Platelet factor 4 enhances CD4\(^+\) T effector memory cell responses via Akt-PGC1\(\alpha\)-TFAM signaling-mediated mitochondrial biogenesis

Shuai Tan\(^1\) | Shuijie Li\(^2\) | Yanan Min\(^1\) | Anton Gisterå\(^3,4\) | Noah Moruzzi\(^5\) | Junhao Zhang\(^1,6\) | Yang Sun\(^7\) | John Andersson\(^8\) | Rickard E. Malmström\(^1,9\) | Miao Wang\(^10\) | Per-Olof Berggren\(^5\) | Susanne Schlisio\(^2\) | Wangjun Liao\(^6\) | Daniel F. J. Ketelhuth\(^3,4\) | Chunhong Ma\(^7\) | Nailin Li\(^1\)

Abstract

Background: Cell metabolism drives T cell functions, while platelets regulate overall CD4\(^+\) T cell immune responses.

Objective: To investigate if platelets influence cell metabolism and thus regulate CD4\(^+\) T effector memory cell (Tem) responses.

Methods: Human CD4\(^+\) Tem cells were activated with αCD3/αCD28 and cultured without or with platelets or platelet-derived mediators.

Results: Polyclonal stimulation induced rapid and marked Th1 and Treg cell activation of CD4\(^+\) Tem cells. Platelet co-culture enhanced Th1 response transiently, while it persistently enhanced Treg cell activation of Tem cells, with an enhancement that plateaued by day 3. Platelet factor 4 (PF4) was the key platelet-derived mediator regulating CD4\(^+\) Tem cell responses, which involved cellular metabolisms as indicated by mass spectrometric analyses. PF4 exerted its effects via its receptor CXCR3, attenuated Akt activity, and reduced PGC1\(\alpha\) phosphorylation, and resulted in elevations of PGC1\(\alpha\) function and mitochondrial transcription factor A (TFAM) synthesis. The latter increased mitochondrial biogenesis, and subsequently enhanced Th1 and Treg responses. Consistent with these observations, inhibition of mitochondrial function by rotenone counteracted the enhancements by recombinant PF4, and TFAM overexpression by TFAM-adenovirus infection mimicked PF4 effects. Furthermore, increased mitochondrial mass elevated oxygen consumption, and enhanced adenosine triphosphate and reactive oxygen species production, which, in turn, stimulated Th1 (T-bet) and Treg (FoxP3) transcription factor expression and corresponding CD4\(^+\) T effector cell responses.
1 | INTRODUCTION

Atherosclerosis involves multiple mechanisms, including inflammation and thrombosis, to which platelets and CD4+ T cells contribute importantly. Platelets are not only a thrombocyte, but have also emerged as an important coordinator between, eg, thrombotic and inflammatory mechanisms in atherogenesis.

Over the last decades, accumulating evidence has demonstrated that platelets contribute importantly to the inflammatory microenvironment of atherosclerosis, such as recruitment of inflammatory cells, macrophage polarization, neutrophil extracellular trap (NET) formation, and inflammation resolution. We and others have shown that platelets can regulate multiple aspects of CD4+ T cell activities. Activated platelets release various CD4+ T cell-active mediators, such as platelet factor 4 (PF4), transforming growth factor β (TGFβ), thromboxane A2 (TxA2) and RANTES (regulated upon activation, normal T cell expressed and presumably secreted). Platelet co-cultures attenuated αCD3/αCD28-induced proliferation of the total CD4+ T cells. PF4 inhibited proliferation of the total CD4+ T cells, but selectively stimulated proliferation of Treg cells. Similarly, TGFβ inhibited cell proliferation of the total CD4+ T cells and Th1 cells, but enhanced Treg and Th17 cell proliferation. Other platelet-derived chemokines RANTES and monocyte chemotactic protein-1 (MCP-1) have been shown to stimulate CD4+ T cell proliferation. Platelet-released TxA2, however, inhibited CD4+ T cell proliferation. Importantly, these platelet-derived mediators also regulate CD4+ T cell functions. It has been shown that PF4 and TGFβ inhibited Th1 differentiation and cytokine production, but enhanced Treg differentiation and activation. RANTES and MCP-1, however, enhanced Th1 differentiation and cytokine production, while TxA2 had inhibitory effects. We have shown that platelets regulate CD4+ T effector cell responses of the total CD4+ T cells with distinct dynamics, i.e., with a bi-phasis regulation on T effector (Th1 and Th17) responses but with a persistent enhancement on Treg cell responses. However, it is unclear how platelets exert the distinct regulations in individual CD4+ T cell subsets, and what are the mechanisms underlying the distinct regulation of individual CD4+ T cell subsets.

Cell metabolism drives T cell development, activation, differentiation, function, and survival. It has been recognized that mitochondrial energy metabolism, namely adenosine triphosphate (ATP) production, during the first 24 to 48 hours after T cell activation is critical for optimal T effector cell responses, and that mitochondrial metabolism is important for T cells to exit their quiescence.

Conclusions: Platelets enhance CD4+ T cell responses of Tem cells through PF4-dependent and Akt-PGC1α-TFAM signaling-mediated mitochondrial biogenesis. Hence, PF4 may be a promising intervention target of platelet-regulated immune responses.

KEYWORDS
CD4+ T effector cell responses, CD4+ T effector memory cells, mitochondria, platelets, platelet factor 4

Essentials
- Platelets enhance T effector cell responses of CD4+ T effector memory (Tem) cells.
- The effects are exerted by platelet factor 4 and via Akt-PGC1α-TFAM signaling.
- The effects are achieved by enhancing mitochondrial biogenesis and metabolism of Tem cells.
- PF4 can be a promising intervention target of platelet-regulated immune responses.

