyser (CPA) procedure has been developed to detect platelet adhesion and aggregation of whole blood on a plate coated with subendothelial matrix proteins (114). It can also be used to observe platelet response to anti-platelet drugs (115). The main procedure of CPA is illustrated in figure 4.

Briefly, polystyrene wells were coated with different subendothelial matrix proteins. Afterwards, the wells were blocked and then washed three times with phosphate-buffered saline (PBS). Blood aliquots (130 or 200 µl) were added to the coated wells, and incubated without or with blocking agents at 22°C for 5 min. The blood samples were then subjected to shear stress in the Cone-and-Plate(let) Analyser (CPA) Impact-R (17) for 5 min. Thereafter, blood samples were taken for flow cytometric analysis, and the wells were stained with May-Grünwald stain, and cell adhesion was visualised using an inverted light microscope. Total surface coverage (SC) by adhered blood cells was assessed using Impact-R software (DiaMed AG).

### 3.1.2 Murine model of in vivo arterial thrombosis

Ferric chloride-induced thrombus model is one of the most applied methods to observe thrombus formation in vivo. In the present work, arterial thrombus formation of the mesenteric arteries was induced by applying a piece of filter paper saturated with 10% ferric chloride on the mesenteric artery for 3 minutes. Afterwards, thrombus building-up and lymphocyte recruitment

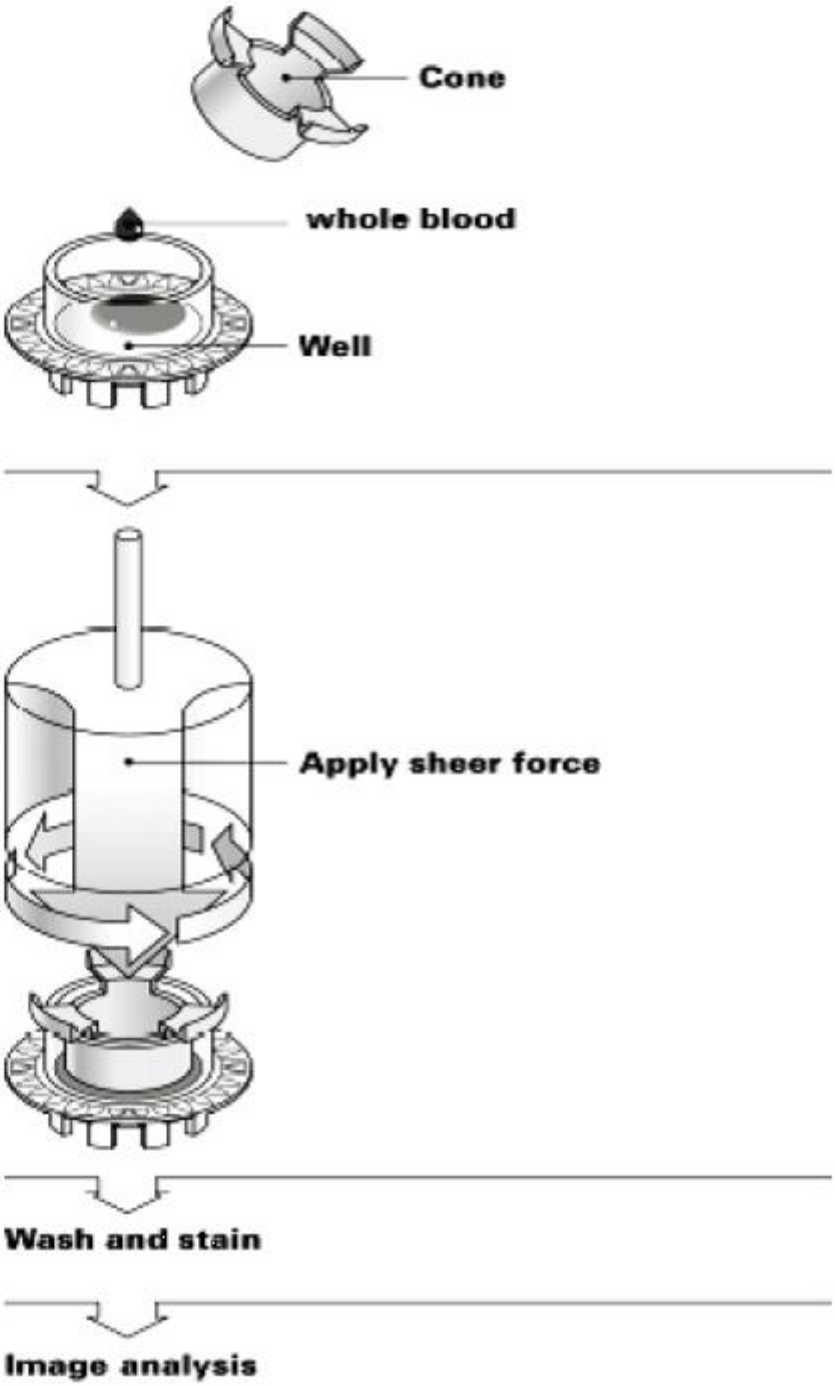



**Figure 4. Cone-and-Plate(let) Analyser (CPA) procedure. Adapted from Savion et al.(115)**

were observed in real time and recorded for up to 50 min after vessel wall injury, using an intravital fluorescence microscope. The mechanism of our ferric chloride-induced arterial thrombus formation is associated with superoxide anion-induced endothelial damage. The occlusive thrombus mainly contains activated platelets (116), and antiplatelet treatment can significantly inhibit thrombosis of the model (117).

## 3.2 Platelet-CD4$^+$ T cell co-culture

Cell co-culture is a common method to study the interaction between/among different cells (118). Both platelets and T cells are present in atherosclerotic lesions. Therefore, we used platelet-T cell co-culture to investigate potential platelet-T cell interactions during atherosgenesis. We employed both direct co-cultures and trans-well co-cultures. The latter enabled us to dissect the influences of platelet-released mediators and direct cell-cell contact on T cell functions.

### 3.2.1 CD4$^+$ T cell isolation

CD4$^+$ T cells were isolated by a positive isolation procedure using Dynal CD4$^+$ beads. It has been reported that a positive selection may achieve a higher purity of target cells as compared to a negative selection, which can lead to an altered profile of gene expression due to the contamination with other cell types (119). The shortcoming of positive selection is that CD4$^+$ is also expressed on monocytes, macrophages, and dendritic cells (120, 121).

### 3.2.2 CD4$^+$ T cell activation

Isolated CD4$^+$ cells were activated by immoblized anti-CD3 MAb (3 μg/ml) and soluble CD28 MAb (0.3 μg/ml). As a classical way to activate T cells, anti-CD3 and anti-CD28 MAb co-stimulation activates TCR complex, and simultaneously activates signal 1 and signal 2 without triggering early cell death (122). Comparing to the method of using natural antigen-presenting cells (APC), anti-CD3/CD28 MAb stimulation is more efficient and stronger, and evokes quick CD4$^+$ T cell activation (123). In our experiments, CD4$^+$ T cells were activated and monitored mostly within 5 days, we thus chose anti-CD3/CD28 stimulation for its high efficiency.

### 3.2.3 CD4$^+$ T cell proliferation assay

We used two methods to measure T cell proliferation.

#### *3.2.3.1 Methyl-$^3$H-thymidine incorporation*

Methyl-$^3$H-thymidine incorporation is a widely used method to detect cell proliferation (124). The mechanism is based on that, during cell proliferation, $^3$H-thymidine in the culture medium is incorporated into the newly synthesized DNA. Thus, measurement of the $^3$H-thymidine radioactivity in DNA reflects the extent of cell proliferation. However, the Methyl-$^3$H-thymidine incorporation can only determine cell proliferation of the total T cells, but can not differentiate

cell proliferation of different cell subsets in the same culture mass. To detect the cell proliferation of individual CD4 T cell subsets, we have to induct another cell proliferation assay described below.

