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PLATELET-REGULATED CD4$^+$ T EFFECTOR CELL RESPONSES IN ATHEROSCLEROSIS

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Platelet-regulated CD4$^+$ T effector cell responses in atherosclerosis
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Dedicated to my beloved family

献给我最亲爱的人

ABSTRACT

Atherosclerosis (AS) is a thrombotic and inflammatory disease of the medium- and large-sized arteries. Platelets participate in all stages of its formation, forming thrombus at the ruptured plaque; meanwhile, $CD4^+$ T cells are a key driving force of inflammation, plaque formation and instability. Therefore, the interaction between $CD4^+$ T cells and platelets are important for AS. It is vital and urgent to explore effective prevention and new treatment methods. Our earlier studies demonstrated that platelets had profound regulatory effects on $CD4^+$ T cells. However, the mechanisms of interaction between platelets and T cell subsets, such as $CD4^+$ effector memory T cells (Tem), $CD4^+$ central memory T cells (Tcm), and $CD4^+$ naïve T cells (Tn), is still elusive. The present doctoral thesis research is to elucidate the inflammatory mechanism of platelet regulation in atherosclerosis and to identify potential intervention sites for the development of a new generation of antiplatelet/antiatherosclerotic drugs. The specific goals of this thesis are: (1) To dissect the mechanisms of platelets regulating in individual $CD4^+$ T cell subsets of Tem, Tcm, and Tn; and (2) To elucidate the effects of individual platelet-derived mediators such as transforming growth factor β (TGFβ); to investigate the impact of platelet-derived TGFβ1 deficiency in AS and $CD4^+$ T effector responses with a murine model.

Firstly, we conducted a study on the regulation of platelets on $CD4^+$ Tem. Specifically, when platelets were co-cultured with $CD4^+$ Tem, they enhanced the type 1 T helper (Th1) response transiently, while continuously enhanced the activation of regulatory T (Treg) cells. Platelet factor 4 (PF4) was the key to regulate Tem response. PF4 acted through the receptor CXCR3 to attenuate the activity of protein kinase B (Akt) and reduce the peroxisome proliferator-activated receptor γ coactivator 1 (PGC1α) phosphorylation, leading to elevated mitochondrial transcription factor A (TFAM) expression and mitochondrial biogenesis. The latter increased adenosine triphosphate (ATP) and reactive oxygen species (ROS) production and subsequently enhanced Treg and Th1 responses.

Similar to that for Tem, PF4 exerted regulatory effects on Tcm via CXCR3-initiated intracellular signalling and mitochondrial biogenesis and enhanced Th1 and Treg responses. Unlike Tem, we found that platelets and PF4 enhanced Tcm responses mainly via cell proliferation, which was related to mitochondrial biogenesis and metabolism. Hence, platelets and PF4 enhanced Tcm responses through cell proliferation, while potentiated Tem responses via cell activation.

After exploring the regulation mechanisms of platelets on the memory T cell (TM), we continued to explore the interaction between Tn and platelets. Platelets regulated the effector responses of Th1, Th2, Th17, and Treg cells in a complex manner that involved close cooperation of TGFβ and PF4. Briefly, PF4 with low concentrations enhanced TGFβ signalling through heteromerizing with type III TGFβ receptor (TGFBRIII) and subsequently enhanced type II TGFβ receptor (TGFBRII) expression and TGFβ signalling. However, high concentrations of PF4 directly bound to TGFBRII, blocked TGFβ-TGFBRII ligation, thereby inhibited TGFβ signalling and produced an opposite effect. In addition, the blockade of platelet-Tn cell contact weakened platelet effects, and the injection of PF4-immobilized particles into the spleen of PF4-deficient mice mimicked the platelet effect, indicating the importance of direct platelet-Tn contact and platelet-bound PF4 for optimal regulation of platelets. Thus, platelets exerted a context-dependent regulation on the effector response of Tn cells through a PF4-TGFβ duet.

As interruption of TGFβ signalling exacerbates atherosclerosis, the effect of platelet-derived TGFβ on atherosclerosis is, however, still unclear. So the fourth research aim was to clarify the effect of platelet-specific TGFβ1 deficiency on $CD4^+$ T effector cell responses and atherosclerosis with a murine atherosclerosis model. We created a murine strain with platelet-specific TGFβ-deficiency (plt-$TGF\beta^{-/-}$), and established the mouse model of atherosclerosis through low-density lipoprotein receptor (LDLR) functional knockout and a high-fat diet during 10-15 weeks, in plt-$TGF\beta^{-/-}$ mice and their control littermates. We found that plt-$TGF\beta^{-/-}$ increased the total cholesterol (Cho), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG), and that plt-$TGF\beta^{-/-}$ was related to decreases of Treg cells and Treg cell activities, as well as altered Th1 and Th17 activation. The aortic root cryosections and en face oil red O (ORO) staining of the aorta showed significantly enhanced atherosclerotic lesion formation in plt-$TGF\beta^{-/-}$ mice. RNA sequencing and proteomic analyses also showed signs of $CD4^+$ T effector cell and macrophage activation in plt-$TGF\beta^{-/-}$ mice. Therefore, platelet-derived TGFβ has a protective effect against atherosclerosis.

Together, the thesis work shows how platelets regulate the different $CD4^+$ T cell subsets Tem (**Paper I**), Tcm (**Paper II**), and Tn (**Paper III**); and that platelet-selective TGFβ deficiency aggravates atherogenesis in vivo (**Paper IV**).

LIST OF SCIENTIFIC PAPERS

I. **Shuai Tan**, Shuijie Li, Yanan Min, Anton Gisterå, Noah Moruzzi, Junhao Zhang, Yang Sun, John Andersson, Rickard E. Malmström, Miao Wang, Per-Olof Berggren, Susanne Schlisio, Wangjun Liao, Daniel F. J. Ketelhuth, Chunhong Ma, Nailin Li.
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II. **Shuai Tan**, Junhao Zhang, Yang Sun, Anton Gisterå, Zi Sheng, Rickard E. Malmström, Ming Hou, Jun Peng, Chunhong Ma, Wangjun Liao, Nailin Li.
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CONTENTS

LIST OF ABBREVIATIONS

Akt	Protein kinase B
APC	Antigen-presenting cell
AS	Atherosclerosis
ATCC	American type culture collection
ATP	Adenosine triphosphate
AV	Adenovirus
BGI	Beijing Genomics Institute
BSA	Bovine serum albumin
BV	Brilliant violet
CBA	Cytometric bead array
CD40L	CD40 ligand
Cho	Cholesterol
COVID-19	Coronavirus disease 2019
DC	Dendritic cell
GAG	Glycosaminoglycans
GFP	Green fluorescent protein
GO	Gene ontology
HDL-C	High-density lipoprotein cholesterol
HDM	House dust mites

HEV	High endothelial venule
HS	Heparan sulfate
HT-BiFC	High throughput bimolecular fluorescence complementation
imPF4-MPs	PF4-immobilized microparticles
ITP	Immune thrombocytopenia
KEGG	Kyoto encyclopedia of genes and genomes
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LNs	Lymph nodes
LPS	Lipopolysaccharide
LRP1	Lipoprotein receptor-related protein 1
MACS	Magnetic activated cell sorting
MFI	Mean fluorescence intensity
MHCII	Major histocompatibility complex II
MHCI	Major histocompatibility complex I
MI	Myocardial infarction
MS	Multiple sclerosis
NAC	N-acetyl-L-cysteine
OCR	Oxygen consumption rate
OPLS-DA	Orthogonal projections to latent structures discriminant analysis

ORFs	Open reading frames
ORO	Oil Red O
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCC	Pearson Correlation Coefficient
PCSK9	Proprotein convertase subtilisin/kexin type 9
PD-1	Programmed cell death protein 1
PF4	Platelet factor 4
PFA	Paraformaldehyde
PGC1α	Peroxisome proliferator-activated receptor γ coactivator 1
PGE_1	Prostaglandin E_1
PGI_2	Prostaglandin I_2
PMA	Phorbol myristate acetate
PPI	Protein-protein interaction
PRP	Platelet-rich plasma
RA	Rheumatoid arthritis
rh	Recombinant human
ROS	Reactive oxygen species
RT	Room temperature
SFB	S protein-FLAG-streptavidin binding peptide

SLE	Systemic lupus erythematosus
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
TFAM	Mitochondrial transcription factor A
TFAM-AV	Mitochondrial transcription factor A adenovirus
TG	Triglyceride
TGFBRI	Type I TGFβ receptor
TGFBRII	Type II TGFβ receptor
TGFBRIII	Type III TGFβ receptor
TGFβ	Transforming growth factor β
Th	T helper cells
Th1	Type 1 T helper cells
Th17	Type 17 T helper cells
Th2	Type 2 T helper cells
TLR	Toll-like receptor
TM	Memory T cell
Tn	Naïve T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cells

TXA_2	Thromboxane A_2
WHO	World health organization
YFPc	C-terminal fragment of YFP
ΔTGFBRIII	TGFBIII knock-out

1 INTRODUCTION

Atherosclerosis (AS) related cardiovascular diseases are one of the most common causes of human death according to the World Health Organization (WHO) data [1]. So, it is very crucial and meaningful to find a novel and effective prevention/treatment against atherosclerosis. For every stage of atherosclerosis, the participation of platelets is very essential, and platelets can cause fatal attacks by forming a thrombus at the ruptured plaques [2]. AS is a chronic inflammatory disease that occurs in the medium- and large-sized arteries. It is characterized by lipid deposition in the vessel wall, accompanied by immune cell infiltration and smooth muscle cell proliferation. What is more, it is noted that both innate and adaptive immune responses, involving neutrophils, macrophages, and mainly $CD4^+$ T cells, take part in the development of atherosclerosis.

After exposure to specific antigens, $CD4^+$ T cells undergo cell activation, polarization, and differentiation, and contribute to adaptive immune responses. For instance, if the people are infected by the coronavirus during the COVID-19 pandemic, the activation, proliferation and differentiation of $CD4^+$ T cells will occur to control and help eliminate infection; afterwards, most of the effector $CD4^+$ T cells undergo apoptosis. The surviving cells can differentiate into long-lived memory cells, such as Tem and Tcm [3,4,5]. Because $CD4^+$ T cells are a vital cellular component in atherosclerotic lesions, their roles in atherosclerosis get more and more attention in recent years. They are a major driving force in lesion inflammation, plaque formation and destabilization, in which Th1 and Treg cells are pro- and anti-atherosclerotic, respectively [6,7].

The occurrence and development of AS involve many complex mechanisms, among which platelets/thrombosis, $CD4^+$ T cells/inflammation, and their interactions play important roles [6-9]. In addition, the development, activation, differentiation, function and survival of T cells all need cell metabolism and energy production. Mitochondria are the energy plant of cells, which are important for the exit of the quiescent state and initial 24-48 hours after T cell activation [10-12]. Therefore, to study the interaction between platelets and T cells, the function of mitochondria cannot be ignored.

2 LITERATURE REVIEW

Atherosclerosis is a chronic vascular disease, involving a variety of mechanisms such as thrombosis, inflammation, and hyperlipidemia [13,14]. Platelets and $CD4^+$ T cells are involved in these complex mechanisms. In particular, platelets have become a crucial coordinator between thrombosis and inflammation.

2.1 PLATELET PHYSIOLOGY

Platelets are anucleate discoid cells with a median size of (2.0-5.0)×0.5 μm, mean cell volume 6-10 fl, blood concentration 200-300×10^9/L, and a life span of ≈10 days [15]. The most well-known function of platelets is in hemostasis and thrombosis. Platelets are not only an important cell accounting for myocardial infarction (MI), which is mainly caused by thrombus formation; but also have a correlation with some immune system diseases like the virus or bacterial infection in patients with idiopathic thrombocytopenic purpura [16]. Recent evidence suggests that these cells may exhibit immune functions via Toll-like receptors (TLR) [17-21].

Alpha-granules (α-granules) are secreted upon platelet activation, which is associated with major histocompatibility complex I (MHCI) molecules. For instance, MHCI expression can be observed in the platelet plasma membrane and cytosol, although it is highly unstable [22,23]. Additionally, PF4/CXCL4, TGFβ, and some other mediators are also secreted by α-granules, and participate in thrombosis, inflammation, immunity, and aggravate vascular intimal injury [24]. PF4 exhibits a strong chemotactic effect on neutrophils by binding heparan sulfate on the surface of the vascular endothelium, which attenuates thrombin inactivation. TGFβ levels in circulation are mainly derived from platelets and are considered to be a specific indicator of platelet activation in vivo [25-28].

For clinical prevention and treatment of atherosclerotic diseases, antiplatelet therapy has been established as a broad clinical consensus and therapeutic cornerstone for a long time [2]. Current antiplatelet therapies, typically targeting the activation of platelets per se with, e.g., the COX-inhibitor aspirin or/and the ADP P2Y12 receptor antagonist clopidogrel or ticagrelor, provides approximately 30-40% protection for acute coronary syndrome patients. Current attempts to improve antiplatelet treatments have, however, reached their therapeutic plateau, as evidenced by limited additive developments (e.g., ticagrelor superior to clopidogrel by only 2% in primary endpoint protection during one year) [29,30]. Thus, there is an urgent need for other kinds of antiplatelet drugs. New antiplatelet drug development may anchor to platelet engagements in inflammatory mechanisms of atherosclerosis. Two

antiplatelet drugs, respectively targeting platelet activation and inflammatory functions, could likely produce marked additive or even synergistic effects.

2.2 $CD4^+$ T CELL

$CD4^+$ T cell is a type of white blood cell, which is responsible for helping to eliminate and control a variety of infections. $CD4^+$ T cells are vital to the human immune system and could be envisioned as a "command centre". If $CD4^+$ T cells need to become functional, they must be activated. A two signal process controls $CD4^+$ T cell activation. After the antigen processing and presentation, the antigen-presenting cells (APC) will form the major histocompatibility complex II (MHCII)-peptide complex on their surface, which can be recognized and ligated by T cell receptor (TCR) and CD4, called the first signal of $CD4^+$ T cell activation. The second signal is the costimulation by costimulators including cytokines or a pair of plasma membrane molecules such as B7 (CD80) on the APC surface and CD28 on the $CD4^+$ T cell surface. Without costimulation, the $CD4^+$ T cells get "no energy". The activated $CD4^+$ T cells will synthesize and secrete IL-2 and will proliferate and differentiate. $CD4^+$ T cells are activated by the antigen to become effector cells, and then most of them will die. A small number of cells will eventually survive as memory cells with long-term memory function for the antigen.

2.2.1 Th and Treg cells

$CD4^+$ T cells can differentiate to Th cell subsets, such as Th1/Th2/Th17 and Treg cells that participate in various facets of immunity to eliminate pathogens. They are induced via the transcriptional activity of T-bet, GATA3, RORγt, and FoxP3, respectively [31,32]. In addition, some chromatin modifiers can also affect T cell function at the gene level [33].

Under a normal physiological context, Th1 and Th2 exist in homeostasis. These subset cells can also change their phenotypes under certain conditions [34,35]. Several factors are associated with Th1 differentiation and activity, including IL-12, CXCR3, and IFNγ. Th1 cells mainly secrete pro-inflammatory cytokines—such as IFNγ—to mediate cellular immunity, and may induce the activation of other cell types, such as macrophages [36-40]. The function of Th2 is not the same as that of Th1. The survival and function of B cells such as proliferation, maturation, antibody production, and mediation of humoral immunity require IL-4, IL-5, and IL-13 secreted by Th2. Importantly, the two subsets regulate the activities of each other to maintain immune homeostasis, as IL-4 and IFNγ can inhibit the functions of Th1 and Th2 cells, respectively [41]. Moreover, there also exists a specialized subtype of "Th2/Th1" cells, which express IL-4, IFNγ, GATA3, and T-bet. Interestingly, Papain and House Dust Mites

(HDM) antigens can induce Th2 cells through the non-MHC-II pathway, suggesting that Th2 cells may also have an intrinsic function in adaptive immunity [42,43].

Th17 cells were first detected in an experimental autoimmune encephalomyelitis mouse model and characterized by their expression of IL-17 (IL-17$^+$ IFNγ$^-$). During differentiation, the transcription factor HIF1α induces the expression of RORγt and promotes glycolysis pathways to facilitate Th17 cell polarization [44,45]. IL-17 is an important pro-inflammatory factor with multiple biological functions, such as inducing neutrophil proliferation and maturation and inducing pro-inflammatory cytokine expression in various cell types. As such, these cells can affect the pathophysiology of several health conditions, including infection, cancer, autoimmunity, aplastic anaemia, rheumatoid arthritis (RA), and others [46-48]. Moreover, Th17 cells also co-produce IL-17 with IFNγ in response to candida albicans [49].

Treg cells are an immunosuppressive T cell subset induced by IL-2-stimulated FoxP3 transcriptional activity, which maintains peripheral immune tolerance, and controls autoimmune responses [50-54]. They not only inhibit adaptive immune reactions but also inhibits innate immune activity. Some immune cells like neutrophils, macrophages, and monocytes are inhibited by Treg cells [55,56]. Treg cells can be characterized by several classification schemes. For instance, Treg cells can be divided into tTreg and pTreg based on their derivation from the thymic or peripheral T cell compartments as determined by the expression of neuropilin-1 and helios, respectively [57]. It should also be noted that Treg cells can alter the activity of other Th subsets. For instance, T-bet$^+$/IFNγ$^+$, IRF4$^+$, and TIGIT$^+$ Treg cells inhibit the Th1, Th2, and Th17 responses, respectively [58-62].