Quiescent CD4+ T cells in peripheral blood exist as naïve, effector memory, and central memory cells. CD4+ T effector memory (Tem) cells are antigen-experienced cells, and can mount a quick and strong response upon re-encountering cognate antigen. Therefore, we asked how important mitochondrial metabolism is in platelet-regulated CD4+ T effector cell responses of Tem cells. Indeed, we demonstrated that mitochondrial ATP and reactive oxygen species (ROS) metabolisms played a critical role in platelet-regulated Tem cell responses. Here we showed that platelet-CD4+ T effector memory (Tem) cell co-culture gave rise to a transient enhancement of Th1 effector responses, and a continuous enhancement on Treg activation of Tem cells. We identified PF4 as the primary mediator for platelet-regulated CD4+ T effector cell responses. PF4 bound to its receptor CXCR3, elevated mitochondrial transcription factor A (TFAM) expression, increased mitochondrial biogenesis, and enhanced ATP and ROS production. Consequently, ATP and ROS stimulated T-bet and FoxP3 expression and promoted Th1 and Treg responses of Tem cells.

2 | METHODS AND MATERIALS

2.1 | Study subjects

Fifty-eight healthy volunteers, 23 females, 35 males, aged 23 to 55 years, gave informed consent to donate blood samples for platelet isolation in the study, which was approved (Dnr 94-146) by the Ethics Committee of Karolinska Institutet and in accordance with the Declaration of Helsinki. All volunteers disclosed not taking any medication during 2 weeks prior to blood sampling.
2.2 | Co-cultures of CD4+ Tem cells and platelets

Isolation of CD4+ Tem cells and platelets were detailed in Materials and Methods of Appendix S1 in supporting information. CD4+ Tem cells and platelets were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. CD4+ Tem cells were cultured in the presence or absence of platelets (Tem:platelet ratio at 1:250, which is similar to their ratio in circulation) at 37°C and 5% CO2 for up to 7 days. Notably, direct Tem cell-platelet co-culture was used in the present study for the optimal effects of platelets, as indicated by our earlier work showing that CD4+ T cell-platelet transwell co-culture (ie, acting via soluble platelet-derived mediators but not cell-cell contact) provides suboptimal effects.19

Washed platelets were used in the co-culture to avoid the potential influences of non-platelet-derived mediators in platelet-rich plasma or preservatives used in platelet concentrates. CD4+ T cell activation was induced by coated anti-CD3 antibody (3 µg/mL, 100 µL/well at 37°C for 3 hours) and soluble anti-CD28 antibody (0.3 µg/mL). Significant platelet activation occurred during co-culture, with platelet P-selectin expression increased from 3.4 ± 1.2% to >70% (Figure S2C in supporting information). According to experimental design, the supernatants and cultured CD4+ T cells were collected at day 0, 1, 3, 5, and 7 for cytokine cytometric bead array (CBA) analyses and flow cytometric phenotyping. CD3/CD28-stimulated CD4+ T cells were also re-challenged by phorbol myristate acetate (PMA; 50 ng/mL) and Ionomycin (1 µg/mL) in the presence of protein transport inhibitor BD GolgiStop 5 hours before harvesting cells for phenotyping.

2.3 | Flow cytometry, mass spectrometry, confocal microscopy, and western blotting

Flow cytometry for CD4+ T cell and platelet phenotyping mass spectrometry,24 confocal microscopy,25,26 and western blotting25,26 used in the present study have been described previously. These methodologies have been detailed in Appendix S1.

2.4 | CD4+ Tem cell CXCR3 knock-down

CD4+ Tem cells in Opti-MEM™ Reduced Serum Medium (Thermo Fisher, cat# 31985070) were transfected with ON-TARGETplus Human CXCR3 siRNA SMARTPool (L-005472-00-005; a mixture of 4 siRNAs; final concentration 100 nM) and corresponding ON-TARGETplus Non-targeting Control Pool (D-001810-10-05; Horizon Discovery) for 72 hours using a Lipofectamine™RNAiMAX Transfection Reagent kit (Thermo Fisher, cat# 13778030). Afterward, Tem cells were harvested for flow cytometric analysis of CXCR3 expression and for further experiment of αCD3/CD28 stimulation in the absence or presence of rhPF4 (5 µg/mL) for 3 days and flow cytometric phenotyping of Th1 and Treg cells.

2.5 | ATP assay

Cellular ATP levels were measured using the ADP/ATP Ratio Assay Kit (#ab65313; Abcam) according to manufacturer’s instructions. Samples containing 10^4 Tem cells in 20 µL phosphate buffered saline (PBS) were plated in a 96-well microplate with 100 µL of reaction mix consisting of 5 µL ATP monitoring enzyme and 95 µL nucleotide releasing buffer. Samples were incubated for 2 minutes, and then read in a luminometer to assess ATP levels according to luminescence intensities.

2.6 | Seahorse assay

CD4+ Tem cells without or with αCD3/αCD28-stimulation or rhPF4 were harvested after 3-day culture. Cells (1-2 x 10^5 cells) were seeded onto poly-D-lysine coated 96-well XF plates containing RPMI without bicarbonate supplemented with 11 mM glucose, 2% FBS, and 2 mM L-glutamax. After 1-hour culture in a non-CO2 incubator, the oxygen consumption rate (OCR), an indicator of mitochondrial respiration, was assessed using a Seahorse XF 96 Analyzer (Seahorse Biosciences, Inc). OCRs were measured for 6 minutes at baseline and after addition of antymycin A and rotenone (2.5 and 2.5 µM; to assess non-mitochondrial OCR). ATP-coupled and the maximum respiration were not assessed due to the low respiration and the number of Tem cells available for this assay. Data were normalized on the number of cells per well. Thus, the cells were stained with the nucleic dye Hoescht 33342 (Molecular Probes) for 10 minutes. Cell numbers per well were assessed by individual well imaging with a 20× objective using BD Pathway 855 Bioimaging System and the cell profiler software (BD Biosciences).

