Supplementary Material to Chen, Rivera et al. “PreImplantation factor prevents atherosclerosis via its immunomodulatory effects without affecting serum lipids” (Thromb Haemost 2016; 115.5)

Suppl. Figure 1: Gene expression of VCAM-1 and IFN-γ is down regulated by PIF in the TNF-α stimulated RAW cells. Graph represents VCAM-1 and IFN-γ gene expression compared to the 18S house keeping gene at time points 0, 1, 8, 12 and 24 hours. PIF treatment is represented by the circle dot line showing down regulation, compared to the scPIF control as shown by the square dot line (n=9; mean ± SD; p=0.0074 in VCAM-1 and p=0.049 in IFN-γ).
**Suppl. Figure 2: PIF does not affect H$_2$O$_2$ production in monocytes.** PIF (1 µM, 10 µM) treated monocytes do not lose the ability to release the reactive oxygen species (ROS) hydrogen peroxide. Leukocytes from mouse whole blood were stained with Ly6C then subsequently treated with either PIF (1 µM, 10 µM) or scPIF (1 µM, 10 µM) for H$_2$O$_2$ production. PIF did not have effects on the hydrogen production compare to scramble PIF peptide control or the PBS control (n=3). DHR: dihydrorhodamine 123.
Suppl. Figure 3: PIF reduced CD4T cells in blood and does not affect other immune cell subtypes in spleen or peritoneal fluid. PIF (10 µM) treatment of mice results in a significant reduction of CD4 T cell numbers in blood compared to scPIF treatment (10 µM). However, CD4 T cells do not change in spleen and peritoneal fluid. M1 (CD11b+ Ly6C high), M2 (CD11b+ Ly 6C low) macrophages, B1 (CD5+ CD19+), B2 (CD5- CD19+), CD4 (TCRβ+ CD4+), CD8 (TCRβ+ CD8+), NK (TCRβ- NK1.1+), and NKT (TCRβ+ NK1.1+) lymphocytes remain unchanged between two groups.
Suppl. Figure 4: Proliferation marker Ki67 and apoptosis marker Annexin V did not change by PIF treatment in atherosclerosis. CD68 is significantly reduced by PIF treatment. Counting CD68 (A) positive cells per microscope view shows consistent reduction of foam cells in the PIF treated group. Isotype control antibodies do not show background staining (B). PIF (10 μM) treated mice have similar Ki67 (C) and Annexin V (D) expression compared to scPIF (10 μM) in the atherosclerotic area of the aortic sinus.
Suppl. Methods

H$_2$O$_2$ production in leukocytes

Whole blood (100 µl) of mice was pretreated with PIF (1 µM, 10 µM), scPIF (1 µM, 10 µM) or PBS (as vehicle control). Leukocytes were purified by lysing the red blood cells and incubating with Dihydrorhodamine 123 (DHR, Invitrogen) for 5 mins at 37 °C before PMA was added to activate leukocytes (10 min at 37 °C). As a positive control leukocytes were incubated with PBS (as vehicle control) instead of peptides. As negative control, leukocytes were not activated with PMA. Hydrogen peroxide oxidizes DHR to the highly fluorescent rhodamine. Leukocyte populations were identified with Ly6C (BD). Rhodamine was detected by flow cytometry in the FITC channel and analyzed using CantoII.

TNF stimulated Raw cells gene expression

RAW 264.7 cells were grown in DMEM with 10 % fetal bovine serum and calculated to ~ 1 million/ml using the Z2 Coulter® Particle Count and Size Analyzer. Raw cells were treated with 10 µM PIF, 10 µM scPIF or PBS of the equivalent volume and incubated for 48 h. After incubation, each flask was divided between 5 wells containing 2 mL of media each. PIF and scPIF treated cells were stimulated with 10 µg/mg of TNF-α (SIGMA, Missouri, USA) and collected at 0, 1, 8, 12 and 24 h time points. The unstimulated PBS control cells were collected at the corresponding time points. The cells were centrifuged at 1500 rpm for 10 min at room temperature. A cell pellet formed at the base and the above media was discarded. Cell pellets were then washed with PBS and re-spun for 10 min at 1500 rpm. To extract mRNA, 700µl of QIAzol® Lysis Reagent (QIAGEN, Doncaster, Australia) was added to the cell pellets for cell rupture, which was followed by the application of the RNeasy mini kit (QIAGEN, Doncaster, Australia). RNA was then frozen at -80 °C for later RT-PCR.
PIF binding probability to different potassium channels families