Importantly, the balance between Treg and other Th subsets prevent autoimmunity by blocking the activation of autoreactive T cells, and the expression of cytokines. Consistently, Ait-Oufella et al. found that CD4$^+$ CD25$^+$ Tregs can inhibit the formation of AS [63]. In addition, lipopolysaccharide (LPS) stimulation induces CD69 and B7 expression and enhances Treg survival and proliferation. A recent study shows that miR-27 is a critical regulator of Treg function and immunological tolerance. miR-27 can target IFNγ directly to attenuate T cell responses and will impair Treg-mediated immunological tolerance and promote T cell-mediated autoimmunity [64]. Moreover, Tregs also express IL-10 and TGFβ to inhibit macrophage function after TLR4 activation [65-67]. IL-10 can downregulate T cell-mediated immune responses, such as repressing the proliferation of Th1 and Th2. TGFβ can regulate the functions of immune cells [68,69]. Additionally, Th17 cells can convert to Treg

cells by inducing TET protein recruitment, DNA methylation, and subsequent FoxP3 expression via TGFβ expression of Treg [70,71].

2.2.2 Naïve and memory T cells

T cell subsets can reflect the status of immune function. $CD4^+$ cells secrete a large number of cytokines and promote B cell antibody production to fulfil their immunological function in the humoral and cellular immune systems. A vast diversity of T cell phenotypes exists in human peripheral blood, based on cell surface marker expression [72-74]. In particular, $CD4^+$ T cells comprise a variety of phenotypes and functional cell subsets, such as naïve T (Tn) and Memory T cells (TM). CD45RA and CD45RO are two variants of the leukocyte common antigen CD45, which are essential for lymphocyte signal transduction, maturation, and regulation of function. As such, we can classify T cells as Tn ($CD45RA^+$ and $CD45RO^–$) or TM ($CD45RA^–$ and $CD45RO^+$) based on the expression of either variant [75].

Tn matures in the thymus and migrates to the spleen and the lymph nodes (LNs), i.e., peripheral secondary lymphatic organs/tissues. Tn is a type of T cell with surface molecules such as TCR/CD3, CD4, CD28, etc., which is developed in the thymus and circulate quiescently before antigen stimulation. It can be further differentiated into Th1, Th2, Th17, Treg, etc. by interactions with APC, such as dendritic cell (DC). For Tn, Tcm and Tem, these three kinds of cells, the capacity for strength of APC functional differentiation is gradually enhanced; the proliferative capacity and antitumor efficacy are decreased gradually [76]. Most of the effector cells will die by apoptosis during a contraction phase after they have cleared the infection. A small part of them persists and differentiates into memory cells. Some of the effector T cells become Tcm and Tem directly, and some of Tem are converted from Tcm [32,77,78]. Compared with Tn, TM initiates and performs a faster immune response after being re-infected by pathogens. These cells are important for immune functions, such as tumour immunity, host defence, and other immune responses; and are also mediating memory after vaccine responses [79,80].

Memory T cells can be further divided as Tem and Tcm, according to the expression of cell surface molecules such as CD62L and CCR7. CD62L is a lymph node homing receptor related to cell migration, whereas CCR7 is a chemokine receptor that participates in T cell recirculation and effector function [72,81,82]. The Tcm localize to the secondary lymphoid tissues and are involved in recirculation like Tn, whereas Tem preferentially migrate and are distributed throughout the non-lymphoid tissue and local immune response areas to facilitate a quick immune response by expressing cytokines, chemokines, and adhesion molecules

upon contact with their cognate antigens [83-88]. Further, Tcm and Tem differ from Tn in morphology, biological functions, and surface adhesion molecule expression—such as CD62L that gradually decreases with repeated cell activation [89]. Tcm and Tem also play distinct roles during different stages of the protective immune response against foreign pathogens. Consistent with their tissue distribution, Tem participate during in early stages of infection, whereas Tcm predominate during later stages because of their strong proliferative potential and extended period of effector function.

Memory cells are key mediators of vaccine immunity [79]. Tcm highly express CD45RO, CD62L, CD28, CD44, CD11a, and IL-12R (β1 subunit) and exhibit a stronger proliferative potential and anti-tumour immunity as compared to Tem [76,83,90]. These cells pass through the high endothelial venule (HEV) to migrate to the secondary lymphoid organs. In comparison, Tem are distributed in the extra-lymphoid effector sites, such as the intestine and lung, and express low levels of CD62L and CCR7 on their surface. Because of their limited CCR7 expression, Tem do not recirculate [91], but quickly localize to inflamed tissues via a chemotactic gradient and express pro-inflammatory factors, such as IL-4, IL-5, IFNγ, and perforin. Importantly, some autoimmune diseases including psoriasis, multiple sclerosis (MS), and RA are related to Tem function. For instance, a large number of Tem cells can be observed in the synovial fluid or skin of RA and psoriasis patients during clinical diagnosis and treatment, respectively [92-94]. It is also important to note that Tcm and Tem are capable of transforming to their counterpart subtype in response to antigen stimulation [95]. Tcm cells are considered to exhibit characteristics similar to those of stem cells, which have the ability of self-renewal. In addition, some of Tem can also be generated from Tcm in response to antigen. Tem cells function via the secretion of various factors [96]. Tem cells have also been implicated in AS development. Tn decreased, while Tem increased during the course of the disease [97-101].

2.2.3 Memory T cells facilitate immunological memory

The vast majority of effector T cells at the peak of immune activation, as much as up to 90 to 95 percentage, will decrease via apoptosis after the antigen is cleared [102,103]. Maintaining long-term self-tolerance during rapid cellular expansions and contractions is a major challenge to the immune system [104]. Immunological specificity and memory is, therefore, a primary facet of the human immune system. Following bacterial or viral clearance, a majority of effector $CD4^+$ T cells die, leaving only a small number of memory T cells with a lower proliferation rate and increased apoptotic resistance, compared to naïve counterparts [105-108].

Upon secondary challenge, when a pathogen invades again, it will be recognized and killed by a rapid and strong immune response initiated by the remaining cells, which is also the main component of anti-tumour specific immunity. The number of cells, cell surface molecule expression, and cytokine production change accordingly. What is more, TM is related to cognate antigen-independent steady-state of cellular turnover in response to IL-7/IL-15 [84, 109,110]. This explains how immune memory can continue to be sustained for long periods [111].

However, whether memory T cell maintenance requires antigen stimulation has been controversial [112]. Memory and effector T cells express many common surface molecules, indicating that the long-term maintenance may be derived from contact between memory T cells and antigens [113]. Since antigen-activated T cells exudate to the peripheral organs to elicit an immune response, memory maintenance may be closely related to antigenic stimulation. In this mechanism, the capacity for memory cell differentiation decreases in the absence of continuous stimuli [114, 115].

An alternative school of thought suggests that immunological memory may be associated with T cell longevity [111]. Some scientists hold a conservative view that memory is unlikely to be maintained by long-lived memory cells [116]. The antigens and similar stimuli are necessary to maintain long-term memory, as some reports suggest that weak antigen stimulation facilitates memory T cell persistence [117]. Furthermore, some evidence also indicates that inflammatory signalling contributes to the production of effector cells, whereas a lack of inflammation favours memory cell development [111].

2.3 Platelet-T cell interaction and their impact on atherosclerosis

Significantly, T cell function can also affect the pathophysiology of atherosclerosis. Atherosclerotic plaques are inflammatory sites and contain both macrophages and lymphocytes. Most T cells in plaques exhibit a Th1 phenotype and secrete IFNγ. Several chemokines are also involved in the disease process. As mentioned before, some Th can reduce inflammation by altering chemokine receptor expressions [118,119]. Therefore, further investigation into the relationship between platelets and immune cells could be very valuable research with clinical significance. Platelets are not only predominately involved in thrombosis but are also closely involved in inflammatory mechanisms of AS [8,9]. Platelets are “sensitive sentinels”, which easily become active or even overreact to stimuli such as inflammatory mediators and infection. Activated platelets release various growth factors and cytokines—such as PF4, TGFβ, etc.— and not only promote blood coagulation,

inflammation, and angiogenesis but released particles also form aggregates with other platelets. Platelet particles contain antimicrobial peptides, which can trap and absorb pathogens. What is more, activated platelets also interacts with white blood cells, leading to their recruitment and activation, thereby enhancing the immune response and influencing T cell function [120-122]. For instance, PF4 may be anti-atherosclerotic [123], by inhibiting T cell proliferation and cytokine release by binding glycosaminoglycan [81,82,124,125].

TGFβ has three widely expressed receptors including Type I TGFβ receptor (TGFBRI), TGFBRII and TGFBRIII. After ligand binding, TGFBRII recruits and phosphorylates TGFBRI, which then phosphorylates downstream Smad2/3, leading to recruitment of Smad4 and translocation of a Smad-complex to the nucleus, regulating transcription of various genes [126]. TGFBRIII can sequester TGFβ and present it to TGFBRII. In the process of atherosclerosis, platelets regulate the response of $CD4^+$ T cells: $CD4^+$ T cells recruitment into the arterial wall is facilitated by platelets adherence and activated platelets release a variety of soluble mediators regulating Th/Treg cell responses [7,9]. Hence, platelet-regulated T cell responses are of great interest for atherosclerosis research. As mentioned above, PF4 and TGFβ are important mediators derived from platelets. How they regulate effector responses of T cell subsets is still unclear. Dissection of mechanisms of platelet-regulated T cell subset responses may identify novel intervention sites for future therapeutic development for atherosclerotic diseases.

3 RESEARCH AIMS

Overall, the aim of this thesis is to elucidate platelet-regulated inflammatory mechanisms in atherogenesis and to identify potential sites of intervention for the therapeutic development of a new generation of antiplatelet/anti-atherosclerotic drugs. The specific aims are:

- To dissect the mechanisms of how platelets regulate individual $CD4^+$ T cell subsets: Tem (**Paper I**), Tcm (**Paper II**), and Tn (**Paper III**).
- To elucidate the effects of platelet-derived TGFβ on $CD4^+$ T effector responses and atherogenesis using a murine atherosclerotic model with platelet-selective TGFβ1 deficiency (**Paper IV**).

4 MATERIALS AND METHODS

4.1 ETHICAL CONSIDERATIONS

The studies of the present doctoral thesis were performed with strict adherence to ethical principles, the Declaration of Helsinki, and EU guidelines and regulations on human/animal ethics and practices.

The in vitro studies using human blood samples have been reviewed and granted ethical approvals (Dnr 94-146, Dnr 2015-06-A, and Dnr 2019-04340). The in vivo studies using murine models have been reviewed and granted ethical approval (Dnr SDUQH-20161010-01). The life and welfare of the animals were treated with respect and care. The animals were housed and supported under standard conditions.

4.2 IN VITRO STUDIES

4.2.1 Blood donation and platelet isolation

All healthy volunteers gave informed consent to donate blood samples and denied taking any antiplatelet or immunosuppressive medications during two weeks before blood sampling.

A 10 ml vacutainer containing sodium citrate (final concentration 0.32%) was used to collect blood from an antecubital vein. Blood was centrifuged (190×g, 20 min, 22°C), and the upper 2/3 of platelet-rich plasma (PRP) was collected. After adding prostaglandin E_1 (PGE_1) or prostaglandin I_2 (PGI_2), PRP was re-centrifuged, and the supernatant was further spun (1000×g, 10 min, 22°C). The pellet was resuspended with Tyrode's HEPES buffer containing 10 μM indomethacin and 0.1 μg/ml PGE_1 (or PGI_2), and then re-centrifuged. The platelet pellet was then resuspended in basal medium (1.25×10^9/ml) and kept at room temperature (RT) for 30 min to recover platelet reactivity.

4.2.2 Isolation of $CD4^+$ Tem, Tcm and Tn cells

The buffy coats were obtained from the blood centre of Karolinska University Hospital. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque®-1077. Magnetic activated cell sorting (MACS) $CD4^+$ effector memory T cell isolation kit, $CD4^+$ central memory T cell isolation kit, and Naive $CD4^+$ T cell isolation kit II were used to isolate Tem, Tcm, and Tn cells, respectively, according to manufacturer's instructions. RPMI 1640 medium was used to re-suspend $CD4^+$ T cells (5×10^6/ml) and for cell culture.

4.2.3 $CD4^+$ T cell-platelet co-cultures

$CD4^+$ T cells, i.e., Tem, Tcm, and Tn, were cultured with or without platelets (T cell-platelet ratio 1:250) at 37℃ and 5% CO_2 for seven days. T cells were stimulated with αCD3 (3 μg/ml) and αCD28 antibodies (0.3 μg/ml).

4.2.4 Flow cytometry

Flow cytometry was used for Th/Treg cell phenotyping, mitochondrial mass measurement, and T cell cytokine production analyses. Flow cytometric samples were prepared as previously described [127], and analyzed using CyAn ADP®, BD FACS Canto™ II, and Beckman-Coulter Galios® flow cytometers. FlowJo version 10 was used for data analyses.

4.2.5 Western Blotting

To monitor the Akt-PGC1α-TFAM signalling pathway, T cell lysates were separated with 10% SDS-PAGE by loading the same amount of proteins. After transfer to a nitrocellulose membrane, the membrane was incubated with TBST blocking buffer (Tris-buffered saline: 25 mM Tris, 0.1% Tween 20, 3% non-fat dry milk powder; 22°C, 1 h), the primary antibody, such as Akt, PGC1α, and TFAM antibodies (4°C, overnight), and the secondary antibody (22°C, 1 h), with thorough washing (10 min×3) between incubations. Immunoreactivity bands were detected using an enhanced chemiluminescence kit [128,129].

4.2.6 CXCR3 knock-down

The PF4 receptor CXCR3 silencing/knock-down was performed in Tem and Tcm cells using Opti-MEM™ Reduced Serum Medium, Lipofectamine™ RNAiMAX Transfection Reagent kit, and ON-TARGETplus human CXCR3 siRNA SMARTPool (a mixture of 4 siRNAs) and corresponding ON-TARGETplus non-targeted Control Pool.

4.2.7 Immunofluorescent imaging

To assess mitochondrial biogenesis, Tem cells were stained with MitoTracker Red CMXRos (100 nM, 37°C, 30 min), and mounted using ProLong Diamond Antifade Mountant with DAPI for nuclear staining (22°C, 30 min). The confocal images were acquired with a Zeiss LSM 700 laser scanning confocal microscope.

To demonstrate PF4 and TGFBRII ligation, 48 hours before imaging, 293T cells were transfected with 1 μg of plasmids encoding SFB-tagged PF4 and GFP-tagged TGFBRII for 6

hours. After immunofluorescence staining and nucleic labelling with DAPI (1ng/ml, 2 min), fluorescent images were acquired using a Nikon Eclipse Ti-E microscope.

4.2.8 Mass spectrometry

Tem whole-cell lysates were prepared for mass spectrometry analysis, using a sample preparation procedure described previously [130]. The samples were analyzed by shotgun proteomics using nanoLC-MS/MS with UltiMate 3000 nanoLC system and Orbitrap Elite mass spectrometer. The raw data of mass spectrometry were analysed MaxQuant programme. The protein mass fold change was set at 1.5 and -1.5 as the up-regulation and down-regulation threshold, respectively. Functional clustering of protein interactions was analysed using the online database String (https://string-db.org/).

4.2.9 Seahorse Assay

Seahorse assay was used to monitor mitochondrial respiration by measuring oxygen consumption rate (OCR), thus detecting the level of cell metabolism. Tem cells were cultured three days without or with polyclonal stimulation and in the absence or presence of PF4 supplementation. Tem cells were seeded (1-2×10^5/well) on a 96-well XF plate for OCR measurements by using the Seahorse XF 96 analyzer.

4.2.10 Protein-protein interaction (PPI) screening by high-throughput bimolecular fluorescence complementation technology (HT-BiFC)

HT-BiFC assay was employed to demonstrate PF4 and TGFBRIII interactions by using the stable bait HTC-75 cells expressing PF4-YFPn and C-terminal fragment of YFP (YFPc)-labelled prey retrovirus, which was used to infect stable YFPn-labeled PF4 bait cells. A stable cell line co-expressing YFPn-labeled PF4 and YPFc-labeled prey was obtained by the selection of 1 μg/mL puromycin. HTC-75 cells only infected with YFPc-EV retrovirus were produced as a negative control [131].

4.3 IN VIVO STUDIES

4.3.1 Mice

Animal research protocols have been reviewed and approved by the Animal Ethics Committee of Shandong University Cheeloo Medical College (Jinan, China; Dnr SDUQH 2016-10-13-1).

All mice used in the present thesis work were on the C57BL/6 background. Platelet-specific TGFβ1-deficient (Plt-TGFβ$^{-/-}$) mice were established by crossing mice carrying a "floxed" TGFβ1 allele (*Tgfb1*^{flox}; Jackson Laboratory) to mice expressing Cre recombinase under the control of the megakaryocyte/platelet-specific *Pf4* promoter (*Pf4*-Cre^{+} transgenic mice) (Model Animal Research Center of Nanjing University, Nanjing, China). PF4 deficient (PF4$^{-/-}$) mice were generated using the CRISPR/Cas 9 system (Cyagen Biosciences Inc., Guangzhou, China). TCR transgenic OT-II mice that recognize $OVA_{323-339}$ were purchased from the Jackson Laboratory (Shanghai, China) and backcrossed onto the mice with CD45.1 background, and served as donor mice in adoptive transfer of OT-II T cells. All mice were bred and housed in a specific pathogen-free animal research facility at Shandong University.

4.3.2 Mouse models of platelet depletion and adoptive transfer of OT-II T cells

Mouse platelet depletion was carried out using rat anti-mouse GPIbα/CD42b antibody R300 (4 μg/g i.p. on day -2, followed by 2 μg/g i.p. on day 0, 3 and 5; non-specific rat IgG C301 was used as the control) [132]. To elucidate the impact of platelets on Tn cell responses *in vivo*, platelet-depleted mice, Plt-TGFβ$^{-/-}$ mice, and PF4$^{-/-}$ mice were used as the recipient mice of OT-II T cell adoptive transfer (Fig 1). CD45.1 OT-II T cells were isolated from the spleens and LNs of OT-II mice and were injected (4×10^6/0.2 ml) via the tail vein on day -1. Recipient mice were immunized *i.p.* with 130 μg of OVA in 200 μl of alum adjuvant on day 0. The mice were sacrificed at designated time points. Blood samples were collected for plasma cytokine analyses with a CBA kit, and mononuclear cells were isolated from the spleen or LNs for flow cytometric T cell phenotyping. For the bioinformatic analyses, we used protein sequences and functional information from the Uniprot Database (http://www.uniprot.org/) and DAVID Bioinformation Resources 6.8 [133] to define PF4-interacting proteins/biological functions of PF4 interactome with "gene ontology (GO) enrichment analysis", and "Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis"; we used the search tool for the retrieval of interacting gene/proteins (STRING) version 11.0 database [131], and Cytoscape version 3.8.0 [134] for PPI network.