2.7 | Statistical analyses

The data were presented as mean ± standard error of the mean. Platelet influences on CD4+ effector cell responses over time were analyzed with repeated measurements analysis of variance (ANOVA) using SuperANOVA program (Abacus Concepts). Multiple comparison between the treatments were analysed by one-way ANOVA followed by post hoc Tukey tests using SPSS 22 and Graphpad Prism 5. A P value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Platelets promoted Th1 and Treg cell activation of human CD4+ Tem cells

αCD3/αCD28 stimulation evoked activation of CD4+ Tem cells, as evidenced by marked T cell aggregation (Figure S1B in supporting information). CD4+ Tem cell activation also increased cell sizes (Figure S1B-C), seen as increased forward scattering signals of activated Tem cells. Expression of the T cell activation marker CD25 was markedly
Increased upon stimulation and was further enhanced by platelet co-culture (Figure S1D). Thus, percentages of CD25 positive cells among the total cells were increased from 1.3 ± 0.7% of unstimulated cells to 40.7 ± 17.1% of αCD3/αCD28-stimulated cells on day 3 and were further increased to 76.0 ± 5.3% in the presence of platelets. When cultured Tem cell counts were monitored, it was clear that unstimulated Tem cells did not proliferate and had a slight decrease of cell counts during 7-day culture. CD3/CD28 polyclonal stimulation induced limited cell proliferation, with mild but yet significant elevations of cell counts of Tem cells during 5-day culture without or with platelet co-cultures, while the cell count tended to decrease on day 7 (Figure S1F).

The results obtained using a CellTrace™ Violet Cell Proliferation Kit also suggested that cell activation did not induce marked cell proliferation (Figure S3 in supporting information).
CD4+ T effector cell responses of αCD3/αCD28-stimulated Tem cells were demonstrated by flow cytometric phenotyping of IFNγ+ Th1, IL-17A+ Th17 cells, CD25+FoxP3+ Treg cells (Figure 1A), as well as IL-4+ Th2 cells (not shown). IFNγ+ Th1 cells were elevated by αCD3/αCD28 stimulation through day 1 to day 5, but then declined (Figure 1B). Platelet co-culture induced a biphasic regulation on Th1 activation, seen as a rapid and transient enhancement of Th1 response during 3 days, which was followed by a secondary suppression or a phase of quick decline. αCD3/αCD28 stimulation mildly enhanced Th2 and Th17 activation, while platelet co-culture had little effect on Th2 or Th17 response (Figure 1B, lower panels). αCD3/αCD28 stimulation evoked a marked increase of Treg cell activation during 3 days, which was followed by a regression. In contrast to the platelet effects on Th1 effector responses, platelet co-culture persistently enhanced Treg phenotype. The enhancement reached a maximum for Treg cell responses by day 3, and platelets could maintain high Treg cell levels during the prolonged incubation.

3.2 | PF4 dominated in platelet-regulated effector cell responses of CD4+ Tem cells

We have shown earlier that multiple platelet-derived soluble mediators contribute to CD4+ T cell responses.11 To elucidate what mediators exert the regulatory effects in Th1/Th17/Treg responses in Tem cells, a panel of neutralizing/inhibiting agents were used, which were chosen based on their regulatory potencies and/or physiological significance as indicated by research literature.3,5,11 Three-day co-culture, when platelet-dependent enhancement reached its plateau, was chosen as the observation window. PF4 blockade by a neutralizing antibody abolished platelet-enhanced Th1 responses, whereas TGFB1 neutralization had no effect on Th1 responses or did not offer additive effect on the inhibition by PF4 neutralization (Figure 1C). RANTES neutralization or aspirin treatment of platelets (ie, inhibition of TXA2 synthesis) did not influence platelet-enhanced Th1 responses. Similar trend of PF4/TGFB/ RANTES neutralization and aspirin treatment were also seen with Th17 responses (Figure 1E). As for Treg responses (Figure 1D), TGFB1 neutralization tended to reduce platelet-enhanced Treg responses (P = .08), while PF4 neutralization more markedly inhibited the enhancement. TGFB1 neutralization had no additive effect to the inhibition by PF4 neutralization. RANTES neutralization and aspirin treatment also mildly reduced platelet-enhanced Treg cell activation. As expected, rabbit or goat nonspecific polyclonal antibodies had no effects on platelet-enhanced CD4+ T cell responses (data not shown). Together, PF4 seems to play a dominant role in platelet-regulated CD4+ T effector responses of Tem cells. Further work of the present study was therefore focused on PF4-mediated regulation of Tem responses. Moreover, αCD3/αCD28 stimulation induced limited, and platelet co-culture had little influence on Th2 or Th17 responses in human Tem cells. Further work was thus focused on Th1 and Treg cell activation.

3.3 | Mass spectrometric analyses linked PF4-enhanced Tem activation with cell metabolism

Mass spectrometric analyses of Tem cell samples (three groups: unstimulated, αCD3/αCD28-stimulated, and αCD3/αCD28-stimulated/rhPF4-treated Tem cells) identified 1399 proteins. To demonstrate the protein mass difference between αCD3/αCD28-stimulated Tem cells without and with rhPF4, the orthogonal projections to latent structures-discriminant analysis (OPLS-DA) plot was generated, and showed a clear impact of rhPF4 treatment (Figure 2A). With the protein mass differences set at P = .05 and the fold change (FC) at 1.5, a volcano plot (Figure 2B) was generated. The plot identified that 77 proteins were up-regulated, and that 16 proteins were down-regulated by rhPF4 treatment. Among the top-50 up-regulated proteins, protein-protein interaction analyses with the String database demonstrated that metabolism-related proteins (colored) accounted for approximate one third of those up-regulated proteins, and that the metabolism-related proteins were functionally linked (Figure 2C). Moreover, the key transcription factor of mitochondrial biogenesis, TFAM, was also elevated significantly. These analyses indicated that cell metabolism may be the key in PF4-enhanced effector responses of Tem cells.