#### *3.2.3.2 CFSE cell proliferation assay*

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) is nonfluorescent, and can easily diffuse into cells. When it enters into cell cytoplasm, its acetate groups are cleaved by intracellular esterase to yield highly fluorescent but membrane-impermeable CFSE. Thus, CFSE is well retained in the cells, and can pass on to the daughter cells. With each round of cell division, the relative fluorescence intensity of CSFE decreases by half. Thus, number of cell divisions can be determined by a flow cytometer.

An important advantage of CFSE assay is that it can be combined with surface or intracellular staining, to monitor cell proliferation of different T cell subpopulations. For instance, Treg and Th1 cell proliferation can be monitored by combining CFSE staining with FoxP3 and IFNγ intracellular staining.

### 3.3 Flow cytometry and $CD4^+$ T cell phenotyping

Flow cytometry was used for the analyses of lymphocyte subpopulations, $CD4^+$ T cell subsets, as well as semiquantitative analysis of platelet and lymphocyte surface expressed antigens. After surface and/or intracellular staining of target antigens, samples were mildly fixed with 0.5% paraformaldehyde, and analysed using a FC500, a Cyan ADP, or an EPICS XL-MCL flow cytometer (Beckman-Coulter Corp., Hialeah, FL). Briefly, all leukocytes were gated by PE-CD45. $CD4^+$ and $CD8^+$ T cells were separated by combining PC5-CD3 signal with ECD-CD4 or ECD-CD8. In separate samples, B cells and NK cells were differentiated by ECD-CD19/ECD-CD20 and PC5-CD16/PC5-CD56, respectively. To identify $CD4^+$ T cell subsets, intracellular staining of cytokines and transcription factors was employed. With the help of the protein transport inhibitor GolgiStop®, intracellular accumulation of IFNγ, IL-4, IL-17a was monitored for the phenotype of Th1, Th2, and Th17, respectively. The surface marker CD25 of Th cell activation and intracellular staining of the transcription factor FoxP3 were used to gate Treg cells. Intracellular staining of the transcription factor T-bet was also used to phenotype Th1 cells.

# 4 RESULTS AND GENERAL DISCUSSION

## 4.1 PLATELET-DEPENDENT LYMPHOCYTE ADHESION UNDER ARTERIAL FLOW CONDITIONS (PAPER I)

### 4.1.1 Platelets promote lymphocyte adhesion under arterial flow conditions

It has not been defined if platelets could support lymphocyte adhesion under arterial flow conditions. We thus used reconstituted blood containing florescence labeled platelets and lymphocytes and a flow chamber to observe how platelets enhance lymphocyte adhesion under laminar flow conditions. It was observed that platelets adhered on collagen-coated surface within seconds of perfusion, and that platelet deposition decreased as flow rates increased from venous (200 $s^{-1}$), arterial (500 $s^{-1}$), and to narrowed arterial (1000 $s^{-1}$). Shortly after platelet adhesion, lymphocytes started to roll, tether, and adhere on adhered platelets, and lymphocyte adhesion was proportional to platelet deposition. These observations suggest that platelet adhesion is prior to and supports lymphocyte adhesion.

Adhered lymphocytes were mostly co-localized with adhered platelets or platelet aggregates, especially under arterial flow conditions. To elucidate the importance of platelets in lymphocyte adhesion further, lymphocyte adhesion was compared using reconstituted blood with and without platelets. Fig 5A shows that lymphocyte adhesion was markedly decreased in the absence of platelets, and the decreases were much more marked at arterial shear rates of 500 $s^{-1}$ and 1,000 $s^{-1}$. These results suggested that platelets were important in lymphocyte adhesion, especially at the high shear rates.

### 4.1.2 Platelets promote lymphocyte adhesion via multiple adhesion molecules

Multiple adhesion molecules are involved in platelet-dependent lymphocyte adhesion. Blockade of P-selectin and CD40L attenuated platelet adhesion by approximately 20%, while GPIIb/IIIa inhibition reduced platelet adhesion by 50% at the shear rate of 500 $s^{-1}$. The blockade significantly reduced platelet-dependent lymphocyte adhesion, and the most marked inhibition was seen with GPIIb/IIIa blockade (Fig 5B), which was consistent with the data of platelet adhesion. These data imply that multiple adhesion molecules contribute to platelet-dependent lympocyhe adhesion, and that the integrin GPIIb/IIIa is critical in supporting lymphocyte adhesion in an arterial flow.

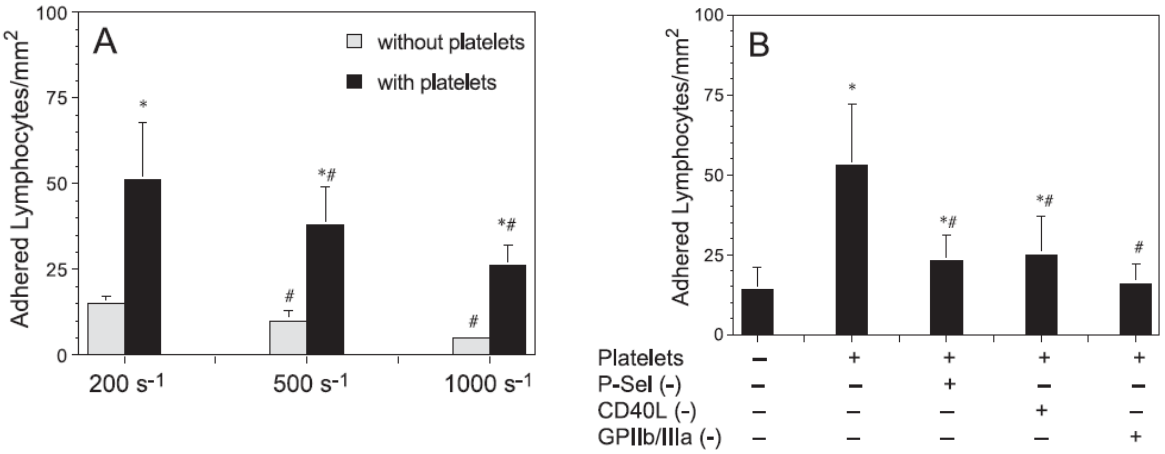



**Figure 5. Impacts of platelets and platelet-related adhesion molecules on lymphocyte adhesion on collagen-coated surface**. A) Reconstituted blood without or with platelets was perfused through collagencoated surface under different shear rates for 5 min. After washed with 1 min perfusion of PBS, the numbers of adhered lymphocytes were counted in 10 randomly selected 10 x fields. Data plotted are mean ± SEM from nine experiments. *p<0.05 compared to the corresponding samples without platelets; #p<0.05 compared to the samples perfused at 200 $s^{-1}$. B) Adhesion molecules involved in platelet-dependent lymphocyte adhesion were investigated using corresponding blocking antibodies [(-)]. Washed platelets were pre-incubated with blocking antibodies to P-selectin (10 μg/ml), CD40L (10 μg/ml), or the GPIIb/IIIa inhibitor tirofiban (12.5 μg/ml) before blood reconstitution. Afterwards, reconstituted blood without or with platelets was perfused through collagen-coated surface at the shear rate of 500 $s^{-1}$ in the presence of the same concentrations of blocking agents. Data plotted are mean ± SEM; n=4. *p<0.05 compared to without platelets, #p<0.05 compared to the platelet-containing blood without blocking agents.