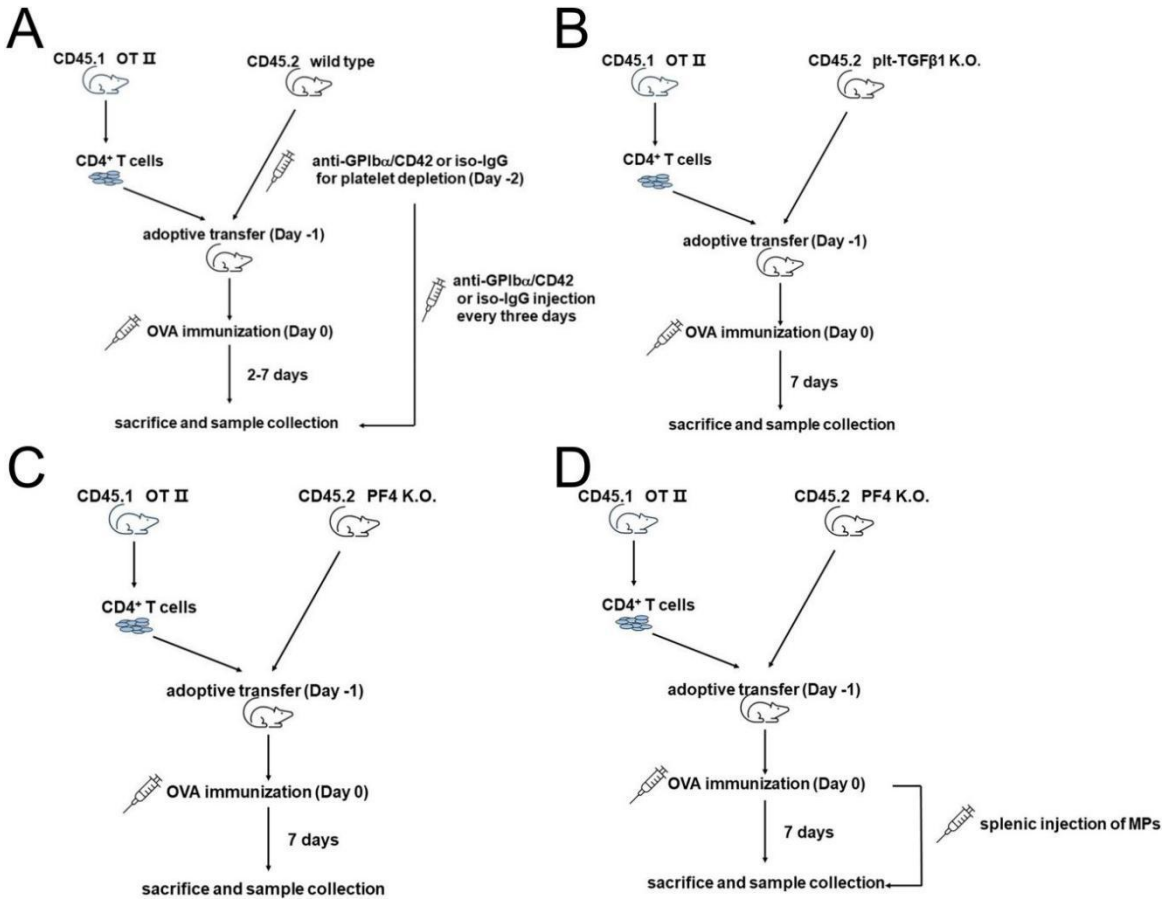

Figure 1. The experimental designs of the murine model in the Tn study. Panel A: the experimental design for adoptive transfer of OT-II T cells, and platelet depletion. $CD4^+$ T cells were isolated from the lymphatic organs of CD45.1 OT II mice. The cells were transferred to CD45.2 mice on day-1, and then injected intraperitoneally with rat anti-mouse GPIbα/CD42b antibody R300 (4 μg/g i.p. on day-2, followed by 2 μg/g i.p. on day 0, 3 and 5); Panel B: platelet-specific TGFβ1 knockout mice or control mice were adoptively transferred with $CD4^+$ T cells from CD45.1 OT-II mice, then challenged with OVA; Panel C: PF4 knockout mice and the littermates (control) mice received CD45.1 OT-II $CD4^+$ T cell adoptive transfer and then OVA challenge; Panel D: C57BL/6 mice received OT-II T cell adoptive transfer and OVA-immunization. One day later, imPF4-MPs or control MPs were injected into the spleen. After incubation for one week, mice were sacrificed, and mononuclear cells were isolated from the spleen and the LNs.

4.3.3 Atherosclerosis mouse model

Eight-week-old male Plt-TGFβ$^{-/-}$ mice (PF4-Cre$^+$-TGFβ1$^{f/f}$) and their littermate control (PF4-Cre$^-$-TGFβ1$^{f/f}$) received proprotein convertase subtilisin/kexin type 9 (PCSK9)-encoding rAAV8 vector (1.0×10^{11} vector genomes/mouse) injection through the tail vein. After 1-week incubation allowing the functional abrogation of LDLR, the mice were fed with a cholate-free Western-type high-fat diet for 10 to 15 weeks. The mice were then sacrificed, Blood was collected by cardiac puncture. The heart, the entire aortic tree, spleen and LNs were collected under an anatomy microscope.

4.3.4 Flow Cytometry

The spleens and LNs were mashed with slides. The cells were resuspended in cold PBS and filtered. Splenocytes underwent red cell lysis using BD PharmLyse™ Lysing Buffer. The cells were washed with PBS, spun at 400*g*, and then resuspended in PBS.

For cell surface marker staining, 100 µl cell suspension (1×10^6 cells) was stained with the corresponding fluorescent antibodies, 30 min, 4°C. For intracellular staining, the cells were first stimulated with PMA (50 ng/ml)/ionomycin (1 µg/ml) for 2 h; brefeldin A was then added to block cytokines secretion, and the cells were incubated for four more hours. Afterwards, the cells were fixed, permeabilized, washed, and then stained with corresponding fluorescent antibodies. The stained cells were resuspended in PBS and analyzed using CytoFLEX S or Galios® flow cytometers.

4.3.5 Cryosectioning

The aortic root in OCT was mounted on the specimen holder of the cryomicrotome, and the proximal 800 µm of the aortic root was serially sectioned [135]. Cryosections (10 µm) were collected on Superfrost Plus microscope slides, fixed with cold acetone (10 min), air-dried (30 min, RT), and stored at -20°C until staining.

4.3.6 Oil red O (ORO) staining

The aorta samples were inserted into 2 ml ORO staining solution (5 mg/ml ORO in isopropanol, diluted 3:2 with water before use), and stained for three hours at RT. The samples were then washed with 70% ethanol and stored in PBS at 4°C. After pinning on a wax plate, the aortas were photographed. The cryosections of the aortic root were stained in ORO solution (20 min, RT), rinsed with water, re-stained with hematoxylin QS for 40 s, rinsed again with water, and mounted with a water-based mounting medium. Micrographs of the cryosections were obtained using an OLYMPUS BX61VS microscope. Lesion size/area of the aorta and the cryosections were analyzed using the programs OlyVIA Version 2.9 and ImageJ 64bit.

4.3.7 RNA sequencing and proteomic analyses

Total RNA of the aortas was extracted using the RNeasy Mini Kit. After taking the RNA-containing part of the upper layer of the Qiazol reagent, the organic pink part was used for protein separation. Briefly, absolute ethanol was added to the pink organic fraction, thoroughly mixed, and incubated at room temperature for 5 min. DNA was then sedimented by centrifugation (2,000 *g*, 5 min, 4°C). The protein-containing supernatant was collected,

and proteins were precipitated by adding isopropanol and incubated at room temperature for 10 min. After centrifugation (12,000 *g*, 10 min, 4°C) and removing the supernatant, the protein pellet was washed with 0.3 M Guanine HCL in 95% ethanol, and then stored at -80°C. The RNA sequencing and mass spectrometry/proteomics analyses were performed by the Beijing Genomics Institute (BGI). The data were analyzed using the Dr. Tom program.

4.4 DATA PRESENTATION AND STATISTICAL ANALYSES

The data are presented as mean±SEM. According to the nature of statistical analyses, a number of analyses were applied. For example, Student's *t*-test and Wilcoxon signed-rank test were used to comparing differences between groups. Multiple comparisons were analyzed by one-way ANOVA followed by post hoc Tukey tests. SPSS 22 and Graphpad Prism 5 were used. A p-value<0.05 was considered statistically significant.

5 RESULTS

5.1 PF4 REGULATES CD4$^+$ TEM IMMUNE RESPONSES THROUGH AKT-PGC1α-TFAM SIGNALING PATHWAY-MEDIATED BY MITOCHONDRIAL BIOGENESIS (PAPER I)

5.1.1 CD4$^+$ Tem immune responses are enhanced by platelets

Flow cytometric analyses of Th1, Th2, Th17, and Treg phenotypes clearly showed that platelet co-cultures regulated CD4$^+$ T effector cell responses (Fig 2A). During the 7-day co-cultures of platelets and Tem (Fig 2B), αCD3/αCD28-stimulated Th1 cell activation was first enhanced by platelets but then decreased, indicating that platelet regulation of Th1 was

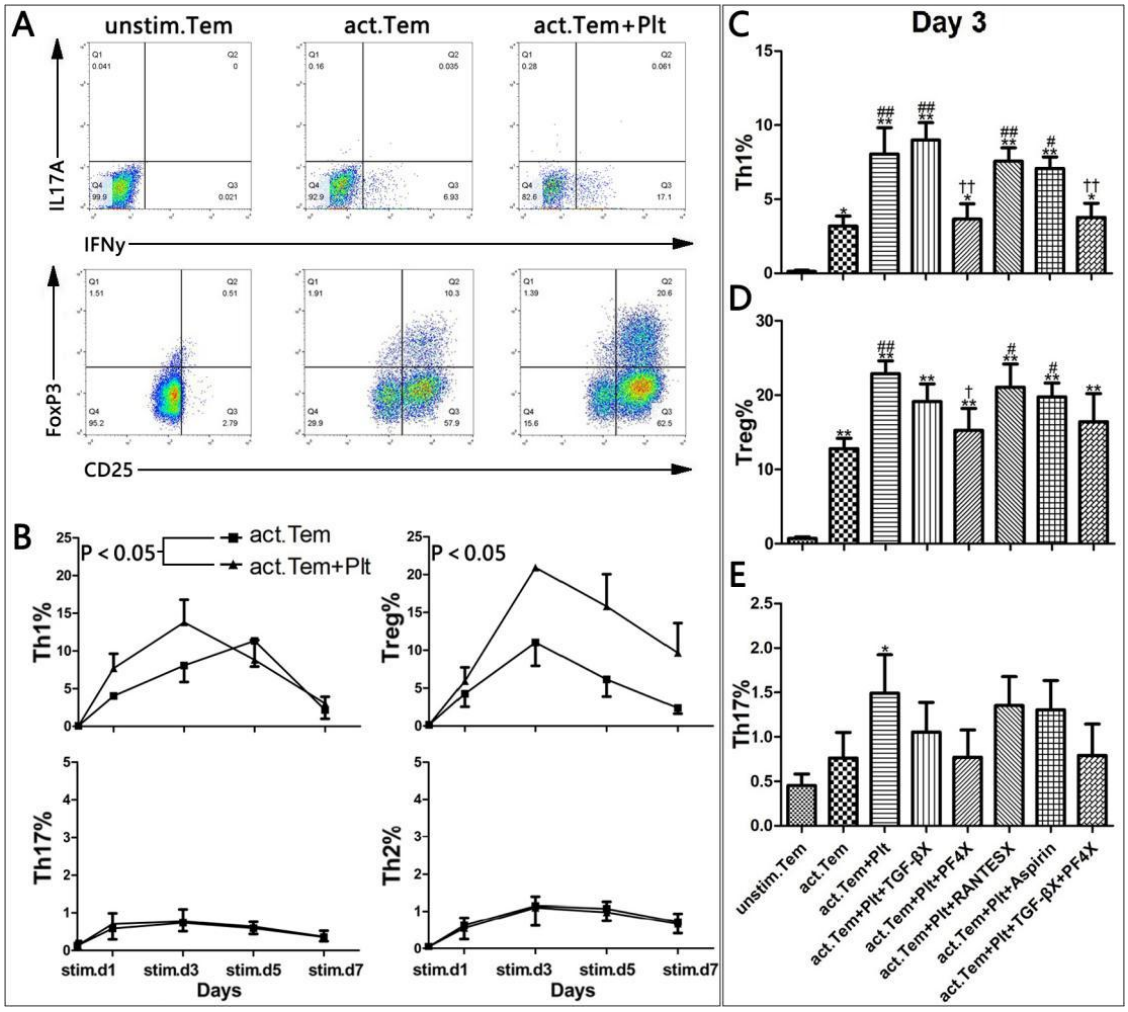

Figure 2. The effector cell responses of Tem were elevated by platelets and PF4. Panel A: representative flow cytometry dot plots for Th1/Th17/Treg phenotypes. Panel B: the dynamic changes for Th1/Th2/Th17/Treg cells without or with platelets, co-cultures for 7 days. Mean±SEM, n=5. Panel C-E: PF4 was the dominant factor for platelet-regulated CD4$^+$ Tem responses. Unstimulated and activated Tem cells were cultured in the absence or presence of platelets and without or with TGFβ neutralizing antibody (20 μg/ml), PF4 neutralizing antibody (25 μg/ml), RANTES neutralizing antibody (10 μg/ml), or aspirin (100 μM) for three days. Mean±SEM, *P<0.05, **P<0.01, compared with unstimulated Tem; #P<0.05, ##P<0.01, compared with activated Tem; †P<0.05, † †P<0.01, compared with the activated T cells and platelet co-culture; n=6 [136].

biphasic, i.e., a rapid enhancement in the first three days, followed by a rapid declining phase. In contrast, platelets continued to enhance Treg cell activation throughout the co-culture period. For Th2 and Th17 responses, platelets had almost no effects (Fig 2B).

5.1.2 PF4 and Akt-PGC1α-TFAM signalling pathway play a regulatory role

5.1.2.1 Platelets regulate $CD4^+$ Tem responses mainly through PF4

Platelets release a number of $CD4^+$ T cell-active mediators, we selected the neutralization approach to find out the effect of each mediator [9,137,138]. On day 3 of platelet-Tem cell co-culture, PF4 neutralization eliminated the enhancement of Th1 by platelets, which was not influenced by the blocking of TGFβ (Fig 2C). Blocking of either PF4 or TGFβ reduced platelet-elevated Treg cell responses, but the effect of PF4 neutralization was more obvious; in addition, TGFβ blockade did not provide additive effects to Th1/Treg inhibition by PF4 neutralization (Fig 2D). Because PF4 was dominant in the platelet regulated $CD4^+$ Tem effector responses, and Th2 and Th17 responses were not markedly changed, therefore, PF4-regulated Th1/Treg cell activation was our focus in the follow-up work.

5.1.2.2 Platelets and PF4 enhance Tem mitochondrial biogenesis via Akt-PGC1α-TFAM signalling pathway

To figure out the mechanisms of platelet-/PF4-regulated Tem responses, mass spectrometric analyses of PF4-treated Tem cells were carried out. Among the total of 1399 proteins identified, 77 up-regulated and 16 down-regulated proteins were found after PF4 treatment. In the top 50 up-regulated proteins, about 1/3 of them were related to metabolic functions, suggesting that PF4 regulation might be linked to cellular aerobic respiration and energy metabolism.

Mitochondria are the “energy plant”, which are important for T cell survival and development such as activation, differentiation, and proliferation [139]. We found that Tem cell activation increased mitochondria mass, and that platelet co-cultures and PF4 supplementation further enhanced this effect (Fig 3B&D). Moreover, platelets and PF4 enhanced the expression of TFAM in activated T cells (Fig 3A&C), and the increases of TFAM expression were correlated with increased mitochondrial mass (Fig 3B&D). To elucidate the underlying signalling mechanisms, we cultured Tem cells with and without PF4 supplement for six hours, and monitored Akt and PGC1α signalling. PF4 decreased the phosphorylated Akt of activated Tem, and subsequently attenuated PGC1α phosphorylation (Fig 3E). As reduced PGC1α

phosphorylation is related to an enhanced PGC1α activity [140], PF4-reduced PGC1α phosphorylation enhanced the expression of TFAM in Tem cells (Fig 3C).