3.4 | Cell activation and platelet co-culture increased mitochondrial mass of Tem cells

As the power plant of cells, mitochondria are important for T cell survival, activation, differentiation, and proliferation.27 We investigated mitochondrial mass of CD4+ Tem cells upon αCD3/αCD28 stimulation without and with platelet co-culture. T cell stimulation markedly increased MitoTracker mean fluorescence intensity (MFI), indicating elevated mitochondrial mass, which was further enhanced in the presence of platelets (Figure 2D). Consistently, confocal microscopic analyses (Figure 2E) showed that unstimulated Tem cells had dim mitochondrial fluorescence, that stimulated Tem cells had elevated mitochondrial fluorescence, and that platelets markedly intensified mitochondrial fluorescence of activated Tem cells.

To illustrate platelet-derived mediators accountable for platelet-enhanced Tem mitochondrial mass, the above neutralizing/inhibiting agents were used. PF4 neutralization was found to dramatically decrease mitochondrial mass, not only abolishing the increment by platelets but also decreasing the elevation by Tem cell activation (Figure 2F). TGFB1 and RANTES neutralization, and aspirin treatment of platelets each attenuated the increase of mitochondrial mass by platelet co-culture, while combination of PF4 and TGFB1 neutralization did not lead to a stronger inhibition compared to PF4 neutralization alone. The flow cytometric results were further confirmed by
FIGURE 2  Platelets and platelet factor 4 (PF4) enhance metabolic protein clustering and mitochondrial mass of activated Tem cells. A, Quality and difference control of mass spectrometric samples. n = 3. B, Volcano plot of mass spectrometric analyses depicting PF4-regulated protein expression in activated Tem cells after 3-day cultures. C, Functional cluster of PF4-up-regulated proteins in activated Tem cells using the online database String. Dots related to cell metabolism were colored. D, Tem cells were cultured under the conditions stated for 3 days, and stained with MitoTracker® Deep Red FM (25 nM, 30 min at 37°C). The cells were analyzed using a Galios flow cytometer, and MitoTracker® mean fluorescence intensities (MFIs) were plotted as mean ± standard error of the mean (SEM), *P < .05, **P < .01, compared to unstimulated Tem cells; #P < .05, compared to activated Tem cells; n = 4. E, After 3-day culture, Tem cells were stained with MitoTracker® Red CMXRos (100 nM, 30 minutes at 37°C) and DAPI (1 µg/mL; Sigma). Fluorescent images were acquired using Zeiss LSM 700 laser scanning confocal microscopes (Carl Zeiss AG). A representative set of confocal images from five independent experiments is shown. F, Tem cells were cultured for 3 days without or with neutralizing antibodies for transforming growth factor β (TGFβ), PF4, regulated upon activation, normal T cell expressed and presumably secreted (RANTES), and platelet treatment with aspirin (100 µM, 30 minutes). Afterwards, T cells were stained with MitoTracker®, and mitochondrial mass was assessed by flow cytometry; mean ± SEM, **P < .01, compared to unstimulated Tem cells; ††P < .01, compared to activated Tem cells; **P < .05, ††P < .01, compared to activated Tem cells with platelet co-culture; n = 5. G, Representative fluorescence images of Tem cell mitochondrial staining upon inhibition of platelet-derived TGFβ, PF4, RANTES, and thromboxane A₂.
Platelet factor 4 (PF4) enhances CD4+ Tem activation and mitochondrial biogenesis via its chemokine, but not pro-coagulant, activities. Tem cells were cultured without or with αCD3/CD28 stimulation, in the absence or presence of recombinant human PF4 (rhPF4; 5 μg/mL), without or with the PF4 receptor CXCR3 inhibitor (±)-AMG487 (1 μM) or recombinant hirudin (20 μg/mL) for 3 days. Flow cytometric analyses were performed using a Galios flow cytometer. A, Representative overlap histogram of Tem cells stained with Brilliant Violet 510™ anti-human CD183/CXCR3 MAb (Cat# 353725; BioLegend) and the corresponding isotypic control antibody. B, The cells were stained with MitoTracker®, and analyzed using a Galios flow cytometer. MitoTracker® mean fluorescence intensities (MFIs) were plotted. C, D, The cells were intracellularly stained for the phenotyping of Th1/IFNγ and Treg/CD25+FoxP3+. Mean ± standard error of the mean (SEM), n = 6; *P < .05, **P < .01, compared to unstimulated Tem cells; †P < .05, compared to activated Tem cells, ††P < .05, †††P < .01, compared to activated Tem cells with rhPF4. E, Tem cells were cultured with vehicle or transfected with CXCR3-SmartPool siRNAs or Plus control-transfected Tem cells were monitored by flow cytometry, and presented as CXCR3 expression index (= CXCR3-positive % × CXCR3 MFI/100; panel E; mean ± SEM, n = 3; *P < .05, compared to non-transfected Tem cells; #P < .05, compared to CXCR3-siRNA transfected Tem cells). F, G, Vehicle-treated or transfected Tem cells were then subjected to αCD3/αCD28 stimulation in the absence or presence of rhPF4 (5 μg/mL) for 3 days. After this time, flow cytometric phenotyping of Th1 (F) and Treg cells (G) were performed. Mean ± SEM, n = 3; *P < .05, **P < .01, compared to activated Tem cells; †P < .05, ††P < .01, compared to activated Tem cells with rhPF4; †††P < .05, ††††P < .01, compared to activated CXCR3-siRNA transfected Tem cells with rhPF4. H, Unstimulated and activated Tem cells were cultured without and with platelets (1:250 Tem:platelets) or rhPF4 (5 μg/mL). PF4 binding of Tem cells were assessed by flow cytometry after surface staining of PF4 using PE-conjugated CXCL4/PF4 antibody (IC7952P; R&D Systems). Mean ± SEM, n = 5; **P < .01, compared to unstimulated Tem cells; ††P < .01, compared to activated Tem cells, †††P < .01, compared to activated Tem cells with platelet co-culture. I, J, Tem cells cultured for 3 days without or with transforming growth factor β or PF4 neutralization antibodies or in the absence or presence of the PF4 receptor CXCR3 inhibitor (±)-AMG487 (1 μM). Afterward, Tem cells were were stained for flow cytometric analyses of PF4 binding. Mean ± SEM, n = 5 for both; **P < .01, compared to unstimulated Tem cells; †P < .05, ††P < .01, compared to activated Tem cells, †††P < .05, ††††P < .01, compared to activated Tem cells with platelet co-culture or rhPF4. K–M, Tem cells were stained with MitoTracker®, or intracellularly stained for the phenotyping of Th1/IFNγ and Treg/FoxP3+. Mean ± SEM, n = 7/panel K, n = 14/panels L-M; **P < .01, compared to unstimulated Tem cells; ††P < .01, compared to activated Tem cells, †††P < .05, compared to activated Tem cells with rhPF4.
confocal microscopy (Figure 2G). These findings confirm that PF4 is the key platelet-derived mediator in platelet-regulated Tem cell activation. The data also suggest that increased mitochondrial biogenesis is a common node of platelet regulation in Tem cell responses, as each of the neutralizing agents reduced mitochondrial mass in CD4+ Tem cells at day 3.