### 4.1.3 Platelet-dependent lymphocyte adhesion is selective

When numbers of adhered lymphocytes were analysed by whole blood flow cytometry using Rainbow® counting beads, it was found that platelets could support adhesion of all lymphocyte subpopulations of $CD4^+$ T, $CD8^+$ T, B and NK cells at the arterial flow rate of 500 $s^{-1}$. Interestingly, platelet-dependent T cell adhesion was more marked among large, monocyte-sized T cells, whilst the enhancement was seen in small B cells but not in large B cells. The difference is physiologically and pathophysiologically meaningful. Difference of T cell adhesion may lead to selective recruitment of activated T cells at the sites of arterial injuries and/or atherosclerotic lesions, which may contribute to the predominance of T cells in the lesions (1). Moreover, preferential adhesion of small B cells may help their homing at peripheral lymphoid tissues.

### 4.1.4 Platelets support lymphocyte recruitment into arterial thrombi

We have also confirmed our in vitro findings using a mouse model of arterial thrombosis. Fig 6

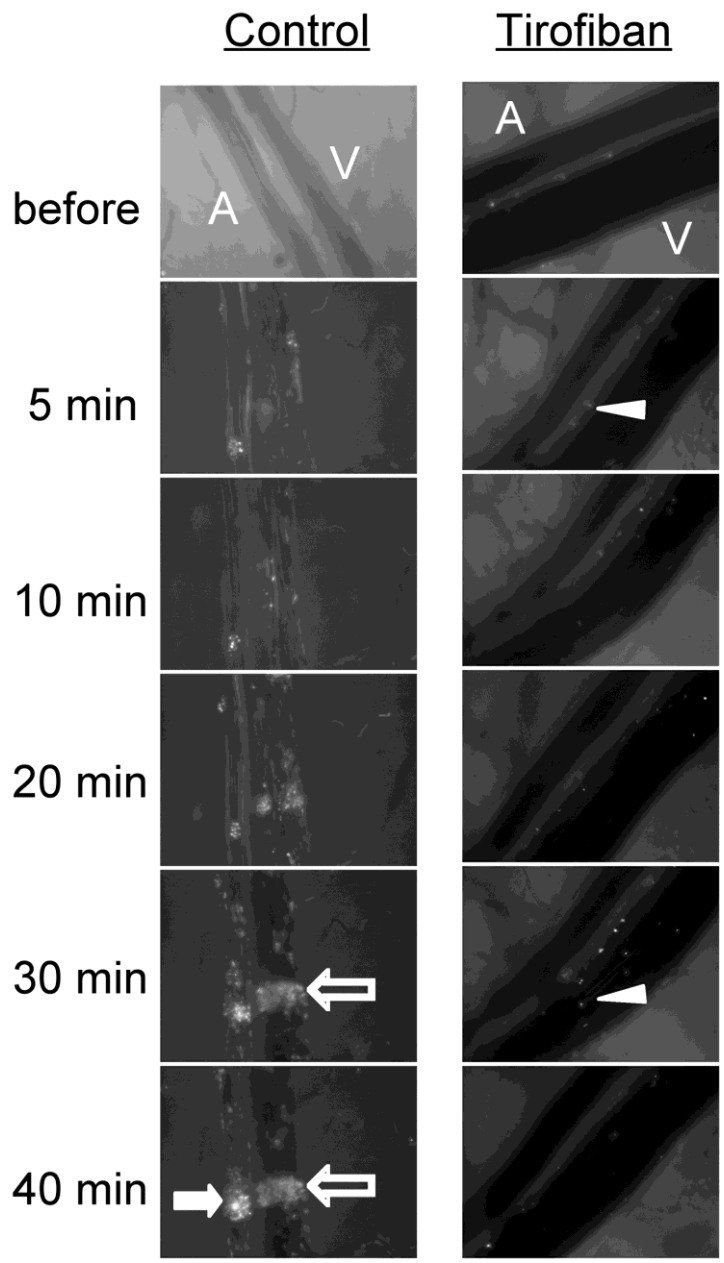



**Figure 6. Lymphocyte adhesion in the mesenteric arterioles and venules of a mouse thrombosis model.** A bolus of fluorescently labelled murine lymphocytes was injected via a jugular vein catheter of a C57BL/6 mouse under chloraldurat anesthesia, and a pair of mesenteric arteriole and venule was treated with 10% FeCl3 for 3 min. Afterwards, ferric chloride-induced thrombus formation and lymphocyte-vessel wall interaction were observed in real time under a Nikon intravital fluorescence microscope and recorded≥50 min. The treatments observed were vehicle (normal saline, i.e. control) and the GPIIb/IIIa inhibition by tirofiban (200 μg/kg bolus followed 0.67 μg/kg/min infusion). FeCl3-induced thrombi with lymphocyte incorporation were seen in both arterial and venous sides in control animals, and occlusive thrombi were first seen in the vein (open arrows) and then formed in the artery approximately after 40 min (solid arrow). In tirofiban-treated animals, there was no thrombus formation, and tethering lymphocytes were only seen on the venous side (arrow heads). The images were representatives from 3–4 observations.

shows that, before $FeCl_3$ treatment, few lymphocytes rolled on the vessel wall. With $FeCl_3$ treatment, lymphocyte rolling increased dramatically within 5 min and on both venous and arterial sides. As the stable and occlusive thrombi gradually building up in both venous (within 30 min) and arterial sides (40 min), fluorescent lymphocytes were recruited into the thrombi. In contrast, pretreatment of the mice with the GPIIb/IIIa inhibitor tirofiban prevented arterial thrombus formation and subsequently abolished lymphocyte rolling, adhesion, or recruitment at the site of $FeCl_3$ treatment. Hence, all our data support the notion that lymphocytes may adhere under arterial flow conditions, and that the adhesion is largely platelet-dependent. Moreover, platelet-dependent lymphocyte adhesion under arterial flow conditions is selective among large T cells, but small B cells.

## 4.2 PLATELETS ENHANCE LYMPHOCYTE ADHESION ON SUBENDOTHELIAL MATRIX UNDER ARTERIAL FLOW CONDITIONS (PAPER II)

### 4.2.1 Subendothelial matrix proteins induce platelet activation and deposition

Vessel injuries expose subendothelial extracellular matrix, a thrombogenic surface containing collagen, fibrinogen, VWF and fibronectin (125). Our follow-up study was thus to investigate how subendothelial matrix proteins (SEMPs) support platelet and lymphocyte adhesion using a cone-and-plate(let) analyzer (CPA). Whole blood sheared (500 $s^{-1}$ for 5 min) on the plates coated with different SEMPs resulted in marked platelet deposition on collagen-, fibrinogen- and VWF-coated plates, but not on fibronectin-coated plates, and the most marked deposition was seen with collagen-coated surfaces. Platelet deposition was seen as aggregates of various sizes and in different patterns. On collagen-coated surfaces, platelet aggregates/thrombi were distributed alongside collagen fibers. The platelet aggregates were often tightly packed and larger as compared to those on fibrinogen- and VWF-coated surfaces. Platelet deposition on fibrinogen-coated surfaces often appeared as loose platelet aggregates or clouds, while platelet deposition on VWF-coated surfaces was seen as evenly dotted and small sized platelet aggregates.