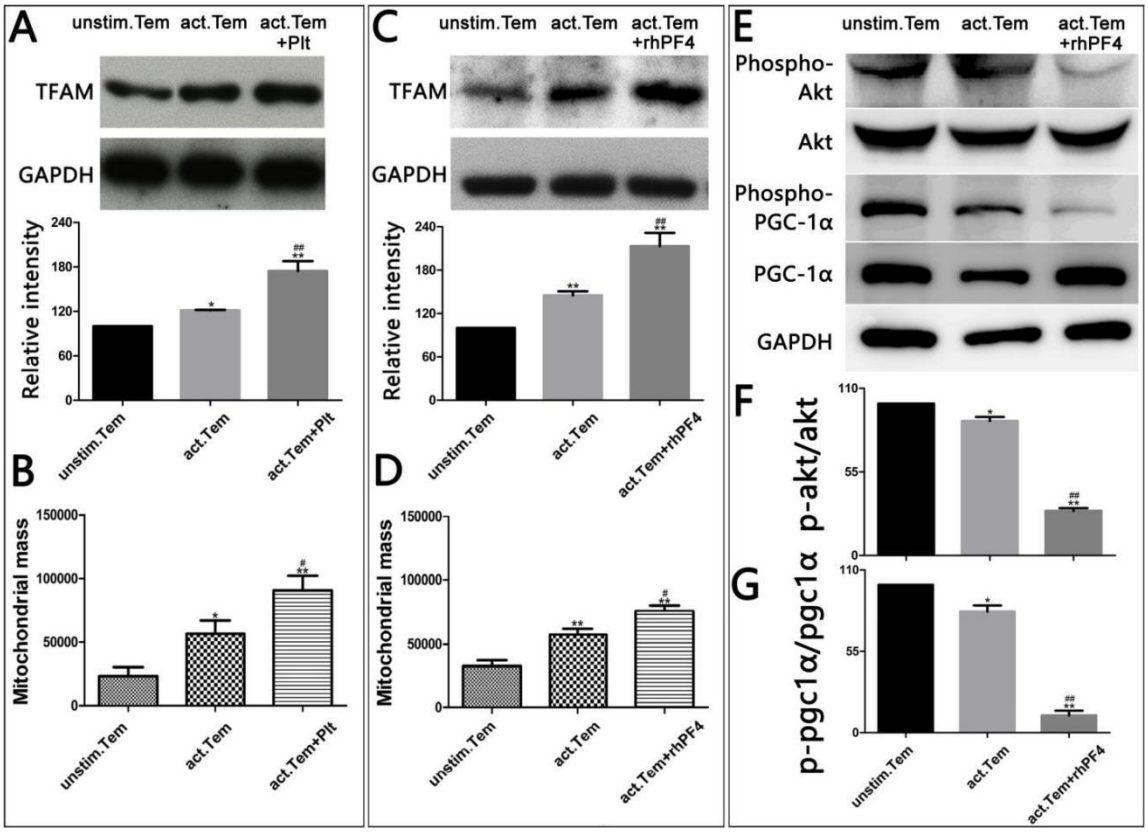

Figure 3. PF4 enhances the mitochondrial biogenesis of CD4⁺ Tem through the Akt-PGC1α-TFAM signalling pathway. Tem cells were cultured for three days in the absence or presence of αCD3/αCD28 stimulation and the absence or presence of platelets (1:250) or rhPF4 (5 μg/ml). Panel A & C: whole cell lysates of Tem for western blotting of TFAM (anti-TFAM antibody, ab131607; Abcam). GAPDH was used as an internal control (anti-GAPDH antibody 6C5, cat#ab8245; Abcam). Representative images from three independent experiments are shown as western blot results. The immunoblot intensities of TFAM were plotted as the mean ± SEM. *P<0.05, compared with Tem cultured alone. Panel B & D: Tem were stained with MitoTracker® and analyzed for MFI using a Galios flow cytometer. The data plotted were the mean±SEM, n=4. *P<0.05, **P<0.01, compared with unstimulated cells; #P<0.05, compared with αCD3/αCD28 stimulated cells. Panel E: unstimulated and αCD3/αCD28 stimulated Tem were cultured in the absence or presence of rhPF4 (5 μg/ml). After six hours of incubation, whole-cell lysates of Tem were prepared for western blotting of Akt/p-Akt and PGC1α/p-PGC1α. The western blot bands were representative images from 3-4 independent experiments. The relative immunoblot intensities of phosphorylated Akt (F) and phosphorylated PGC1α (G) were expressed as their ratio to the corresponding unstimulated Tem. Mean ± SEM; *P<0.05, **P<0.01, compared with unstimulated Tem; ##P<0.01, compared with αCD3/αCD28 stimulated Tem cultured without rhPF4 [136].

5.1.3 The activation of Tem and mitochondrial biogenesis were elevated by PF4 and CXCR3

CXCR3 is a functional receptor for PF4 [141] and was expressed on CD4⁺ Tem cells (Fig 4A). The CXCR3 inhibitor (±)-AMG487 had no effect on Tem activation-increased mitochondrial mass, but it attenuated the enhancement effect by PF4 (Fig 4B) and subsequently eliminated PF4-enhanced Th1/Treg cell responses (Fig 4C-D). In line with these observations, CXCR3 specific siRNA transfection reduced the CXCR3 expression (Fig 4E), and CXCR3 knockdown reduced PF4-enhanced Th1 and Treg cell activation (Fig 4F-G). Hence, these

data supported that PF4 exerted its regulatory effects on Tem cells via CXCR3.

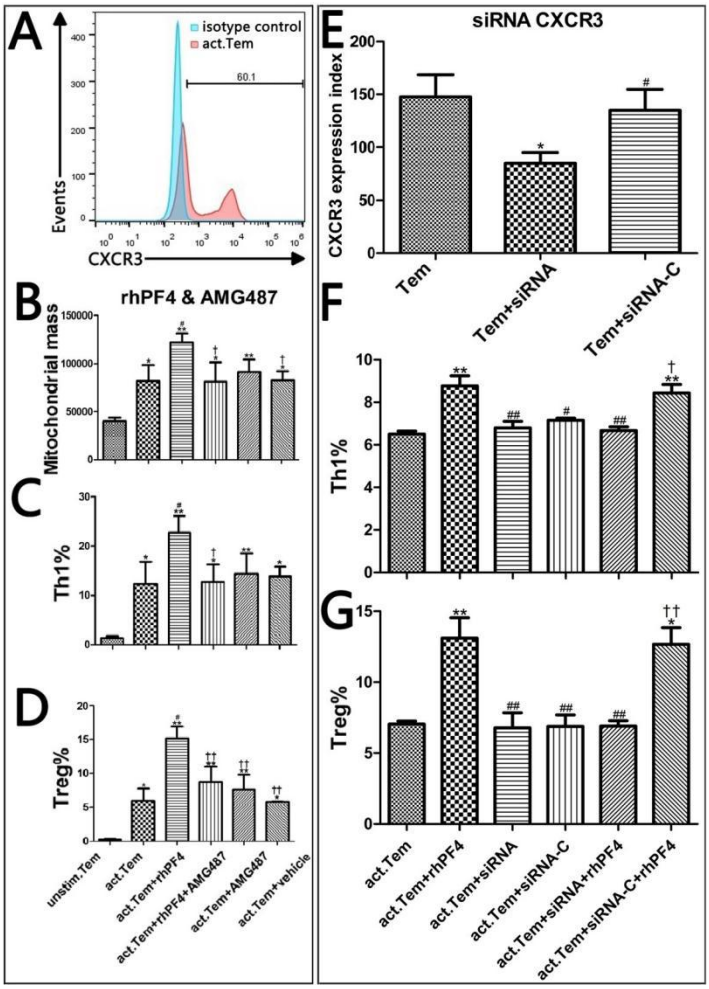

Figure 4. PF4 enhances CD4$^+$ Tem activation and mitochondrial biogenesis through the chemokine receptor CXCR3. Tem were cultured for three days without or with αCD3/αCD28 stimulation, without or with rhPF4 (5 µg/ml), without or with PF4 receptor CXCR3 inhibitor (±)-AMG487 (1 µM). Galios flow cytometer was used for flow cytometry analysis. Panel A: representative overlapping histograms of Tem stained with Brilliant Violet 510™ anti-human CD183/CXCR3 MAb (Cat# 353725; BioLegend) and corresponding isotype control antibodies. Panel B: Tem cells were stained with MitoTracker® presented of MFI as mitochondrial mass. Panel C & D: intracellular staining was used for the phenotype of Th1 and Treg. Mean±SEM, n=6; *P<0.05, **P<0.01, compared with unstimulated Tem; #P<0.05, compared with activated Tem, †P<0.05, ††P <0.01, compare activated Tem with rhPF4. Panel E: LipofectamineTM RNAiMAX transfection kit and Opti-MEM medium (ThermoFisher Scientific) were used for culture of Tem with vehicle or CXCR3-SmartPool siRNA or the corresponding On-TargetPlus control (Horizon Discovery; Cambridge, UK) for 72 hours transfection, then Tem were washed. The CXCR3 expression of vehicle-treated, siRNA- and Plus-control-transfected Tem was monitored, which were expressed as CXCR3 expression index (= CXCR3-positive% × CXCR3-MFI / 100; mean±SEM, n=3; *P <0.05, compared with untransfected Tem; #P<0.05, compared with CXCR3-siRNA transfected Tem). Panel F and G: in the absence or presence of rhPF4 (5 µg/ml), the vehicle-treated or transfected Tem were stimulated with αCD3/αCD28 for 3 days. Thereafter, flow cytometric analysis of Th1 (F) and Treg (G). Mean±SEM, n=3; *P<0.05, **P<0.01, compared with activated Tem; #P<0.05, ##P<0.01, compared with activated Tem with rhPF4; † P<0.05, ††P<0.05, compared with Tem transfected with activated CXCR3-siRNA with rhPF4 [136].

5.1.4 Mitochondrial function is essential for platelet-enhanced Tem cell responses

Rotenone blocks mitochondrial complex I [142] and thus mitochondrial function. We found that platelet- and PF4-enhanced mitochondrial mass was abolished by rotenone treatment,

which subsequently resulted in the elimination of platelet- or PF4-enhanced Th1 and Treg cell responses.

Next, we enhanced mitochondrial function by TFAM overexpression via infection of TFAM-adenovirus. The results indicated that TFAM overexpression increased mitochondrial mass to a similar level of rhPF4 supplementation, and also enhanced Th1 and Treg cell activation similarly.

5.1.5 PF4 enhances Tem response via mitochondrial production ATP and ROS

The ROS scavenger N-acetyl-L-cysteine (NAC) and the ATP synthase inhibitor oligomycin were used to illustrate the effects of PF4 on enhanced mitochondrial metabolism. PF4 enhanced the production of ROS and ATP (Fig 5A-B). NAC decreased ROS production of activated Tem cells without PF4 and eliminated the increase by PF4. Oligomycin decreased ATP production of activated Tem cells regardless of PF4 supplementation (Fig 5A-B).

ROS scavenging by NAC abolished PF4-elevated T-bet expression of activity Tem cells and oligomycin inhibition decreased T-bet expression of Tem cells to a similar level of unstimulated Tem (Fig 5C&E). NAC scavenging did not decrease FoxP3 expression of activated Tem cells, while oligomycin inhibition reduced FoxP3 expression of activated Tem cells to a similar level of unstimulated Tem cells (Fig 5D&F). These findings indicated that the production of mitochondrial ROS mainly affected the PF4-enhanced Treg response.

OCR, as measured by the seahorse assay, of activated Tem was elevated by PF4 treatment, (Fig 5G-H), further supporting that PF4 enhances mitochondrial metabolism of activated Tem cells.

In summary, platelets and platelet-derived PF4 elevated mitochondrial biogenesis and metabolism of activated Tem cells, increased their oxygen consumption and ATP and ROS production, resulting in enhanced T cell transcription factor expression and effector cell responses.

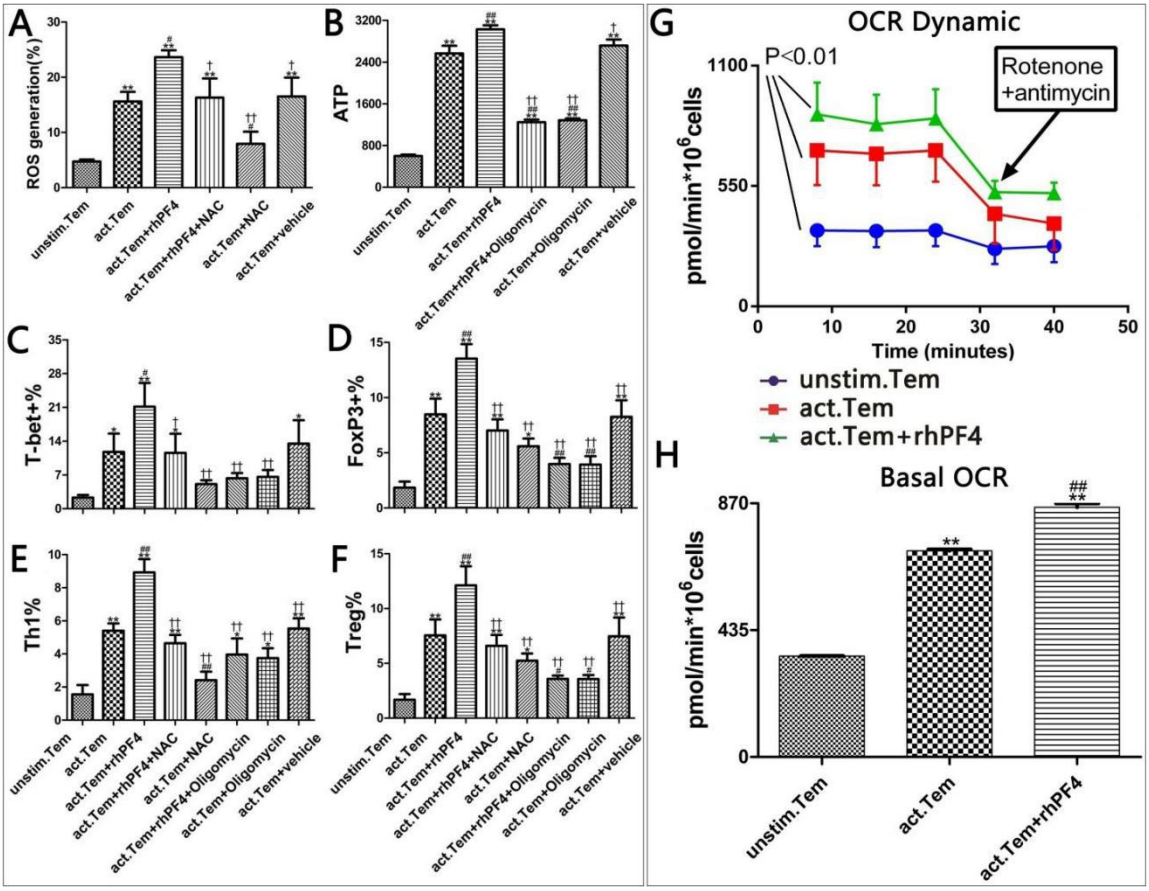

Figure 5. PF4 enhances Th1 and Treg activation of CD4$^+$ Tem through mitochondrial ATP and ROS production. Panel A-F: unstimulated Tem and αCD3/αCD28 stimulated Tem in the presence or absence of rhPF4 (5 µg/ml), oligomycin (0.1 nM), NAC (1 mM) and vehicle (0.01% DMSO) were cultured for three days. After that, Tem were harvested and loaded with the ROS probe DCFH-DA (10 µg/ml, 20 minutes at 37°C) to monitor ROS (A). The ADP/ATP ratio assay kit was used to measure ATP (B). Flow cytometry was used to detect the expression of Th1 transcription factor T-bet (T-bet$^+$) (C) and Th1 phenotype (IFNγ$^+$) (E), and the expression of Treg transcription factor FoxP3 (FoxP3$^+$) (D) and Treg phenotype (CD25^{++}-FoxP3$^+$) (F). Mean ± SEM, n=6. *P<0.05, **P<0.01, compared with unstimulated Tem; #P<0.05, ##P<0.01, compared with αCD3/αCD28 stimulated Tem; †P<0.05, ††P< 0.01, compared with activated Tem with rhPF4. Figure G & H: Tem were cultured for three days without or with αCD3/αCD28 stimulation and in the presence or absence of rhPF4 (5 µg/ml). The cells were then harvested and seeded in 96-well XF plates at 1-2×10^5 cells/well. After baseline and addition of 2,5 µM antimycin A and 2,5 µM rotenone (G), the Seahorse XF 96 analyzer was used to evaluate the basic OCR of Tem. Basal OCR was calculated after correction for non-mitochondrial respiration (H). Mean±SEM, n=7-8; P<0.01, as analyzed by repeated measurement analysis of variance, the comparison of activated Tem treated with rhPF4 and activated Tem or unstimulated Tem. The basic OCR was expressed as the mean ± SEM (H). **P <0.01, compared with unstimulated Tem; ##P <0.01, compared with αCD3/αCD28 stimulated Tem; n=7-8 [136].

5.2 PLATELETS ENHANCE CD4$^+$ TCM RESPONSES THROUGH PF4-DEPENDENT MITOCHONDRIAL BIOGENESIS AND CELL PROLIFERATION (PAPER II)

5.2.1 CD4$^+$ Tcm immune responses are enhanced by platelets

We further investigated the effects of platelets on CD4$^+$ Tcm cells. In the first three days, platelet co-cultures had limited effects on IFNγ$^+$ Th1 cell activation (Fig 6A-B) or CD25^{++}-FoxP3$^+$ Treg cell responses (Fig 6C-D) during the first three days. Platelets enhanced Th1 cell activation on day 5, while they promoted Treg cell activation from day 3 onwards. These results indicated that platelets had significant regulatory effects on Th1/Treg cell activation of Tcm cells, and day 5 was selected as the observation time point in subsequent experiments.