3.5 | PF4 acts through CXCR3

The chemokine receptor CXCR3 has been identified as a functional receptor of PF4.

The expression of CXCR3 on CD4+ Tem cells was confirmed by flow cytometry, with a CXCR3 expression rate at 56.9 ± 6.7%, compared to 2.3 ± 0.1% in the cells stained with isotypic control antibody (n = 3; Figure 3A). The CXCR3 inhibitor (±)-AMG487 blocked recombinant human PF4 (rhPF4)-increased mitochondrial mass of activated Tem cells, but had no effects on αCD3/αCD28-elevated mitochondrial mass (Figure 3B). Accordingly, (±)-AMG487 abolished rhPF4-enhanced Th1 and Treg responses of stimulated Tem cells (Figure 3C,D). Furthermore, CXCR3-specific siRNA transfection decreased, but the random RNA control had no effect on CXCR3 expression on Tem cells (Figure 3E). CXCR3 knock-down by siRNAs abolished the enhancements of rhPF4 on Th1 and Treg responses of Tem cells was confirmed by flow cytometry, with a CXCR3 expression rate at 56.9 ± 6.7%, compared to 2.3 ± 0.1% in the cells stained with isotypic control antibody (n = 3; Figure 3A). The CXCR3 inhibitor (±)-AMG487 blocked recombinant human PF4 (rhPF4)-increased mitochondrial mass of activated Tem cells, but had no effects on αCD3/αCD28-elevated mitochondrial mass (Figure 3B). Accordingly, (±)-AMG487 abolished rhPF4-enhanced Th1 and Treg responses of stimulated Tem cells (Figure 3C,D). Furthermore, CXCR3-specific siRNA transfection decreased, but the random RNA control had no effect on CXCR3 expression on Tem cells (Figure 3E). CXCR3 knock-down by siRNAs abolished the enhancements of rhPF4 on Th1 and Treg responses of Tem cells.
**FIGURE 5** Inhibition of mitochondrial function by rotenone abolishes platelet- or recombinant human platelet factor 4 (rhPF4)-enhanced CD4⁺ T effector cell responses of Tem cells. Unstimulated and stimulated Tem cells were cultured for 3 days in the absence or presence of vehicle, 0.01, 0.1, and 1 μM rotenone. A, Phase contrast images of cultured Tem cells were captured using an Olympus CKX41 inverted light microscope (Olympus Corporation) equipped with a Nikon D5100 camera (Nikon Corporation). B, Cell proliferation of cultured Tem cells was monitored using an ABX Micros 60 Cell Counter. Data plotted are mean ± standard error of the mean (SEM), n = 3. C, Tem cells were stained with MitoTracker® Red, and analyzed using a Zeiss LSM 700 laser scanning confocal microscope. D–F, unstimulated and activated Tem cells were cultured without or with platelets or rhPF4, and in the absence or presence of 0.01 μM rotenone. After 3-day culture, Tem cells were stained for flow cytometric phenotyping of mitochondrial biogenesis/MitoTracker® (D), Th1/IFNγ⁺ (E), and Treg/CD25⁺⁺-FoxP3⁺ (F). Data plotted are mean ± SEM, n = 6; *P < .05, **P < .01, compared to unstimulated Tem cells; †P < .05, ††P < .01, compared to activated Tem cells; #P < .05, ##P < .01, compared to activated Tem cells with platelet co-culture.
cells, but had no influence on Th1 or Treg responses of Tem cells stimulated without rhPF4 (Figure 4F,G). Altogether these data support that PF4 exerts regulatory effects via CXCR3 on Tem cells (Figure 3A-G).

Flow cytometric analyses showed that Tem cell activation per se did not increase PF4 binding on the cell surface. However, PF4-binding on activated Tem cells was increased in activated Tem cells co-cultured with platelets, and was increased even more markedly in the presence of rhPF4 (Figure 3H). When intracellular staining was performed, PF4 MFIs did not differ from those of surface staining (data not shown). These observations indicated that PF4 exerted its action on Tem cell surface, and that PF4 was from external resources. Moreover, PF4 neutralization by an antibody and PF4 receptor blockade by (±)-AMG487 either abolished or markedly reduced PF4 binding on activated Tem cells (Figure 3I, J), while TGFβ neutralization or vehicle had no influence on PF4 binding of Tem cells.