### 4.2.2 Platelets selectively facilitate lymphocyte adhesion on subendothelial matrix protein-coated surfaces

Platelet deposition also enhanced significant lymphocyte adhesion, as well as other leukocytes. Similar to those shown in paper I, adhered lymphocytes were in close contacts with platelet aggregates. Collagen induced most marked platelet deposition, and thus most efficiently supported lymphocyte adhesion at the shear rate of 500 $s^{-1}$. The latter was evidenced by that collagen supported significant cell adhesion of all lymphocyte subpopulations. Fibrinogen was second to collagen in its abilities to support lymphocyte adhesion, and could enhance cell adhesion of both T cells and NK cells. NK cells are most prone to adhesion, as significant adhesion could be seen with all collagen-, fibrinogen- and VWF-coated surfaces. B cells are less adhesive, because significant B cell adhesion was only seen with collagen-coated surfaces. Importantly, this set of experiments also confirmed our findings of paper I that platelets selectively enhanced cell adhesion of large T cells and small B cells.

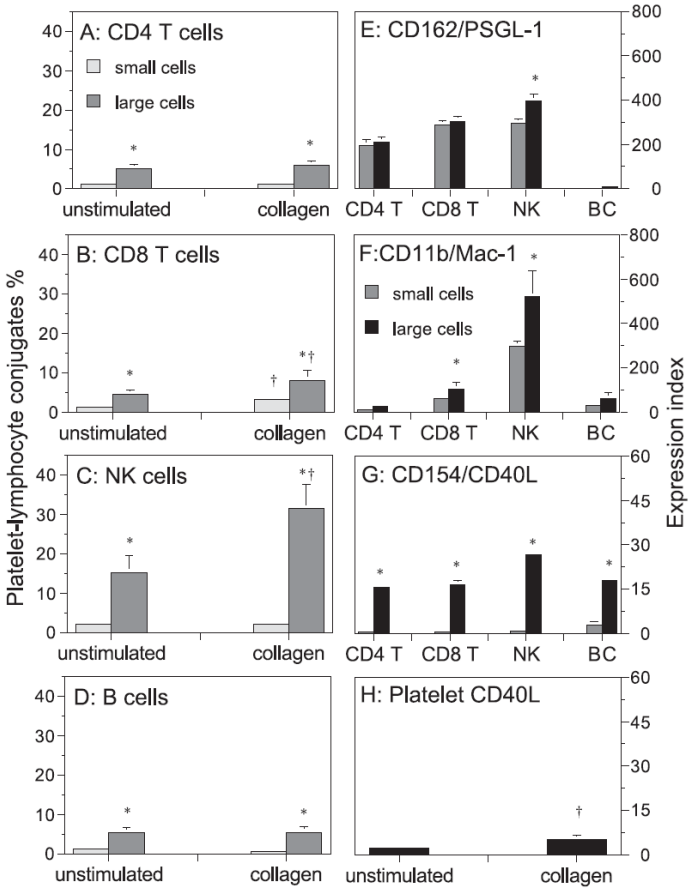



**Figure 7. Differential platelet-conjugating potentials and adhesion molecule expression profiles among different lymphocyte subsets.** Hirudin-anticoagulated whole blood was aliquoted and stained before (unstimulated) and after exposure to shear treatment (500 $s^{-1}$; 5 min) in collagen-coated plates (collagen). Platelet-conjugated CD4 T cells (panel A), CD8 T cells (panel B), NK cells (panel C), and B cells (panel D) were monitored by whole blood flow cytometry, and the results were reported as the percentages of platelet conjugation among small (open bars) and large cells (grey bars). * P<0.05 compared to corresponding small cells, † P<0.05 compared to corresponding cells without collagen stimulation; n=6. Basal CD11b/Mac-1 (panel E), PSGL-1 (F), and CD40L expression (G) of different lymphocyte subsets were measured by whole blood flow cytometry. The data are presented as expression index (EI= positive percentages × mean fluorescence intensity). * P<0.05 compared to corresponding small cells, n=4–7. Platelet CD40L expression was also analysed before and after sheared on collagen-coated surface (500 $s^{-1}$; 5 min) (panel G). † P<0.05 compared to unstimulated platelets; n=5.

### 4.2.3 Platelet-supported lymphocyte adhesion correlates to adhesion molecule profiles of lymphocyte subsets

The work also provides us with a strong support that cell adhesion capacity under arterial flow conditions is closely linked to the expression profiles of adhesion molecules and platelet-conjugating potentials. As can be seen in fig 7, adhesion-prone NK cells and T cells, especially the large cells, are associated with higher expression of PSGL-1, CD11b and CD40L. Those adhesion-prone cell subsets also showed higher percentages of platelet-leukocyte conjugation.

Therefore, these characteristics of platelet-dependent lymphocyte adhesion under arterial flow conditions may direct cell recruitment of selective lymphocyte subsets, for example large and activated $CD4^+$ T cells, at the sites of arterial injury and/or inflammation (e.g., atherosclerotic plaques). As having been pointed out by the editorial accompanied with this paper (126), our observations suggest a possible mechanism how platelets promote chronic inflammatory process of atherosclerosis, and how platelets can constitute a bridging between thrombosis and inflammation.

## 4.3 PLATELETS REGULATE CD4$^+$ T-CELL ACTIVATION (PAPER III)

### 4.3.1 Platelets attenuate CD4$^+$ T cell proliferation

Platelets not only facilitate lymphocyte adhesion but also regulate lymphocyte functions (2, 127). We have thus used a system of autologous platelet-CD4$^+$ T cell co-culture to investigate the influences of platelets on CD4$^+$ T cell function. Polyclonal stimulation with anti-CD3/CD28 MAbs induced marked proliferation of CD4$^+$ T cells. The cell proliferation was, however, inhibited in the presence of platelets, as evidenced by decreased $^3$H-Thymidine incorporation. Platelets contain and release both stimulatory mediators, such as IL-1β (128) and RANTES (88), and inhibitory mediators, such as PF4 (93) and $TxA_2$ (129), for CD4$^+$ T cell proliferation. Our results suggest that, with the particular experimental settings, proliferation-inhibiting activities of platelets predominated the regulation of CD4$^+$ T cell proliferation by platelets.

### 4.3.2 Platelets enhance CD4$^+$ T cell activation via both soluble mediators and direct cell-cell contact

Upon CD3/CD28 stimulation, T cells displayed multiple signs of cellular activation, including CD69$^+$ expression, T cell-platelet conjugation, and cytokine secretion. Hence, CD3/CD28-stimulation induced marked IFNγ secretion, a sign of Th1 cell activation. The IFNγ production was further elevated by more than two-folds in the presence of platelets. Platelet-enhanced IFNγ production of CD4$^+$ T cells increased/accumulated over time, and was dependent on platelet/T cell ratio in the co-cultures (fig 8A and B). The optimal enhancement of IFNγ production by platelets was seen with direct platelet-T cell co-cultures. Separation of the two types of cells by a transwell co-culture (fig 8C) and supplementation of supernatant from activated platelets (fig 8E) retained partial enhancements by platelets. Blocking antibodies against P-selectin, CD40L, and GPIIb/IIIa, which inhibit platelet-T cell conjugation (74), also attenuated platelet-enhanced IFNγ production (fig 8D). These data indicate that both direct cell-cell contact and platelet derived soluble mediators contribute to platelet-enhanced activation of T cells.