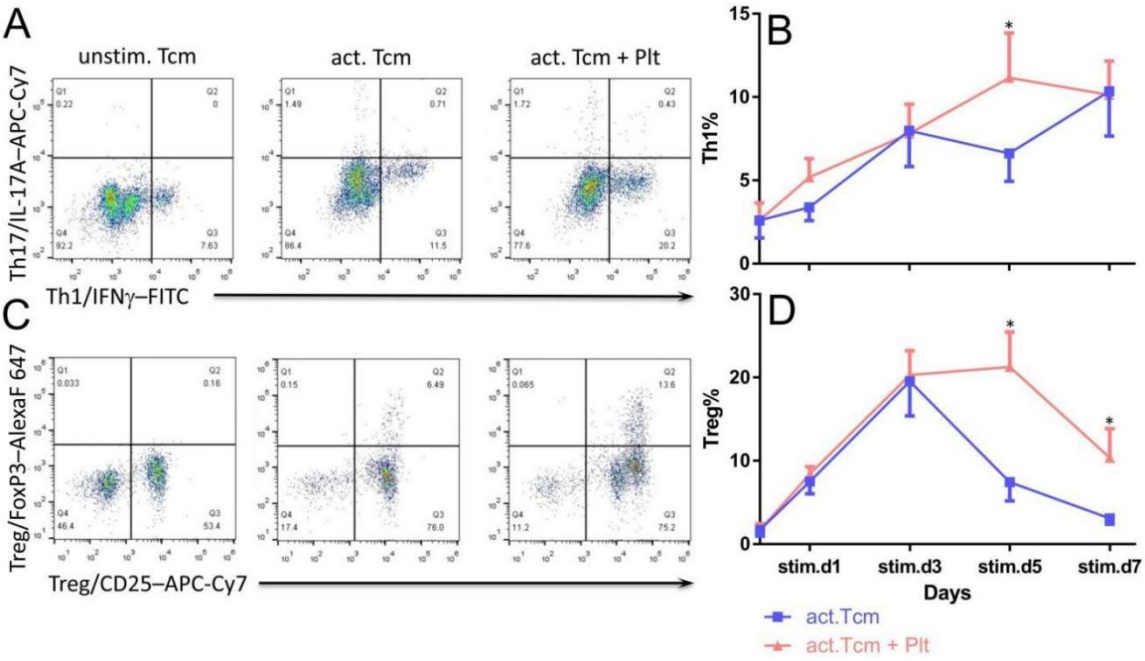

Figure 6. Platelets enhance the effector cell response of Tcm. Panel A: representative flow cytometry dot plots of Th1/FITC-IFNγ and Th17/APC-Cy7-IL-17A phenotypes at day 5. Panel B: in the absence of platelets or the presence of platelets, the dynamic change of Th1 cell phenotype (%) during seven days of co-culture of Tcm and platelets. Plot the mean±SEM, *P<0.05, Tcm-platelet co-culture vs. Tcm cell culture alone, n=8. Panel C: representative dot plot of Treg/APC-Cy7-CD25/Alexa Fluor 647-FoxP3 flow cytometry phenotype at day5. Panel D: in the absence of platelets or the presence of platelets, the dynamic change of Treg cell phenotype (%) during seven days of co-culture of Tcm and platelets. Mean±SEM, *P<0.05, n=5 [143].

5.2.2 PF4 markedly enhances effector cell responses of CD4$^+$ Tcm cells via the CXCR3 receptor

PF4 neutralization not only eliminated the platelet-enhanced Th1 and Treg responses of Tcm cells but also counteracted αCD3/αCD28-stimulated Th1 and Treg activation (Fig 7A-B). In contrast, neutralization of TGFβ, RANTES, or thromboxane synthesis inhibition by aspirin was much less effective.

PF4 supplementation mimicked platelet enhancement on Th1 and Treg activation of Tcm cells. CXCR3 blockade by (±)-AMG 487 eliminated the enhancements by PF4 but did not influence the Th1 or Treg cell activation by αCD3/αCD28 stimulation (Fig 7C-D). Similar to the findings with Tem cells, CXCR3 knockdown by siRNA transfection significantly reduced CXCR3 expression of Tcm cells and eliminated PF4-enhanced Th1 and Treg response.

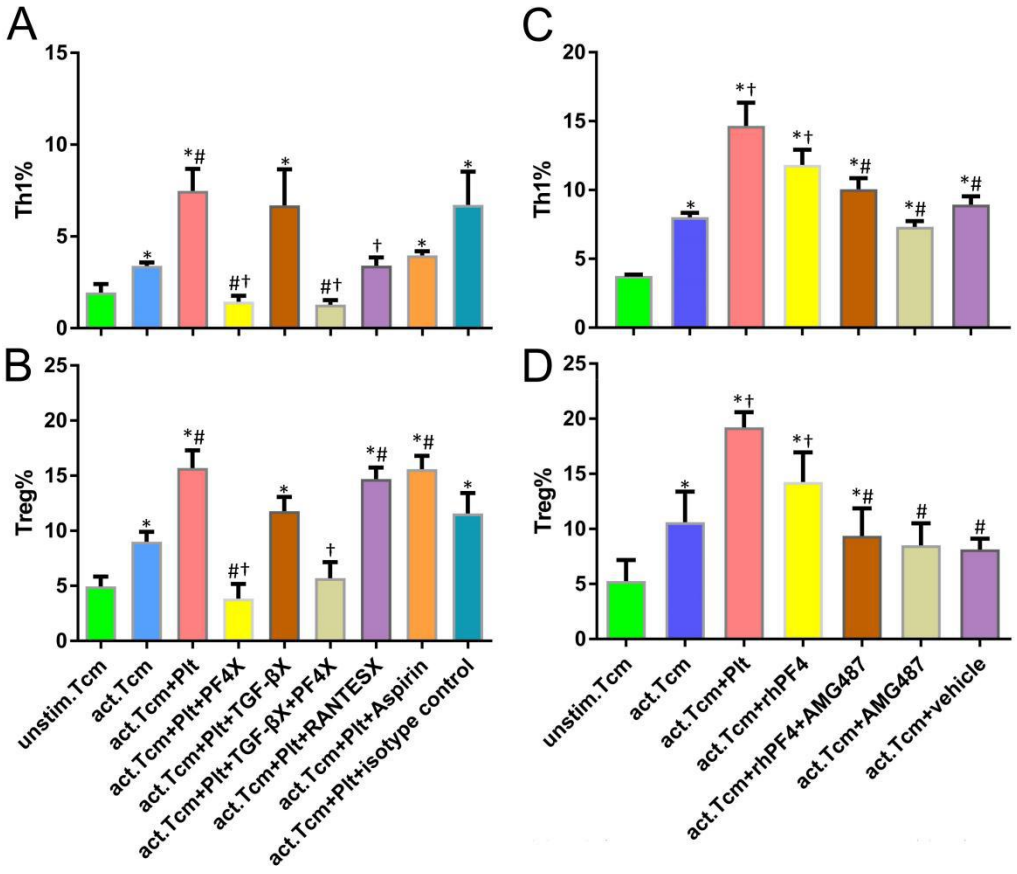

Figure 7. Platelet-derived PF4 and TGFβ enhance Th1 and Treg activation of Tcm. Panel A & B: the Tcm in the absence or presence of αCD3/αCD28 stimulation and platelets, and in the absence or presence of neutralizing antibodies against PF4 (25 μg/ml), TGFβ (20 μg/ml), or RANTES (10 μg/ml), with aspirin (100 μM, 30 min), or isotype control antibody (rabbit IgG, 25 μg/ml) incubated for five days, then Th1 and Treg phenotype analysis was performed. Plot the mean±SEM, *P<0.05, compared with unstimulated Tcm; †P<0.05, compared with αCD3/αCD28 activated Tcm; #P<0.05, compared with activated Tcm and platelet co-culture; n=7. Panel C & D: Tcm were cultured for five days without or with αCD3/αCD28 stimulation, without or with platelets or human rhPF4 (5 μg/ml), and without or with CXCR3 inhibitors (±)- AMG487 (1 μM) or vehicle (0.01% DMSO). After five days of culture, the Th1 and Treg cell phenotypes were analyzed by flow cytometry. Mean±SEM, *P<0.05, compared with unstimulated Tcm; †P<0.05, compared with αCD3/αCD28 activated Tcm; #P<0.05, compared with co-cultured with activated Tcm and platelets; n=4 [143].

5.2.3 Platelets and PF4 markedly enhance Tcm cell mitochondrial biogenesis and proliferation

Cell proliferation is critical for Tcm cell responses [5]. We showed that platelet co-cultures and PF4 supplementation markedly increased Tcm cell proliferation, as evidenced by increased cell densities and cell counts in Tcm cell culture and by the elevated Tcm cell

expression of Ki-67, a cell proliferation marker (Fig 8A). Moreover, PF4 receptor CXCR3 blockade by (±)-AMG487 counteracted PF4-enhanced Tcm cell proliferation (Fig 8B). Furthermore, it was shown that platelets and PF4 enhanced Tcm cell responses and proliferation through the CXCR3-receptor-mediated Akt signalling pathway and mitochondrial biogenesis. Thus, platelets and PF4 supplementation elevated mitochondrial mass of activated Tcm cells in a similar manner seen in our Tem cell work [136]. Rotenone treatment reduced mitochondrial mass, and subsequently inhibited platelet- and PF4-enhanced Th1 and Treg responses of Tcm cells.

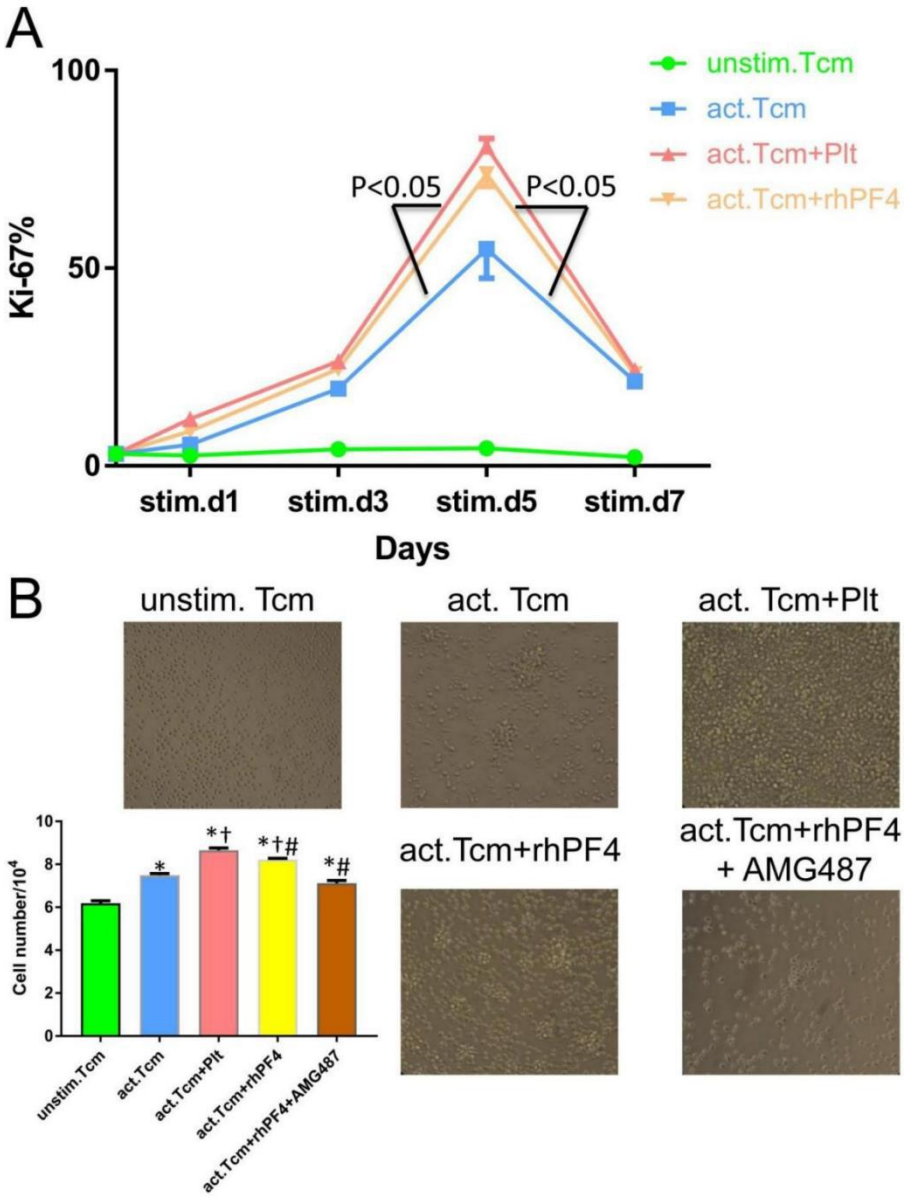

Figure 8. Platelets and PF4 enhance CD4⁺ Tcm cell proliferation. Panel A: unstimulated and αCD3/αCD28 stimulated Tcm cells were cultured for seven days in the absence or presence of platelets and the presence or absence of rhPF4 (5 μg/ml). Ki-67⁺ Tcm were monitored before (day 0) and after culture for 1, 3, 5, and 7 days. Plot the mean±SEM, n=3. The P-value was evaluated by repeated-measures analysis of variance. Panel B: phase-contrast images of cultured Tcm were captured with an Olympus CKX41 inverted light microscope (Olympus Corporation, Tokyo, Japan) equipped with a Nikon D5100 camera (Nikon Corporation, Tokyo, Japan). The bar graph depicted the relative increase in the number of Tcm cells after five days of culture [143].

5.3 PLATELETS FINE TUNE CD4$^+$ TN CELL RESPONSES VIA PF4-REGULATED TGFβ SIGNALING (PAPER III)

5.3.1 Platelets regulate Tn response in a cell concentration-dependent manner and via both soluble mediators and cell-to-cell contact

Platelet-Tn cell co-cultures showed that platelets, generally in a cell concentration-dependent manner, regulated CD4$^+$ T effector cell responses of Tn cells, namely enhancing Treg but inhibiting Th1 cell responses (Fig 9A). Platelets enhanced the production of pro-inflammatory cytokines (TNF/IFNγ/IL-17A/IL-6/IL-2) but tended to suppress IL-10 production (Fig 9B). Dynamic observations during 7-day co-cultures showed that platelets

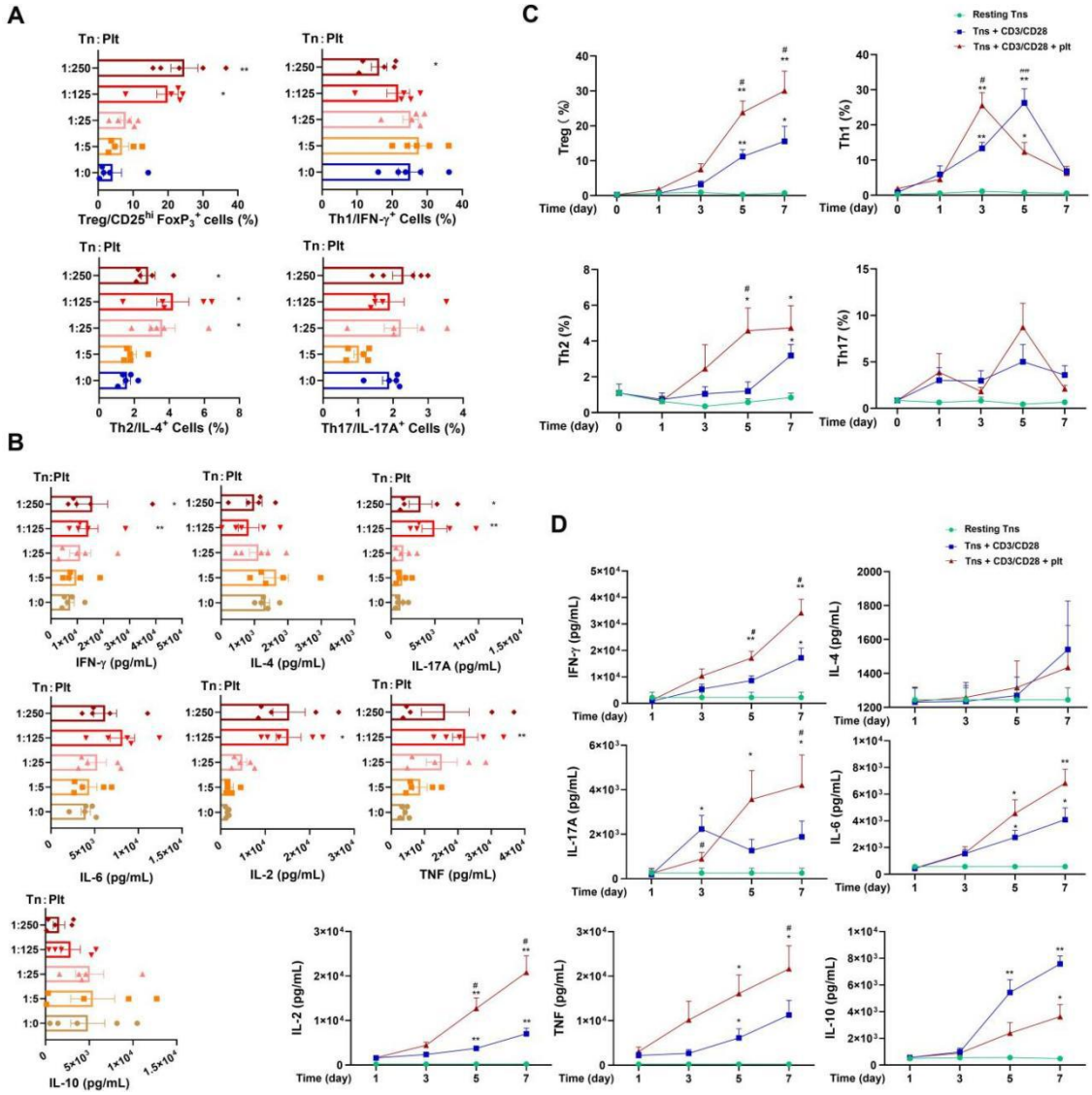

Figure 9. Platelets modulate the CD4$^+$ Tn effector cell response in a time- and concentration-dependent manner. Tn were cultured in the absence or presence of αCD3/αCD28 stimulation and in the absence or presence of platelets. Panel A&B: αCD3/αCD28 stimulated Tn and platelets were co-cultured with a ratio of 1:0 to 1:250 for five days. Flow cytometry phenotype analysis of Treg/CD25hiFoxP3$^+$, Th1/IFNγ$^+$, Th2/IL-4$^+$ and Th17/IL-17A$^+$ cells (A) and the CBA measurement of cytokine levels in supernatants (B). Mean±SEM, n=5;

differences of co-culture conditions were assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *P<0.05, **P<0.01, as compared with Tn cultured alone. Panel C&D: Tn were cultured for seven days at the ratio of 1:250 in the absence ( ) or αCD3/αCD28-polyclonal stimulation ( ) and absence ( ) or presence ( ) of platelets. Flow cytometry phenotype analysis of Treg, Th1, Th2, and Th17 cells (C) and the CBA measurement of cytokine levels in supernatants (D). Mean ± SEM, n=5. Comparisons were made by two-way analysis of variance and Tukey's multiple comparison test. *P<0.05, **P<0.01, as compared with unstimulated Tn ( ); #P<0.05, as compared with αCD3/αCD28 stimulated Tn alone ( ).

continuously enhanced Treg cell activation, while they exerted bi-phasic regulations on Th1 cell responses of Tn cells (Fig 9C). Although platelets/PF4 increase IL-10 level in Treg, this increase is much smaller than the decrease via PF4 in $CD4^+CD25^-$ non-regulatory T cells [137, 144].

