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Chen YC, Rivera J, Fitzgerald M, Hausding C, Ying YL, Wang X, Todorova K, Hayrabyan S, Barnea ER, Peter K. Preimplantation factor prevents atherosclerosis via its immunomodulatory effects without affecting serum lipids. Thromb Haemost 2016;115(5):1010-24.

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PreImplantation Factor Prevents Atherosclerosis via its Immunomodulatory Effects without Affecting Serum Lipids

Journal:	<i>Thrombosis and Haemostasis</i>
Manuscript ID	TH-15-08-0640.R1
Manuscript Type:	Original Article: Atherosclerosis and Ischaemic Disease
Category:	Basic Science
Date Submitted by the Author:	n/a
Complete List of Authors:	Chen, Yung-Chih; Baker IDI Heart and Diabetes Institute, Rivera, Jennifer; Baker IDI Heart and Diabetes Institute, Fitzgerald, Melissa; Baker IDI Heart and Diabetes Institute, Hausding, Christian; Baker IDI Heart and Diabetes Institute, Ying, Ya-Lan; Baker IDI Heart and Diabetes Institute Wang, Xiaowei; Baker IDI Heart and Diabetes Institute, ; Monash University, Departments of Medicine and Immunology Todorova, Krassimira; Academician Bratanov Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences Hayrabyan, Soren; Academician Bratanov Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences Barnea, Eytan; Society for the Investigation of Early Pregnancy (SIEP) and BioIncept, LLC, Peter, Karlheinz; Monash University, Departments of Medicine and Immunology
Keywords:	Atherosclerosis, Inflammatory mediators, Macrophage

PreImplantation Factor Prevents Atherosclerosis via its Immunomodulatory Effects without Affecting Serum Lipids

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Word count: 5,467

Abstract:

Aims: PreImplantation factor (PIF) is a 15-amino acid peptide endogenously secreted by viable embryos, regulating/enabling maternal (host) acceptance/tolerance to the “invading” embryo (allograft) all-while preserving maternal immunity to fight infections. Such attributes make PIF a potential therapeutic agent for chronic inflammatory diseases. We investigated whether PIF’s immunomodulatory properties prevent progression of atherosclerosis in the hyper-cholesterolemic ApoE-deficient murine model.

Methods/Results: Male, high-fat diet fed, ApoE-deficient (ApoE^{-/-}) mice were administered either PBS, scrambled PIF (0.3-3mg/kg) or PIF (0.3-3mg/kg) for 7 weeks. After treatment, PIF (3mg/kg)-treated ApoE^{-/-} mice displayed significantly reduced atherosclerosis lesion burden in the aortic sinus and aortic arch, without any effect on lipid profile. PIF also caused a significant reduction in infiltration of macrophages, decreased expression of pro-inflammatory adhesion molecules, cytokines and chemokines in the plaque, and reduced circulating IFN- γ levels. PIF preferentially binds to monocytes/neutrophils. *In vitro*, PIF attenuated monocyte migration (MCP-1-induced chemotaxis assay) and *in vivo* in LPS peritonitis model. Also PIF prevented leukocyte extravasation (peritonitis thioglycollate-induced model), demonstrating that PIF exerts its effect in part by modulation of monocyte function. Inhibition of the potassium channel KCNAB3 (Kv1.3) and of the insulin degrading enzyme (IDE) was demonstrated as potential mechanism of PIF’s immunomodulatory effects.

Conclusions: PIF regulates/lowers inflammation and prevents atherosclerosis development without affecting circulating lipids. Overall our findings establish PIF as a strong immunomodulatory drug candidate for atherosclerosis therapy.

Keywords: Atherosclerosis, immune cells, ApoE-deficient mice, PreImplantation Factor (PIF), macrophage, immunomodulatory therapy.