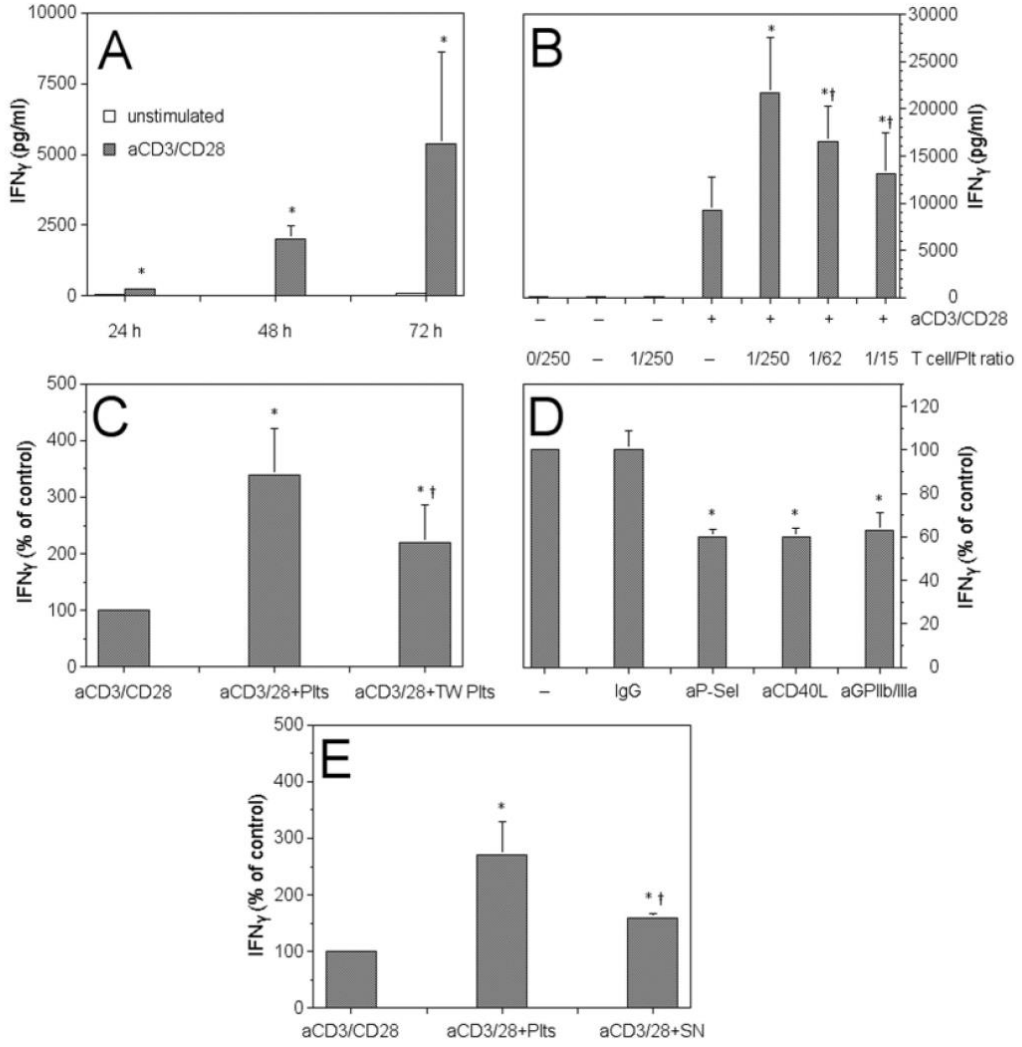



**Figure 8. Platelets promote IFNγ production by CD4$^+$ T cells.** Unstimulated and CD3/CD28-stimulated CD4$^+$ T cells were cultured for 24, 48, and 72 h (A; n=5, *P<0.05 compared to the corresponding unstimulated cells) and at different T cell:platelet ratios (B; n=9; *p<0.05 compared to activated CD4$^+$ T cells cultured without platelets, and †p<0.05 compared to co-cultures with a T cell:platelet ratio of 1:250). CD3/CD28-stimulated CD4$^+$ T cells were also cultured using transwell plates with platelets in an insert well (TW Plts), i.e. without direct cell-cell contact (C), or co-cultured with platelets in the absence (–) or presence of blocking antibodies against P-selectin (aP-Sel; clone 9E1, 20 µg/ml; R&D Systems), CD40L (aCD40L; clone 40804, 10 µg/ml; R&D Systems), GPIIb/IIIa (c7E3, 20 µg/ml; Centocor BV, Leiden, the Netherlands) or with a control mouse IgG antibody (20 µg/ml; Sigma) (D). Supernatants (SN) from activated platelets were also added to activated CD4+ Tcell cultures (E). Culture media were collected after 72 h unless otherwise indicated for measurements of IFNγ. Data in panels C and E are relative changes compared to activated CD4$^+$ T cells cultured alone (*p<0.05 compared to the activated CD4$^+$ T cell cultured without platelets, †p<0.05 compared to the co-culture of mixed cells; n=4–5). Panel D depicts percentages compared to IFNγ levels in platelet-activated CD4$^+$ T cell co-cultures (*p<0.05 vs. without blocking antibody; n=4).

### 4.3.3 Platelets enhance Th1, Th17, and Treg cell activation

Using intracellular staining of cytokines and the Treg transcription factor FoxP3, CD3/CD28 polyclonal stimulation was found to increase Th1 ($IFN\gamma^+$) and Treg ($FoxP3^+$) phenotypes markedly, and also slightly but significantly elevated Th2 ($IL\text{-}4^+$) and Th17 ($IL\text{-}17^+$) activation. Platelets significantly increased Th1, Th17, and Treg cell phenotyping, but had no influence on Th2 phenotyping. The enhancements were accompanied by elevated productions of corresponding cytokines.

### 4.3.4 Influences of PF4, TGFβ and RANTES on $CD4^+$ T cell activation

We next asked how PF4, TGFβ and RANTES, which are known to have diverse influences on different $CD4^+$ T cell subsets (2), modulate the balance between pro- and anti-inflammatory $CD4^+$ T cell responses. For this purpose, we monitored the production of TNFα (a Th1 cytokine) and IL-10 (largely reflecting Treg response in our setting) in the presence of neutralizing antibodies or recombinant cytokines. PF4 neutralisation abolished platelet-enhanced Th1 and Treg responses, while addition of recombinant PF4 mimicked the effects of platelets on both TNFα and IL-10 production (Fig 9A and B). These findings indicate that PF4 has major influences on both Th1 and Treg responses, and that the final readout of PF4 effects is likely dependent on the negotiation of these counteracting effects.

TGFβ neutralization did not affect platelet-enhanced TNFα production, while recombinant TGFβ only slightly hamper production of the Th1 cytokine TNFα (fig 9C), suggesting that TGFβ likely have a mild impact on Th1 responses in our experimental setting. On the other hand, TGFβ is an important factor in Treg cell differentiation (130). TGFβ neutralization did not, however, influence IL-10 production in platelet-$CD4^+$ T cell co-culture (fig 9D), indicating that other platelet-derived mediators (e.g., PF4) may have a strong influence on Treg responses. Indeed, recombinant TGFβ only partially mimicked the effect of platelet-enhanced IL-10 production in stimulated $CD4^+$ T cells (fig 9D).