PF4 is best known for its ability to neutralize heparin-like molecules, and thus enhances thrombin activity and blood coagulation. To further confirm the PF4 effect and to understand if pro-coagulant activities of PF4 are involved in PF4-regulated Tem responses, a follow-up experiment was performed using rhPF4 and the thrombin inhibitor hirudin. Figure 3K shows that rhPF4 enhanced CD3/CD28-stimulated elevations of mitochondrial mass, and that the elevated mitochondrial mass was not affected by hirudin. Consistently, Th1 and Treg responses were not influenced by hirudin (Figure 3L, M). The results also showed that thrombin inhibition by hirudin did not significantly attenuate the enhancements by rhPF4, indicating that the procoagulant activity of PF4 has little impact on PF4 regulation of Tem responses.

### 3.6 Platelets enhanced mitochondrial biogenesis via Akt-PGC1α-TFAM signaling

TFAM is synthesized in the nucleus and then transported to mitochondria, for which TFAM serves as the key activator of mitochondrial transcription and genome replication. Tem cell activation increases TFAM expression, as evidenced by an intensified TFAM immunoreactive band, and the increase was further enhanced in the presence of platelets (Figure 4A, B). Similarly, supplement of rhPF4 to CD3/CD28-stimulated Tem cells also markedly enhanced TFAM expression (Figure 4D, E). As expected, elevations of TFAM expression were correlated to increases of Tem cell mitochondrial mass (Figure 4C, F).

To further illustrate the mechanisms underlying PF4-enhanced mitochondrial biogenesis, Tem cells were stimulated in the absence or presence of rhPF4 for 6 hours. Figure 4G shows that rhPF4 treatment reduced Akt phosphorylation/activity of αCD3/αCD28-stimulated Tem cells, and subsequently attenuated PGC1α phosphorylation. The reduced PGC1α phosphorylation, which is known to be associated with an enhanced PGC1α activity, will therefore enhance TFAM expression in αCD3/αCD28-stimulated Tem cells that has been shown in Figure 4D after 3-day culture.

### 3.7 Inhibition of mitochondrial function counteracted platelet enhancement on Tem responses

Rotenone is a mitochondrial complex I inhibitor that blocks the complex I oxidative respiratory chain and thus inhibits mitochondrial function and ATP production. A range of rotenone concentrations (0.01, 0.1, and 1 µM) was examined during 3-day culture of activated Tem cells. Rotenone at 0.01 µM appeared to influence cultured Tem cells little as monitored by cultured Tem cell morphology (Figure 5A) and cell counts of activated Tem cells (Figure 5B). In contrast, higher concentrations (0.1 and 1 µM) of rotenone significantly hampered Tem cell survival as evidenced by reduced cell densities/counts (Figure 5A, B). Thus, 0.01 µM rotenone was chosen for further experiments. Rotenone abolished platelet-induced increases of mitochondrial mass (Figure 5C, D). The same effects of rotenone were also seen when rhPF4 was supplemented, in which rhPF4-elevated mitochondrial mass was inhibited. Rotenone-inhibited mitochondrial mass subsequently led to a complete inhibition of platelet-enhanced Th1 responses and an even greater inhibition on rhPF4-enhanced Th1 responses (Figure 5E). Moreover, rotenone treatment abolished both platelet- and rhPF4-enhanced Treg cell activation (Figure 5F). Together, these data give further support that PF4 exerts enhancements on CD4+ T effector cell responses of activated Tem cells via increasing mitochondrial biogenesis.

### 3.8 TFAM overexpression enhanced CD4+ effector cell responses

Our data indicated that PF4-increased mitochondrial mass is closely linked to CD4+ Tem cell responses. In order to further confirm that platelets regulate CD4+ Tem responses via the axis of PF4-mitochondria-CD4 T effector responses, we conducted experiments to enhance mitochondrial function via TFAM overexpression by infection of TFAM-adenovirus (TFAM-AV). As shown in Figure 6A-D, TFAM-AV infection (multiplicity of infection [MOI] = 10) markedly increased mitochondrial mass/fluorescence of activated Tem cells, and to an extent not less than the elevated levels in the presence of rhPF4. Transfection of activated Tem cells with control adenovirus did not increase Tem mitochondrial mass, which was similar to activated Tem cells without transfection (Figure 6B, E). TFAM-AV-enhanced mitochondrial mass was also confirmed by flow cytometric analyses (Figure 6F). TFAM-AV infection enhanced mitochondrial mass in activated Tem cells to the level similar to that by rhPF4 supplementation, while the control adenovirus infection did not change mitochondrial mass in activated Tem cells. Consistent with our above observations, the TFAM-AV infection-enhanced Tem mitochondrial mass was associated with enhanced Th1 and Treg cell activation (Figure 6G-H). The levels of enhancements were similar to those by rhPF4 supplementation.
3.9 | Mitochondrial ATP and ROS production enhanced effector cell responses of Tem cells

The present data have clearly demonstrated a strong link between PF4-enhanced Tem effector cell responses and mitochondrial biogenesis. It is natural to ask how the products of mitochondrial metabolism, namely ATP and ROS, regulate T effector cell responses of Tem cells. Aiming to elucidate the impact of PF4-enhanced mitochondrial metabolism, the ATP synthase inhibitor oligomycin was used at a low concentration (0.1 nM), which inhibited ATP production of mitochondria, but largely retained the cell reactivities (Figure S4A, B in supporting information), and N-acetyl-L-cysteine (NAC; 1 mM) was used as the ROS scavenger. As expected, CD3/CD28 stimulation increased, and PF4 supplementation further enhanced ROS and ATP production of Tem cells (Figure 7A, B). NAC abolished PF4-enhanced ROS production of activated Tem cells, but also reduced ROS production of activated Tem cells in similar proportion. Oligomycin markedly reduced ATP production of activated Tem cells without and with PF4 supplementation to the same level (Figure 7A, B).