53 **Introduction**

54 Previously considered merely as a lipid storage condition, atherosclerosis is now well
55 established as a chronic inflammatory disease where dyslipidemia and inflammation equally
56 play a pivotal role. Cells from both the innate and adaptive arms of the immune system,
57 including macrophages, neutrophils, dendritic cells, and T and B lymphocytes, have been
58 shown to contribute to atherogenesis ¹. In particular, extensive evidence has identified
59 monocytes and macrophages as key players in various stages of lesion progression. In the
60 early stages of atherogenesis, damaged or activated endothelium attracts the infiltration of
61 monocytes into the sub-endothelial space, where they differentiate into macrophages and
62 ingest native and modified lipoproteins to become foam cells ². During the intermediate and
63 later stages, macrophages secrete cytokines that attract other leukocytes to the atheroma and
64 amplify the inflammatory response, while apoptosis and necrosis of these macrophages
65 leads to the formation of the ‘necrotic core’, rendering plaques vulnerable and susceptible to
66 rupture ^{1,2}.

67 Current therapies for atherosclerosis and cardiovascular disease in general are based on
68 targeting risk factor management, such as the lipid lowering (statins) or diabetes
69 management ³. However, despite the extensive evidence of the role of inflammation in the
70 pathogenesis of atherosclerosis, there are currently no effective therapies that directly target
71 immune cells/inflammatory pathways. Ideally, anti-inflammatory or immunomodulatory
72 therapy should reduce inflammation without compromising normal immune function, and
73 this still remains a clinical challenge ^{1,3}.

74 An interesting immunological paradox arises during pregnancy, - the mother (host)
75 tolerates and accommodates the “invading” embryo (allograft) while preserving maternal
76 immunity, the ability to fight infections and regulate autoimmune processes ⁴⁻⁶. Hence, the
77 mammalian embryo can be considered a perfect immune regulatory scenario, maintaining

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4 78 basal immune balance and stepping-up when necessary to enhance immunity and acting as a
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6 79 perfect transplant. Furthermore, it is widely acknowledged that during pregnancy symptoms
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8 80 of autoimmune disease are alleviated, but flare up after pregnancy ⁷, suggesting that
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10 81 maternal-derived compounds may play an immunomodulatory role to improve these
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12 82 conditions.

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14 83 Preimplantation factor (PIF) is a novel embryo-specific 15 amino acid, linear
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16 84 peptide, isolated and characterised by Barnea and colleagues ^{8,9}. PIF is secreted and present
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18 85 in maternal circulation throughout viable pregnancy but absent from non-viable embryos ⁴.
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20 86 PIF has been shown to modulate the maternal environment to create a favourable embryo
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22 87 implantation milieu by interacting with immune cells that play a role in decidual changes
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24 88 necessary for appropriate placental formation and trophoblast invasion ^{5, 10-13}. These include
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26 89 antigen presenting, cytokine inducing and phagocytic maternal macrophages and dendritic
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28 90 cells ^{9, 14-17}. These characteristics make PIF an ideal candidate as an immunomodulatory
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30 91 therapeutic.

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34 92 Indeed, the therapeutic potential of the synthetic analog of PIF has been examined in
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36 93 several chronic inflammatory diseases *in vivo*. In experimental autoimmune encephalitis
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38 94 (EAE), a murine model of multiple sclerosis (MS), PIF-treated mice had a higher survival
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40 95 rate, lower pro-inflammatory IL-17 and IL-6 levels and advanced chronic paralysis was
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42 96 reversed ¹⁸. In a non-obese diabetic (NOD) mouse model of type 1 diabetes mellitus,
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44 97 subcutaneous administration of PIF prevented diabetes development while maintaining
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46 98 pancreatic function on a long-term basis, as shown by lowered plasma glucose levels,
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48 99 preservation of pancreatic islet architecture, a reduction in inflammatory cell infiltration and
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50 regulation of Th1/Th2 cytokine secretion ¹⁹. In the PHA-activated PBMC assay, PIF
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52 100 promotes anti-inflammatory IL-10 secretion and reduces pro-inflammatory IFN- γ ²⁰.
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54 101 Similarly, in semi- or allogeneic mouse bone marrow transplant models, two week
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103 administration of PIF reduced acute graft-versus-host disease while maintaining the
104 beneficial graft-versus-leukemia, reducing liver and skin inflammation and colon ulceration,
105 systematically reducing circulating pro-inflammatory IL17- α levels ²¹, while PIF also
106 promoted neuroprotection in a hypoxic-ischemic brain injury model ^{22, 23}.