5.3.2 Combined action of soluble factors and cell-to-cell contact

As a step forward after in vitro studies on platelet regulated Tn cell responses, as well as Tem (Paper I) and Tcm cell studies (Paper II), we next asked how platelets regulate $CD4^+$ T effector cell responses in vivo using a murine model combining OT-II $CD4^+$ T cell adoptive transfer and platelet depletion. Application of the rat anti-mouse GPIba/CD42b antibody R300 dramatically reduced circulating platelets and plasma levels of active TGFβ1, indicating that platelets were vital for the latent TGFβ1 activation.

Platelet depletion impacted OVA-evoked $CD4^+$ T effector cell responses in the spleen and largely abolished Treg and Th2 cell responses and attenuated Th17 cell activation. However, platelet depletion had no influences on OVA-induced T effector cell responses in LNs. These results suggested that direct platelet-T cell contact, which exists in the spleen but not in LNs, is important. Indeed, as compared to direct co-cultures, transwell co-cultures of Tn cells and platelets reduced platelet regulation on T effector cell responses on day 5. The transwell chamber diaphragm also reduced the elevations of IL-6, IL-2, and TNF.

5.3.3 TGFβ influences the effector cell responses

TGFβ and PF4 are two important platelet-derived T cell-acting mediators. Firstly, we studied the impact of TGFβ by using a TGFβ neutralizing antibody that eliminated active TGFβ forms (Fig 10C). TGFβ neutralization eliminated platelet-regulated Th1 and Th17 responses but had no effects on Treg and Th2 responses (Fig 10A). To further clarify the mechanism of TGFβ1 signalling, we then used a TGFBRII blocking antibody to block TGFβ1 signalling. TGFBRII blockade produced more profound effects that counteracted platelet-enhanced Treg cell responses, in addition to the elimination of effects on Th1/Th17 responses (Fig 10B).

Further work with platelet-specific TGFβ1 knockout (plt-TGFβ$^{-/-}$) mice and adoptive transfer of OT-II CD4^{+} T cells showed that platelet-specific TGFβ deficiency did not influence OVA-induced T effector cell responses or p-Smad2/3 signalling of splenic CD4^{+} T cells, but reduced Th1, Th17, and Treg responses and p-Smad2/3 signalling in LNs (Fig 10E-F). Together, these in vitro and in vivo data indicated that there may be other platelet-derived mediators also involved in TGFβ-regulated Tn cell responses and that cell-to-cell contacts also had an important impact on platelet-regulated Tn responses.

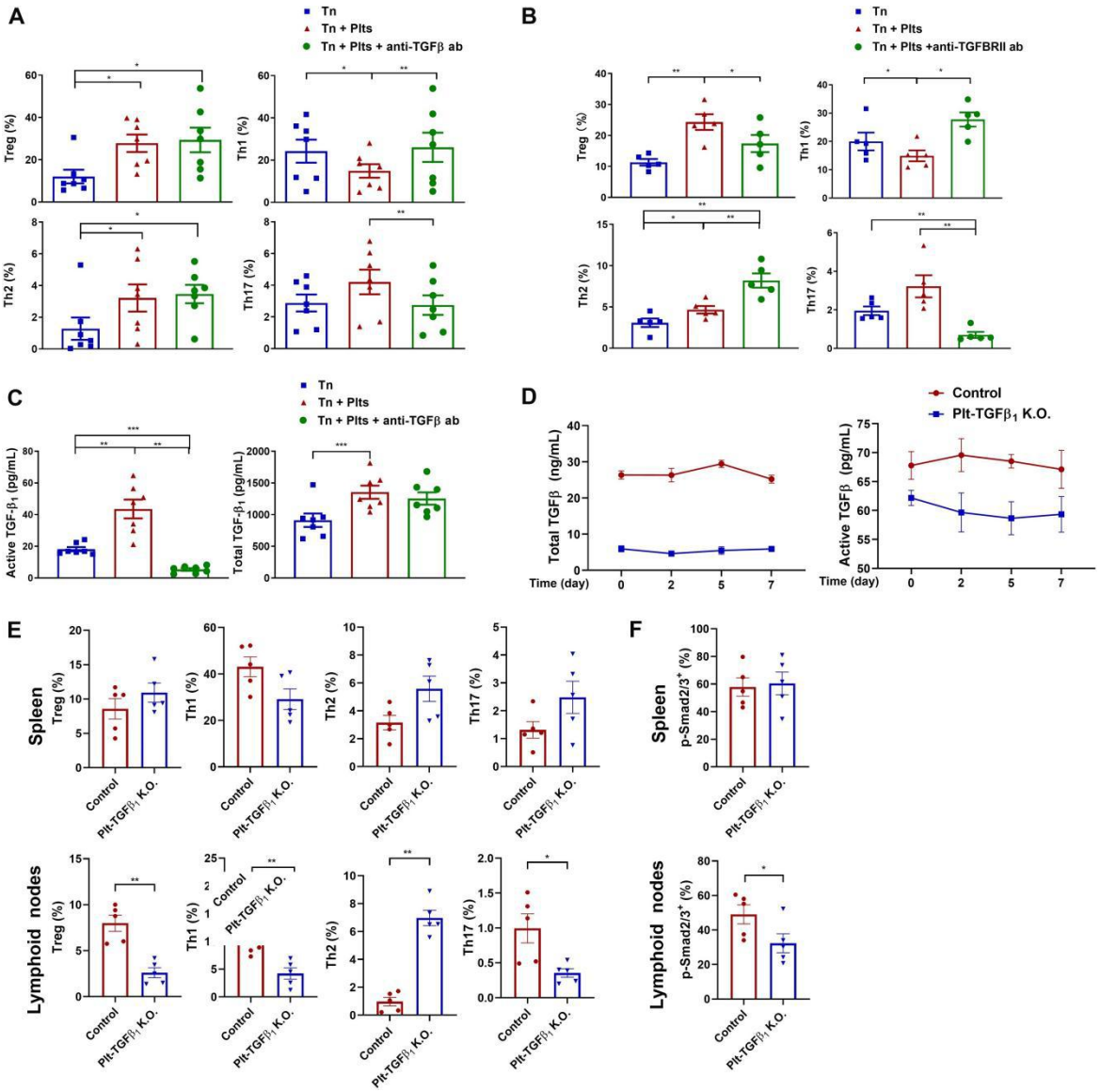

Figure 10. The effect of platelet-derived TGFβ on the response of effector cells. Panel A: stimulate Tn with αCD3/αCD28 antibody in the absence (■) or presence (▲ ●) of platelets (Tn:plt=1:250) cultured for five days with (●) or without (■ ▲) TGFβ neutralizing antibody (20 μg/ml). The bar graphs show Treg/Th1/Th2/Th17 phenotypes and the total/active TGFβ1 levels in the supernatant measured via ELISA, n=7. Panel B: stimulated Tn with αCD3/αCD28 antibody in the absence (■) or presence (▲ ●) of platelets (Tn:plt=1:250), cultured for five days with (●) or without (■ ▲) TGFBRII blocking antibody (15 μg/ml) for five days. The bar graphs show Treg/Th1/Th2/Th17 phenotypes (n=5). Panel C-E: adoptive transfer CD4^{+} T cells from CD45.1 OT-II mice to platelet-specific TGFβ1 knockout or control mice, and then followed via OVA challenge (C). The plasma levels of total/active TGFβ were also monitored during seven days (D; n=5). Mononuclear cells were isolated from the spleen and LNs of recipient mice, and the Treg/Th1/Th2/Th17 cell phenotype (E; n=5) and the phosphorylation levels of Smad2/3 of OT-II T cells were measured (F; n=5). Mean±SEM. For the data shown in

Panel A&B, RM ANOVA and Tukey's multiple comparison test were used for comparison between treatments. For the data shown in Panel D, two-way ANOVA followed by Sidak's multiple comparison test was used to compare groups. For the data shown in Panel E&F, the Mann-Whitney test was used. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

5.3.4 PF4 regulates effector cell responses by regulating TGFβ1 signalling

In search of other platelet-derived mediators involved in TGFβ-regulated Tn cell responses, PF4 is a natural candidate. It is the most abundant chemokine released by platelets and played important regulatory roles for Tem and Tcm cell responses. Surprisingly, PF4 neutralization strengthened platelet-regulated Th1/Th17/Treg responses of Tn cells (Fig 11A), and further elevated platelet-enhanced IFNγ and IL-17A production (Fig 11B).

Adoptive transfer of OT-II $CD4^+$ T cells in PF4 knockout mice showed that OVA-induced Treg and Th17 responses were enhanced significantly both in spleen and LNs (Fig 11D). The latter ware associated with elevated p-Smad2/3 levels, indicating that the TGFβ signalling was also elevated (Fig 11E).

To explain the discrepancy between the readouts of PF4 neutralization/deficiency and PF4 supplementation in PF4-regulated Tn cell responses, gradient PF4 concentrations (0.2, 1, 5, and 25 μg/ml) were introduced to αCD3/αCD28-stimulated Tn cells. PF4 dose-dependently enhanced Treg cell activation from 0.2-5 μg/ml, the regulatory effect of PF4 on Treg was enhanced according to the increase of the concentration, but PF4 at 25 μg/ml abolished the enhancement (Fig 11F). The same trend was also seen with p-Smad2/3 levels (Fig 11F).

Taken together, these results indicated that PF4 conducts complex regulation on $CD4^+$ T effector cell responses of Tn cells and that PF4 seems to exert its effects via regulating the action of TGFβ1 and/or TGFβ signalling.

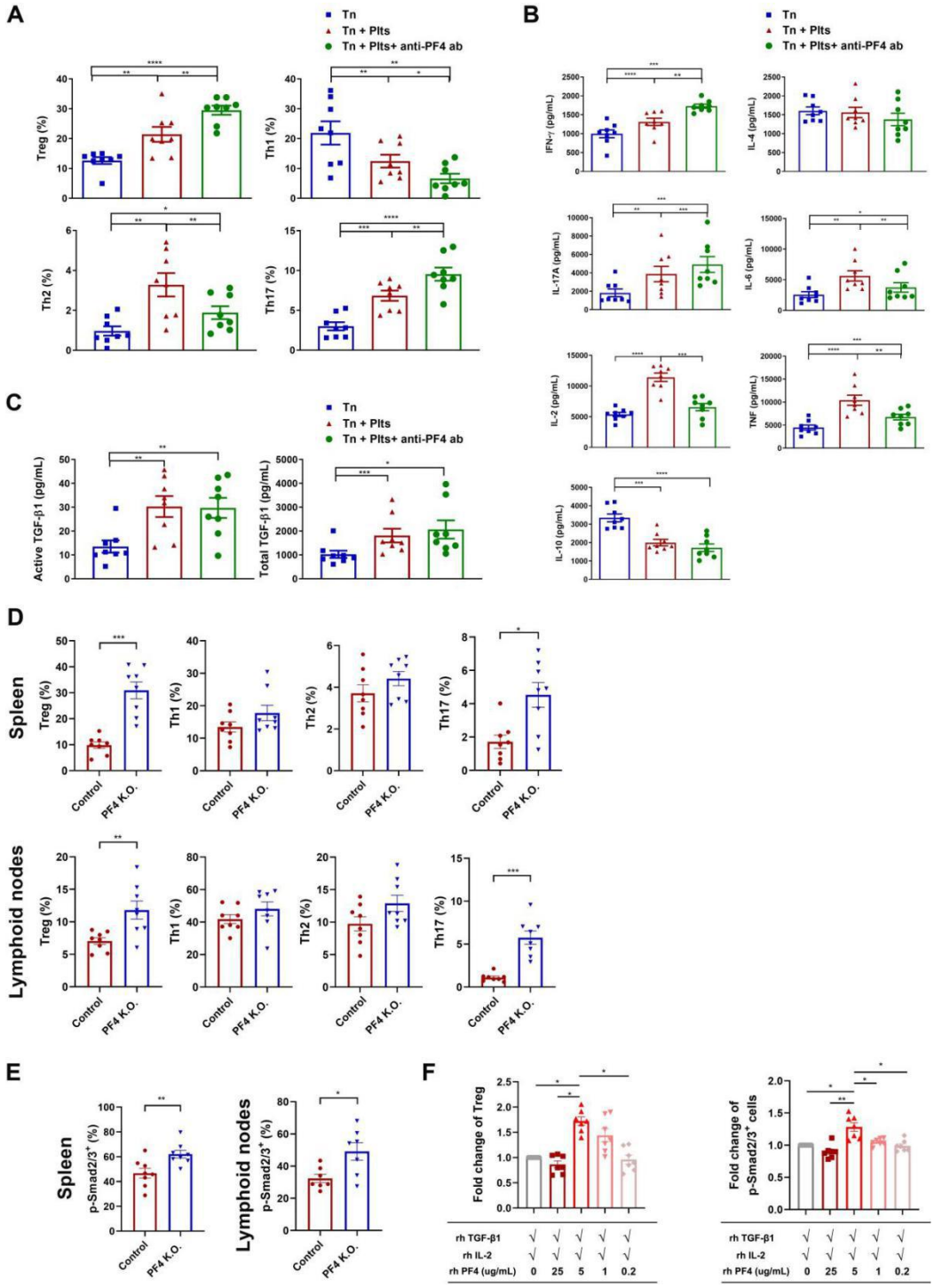

Figure 11. The effect of PF4 on the CD4⁺ Tn response. Panel A-C: in the absence of plts (Tn:plt=1:250) (■) or presence (▲ ●), αCD3/αCD28 antibody was used to stimulate Tn, and cells were cultured for five days with (●) or without (■ ▲) PF4 neutralizing antibody (25 μg/ml). The phenotype of Treg/Th1/Th2/Th17 cells was determined by flow cytometry (A). CBA kit and ELISA kit were used to determine $CD4^+$ T cell cytokines (B) and the total/active TGFβ levels (C) in the supernatant respectively; n=8. Panel D&E: PF4 knockout mice and their littermates (control) mice were adoptively transferred with $CD4^+$ CD45.1 OT-II T cells, and then challenged with OVA. After seven days of incubation, mononuclear cells were isolated from the spleen and LNs of the recipient mice, and the Treg/Th1/Th2/Th17 cell phenotype (D; n = 8) and the Smad2/3 phosphorylation levels of OT-II T cells were detected (E; n=8). Mean ± SEM. For the data shown in Panel A-C, RM ANOVA and Tukey's multiple comparison test were used for comparison between treatments; for the data shown in Panel D&E, a paired t-test was used for comparison between groups; for the data shown in Panel F&G, Mann Whitney's test was applied; for the data shown in Panel H, we used Friedman's test and then Dunn's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

5.3.5 PF4 acts as an accelerator of the TGFβ signalling pathway by binding to the glycosaminoglycan (GAG)-rich region of TGFBRIII

HT-BiFC assay helped to identify the PF4 protein interactome, and GO analysis showed that PF4 interacting proteins were enriched in immune effector and immune system processes (Fig 12A). Among the PF4 interacting proteins, TGFBRIII was highly expressed on T cells and was judged the most likely "partner" of PF4. Hence, a BiFC assay was used to determine the possible PF4-TGFBRIII interaction [145] by co-transfecting PF4-YFPn and TGFBRIII-YFPc vectors. Figure 12C shows that TGFBRIII-PF4 heteromerization was stronger than the CCL5-PF4 heteromerization positive control. Immunofluorescence imaging also confirmed PF4-TGFBRIII interaction and showed that PF4 and TGFBRIII fluorescence were co-localized in TGFBRIII-C-GFP (green fluorescent protein) and PF4-N-SFB (S protein-FLAG-streptavidin binding peptide) co-expressed 293T cells (Fig 12D). Furthermore, TGFBRIII knockout (ΔTGFBRIII) HuT-78 cells had no responses to PF4 supplementation, whilst wild type HuT-78 cells responded with significantly enhanced p-Smad2/3 levels and FoxP3 expression (Fig 12E). The data implied that TGFBRIII and PF4 cooperation is crucial for PF4-enhanced TGFβ1 signalling and downstream FoxP3 expression.

TGFBRIII is rich in heparan sulfate (HS) and chondroitin sulfate glycosaminoglycans (GAGs) domains [146,147], and PF4 contains corresponding binding domains [148]. Enzymatic GAG digestion reduced PF4-enhanced p-Smad2/3 and FoxP3 expression of HuT-78 cells (Fig 12F), confirming the hypothesis that PF4 could bind to the GAG-rich region of TGFBRIII, and thus enhance TGFβ signalling.

Because TGFBRIII has no kinase activity, it binds and presents TGFβ to TGFBRII which is the initiator of TGFβ1 signalling [149,150]. When TGFBRII was blocked, PF4-enhanced Smad2/3 phosphorylation and FoxP3 expression were significantly reduced (Fig 12G), indicating that PF4-TGFBRIII interaction exerts the effect through TGFBRII.

5.3.6 Platelet-enhanced TGFBRII expression occurs through interaction with GAG on the surface of Tn cells via cell-to-cell contact

We next monitored TGFBRII expression under different conditions: (1) Platelet co-cultures increased TGFBRII expression of Tn cells, in which the elevation was diminished in transwell cultures. (2) Heparinase or/and chondroitinase pre-digesting GAG also attenuated the elevation of TGFBRII in HuT-78 cells caused by platelets. (3) PF4 enhanced the

expression of TGFBRII exerted via TGFBRIII, because this effect was eliminated in ΔTGFBRIII HuT-78 cells.