Flow cytometric analyses showed that ROS scavenging by NAC reduced PF4 enhanced T-bet expression and Th1 phenotype to those levels of αCD3/αCD28-stimulated Tem cells, while the scavenger also reduced T-bet expression and Th1 phenotype of αCD3/αCD28-stimulated Tem cells (Figure 7C, E). Inhibition of mitochondrial ATP production by oligomycin not only abolished PF4-enhanced but also reduced αCD3/αCD28-induced expression of the Th1 transcription factor T-bet and Th1 phenotype (Figure 7C, E). Similar to Th1 responses, inhibition of mitochondrial ATP production by oligomycin reduced FoxP3 and Treg levels of activated Tem cells.
cells to the same levels regardless the presence of rhPF4. ROS scavenging by NAC abolished PF4-enhanced FoxP3 expression and Treg phenotype. However, the scavenging did not significantly reduce αCD3/αCD28-stimulated FoxP3 expression and Treg phenotype of Tem cells, albeit a weak trend was observed (Figure 7D,F). Thus, these findings indicate that mitochondrial ROS production primarily exerts its impact on PF4-enhanced Treg responses.

Using the Seahorse assay to measure cellular oxygen consumption (OCR), we found that αCD3/αCD28 stimulation markedly increased basal cellular respiration of Tem cells compared to unstimulated cells, and that rhPF4 supplementation enhanced OCR further (Figure 7G,H). Together, our data showed that PF4 enhanced mitochondrial biogenesis of activated Tem cells, increased oxygen consumption and thus metabolic production of mitochondria, and

**FIGURE 7** Platelet factor 4 (PF4) enhances Th1 and Treg activation of Tem cells via mitochondrial adenosine triphosphate (ATP) and reactive oxygen species (ROS) production. A–F, Unstimulated Tem cells and αCD3/αCD28-stimulated Tem cells were cultured in the absence or presence of recombinant human PF4 (rhPF4; 5 µg/mL), oligomycin (0.1 nM), N-acetyl-L-cysteine (1 mM), and vehicle (0.01% DMSO) for 3 days. Afterward, Tem cells were harvested, and loaded with the ROS probe DCFH-DA (10 µg/mL at 37°C for 20 min) to monitor ROS production using flow cytometry (A). Tem cells were also collected for the measurement of ATP production using an adenosine diphosphate (ADP)/ATP ratio assay kit (B). Flow cytometric intracellular staining was performed to monitor the expression of the Th1 transcription factor T-bet (C) and Th1 phenotype (E), and to monitor the expression of the Treg transcription factor FoxP3 (D) and Treg phenotype (F). Mean ± standard error of the mean (SEM), n = 6. *P < .05, **P < .01, compared to unstimulated Tem cells; †P < .05, ††P < .01, compared to CD3/CD28-stimulated Tem cells; †P < .05, ††P < .01, compared to activated Tem cells with rhPF4. G–H, CD4+ Tem cells were cultured for 3 days without or with αCD3/αCD28-stimulation and in the absence or presence of rhPF4 (5 µg/mL). The cells were then harvested and seeded in 96-well XF plates at 1-2 × 10^5 cells/well. The basal oxygen consumption rates (OCR) of Tem cells were assessed using a Seahorse XF 96 Analyzer at baseline and after addition of antimycin A and rotenone (2.5 and 2.5 µM, respectively) (G). Mean ± SEM, n = 7-8; P < .01, rhPF4-treated activated Tem cells versus activated Tem cells or unstimulated Tem cells, as analyzed by repeated measurements analysis of variance. Basal OCRs were presented as mean ± SEM (H). *P < .01, compared to unstimulated Tem cells; ††P < .01, compared to αCD3/αCD28-stimulated Tem cells; n = 7-8.
subsequently led to the elevations of CD4+ T effector cell transcription factor expression and effector cell responses (Figure 8).

4 | DISCUSSION

The present study demonstrated that PF4-enhanced mitochondrial biogenesis and metabolism play a critical role in platelet-regulated Tem cell responses in vitro using human cells. It was found that platelet co-culture exerted a transient enhancement of Th1 effector responses of Tem cells, but a persistent enhancement on Treg responses of Tem cells. Notably, PF4 was the primary platelet-derived mediator in these regulations and exerted the effects via mitochondrial metabolisms. PF4 attenuated Akt activation of activated Tem cells, reduced PGC1α phosphorylation, resulted in enhanced PGC1α activities, and therefore elevated TFAM expression. The latter increased mitochondrial biogenesis, and thus elevated ATP and ROS production of Tem cells. ATP and ROS subsequently exerted feedback signals to stimulate the expression of Th1/T-bet and Treg/FoxP3 transcription factors and corresponding CD4+ effector cell responses of Tem cells (summarized in Figure 8).

Polyclonal stimulation enhanced T effector responses of Tem cells, characterized by a rapid and marked Th1 response but mild Th2/Th17 responses. αCD3/αCD28 stimulation also evoked rapid and marked Treg activation of Tem cells. Platelets enhanced Th1 responses markedly (two- to three-fold) and transiently (maximized on day 3 and followed by a quick decrease), while platelets continuously enhanced Treg responses of Tem cells with the plateau reached on day 3. The persistent enhancements of Treg responses should arise from the effects that platelets selectively enhance cell proliferation and activation of FoxP3+ CD4+ T cells. The distinct dynamics of platelet-regulated CD4+ T effector cell responses indicate that platelets may promote prompt and strong proinflammatory Th1 responses upon Tem cell re-encountering antigen, while platelet-enhanced persistent Treg cell activation may help inflammation resolution during the subacute phase.