RANTES is thought to be a proinflammatory chemokine [Zernecke, 2008]. Thus, RANTES neutralization reduced TNFα production in the platelet-$CD4^+$ T cell co-cultures, and recombinant RANTES increased TNFα production in activated $CD4^+$ T cells cultured alone (fig 9E). Surprisingly, RANTES supplementation also increased the production of IL-10 by activated $CD4^+$ T cells cultured alone. The data suggest that RANTES may regulate $CD4^+$ T cells in a complex manner, and that RANTES may exert this enhancement by promoting IL-10 production of

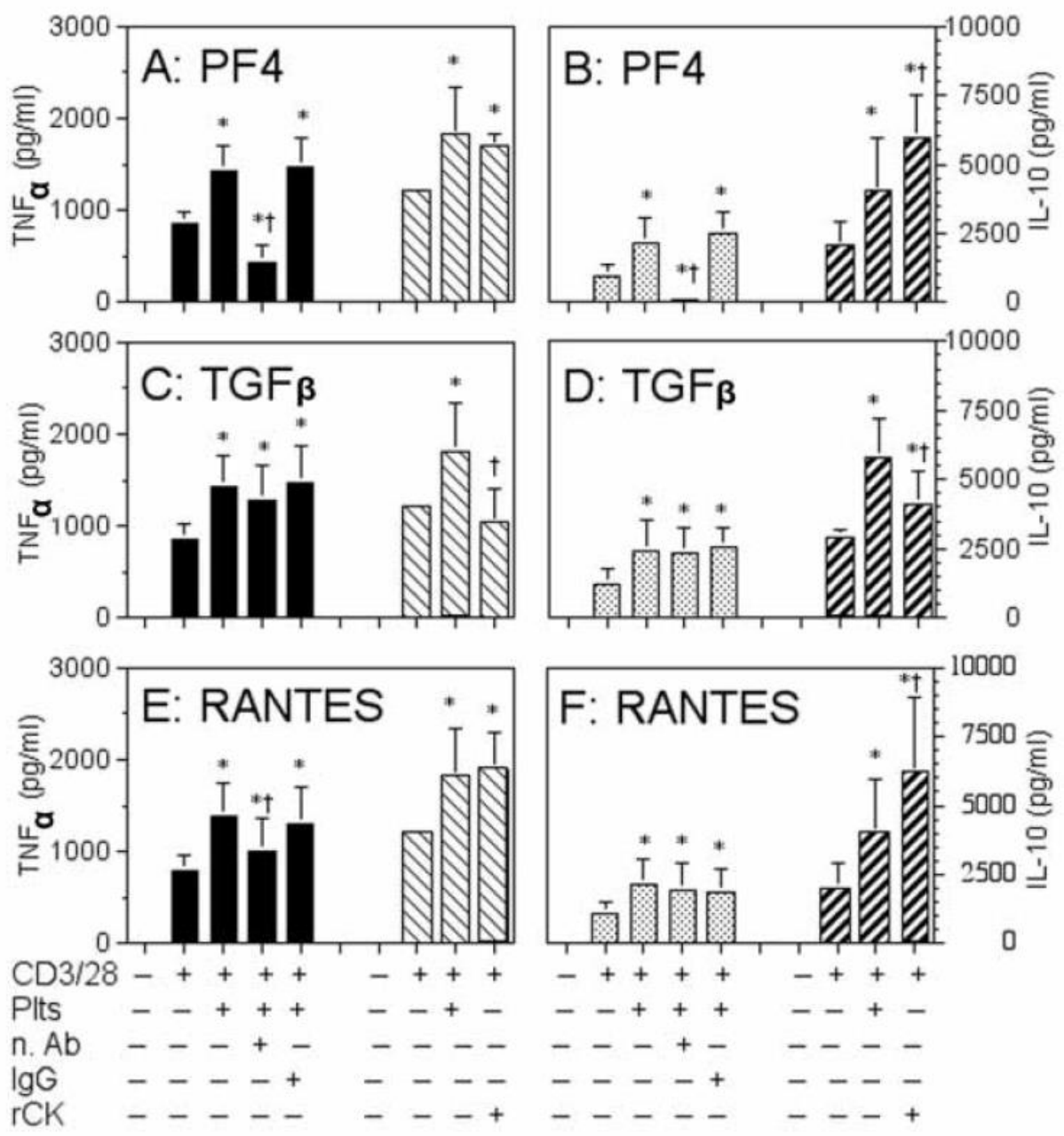



**Figure 9. Influences of platelet-released soluble mediators on TNFα and IL-10 production by CD4$^+$ T cells.** CD4$^+$ T cells were cultured without or with CD3/CD28-stimulation and in the absence or presence of platelets for 48 h. Neutralising antibodies (n. Ab) to PF4 (rabbit anti-human IgG, cat # 500-P05, PepreTech; final concentration 25 μg/ml; A and B), TGFβ (rabbit anti-human IgG, R&D Systems; 20 μg/ml; C and D), RANTES (goat anti-human IgG, AF-278-NA; 10 μg/ml; E and F), or control IgGs (non-specific rabbit IgG, 25 μg/ml; goat IgG, 10 μg/ml, both from Sigma) were added to platelet- CD3/CD28-stimulated CD4$^+$ T cell co-culture (filled bars for TNFα, n=8; dotted bars for IL-10, n=6). The recombinant chemokines (rCK) PF4 (1 μg/ml; A and B), TGFβ (10 ng/ml; C and D), and RANTES (0.1 μg/ml; E and F) were also supplemented to CD3/CD28-stimulated CD4+ T cells cultured in the absence of platelets (hatched bars). Levels of TNFα (A, D, and E) and IL-10 (B, D, and F) were assessed. *P<0.05 compared to unstimulated CD4$^+$ T cells cultured alone, †P<0.05 compared to corresponding platelet-CD3/CD28-stimulated CD4$^+$ T cell co-cultures without or with control IgG.

Treg cells via CCR3 receptor (98, 131).

Together, platelet co-culture can promote Th1, Th17, and Treg responses of $CD4^+$ T cells via both direct cell-cell contact and multiple soluble mediators. Our findings support the notion that platelets can not only regulate adjacent $CD4^+$ T cell activation but also influence the functional responses of remote $CD4^+$ T cells.

## 4.4 DISTINCT ACTIVATION DYNAMICS OF CD4$^+$ T CELL SUBSETS IN THE PRESENCE OF PLATELETS (PAPER IV)

### 4.4.1 Platelets foster distinct activation dynamics of CD4$^+$ T cell subsets

The simultaneous enhancements of Th1, Th17, and Treg cell activation by platelets led us to hypothesize that platelet-enhanced activation of CD4$^+$ T cell subsets may promote the cross-talk among the subsets, such as between Th1 and Treg cells. Therefore, the phenotypes of CD4$^+$ T cell subsets cultured without or with platelets were monitored during 5 days. Fig 10A shows that CD3/CD28 stimulus dramatically increased Th1 phenotype within the first day, and that this nimble activation of Th1 cells was similar in the absence or presence of platelets. The impact of platelets was seen from day 2 when Th1 phenotype in the CD4$^+$ T cells cultured alone decreased markedly and remained low thereafter. In contrast, Th1 phenotype of the CD4$^+$ T cells co-cultured with platelets did not decrease on day 2, but went down more markedly from day 3. A similar biphasic regulation of platelets was also seen with Th17 cell activation (fig 10B). In contrast, platelet-enhanced Treg cell response persisted throughout the observations (fig 10C).

### 4.4.2 Platelets, not Treg cells, predominate the regulation of Th1 responses in the co-cultures

The most interesting finding of above observations is the biphasic Th1 responses in the presence of platelets. To elucidate if enhanced Treg cell activation or platelets are responsible for the biphasic response, we first tested the impact of Treg cells. The FoxP3 activation inhibiting peptide P60 (132) mildly suppressed Treg cell response, and subsequently increased Th1 phenotype. These data suggest that Treg cells in our experimental system were functional, and could attenuate Th1 responses. With platelet-CD4$^+$ T cell co-cultures, however, FoxP3 inhibition by P60 did not alter Treg phenotype, and the same was also true with Th1 cells. These results indicate that Treg intervention by P60 has limited influences in CD4$^+$ T cell responses in the presence of platelets. Furthermore, TGFβ neutralization dramatically increased Th1 phenotype in platelet-CD4$^+$ T cell co-cultures. IL-10 neutralization had no effect, or offered no additive effect on that of TGFβ neutralization. Because TGFβ in our experimental system comes almost exclusively from platelets, it is assumable that platelets predominately regulate Th1 responses in the co-cultures.