107 Based on these findings, we postulated that synthetic PIF may exert an equally
108 beneficial effect on inflammation underlying atherosclerosis. Herein, we aimed to
109 characterize the therapeutic effects of PIF on atherosclerosis progression using the high fat
110 fed ApoE-deficient mouse model. Our results imply that PIF prevents atherosclerotic lesion
111 development independently of any lipid lowering effects via multiple mechanisms inhibiting
112 the potassium channel blocker Kv1.3 and the insulin degrading enzyme (IDE) and by
113 influencing monocyte-induced inflammation and function.

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115 **Materials and Methods**

116 *PIF peptide synthesis*

117 Synthetic PIF, a fifteen-amino-acid peptide (MVRIKPGSANKPSDD), scrambled PIF (con-
118 trol) (scPIF; GRVDPSNKSMPKDIA) were obtained from Biosynthesis Inc. (Texas, USA).

120 *Animals*

121 Male, ApoE^{-/-} mice on a C57BL6/J background were originally obtained from the Animal
122 Resource Centre (Western Australia, Australia). At six weeks of age, mice were placed on a
123 high fat diet (HFD; 22% fat, 0.15% cholesterol; SF00-219, Specialty Feeds, WA, Australia)
124 and tap water *ad libitum* throughout the experimental period. Mice were randomly assigned
125 to receive one of the following treatments: PIF (0.3mg/kg or 3mg/kg), PBS, or scrambled
126 PIF (0.3mg/kg or 3mg/kg). The mice received intra-peritoneal injections three times per
127 week for 7 weeks. All animal procedures were reviewed and approved by the Alfred
128 Medical Research Education Precinct Animal Ethics Committee and conducted in
129 compliance with the National Health and Medical Research Council of Australia's
130 (NHMRC) Guidelines for the Ethical and Humane Use of Animals in research.

132 *Tissue processing and analysis*

133 At the end of the 7 week treatment period, mice were euthanized with Pentobarbitone
134 100mg/kg (Delvet Limited, Seven Hills, Australia) for collection of blood and tissues.
135 Blood samples were collected via cardiac puncture. The aortic sinus and entire aortic arch,
136 were embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Japan),
137 Blood was centrifuged at 400 x g for 10 min and plasma was transferred to an Eppendorf
138 tube and stored at -80°C for measurement of plasma lipid levels and inflammatory markers
139 (ELISA). Blood, spleen and lymph nodes were also collected for subsequent flow cytometry
140 analysis in the immune cell subset study.

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Histology and immunohistochemistry

For histology and immunohistochemistry analysis of atherosclerotic lesions, the aortic arch and aortic sinus were sectioned at 6µm thickness. To allow for quantification of lesion area, lipid content and collagen sections were stained with either standard Mayer's hematoxylin/eosin (Sigma-Aldrich) or oil red O (Sigma-Aldrich) source, all according to manufacturer's instructions.

To assess MCP-1, VCAM-1, CD68, **Ki67 and Annexin V** expression, sections of the aortic sinus and aortic arch were used for immunohistochemistry as previously described ²⁴ Sections were **stained** with one of the following primary antibodies: VCAM-1 (Santa Cruz, CA, USA), MCP-1 (R&D System, Minneapolis, MN, USA), CD68 (AbD Serotec, Oxford, UK), **Annexin V (Abcam, Cambridge, MA, USA) and Ki67 (Abcam)**. Antigens were visualized using 3,3- diaminobenzidine (DAB) and counterstained with hematoxylin. For each mouse, quantification was given as the average of three or more sections of aortic sinus and aortic arch taken at 60µm intervals. The expression of antigens was quantified using Optimus 6.2 VideoPro-32 and the stained segments were expressed as a percentage of the total plaque area.

Plasma cholesterol quantification

Total plasma cholesterol concentrations were measured with a Cobas Integra ® 400 plus Autoanalyzer using the following kits in accordance to manufacturer's instructions: Cholesterol Gen.2, HDL-Cholesterol plus 2nd generation, LDL-Cholesterol plus 2nd generation, and Triglycerides (Roche Diagnostics, Mannheim, Germany).