Compared to the partial inhibition by TGFβ or RANTES neutralization or blockade of TxA2 synthesis by aspirin, PF4 neutralization abolished platelet-dependent enhancements on Th1 and Treg activation of Tem cells. PF4 has thus emerged as the leading platelet-derived regulator of effector cell responses in Tem cells, apart from its regulatory effects on other aspects of immune responses, such as macrophage polarization and function. The notion was further supported by the observation showing that rhPF4 supplement mimicked the enhancements by platelet co-culture. PF4 appeared to exert the effects independently from its pro-coagulant activities, as the direct thrombin inhibitor hirudin had little influence on PF4-enhanced T effector cell responses. In contrast, it seemed to regulate CD4+ T effector cell responses through receptor-dependent chemokine effects. PF4 has several receptors, including glycosaminoglycan (GAG), CXCR3, CXCR4, αV integrins, as well as recently reported αM integrin Mac1/CD11b, PF4-CXCR3.
ligation is accountable in the present observations because both CXCR3 blockade by its inhibitor AMG487 and CXCR3 knock-down by siRNAs diminished rhPF4- or markedly attenuated platelet-enhanced T effector cell responses. Other PF4 receptors are of functionally less importance in the present setting, because GAG and αv are considered as co-receptors, while LRP1 and Mac1/CD11b are poorly expressed on T cells. Notably, PF4-CXCR3 ligation attenuated Akt phosphorylation/activity. This is because CXCR3 has CXCR3-A and CXCR3-B isomers. PF4 selectively binds CXCR3-B that elevates cyclic adenosine monophosphate levels and subsequently inhibits Akt phosphorylation/activity. In contrast, other CXCR3 ligands, such as CXCL9/10/11, selectively or mainly bind CXCR3-A, and activates Akt. PF4-inhibited Akt activity leads to reduced PGC1α phosphorylation, but resulted in enhanced PGC1α activities, and thus enhanced TFAM expression and mitochondrial biogenesis (Figures 3N and 8). It should also be noted that heteromerization of chemokines is of great importance for chemokine functions. Platelets release a number of chemokines. PF4 is known to heteromerize with other platelet-derived chemokines, such as CCL2/MCP-1, CCL5/RANTES, CXCL7/β-thrombogogulin, and CXCL12/stromal cell-derived factor-1α (SDF-1α). It has been shown that PF4-RANTES heteromers promoted monocyte arrest on the vessel wall, while disruption PF4-RANTES heteromerization hampered monocyte arrest and subsequently attenuated atherogenesis in hyperlipidemic mice. RANTES inhibition attenuated platelet-enhanced CD4⁺T effector cell responses in the present work, which may be attributed to RANTES cooperation with PF4.

Cell metabolism drives T cell function. It is clear that mitochondrial metabolism can shape T cell activation, proliferation, and differentiation. Interestingly, the present work demonstrated that platelets regulate CD4⁺ T effector cell responses of Tem cells through mitochondrial biogenesis and metabolism. Platelet-derived mediator PF4 markedly elevated TFAM expression, increased mitochondrial biogenesis/mass, enhanced mitochondrial oxygen consumption, and subsequently ATP and ROS production. In line with findings, TFAM overexpression of Tem cells by TFAM-AV infection increased mitochondrial mass and subsequently enhanced Th1 and Treg responses of Tem cells. Inhibition of mitochondrial respiratory chain and ATP production by the mitochondrial complex I inhibitor rotenone attenuated platelet- and rhPF4-enhanced CD4⁺ T effector responses. Our results also showed that inhibition of PF4-enhanced mitochondrial production of ATP and ROS by the ATP synthase inhibitor oligomycin and the ROS scavenger NAC abolished PF4-dependent elevations of Th1 and Treg transcription factor expression and phenotypes, indicating the importance of mitochondrial metabolism for the PF4 effects. Moreover, ROS scavenging by NAC attenuated the Th1 effector responses more markedly, while inhibition of ATP production by oligomycin reduced Treg cell activation more intensely. Our findings reinforce the notion that the mitochondrial metabolites ATP and ROS are important regulators of effector responses of Tem cells. Our results also indicate that mitochondrial production of ROS and ATP have preferential impacts on Th1 and Treg responses, respectively.

The present work has for the first time, to our best knowledge, demonstrated that PF4 exerts an immune regulatory effect through mitochondrial biogenesis (Figure 8). Our findings have added a good piece of evidence that platelets are a pivotal player in inflammatory responses. It is known that CD4⁺ T cell immunity malfunctions in atherosclerotic patients, seen as increased pro-inflammatory Th1 but decreased anti-inflammatory Treg cell frequencies and activities. Our findings of distinct regulation of Th1 and Treg cell responses by platelets and PF4 suggest potential intervention sites for therapeutic developments targeting platelet inflammatory activities. Moreover, this notion is supported by the recent CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) trial demonstrating that the anti-inflammatory therapy with the anti-IL-1β antibody canakinumab reduces cardiovascular events in high-risk atherothrombotic patients. Furthermore, programmed cell death protein-1 (PD-1) blockade has brought about several therapeutic successes in cancers, relying on enhanced mitochondrial biogenesis and activation of T cells. This implies that manipulation of PF4-mediated CD4⁺T cell mitochondrial biogenesis and activation may be a useful approach of immunotherapies.

In conclusion, platelets enhance CD4⁺ T effector cell responses of Tem cells with a quick and transient Th1 response burst and a persistent Treg cell activation. The enhancement is exerted by PF4-dependent and Akt-PGC1α-TFAM signaling-mediated mitochondrial biogenesis and metabolism. Therefore, the PF4-dependent mechanism represents a new immune-regulatory machinery and may serve as an intervention target for novel therapeutic development of atherothrombotic diseases.

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CONFLICTS OF INTEREST

P.-O. Berggren is the cofounder and CEO of Biocrine AB, Sweden. All the other authors state that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

S. Tan designed the study, performed experiments, interpreted the data, and wrote the manuscript; S. Li, Y. Min, A. Gisterå, N. Moruzzi, J. Zhang, and Y. Sun designed the study, performed research, interpreted the data, and revised the manuscript; J. Andersson, R. Malmström, M. Wang, P.-O. Berggren, S. Schlislo, W. Liao, D. J. Ketelhuth, and C. Ma designed the study, interpreted the data, and revised the manuscript; N. Li designed the study, interpreted the data, organized the research, and wrote the manuscript.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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