### 4.4.3 Platelets differently regulate the proliferation of CD4$^+$ T cell subsets

Next we investigated whether platelets exert distinct regulation on different CD4$^+$ T cell subsets

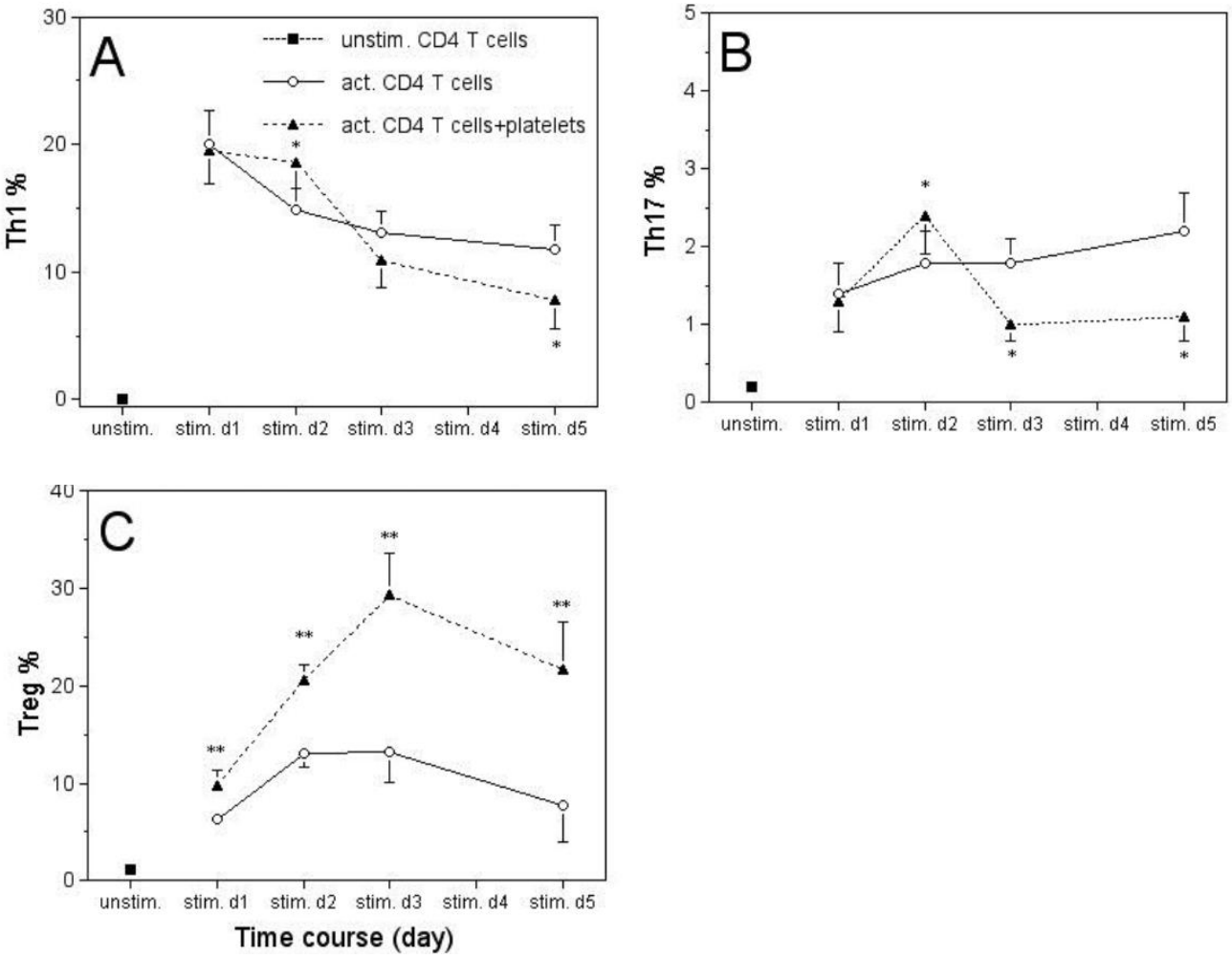



**Figure 10. Dynamics of CD4$^+$ T cell activation with platelet co-cultures. CD4+ T cells were cultured without or with anti-CD3/CD28 MAb stimulation and in the absence or presence of platelets during 5 days.** CD4$^+$ T cell phenotypes of Th1 (panel A), Th17 (B), and Treg cells (C) were assessed by intracellular staining of IFNγ, IL-17, and FoxP3, respectively, using a human Th1/Th2/Th17 phenotyping kit from BD Bioscience and a FoxP3 intracellular staining kit from eBiosciences. Data presented are mean±SEM of the percentages of the subsets in the total CD4$^+$ T cells, n=8; *P<0.05, **P<0.01, compared to corresponding CD4$^+$ T cells cultured without platelets.

through subset-specific regulations of cell proliferation. In paper III, we showed that platelets inhibited $^3$H-thymidine incorporation of total CD4$^+$ T cells. In the present study, we monitored cell proliferation using CFSE-loaded CD4$^+$ T cells that allows simultaneous subset phenotyping by intracellular staining. Fig 11 clearly shows that there were no remarkable differences in cell proliferation activities between FoxP3$^-$ and FoxP3$^+$ CD4$^+$ T cells cultured alone. In the presence of platelets, however, cell proliferation was clearly hampered in FoxP3$^-$/CD4$^+$ T cells, but enhanced in FoxP3$^+$/CD4$^+$ T cells. When proliferation index was assessed by Modfit LT programme, it was clear that platelets markedly reduced proliferation index of FoxP3$^-$ CD4$^+$ T cells, but increased that of FoxP3$^+$ CD4$^+$ T cells, i.e., Treg cells (fig 11C). Furthermore, platelet-