PIF binding to different potassium channel families was evaluated using a bioinformatics workflow including acquiring lists of PDB models (RCSB Protein Data Bank retrieved) corresponding to several potassium channel groups (GO: regulation of potassium ion transmembrane; transporter activity (GO ID:1901016); KCNAB; Potassium channel protein; SCOP: Superfamily of Voltage-gated potassium channels (oligomeric transmembrane alpha-helical proteins); Voltage Gated Potassium Channels; Voltage-dependent K+ channel beta subunit), predicting PIF putative binding to these models using PepSite ver 2 server (Taverna workbench operated RESTful queries on pepsite2.russelllab.org). Each prediction consisted of 10 weights (M, V, R, I, K, P, G, S, A, N) corresponding to first 10 amino acids sequence of PIF (encoding 0 - no binding, 1 - binding to putative target), binding score of PIF to target and p-value representing probability of the prediction. The data was analyzed using data mining suite Orange (http://orange.biolab.si/). Euclidian distance matrix on PIF predictive data were subject to a hierarchical clustering analysis and dendrogram plot. The PIF predictive score, p-value and target Groups were plotted using multidimensional analysis Parallels plot tool. Score was filtered to range 100–80, p-values were filtered to range 0–0.005, resulting in pale colour coded links.

The unsupervised learning mode of an artificial neural network of type self-organizing map (SOM) was used to produce a 2-dimensional, discretized representation of the input space (PIF prediction analysis parameters) in order to create discrimination rules for distinguishing potassium channels based on PIF binding. In Orange, SOM nodes were generated to have two-dimensional regular spacing in a hexagonal grid topology. Associated with each node is a weight vector of the same dimension as the input data vectors, and a position in the map space. Neighbourhood function was Gaussian. The training of the net was done in 100 iterations. Orange was used to visualize the SOM by plotting the hexagonal
nodes using prediction Scores as background colour, and weight vector represented as circle with radius and colour depending on the Majority value of the specific potassium channel group. Interpretation: Because in the training phase weights of the whole neighbourhood are moved in the same direction, similar items tend to excite adjacent neurons. Therefore, SOM forms a semantic map where similar samples are mapped close together and dissimilar ones apart.

**Flow cytometry for immune cell subsets**

For immune cell subset studies lymphocytes were stained with fluorochrome-labeled antibodies (all from BD Biosciences unless otherwise stated). The antibodies used were anti-CD19 (PE-Cat# 12-0193-81, eBioscience), anti-CD5 (APC-Cat# 550035), anti-CD25 (APC-Cy7-Cat# 557658), anti-CD4 (Pacific Blue-Cat# MCD0428, Caltag Laboratories), anti-CD8 (PerCP-Cat# 553036), anti-TCR-β (FITC-Cat# 11–596185, eBioscience), anti-NK1.1 (PE-Cy7-Cat# 552878) and anti-foxp3 (PE-Cat# 12-5773-82, eBioscience). For surface markers, single cell suspensions were stained with multiple antibodies at 4 °C for 30 min, then washed and resuspended in PBS with 1 % FCS. For regulatory T cells, anti-CD4 and anti-CD25 stained cells were fixed, permeabilized and further stained with an anti-foxp3 antibody. FACS Canto II (BD Biosciences) was used to collect data from different fluorochrome-labeled cells. FACS Diva software (BD Biosciences) was used to analyze the data. For Ly6C analysis of monocytes, fluorochrome-labeled antibodies were used as following: anti-CD115 (APC-Cat#17115280), anti-CD11b (PE-Cat#12011281) and Ly6C (V450-BD-Cat#560594). Data was subsequently analyzed using FACS Diva software (BD Biosciences).