IFN- γ and TNF-α ELISA

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4 166 Plasma IFN- γ and TNF- α levels were measured using the mouse IFN- γ Platinum enzyme
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6 167 linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) according to
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8 168 manufacturer's instructions.
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12 170 *Intravital microscopy*
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14 171 For intravital microscopy studies, male C57BL6/J mice, 8-12 weeks old were administered
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16 172 PIF (0.3mg/kg), scPIF (0.3mg/kg) or PBS i.p., followed by administration of LPS (1mg/ml;
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18 173 i.p.). At 16 hours after LPS administration, mice were anaesthetised using ketamine/zylazine
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20 174 (100mg/kg and 10mg/kg body weight respectively). The small intestine and supporting
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22 175 mesentery were exteriorised via the incision and placed onto a specially designed
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24 176 transparent viewing stage. The jugular vein was catheterized to administer the fluorescence
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26 177 dye rhodamine 123 (0.05%, Molecular Probes; Mulgrave, VIC, Australia) to allow for
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28 178 visualization of leukocytes. Two to four vessels were selected for each experiment, and the
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30 179 same section of each vessel was observed throughout the experiment. Leukocyte migration
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32 180 rolling and adhesion was captured on video for up to 5 mins per vessel.
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38 182 *Thioglycollate-induced peritonitis leukocyte visualization*
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40 183 Peritonitis was induced as previously described²⁵. Briefly, 8-12 week old C57BL6/J mice
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42 184 were administered PIF (0.3mg/kg), scPIF (0.3mg/kg), margatoxin (MTX, 1pmol/g, Sigma
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44 185 Alderich) or PBS i.p. followed by 1ml of 4% thioglycollate (*Thioglycollate-Bouillon, Merck*
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46 186 *KGaA*) to induce peritonitis. At 20 hours after thioglycollate administration, mice were
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48 187 euthanized with (Ketamine/Zylazine). The total peritoneal lavage leukocyte content was
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50 188 quantified using a hemocytometer and monocyte numbers were quantified by flow
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52 189 cytometry.
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57 191 *Flow cytometry*
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For quantification of monocytes in the peritoneal fluid, fluorochrome-labelled antibodies F4/80 (rat anti-Mouse PE, eBioscience) and CD11b (Anti-Mouse APC, eBioscience) were used in the aboved mentioned Thioglycollate-induced peritonitis model. Single cell suspensions were stained with the antibody cocktail at 4°C for 30 min, then washed and resuspended in PBS with 1% FCS. FACS Canto II (BD Biosciences) was used to collect data. Data was subsequently analyzed using FACS Diva software (BD Biosciences).

Cell culture

Human acute monocytic leukemia cell line (THP-1) cells were obtained from American Type Culture Collection (ATTC; Manassas, USA) and maintained in RPMI Medium 1640 with 10% fetal calf serum, 1% L-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin (Gibco, Invitrogen, Auckland, New Zealand).

Chemotaxis assay

THP-1 cells (1×10^6 /ml) were pre-treated with either PBS, scPIF (10µM) or PIF (10µM) for 40min at 37°C, then placed in the upper chamber of a cell culture insert with PET membrane (BD; 8µm pore). The insert was placed into individual wells of a 24-well plate containing MCP-1 (10ng/ml) (R&D systems). Samples were incubated with MCP-1 for 4hrs at 37°C, and transmigrated cells were collected and quantified using the hemocytometer.

Kv1.3 and IDE siRNA transfection of THP-1 cells

KCNA3 (Kv1.3) and Insulin Degrading Enzyme (IDE) mRNA knockdown in THP-1 cells was performed using gene-specific siRNA. Briefly, THP-1 cells were transfected with either siGENOME ON-TARGETplus, SMARTpool human KCNA3 siRNA (100nM) or human IDE siRNA (50nM) using DharmaFECT Transfection reagent (all from Dharmacon). At 24

hours after transfection, cells were pre-treated with one of PBS, scrambled PIF (10 μ M) or PIF (10 μ M) for a further 16 hours, followed by stimulation with TNF- α (20ng/ml) for 6 hours before cells were harvested for quantitative real-time PCR analysis.