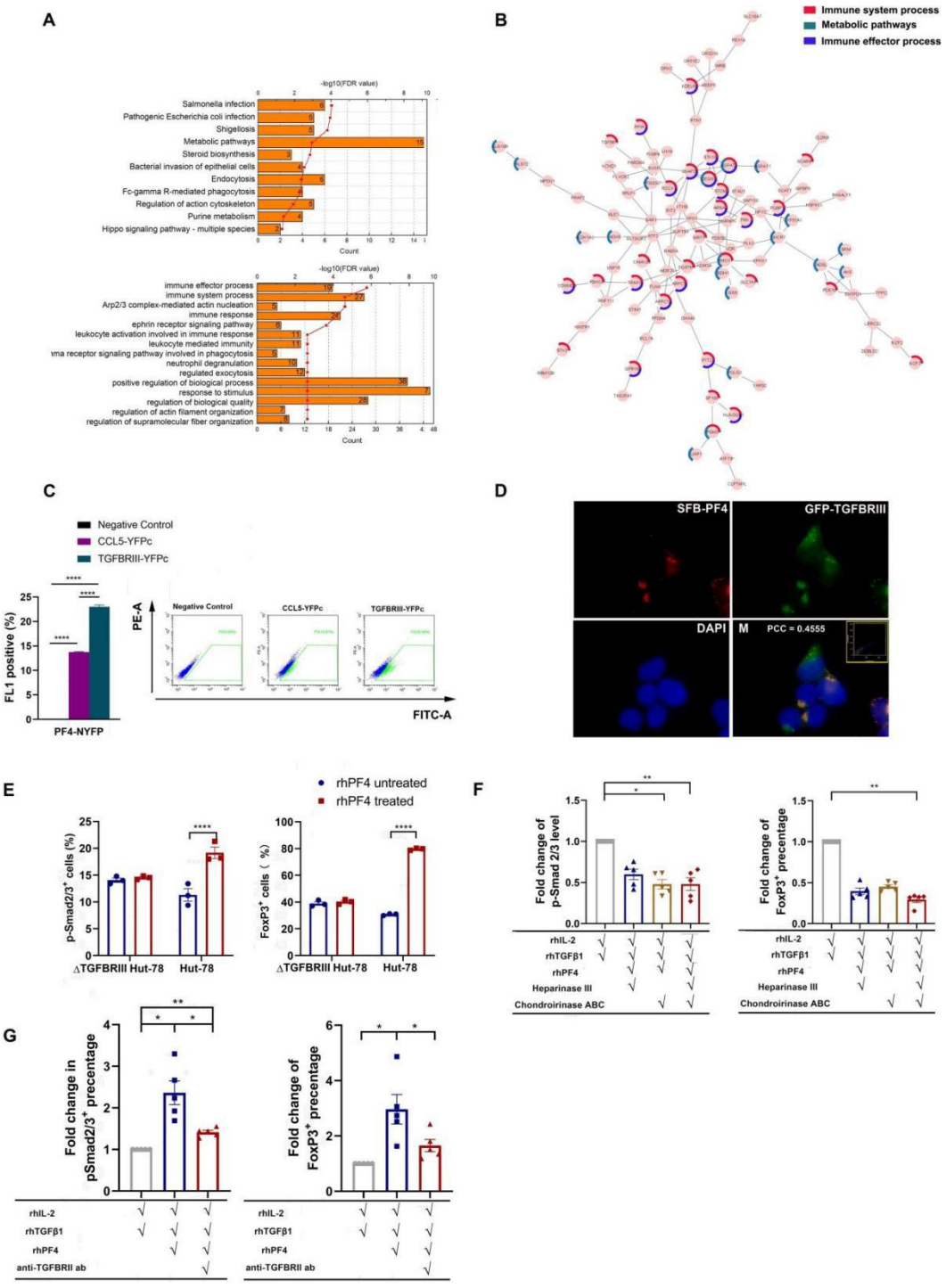

Figure 12. PF4 interactome and its interactions with and impacts on TGFβ signalling of CD4$^+$ T cells. Panel A: DAVID Bioinformatics Resource 6.8 was used for KEGG pathway enrichment analysis. The top 11 enrichment pathways of PF4 interacting proteins, including their corresponding protein counts and p-values, are shown in the bar chart on the upper left. The top 15 protein groups interacting with PF4 in the "biological process" evaluated by GO analysis are shown in the figure below. Panel B: evaluation of the functional interaction of PF4 interacting proteins and networking with STRING. Panel C: confirmation of PF4-TGFBRIII heteromerization of BiFC. Yellow fluorescent signal was phenotyped and performed on HTC75 cells expressing both PF4-YFPn and YFPc-tagged TGFBRIII/CCL5/RANTES (positive control)/non-fused YFPc (negative control); n=3; one-way analysis of variance, and then Holm-Sidak's multiple comparison test was performed. Panel D: revealing the location of TGFBRIII and PF4 by immunostaining. 293T cells were transfected with a

plasmid encoding GFP-tagged TGFBRIII and a plasmid encoding SFB-tagged PF4. The cell nucleus by DAPI staining. M, merged. A scatterplot of red and green pixel intensities is shown in the upper right corner of the merged image. Pearson Correlation Coefficient (PCC) and scatter plot were calculated from compressed pictures using ImageJ software. Panel E: Wild-type and ΔTGFBRIII HuT-78 cells were stimulated with αCD3/αCD28-coated Dynabeads (bead: cell ratio 2:1) for 48 hours, and then without or with rhPF4 (5 μg/mL) incubation for a further 72 hours. The expression levels of P-Smad2/3 and FoxP3 in HuT-78 cells were evaluated by flow cytometry; n=5; comparisons between different treatments in the same cell line were performed by two-way ANOVA followed by Sidak's multiple comparison test. Panel F: HuT-78 cells were pretreated with vehicle, heparinase and/or chondroitinase for two hours, then stimulated with αCD3/αCD28-coated Dynabeads for 48 hours, and then further cultured in the absence or presence of rhPF4 (5 μg/mL) for 72 hours. The expression levels of P-Smad2/3 and FoxP3 in HuT-78 cells were evaluated by flow cytometry; n=5; Friedman test was performed, followed by Dunn's multiple comparison test. Panel G: Hut-78 cells were stimulated with αCD3/αCD28-coated Dynabeads for 48 hours, and then cultured for 72 hours without or with rhPF4 (5 μg/mL) and in the absence or presence of TGFBRII blocking antibody; n= 5; RM ANOVA and Tukey's multiple comparison test was performed to compare treatments. Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

5.3.7 High concentration PF4 binds TGFBRII directly and inhibits TGFβ signalling

To elucidate the mechanisms underlying high concentration PF4 suppressed effector cell responses and TGFβ signalling of Tn cells, a cell model with a high cytoplasmic PF4 level was created using a truncated PF4-YFPn vector [151,152]. It was found that low concentrations of PF4 (intact PF4) had a weak binding to TGFBRII, but that high concentrations of PF4 (truncated PF4) had a significantly increased binding to TGFBRII, which was confirmed by fluorescence co-localization of PF4 (PF4-N-GFP) and TGFBRII (TGFBRII-C-SFB).

Furthermore, molecular docking simulation of TGFBRII and PF4 revealed that TGFBRII provided good topo-matching and docking for PF4 monomers and tetramers and that the docking mainly involved weak hydrogen bonds, which was consistent with the fact that a stable PF4-TGFBRII connection required a high concentration of PF4.

5.3.8 Platelet-bound PF4 plays a key regulatory role in Tn cell responses

Above in vitro and in vivo experimental data indicated the importance of cell-cell contact in platelet-regulated Tn cell responses. Further work was thus carried out to verify the observations. PF4-immobilized microparticles (imPF4-MP) were injected into the spleen of PF4 knockout mice that had received OT-II T-cell adoptive transfer and subsequent OVA immunization (Fig 1D). It was found that splenic injection of imPF4-MPs significantly increased Treg cell response and Smad2/3 phosphorylation, and inhibited Th1 cell response in the spleen. As expected, the splenic injection had no influences on $CD4^+$ T effector cell responses in the LNs.

5.4 PLATELET-DERIVED TGFβ1 AMELIORATES ATHEROGENESIS IN A MURINE MODEL OF ATHEROSCLEROSIS (PAPER IV)

5.4.1 Platelet-specific TGFβ1 deficiency exacerbates atherosclerosis

The en face ORO staining of the aorta showed that platelet-specific TGFβ1 deficiency increased atherosclerotic lesions significantly after 10 weeks of a high-fat diet, compared to the non-TGFβ1-deficient control group. The enhancement was even greater after 15 weeks on a high-fat diet. Atherosclerotic lesions were mainly concentrated in the aortic arch and thoracic aorta, and increased both in terms of absolute lesion area and percentage of lesion area of the total aorta area (Fig 13A-B), while the lesions in the abdominal aorta were much less prominent. In line with the above results, ORO staining of aortic root cryosections also showed that plt-TGFβ$^{-/-}$ mice were associated with more intensive atherosclerotic lesion formation (Fig 13C-D) as compared to control littermates.

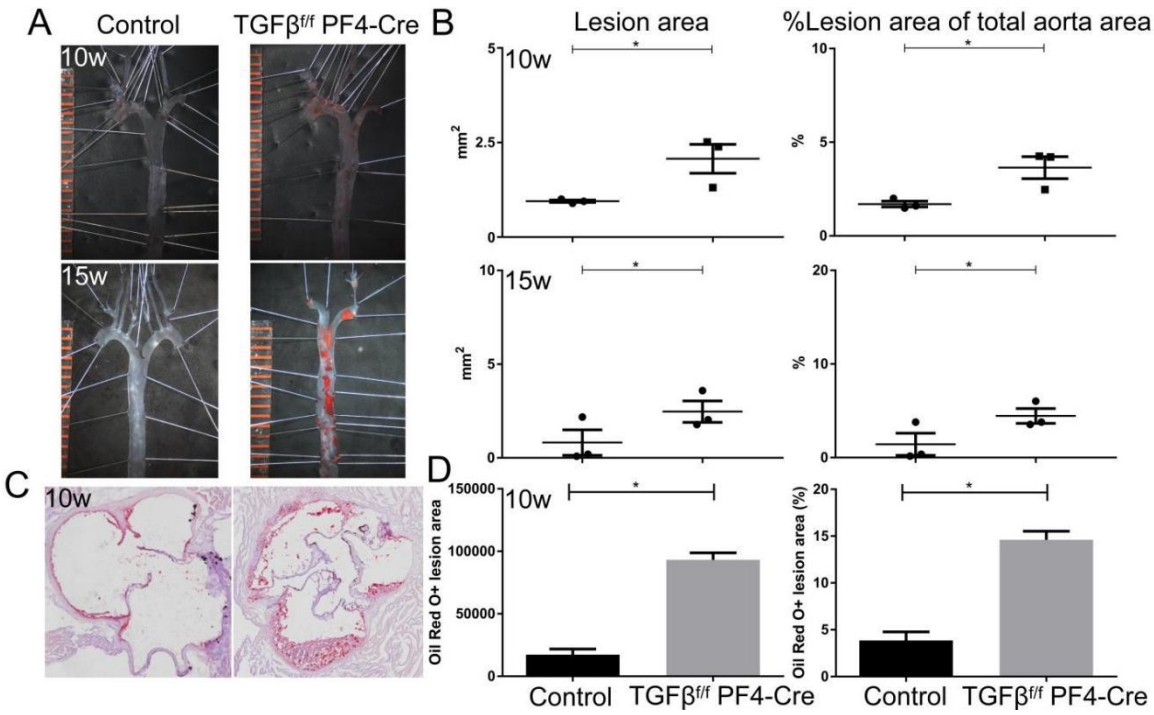

Figure 13. Lack of platelet-specific TGFβ exacerbates atherosclerosis. TGFβ$^{f/f}$ PF4-Cre mice were created by crossing mice carrying the "floxed" TGFβ1 allele (Tgfb1$^{flox/flox}$) with the mice expressing Cre recombinase under the control of the megakaryocyte/platelet-specific Pf4 promoter (Pf4-Cre^{+}). The pro-atherosclerotic mice of LDLR-functional abrogation were created by using 8-week-old TGFβ$^{f/f}$ PF4-Cre male mice and littermate controls with a gain-of-function approach by PCSK9 overexpression. The mice were fed a Western-style high-fat diet (21% fat and 0.21% cholesterol) without cholate for 10 and 15 weeks. Panel A: en face staining of atherosclerotic lesions. The aorta was fixed with 4% PFA in PBS for ≥24 h at 4°C, stained with 3 mg/ml ORO for three hours at RT, washed with 70% ethanol, and stored in PBS at 4°C. Aortas were then pinned on a wax plate and pictures taken. Representative images of aortas after 10 weeks (above) and 15 weeks (below) of a high-fat diet are shown. Scale bar shows orange mm-marks. Panel B: Atherosclerotic burden in the aorta. The Image J program was used to analyze the pictures of ORO stained atherosclerotic lesions. The lesion area in mm^2 and the percentage of atherosclerotic lesions in the total aortic area are shown. P<0.05, compared with control mice; n=3. Panel C&D: Atherosclerotic lesions of the aortic root. Frozen sections of the aortic root were stained with 3 mg/ml ORO solution. Using OLYMPUS BX61VS microscope to observe and take pictures of frozen sections.

The area of lesions in arbitrary units and the percentage of lesions per aortic circumference were shown. Frozen section images were from TGFβ$^{f/f}$ PF4-Cre and control mice after 10 weeks of high-fat diet and the level 300 µm from the aortic sinus was used for quantifications. The area of the lesion was expressed in arbitrary units and used as a percentage of the total blood vessel circumference. * P<0.05, TGFβ$^{f/f}$ PF4-Cre compared to control mice; n=3-6.

5.4.2 Effect of plt-TGFβ$^{-/-}$ on blood lipid levels

Most of the TGFβ in circulation comes from platelets [153], in our experiment, the TGFβ level in plt-TGFβ$^{-/-}$ mice was significantly reduced, confirming that platelet-derived TGFβ was successfully knocked out. Functional knockout of LDLRs and a high-fat diet increased plasma lipid levels in mice. Platelet-selective TGFβ deficiency further elevated plasma levels of total cholesterol (Cho; Fig 14A), low-density lipoprotein cholesterol (LDL-C; Fig 14B), and triglycerides (TG; Fig 14D), but did not alter high-density lipoprotein cholesterol (HDL-C; Fig 14C). These results highlighted that platelet-selective TGFβ deficiency selectively increased levels of Cho, TG, and LDL-C, which are known to aggravate atherosclerotic lesion formation.

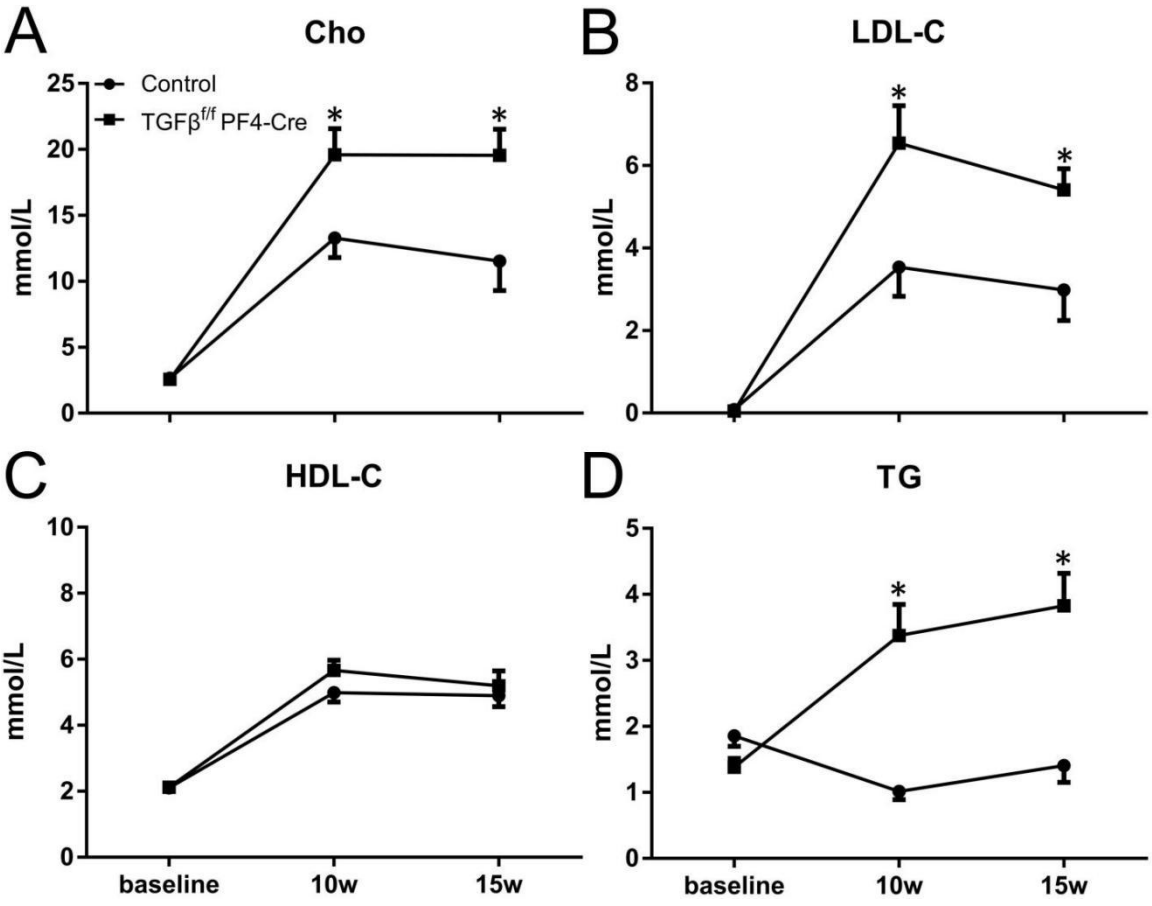

Figure 14. Deficiency of platelet-specific TGFβ increases blood lipid levels. TGFβ$^{f/f}$ PF4-Cre male mice and their littermate controls underwent LDLR functional abrogation by PCSK9 overexpression at week 8 (baseline). The mice were fed with a high-fat diet for 10 to 15 weeks. Collection of plasma samples for analysis of total cholesterol (Cho; A), (LDL-C; B), (HDL-C; C) and triglycerides (TG; D). *P<0.05, TGFβ$^{f/f}$ PF4-Cre vs. control mice; n=3-9.