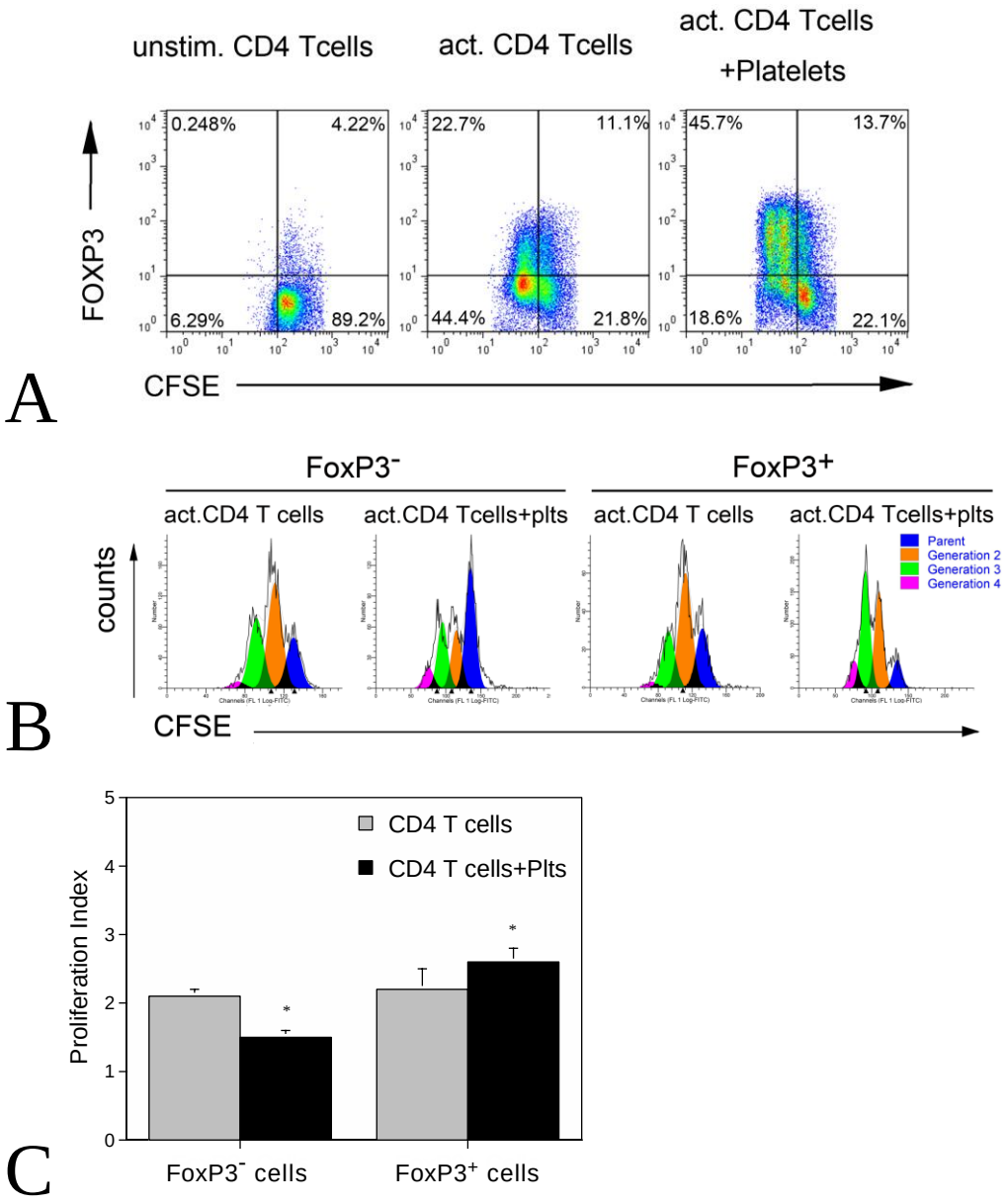



**Figure 11. Platelets inhibit proliferation of FoxP3$^-$/CD4$^+$ T cells.** CFSE-loaded CD4$^+$ T cells were cultured without or with anti-CD3/CD28 MAb stimulation and in the absence or presence of platelets during 5 days. The cells were harvested and stained for APC-FoxP3 on each day for flow cytometric analysis using a Cyan ADP cytometer. Data from the cells harvested on day 3 are shown. CD4$^+$ T cells were subjected to CFSE-APC-FoxP3 analysis that allows the separation of FoxP3$^+$ cells from FoxP3$^-$/CD4$^+$ T cells (panel A). FoxP3$^-$ and FoxP3$^+$/CD4$^+$ T cells were subsequently subjected to CFSE fluorescence analysis to monitor cell proliferation and to assess proliferation index using Modfit LT (Verity Software House; Topsham, ME, USA) (panel B). Proliferation index of activated CD4$^+$ T cells cultured alone (grey bars) and with platelets (solid bars) were presented in panel C. The images shown in panels A and B are representatives from 7 observations. Proliferation index plotted in panel C are Mean±SEMs; n=4. *P<0.05 as compared to corresponding CD4$^+$ T cells cultured alone.

dependent inhibition of FoxP3$^-$ CD4$^+$ T cell proliferation was abolished by TGFβ neutralization, indicating the primary importance of platelet-derived TGFβ in this negative regulation. Platelet-

increased proliferation of FoxP3$^{+}$ cells was, however, not altered by TGFβ neutralization. The phenomenon is likely because other platelet-derived mediators, especially PF4, also have potent stimulatory effects on FoxP3$^{-}$/CD4$^{+}$ T cell proliferation, which have clearly been demonstrated by others (94) and in paper III of the present thesis work.

# 5 SUMMARY AND CONCLUSIONS

Platelets enhance lymphocyte adhesion under flow conditions. Lymphocyte adhesion and recruitment in an arterial flow are platelet-dependent, and have been proven in vivo using a murine model of $FeCl_3$-induced arterial thrombosis.

Platelet-dependent lymphocyte adhesion under arterial flow conditions is selective. Platelets selectively enhance adhesion of large, likely activated, T and NK cells, and small B cells.

The subendothelial matrix protein collagen markedly, while fibrinogen and VWF moderately evoke platelet activation and deposition, and thus support platelet-dependent lymphocyte adhesion under arterial flow conditions.

Platelet-dependent lymphocyte adhesion engages multiple adhesion molecules on both cells, including P-selectin, GPIIb/IIIa, CD40L, PSGL-1 and CD11b. The adhesion potentials of lymphocyte subsets are positively correlated to their platelet-conjugating potentials and expression profiles of CD11b, PSGL-1 and CD40L.

Platelets enhance activation and cytokine production of Th1, Th17, and Treg cells, but not Th2 cells of CD3/CD28-stimulated $CD4^+$ T cells. The enhancements are exerted by both direct cell-cell contact and platelet-derived soluble mediators, including PF4, TGFβ, and RANTES.

Platelets exert distinct regulations on activation dynamics of different $CD4^+$ T effect cells. Hence, platelets constantly promote Treg cell responses, but cast a bi-phasic regulation on Th1 and Th17 cells, seen as a transient enhancement followed by a secondary suppression on Th1/Th17 activation.

The distinct regulations of platelets are achieved by TGFβ-mediated selective inhibition of $FoxP3^-/CD4^+$ T cell proliferation.

Our findings support the notion that platelets are a versatile coordinator of inflammatory responses of $CD4^+$ T cells during atherogenesis.

# 6 FUTURE PERSPECTIVES

The roles of platelets in inflammation have drawn much research attention during the last two decades. Platelets have emerged as a key player in not only thrombotic process but also inflammatory process of atherosclerotic lesion development. Studies of the present thesis work have added novel information of platelet-$CD4^+$ T cell interactions, but also raised new issues to be addressed in our future atherosclerosis research of thrombosis-inflammation cross-talk.

$CD4^+$ T cells are the predominant T cells presented in atherosclerotic lesions. According to the current work, platelets, under arterial flow conditions, support the adhesion of $CD8^+$ T cells and NK cells at least as efficient as $CD4^+$ T cells. It would be of interest to demonstrate how this discrepancy is generated. Moreover, lesional $CD4^+$ T cells are mainly Th1 cells. It would be natural to ask if Th1 cells are selectively recruited. Because $CD4^+$ T cell subset phenotyping can only been done by intracellular staining, the limitation hampers our abilities to differentiate $CD4^+$ T effector cell subsets in live T cell adhesion. Therefore, there is a need to investigate the potentially different adhesion behaviours of different $CD4^+$ T effector cell subsets using differentially polarized and expended $CD4^+$ T cells.

Platelets are important in the recruitment of lesional inflammatory cells. However, many details are still not clear. Dose the importance of platelet-dependent leukocyte recruitment differ in different atherogenetic stages? For example, when the local microvessels have been established in the advanced lesions, is lymphocyte adhesion still dependent on platelets?

$TxA_2$ is an important inflammatory mediator that is synthesized and released by platelets in a large amount. It should be of great interest to elucidate if platelet-derived $TxA_2$ is involved in platelet-$CD4^+$ T cell cross-talk, and if $TxA_2$ may regulate different $CD4^+$ T effector cell subsets differently.

The present thesis work has mainly been carried out in vitro. There is an urgent need for in vivo studies to verify our in vitro findings. For example, it is desirable to demonstrate if and how blockade of platelet adhesion molecules or platelet depletion could influence $CD4^+$ T cell recruitments and/or composition of $CD4^+$ T effector cell subsets in the atherosclerotic lesions.

There is no doubt that antiplatelet therapy is one of the cornerstones for the treatment of atherothrombotic diseases. Further studies of platelet-$CD4^+$ T cell cross-talk in atherosclerosis are warrant, and may be helpful to identify novel therapeutic targets of atherosclerotic diseases.

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