FluxOR™ Potassium Ion Channel Assay

The FluxOR™ Potassium Ion Channel Assay (LifeSciences, US) takes advantage of the well described permeability of potassium channels to thallium (Tl⁺) ions. When thallium is added to the extracellular solution with a stimulus to open channels, thallium flows down its concentration gradient into the cells, and channel or transporter activity is detected with a proprietary indicator dye that increases in cytosolic fluorescence. In this way, the fluorescence reported in the FluxOR™ system becomes an indicator of any ion channel activity or transport process that allows thallium into cells. Acute T cell leukemia derived Jurkat cell line (ATCC, US) was loaded with FluxOR reagent and challenged according to manufacturer protocol using one of the following compounds in various concentration: PIF (10 μ M, 33 μ M, 77 μ M), scPIF (10 μ M, 33 μ M, 77 μ M), Cortisone (2 μ M), or 4-Aminopyridine (4-AP, 100 μ M, 250 μ M, 500 μ M; Tocris Bioscience, US). The background (negative control) fluorescent signal was recorded and then stimulus buffer (containing final concentrations of K₂SO₄ 10 mM and Tl₂SO₄ 2mM) was added and new signal was recorded (FLUOStar Optima, BMG Labtech, Germany). The Potassium flux is represented as F/F₀ (experimental signal normalized to background signal) versus time [sec].

Quantitative real-time PCR

Total RNA was extracted from THP-1 cells using the Bioline Isolate II RNA mini kit (Bioline) followed by cDNA transcription using TaqMan® Reverse Transcription Reagents (Life Technologies), all according to manufacturer's specifications. Subsequently, 20 ng of

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cDNA was used to perform qRT-PCR using a standard SYBR Green detection on an Applied Biosystem 7500 system (Applied Biosystem). Gene expression was normalized relative to the expression of the housekeeping gene 18S ribosomal RNA using the formula $2^{-\Delta\Delta CT}$.

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM). Difference between two data points was analysed using the standard unpaired t-test. Difference between three or more groups was analysed using the repeated measured one-way ANOVA Kruskal Wallis test coupled with Bonferroni post-test comparing all pairs of columns. Data was considered statistically significant at $p < 0.05$.

Results

PIF attenuates atherosclerotic plaque progression

To determine the effect of PIF on atherosclerotic plaque development, atherosclerotic lesions size was assessed in high fat diet (HFD) fed ApoE^{-/-} mice. After seven weeks of chronic treatment, we found that PIF (3mg/kg) significantly reduced plaque size in the aortic sinus when compared with the same scPIF dose ($p < 0.001$), or PBS vehicle control ($p < 0.01$) (Fig. 1A). Furthermore, lesion size in mice administered a lower dose of PIF (0.3mg/kg) appeared smaller when compared with scPIF (0.3mg/kg); however this did not reach significance. Similarly in the aortic arch, only PIF (3mg/kg) significantly reduced atherosclerotic lesion area ($p < 0.01$ and $p < 0.001$ vs. PBS and scPIF, respectively (3mg/kg) (Fig. 1B).

PIF has no influence on serum cholesterol and triglycerides levels

To determine whether PIF attenuates plaque development by influencing plasma lipid profile, plasma levels of total cholesterol, triglycerides, VLDL/LDL, HDL were measured using a commercially available kit. Our results demonstrate that PIF (0.3mg/kg - 3mg/kg) or scPIF (0.3mg/kg - 3mg/kg) had no significant effect on the lipid profile (Table 1).

PIF reduced CD4T cells in blood, but has no influence on other leukocyte subsets

To determine whether PIF causes any immune toxicity, we collected blood, spleen and lymph nodes and examined changes in the population of the following immune cells: M1 (CD11b⁺ Ly6C^{high}), M2 (CD11b⁺ Ly 6C^{low}) macrophages, B1 (CD5⁺ CD19⁺), B2 (CD5⁻ CD19⁺), CD4 (TCRb⁺ CD4⁺), CD8 (TCRb⁺ CD8⁺), NK (TCRb⁻ NK1.1⁺), and NKT (TCRb⁺ NK1.1⁺) lymphocytes. PIF treatment significantly reduced CD4 T cells in blood samples (Supplementary Fig. 4 A & B). However, there are no differences in CD4 levels in spleen or peritoneal fluid. To further investigate the protective effects of PIF, we

examined changes in Treg populations in the spleen. However, no difference was found in Treg cell populations associated with PIF treatment (Supplementary Fig. 4C).