5.4.3 Plt-TGFβ$^{-/-}$ alters CD4^{+} T effector cell activities

TGFβ is an important regulator of CD4^{+} T effector cell responses, which has been shown in our previous work in paper I-III of the present thesis. After being on a high-fat diet for 10 weeks, plt-TGFβ$^{-/-}$ was found to reduce Treg (Fig 15N-O) and Th17 cell frequencies (Fig 15J-K) in the spleen and LNs. Unexpectedly, plt-TGFβ$^{-/-}$ was also shown to reduce Th1 (Fig 15B-C) and Th2 responses (Fig 15F-G). When high-fat feeding reached 15 weeks, splenic Th17 cells were still suppressed (Fig 15L), while Th1 responses were increased in LNs (Fig 15E).

To explore the potential mechanisms underlying the unexpected findings, the relationship between effector cell transcription factor activation and intracellular cytokine production was monitored in the mice after 15 weeks on a high-fat diet. T-bet expression of CD4^{+} T cells of plt-TGFβ$^{-/-}$ mice tended to be higher in the spleen and was significantly increased in the LNs. Albeit FoxP3 expression was similar, intracellular levels of TGFβ, which is a marker of Treg cell immunosuppressive activity, was significantly reduced in plt-TGFβ$^{-/-}$ mice. The discrepancy of transcription factor and intracellular cytokines may suggest that T effector cell activity might be exhausted and Treg cell functionality might be compromised in plt-TGFβ$^{-/-}$ mice.

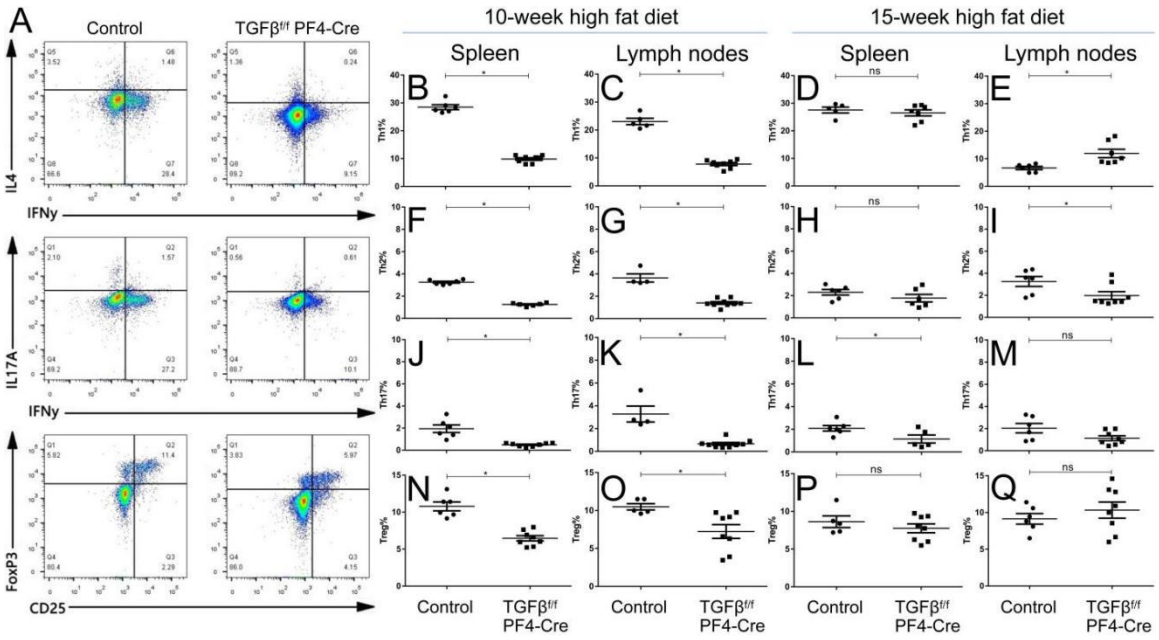

Figure 15. TGFβ$^{f/f}$ PF4-Cre alters CD4^{+} effector cells frequencies in the spleen and LNs. TGFβ$^{f/f}$ PF4-Cre mice and their littermate control mice were fed with high-fat food for 10 to 15 weeks. At baseline, 10 weeks, and 15 weeks, the spleen and LNs were harvested for the preparation of single-cell suspensions. Panel A: flow cytometric analysis of CD4^{+} T effector cells. For cell surface marker staining, the cells were stained with the corresponding fluorescent antibody for 30 minutes at 4°C; for intracellular staining, PMA (50 ng/ml) and ionomycin (1 μg/ml) for two hours firstly, and brefeldin A for four more hours to block the secretion of cytokines were used. Representative dot plots. Panel B-E: Th1/IFNγ^{+}; Panel F-I: Th2/IL-4^{+}; Panel J-M: Th17/IL-

17A^{+}; Panel N-Q: Treg/FoxP3^{+}-CD25^{++}. *P<0.05, comparing TGFβ$^{f/f}$ PF4-Cre mice with littermate control mice, n=4-9.

5.4.4 Plt-TGFβ$^{-/-}$ enhances pro-inflammatory features of the aortic vessel wall

RNA sequencing of the aorta of mice on a 15-week high-fat diet and GO enrichment analysis showed that plt-TGFβ$^{-/-}$ was associated with enhanced the chemotaxis/infiltration and inflammatory responses of lymphocytes and monocytes. KEGG pathway analysis showed that plt-TGFβ$^{-/-}$ promoted the activation of several inflammatory pathways, including cytokine-cytokine receptor interaction, IL-17 signalling and chemokine signalling, as well as arachidonic acid metabolism pathways.

Aortic proteomic analyses of the aorta in the same mice indicated that plt-TGFβ$^{-/-}$ increased the expression of the major histocompatibility complex class II (MHC-II) and a strong enhancement of CD4^{+} T effector cell response, manifested by the enhancements of Th1/Th2/Th17 differentiation pathway and T cell receptor signalling pathway. Hence, the high-throughput analyses of aortic transcriptomics and proteomics supported the notion that plt-TGFβ$^{-/-}$ enhanced inflammatory activities in the arterial vessel wall.

6 DISCUSSION

Effector T cells provide timely and rapid immune protection, but memory T cells will at the same time establish long-term immune protection/memory. The present thesis work has shown that platelets and platelet-derived mediators have profound regulatory effects on all subsets of $CD4^+$ T cells, naïve, effector memory cells, and central memory cells, and that platelet-derived TGFβ is atherosclerosis protective, as plt-$TGF\beta^{-/-}$ aggravated atherosclerotic lesion formation.

Platelets regulated effector cell responses of different $CD4^+$ T cell subsets with distinct mechanisms. For Tem and Tcm cells, PF4 was the key regulator and exerted its effects by enhancing mitochondrial biogenesis and metabolism. PF4 reduced T cell activation-associated Akt phosphorylation and subsequently attenuated PGC1α phosphorylation. The latter resulted in enhanced PGC1α activity, and thereby increased TFAM expression and mitochondrial biogenesis [140]. In Tem cells, PF4-enhanced mitochondrial biogenesis and metabolism primarily promoted effector cell activation. The effects were exerted by elevating ATP and ROS production, stimulating T-bet and FoxP3 expression, and ultimately promoting rapid Th1 and Treg responses. Treg responses seem to be activated continuously because platelets can selectively enhance $FoxP3^+$ $CD4^+$ T cell activation, which leads to uninterrupted enhancement of Treg response [127].

Compared to Tem cells, Tcm cells are a type of self-renewing and long-lived memory "stem cells", which re-activate effector T cell responses fast to offer prompt immune protection [11,154]. Polyclonal stimulation of Tcm cells induced mitochondrial biogenesis and cell proliferation. Platelet co-cultures and PF4 supplementation further enhanced cell proliferation, which was not seen in Tem cells. The response peak of Tcm cells appeared later than for Tem cells, which is consistent with the immunological characteristics of Tcm cells, experiencing significant cell proliferation first rather than inducing immediate effector cell responses [5].

What is more, there are several receptors for PF4, including GAG, αV integrin, lipoprotein receptor-related protein 1 (LRP1), αM integrin Mac1/CD11b, and CXCR3 [141,155,156]. Our results showed that PF4 appeared to modulate the immune response via chemokine receptor function. However, GAG/αV/LRP1/Mac1/CD11b should be limited involved in this environment. GAG/αV are thought to be co-receptors, and the expression of LRP1 and Mac1/CD11b are negligible for T cells; therefore, the interaction between PF4-CXCR3 is the most likely to occur on the T cell surface [141,155,156], which is also consistent with our results of Tem and Tcm: after the CXCR3 inhibitor AMG487 blocked CXCR3 and siRNA knocked

down CXCR3, both reduced the PF4-enhanced T effector cell responses. CXCR3 has two isomers, CXCR3-A and CXCR3-B. Compared to CXCR3-A, PF4 should selectively bind to CXCR3-B, which increases cAMP levels and then attenuates Akt phosphorylation/activity [141,157]. CXCR3-A on the other hand will mainly bind to CXCL9/10/11 and these ligands activate Akt signalling [10].

Cell metabolism is the driving force of the immune response, and the mitochondrial metabolism is critical to the activation, proliferation and differentiation of T cells [10,140,158]. ATP and ROS tend to preferentially affect Th1 and Treg, respectively [10,159]. PF4 regulates pro-inflammatory Th1 activities and anti-inflammatory Treg responses, which could affect inflammation balance and immune homeostasis [127,136,137,144,160]. The present thesis work on platelet-regulated effector cell responses of Tem and Tcm cells accumulate a collection of evidence showing that PF4 exerts an immunomodulatory effect through mitochondrial biogenesis. Programmed cell death protein 1 (PD-1) blocking, which relies on enhanced mitochondrial biogenesis and T cell activation, has achieved certain breakthroughs in cancer treatment. Those achievements suggest that PF4-regulated mitochondria biogenesis and metabolism can be a potential immunotherapy for the future [161]. The notion is supported by the outcome of the recent CANTOS trial showing that anti-inflammatory treatment using the anti-IL-1β antibody canakinumab reduced recurrent cardiovascular events in myocardial infarction patients [162].

Platelets regulate naïve T cells through a PF4-TGFβ duet in a context-dependent manner. Low-concentration PF4 can bind to TGFBRIII to enhance TGFβ signalling. PF4 binds to TGFBRIII with high affinity and could act as a PF4 heterodimerization chemokine [145,163]. PF4 at mildly elevated concentrations served as an accelerator of TGFβ signalling. Hence, moderate-high concentration PF4 bound to TGFBRIII enhanced the presentation of TGFβ to TGFBRII as well as the surface expression of TGFBRII and subsequently enhanced TGFβ signalling. Excessively high concentrations of PF4 acted, however, as a brake of TGFβ signalling. At the site of inflammation and possibly during the early stages of the immune response, activated platelets released a large amount of PF4. Activated macrophages and T cells may also enhance this release [164,165]. Platelets can bind PF4 on the surface as well, which complicates the picture further [166]. Excessively high concentrations of PF4 will bind to TGFBRII directly, which masks TGFβ-binding sites on TGFBRII [125] and, therefore, inhibits TGFβ signalling. To our best knowledge, this is the first piece of evidence showing that PF4 exerts a context- and concentration-dependent modulation of inflammation and immune responses.

After our in-depth research on the mechanisms of platelet-regulated Tem/Tcm/Tn responses, this thesis work further explored the impact of platelet-derived TGFβ on atherosclerosis. The results showed that platelet-derived TGFβ had a protective effect on atherosclerosis. It is well known that platelets are involved in the entire development process of atherosclerosis and that platelets regulate immune cell responses through cell-cell contacts and a variety of soluble mediators such as TGFβ [8,9,137,167-169]. The main source of circulating TGFβ was found to be platelet-derived TGFβ, which can enhance anti-inflammatory Treg activities and inhibit pro-inflammatory Th1 responses. It may also affect other cells in the vasculature, such as smooth muscle cells and macrophages. TGFβ can promote the contractile phenotype of smooth muscle cells and collagen production to enhance atherosclerotic plaque stability. It is also known to hamper monocyte infiltration into the lesion and macrophage activities. In addition, platelets express TGFβ receptor II, which allows for autocrine actions by TGFβ, which leads to platelet inhibition [9,153,167,169-172]. These different effects of TGFβ may collectively synergize into a protective effect of atherosclerosis.

Our results also showed that plt-TGFβ$^{-/-}$ increased plasma lipid levels, including total Cho, LDL-C, and TG. Platelet-derived TGFβ enhanced Treg cell proliferation and response, which can regulate blood lipid levels by enhancing lipoprotein catabolism [127,137,173]. Our results showed that Treg activities were suppressed in plt-TGFβ$^{-/-}$ mice, and the latter may eventually lead to hyperlipidemia. In addition, platelets might also be able to regulate various aspects of hepatocyte functions. We used the PCSK9-mediated functional LDLR knockout method to induce hyperlipidemia and atherosclerosis in our mouse model [174-177]. Platelet-derived TGFβ could trigger intracellular signalling through the pSmad2/3-dependent, MAP kinase, and mTOR pathway in hepatocytes [178]. These pathways directly and/or indirectly regulate the activation of the transcription factor SREBP1/2, which control PCSK9 and LDLR synthesis [176,179,180]. Our results showed that the lack of platelet-derived TGFβ increased plasma lipid levels. Speculatively, the effect may be achieved by that platelet-derived TGFβ enhances LDLR synthesis, but reduces PCSK9 production, which subsequently leads to the reduction of lipid levels.

7 CONCLUSIONS

The present thesis work has provided a collection of novel evidence showing that platelet-regulated $CD4^+$ T effector cell responses are the result of cooperation and synergy of multiple factors, and play an important role in atherosclerosis:

(1) Platelets regulate $CD4^+$ T effector cell responses of the three general $CD4^+$ T cell subsets, namely naïve $CD4^+$ T cells, $CD4^+$ effector memory T cells, and $CD4^+$ central memory T cells. Platelets exert more prominent regulations on Th1 and Treg cell responses with distinct dynamics, i.e., continuous enhancement of Treg cells responses but initial enhancements followed by suppression/reduction of Th1 responses.

(2) Platelets influence $CD4^+$ Tem and Tcm cell responses mainly by PF4-dependent mitochondrial biogenesis and via the PF4 receptor CXCR3-initiated Akt-PGC1α-TFAM signal pathway. Platelets achieve Tem-regulating effects by promoting mitochondrial ATP and ROS production, which stimulate T-bet and FoxP3 expression and subsequently result in enhanced Th1 and Treg responses. In contrast, platelets achieve Tcm-regulating effects mainly through mitochondrial biogenesis and cell proliferation.

(3) Platelets regulated $CD4^+$ Tn cell responses through a context-dependent PF4-TGFβ duet and through both soluble mediators and direct cell-cell contact. PF4 at mildly elevated concentrations binds TGFBRIII, facilitating TGFβ-presentation to and expression of TGFBRII, which subsequently enhances TGFβ signalling and naïve $CD4^+$ effector cell responses. At excessively high concentrations, PF4 directly bind to TGFBRII, blocks TGFβ-TGFBRII ligation, and hampers $CD4^+$ Tn cell responses. Moreover, direct platelet-Tn cell contact is needed for the optimal regulation of platelets.

(4) The atherosclerosis model using platelet specific TGFβ1-deficient mice indicate that platelet-derived TGFβ has a protective effect on atherosclerosis. Platelet-specific TGFβ deficiency hampers Treg cell activities, induces hyperlipidemia, enhances arterial inflammation, and results in aggravated atherosclerosis.

8 POINTS OF PERSPECTIVE

In summary, platelets participate actively in many aspects of the vascular microenvironment, based on the total readout/combination of multiple factors. In addition to TGFβ, PF4 also plays an important role. Our research reveals that the PF4-dependent/PF4-TGFβ duet represents a possible new mechanism of immune regulation, providing new ideas and targets for the treatment of related diseases caused by atherosclerosis in the future.

Platelets not only predominate in thrombotic but are also closely involved in inflammatory mechanisms of atherogenesis. Our research has illustrated the mechanisms underlying platelet-regulated $CD4^+$ Tem, Tcm, and Tn responses, and developed an in-depth understanding of cooperative inflammatory and thrombotic mechanisms in atherogenesis and the importance of platelet-dependent regulations in atherosclerosis.

Among platelet-derived mediators, PF4 has emerged as a key regulator in $CD4^+$ T effector cell responses. The present doctoral thesis research highlights the potential to develop PF4 and/or its signalling as targeted anti-atherosclerotic drugs in the future. Since the PF4-regulatory machinery is complex, there is a remaining need to explore the mechanisms underlying PF4-regulated $CD4^+$ T effector cell responses further. For example, we should study the regulatory effects of different concentrations of PF4 on Tem and Tcm responses to confirm whether its regulatory effects are concentration-dependent. Moreover, cell-to-cell contact is also important for PF4-dependent regulations. We can co-culture Tem/Tcm cells and platelets with or without a transwell system to confirm whether cell-to-cell contact has a major impact on its regulatory mechanisms for Tem and Tcm cells.

What is more, CXCR3 has two forms, CXCR3-A and CXCR3-B. According to our results and evidence in the literature, we believe that CXCR3-B plays a major role. Future studies should be taken to reveal the specific form and mechanisms, which may reveal intervention sites for the future development of platelet-targeted anti-atherosclerotic therapeutics.

Our atherogenic study using plt-TGFβ$^{-/-}$ has highlighted that platelet-derived TGFβ has protective effects on atherosclerosis. It is important to accomplish some further work of the study. For example, immunohistochemical staining of inflammatory cells in lesions should be performed. RT-PCR should be performed to verify the findings of high-throughput transcriptomics analysis. Moreover, because hepatocytes are very important for lipid metabolism, the analysis of hepatocytes should be included in future studies. These

observations will more comprehensively explain the mechanisms of platelet-specific deficiency-aggravated atherosclerosis.

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