Effect of PIF on atherosclerotic lesion characteristics

To gain further insight into how PIF prevented lesion progression in ApoE^{-/-} mice, lesion composition of the aortic sinus and aortic arch were characterized. Lipid content of atherosclerotic plaques was assessed using oil Red O staining. PIF (3mg/kg) significantly reduced plaque lipid content in both the aortic sinus and aortic arch when compared to scPIF 3mg/kg (p< 0.0001, p = 0.0093, respectively Fig. 2A).

As inflammatory cell recruitment contributes to the initiation and progression of atherosclerosis, expression of chemokines and adhesion molecules that act upstream of inflammatory cell infiltration, were also assessed using immunohistochemistry. PIF (3mg/kg) significantly reduced Vascular Cell Adhesion Molecule-1 (VCAM-1) expression in the aortic sinus and aortic arch (p=0.02, p=0.0048, respectively Fig. 2B). Expression of the chemokine, monocyte chemoattractant protein-1 (MCP-1), was also lower in PIF-treated mice (p=0.0012 for both sinus and arch, Fig. 2C). Expression of the macrophage marker CD68 in the aortic sinus decreased (43%) when compared to scPIF 3mg/kg (p = 0.0017, Fig. 2D, and a significant reduction in CD68 expression was also evident in the aortic arch (p = 0.0035, Fig. 2D; Supplementary Fig. 4A).

Effect of PIF on systemic inflammation

To determine whether the protection against atherosclerosis development is limited to local protection, circulating levels of IFN-γ were determined. Interestingly, plasma IFN-γ levels were markedly reduced in PIF (3mg/kg) treated mice as compared to scPIF (3mg/kg) or PBS (Fig. 2E). PIF had no effect on plasma TNF-α levels (data not shown). To further

explore the effect of PIF on proinflammatory cytokine expression, we stimulated RAW cells with TNF- α (5nM). IFN- γ expression was significantly reduced in the PIF treated cells compared to cells treated with scrambled PIF (n=9, p=0.049, Supplementary Fig. 1). The adhesion molecule VCAM-1 was also down-regulated by PIF treatment compare to scramble PIF (n=9, p=0.0074, Supplementary Fig. 1)

PIF acts by regulating activated-innate immune response.

The findings above suggest that a reduction in monocyte/macrophage infiltration may at least partially explain PIF's inhibitory effect on plaque progression. Therefore, we investigated whether PIF can affect monocyte cell function *ex vivo* and *in vitro*.

It has previously been shown that PIF preferentially binds to human peripheral CD14⁺ monocytes, with minimal binding to human lymphocytes¹⁷. Therefore, we set out to determine whether PIF binding to monocytes, translates to a change in monocyte function. The effect of PIF on monocyte function was determined via three different approaches. Firstly, using the THP-1 human monocytic cell line, we examined PIF's effect on monocyte migration *in vitro*. Pre-incubation with PIF (1 μ M & 10 μ M) significantly reduced MCP-1 (10 ng/ml)-induced chemotaxis *in vitro* when compared to PBS or corresponding doses of scPIF (p<0.01; Fig. 3A). To examine PIF's effect on monocyte migration *ex vivo*, we quantified these cells rolling and adhesion in the mesenteric venules (extravasation) using intravital microscopy. Pre-treatment with PIF (0.3 mg/kg) attenuated LPS-induced adhesion and migration of monocytes in the mesenteric venules of C57BL/6 mice when compared to PBS or scPIF (Fig.3B). As a second *in vivo* model, we examined leukocyte migration in the peritoneal cavity after induction of peritonitis in C57BL/6 mice using thioglycollate. Peritoneal fluid was collected at 20 hours after induction of peritonitis and F4/80⁺CD11b⁺ monocytes/macrophages were quantified via flow cytometry. In line with our intra-vital microscopy findings, both doses of PIF used (0.3mg/kg and 3 mg/kg) caused a significant

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reduction in influx of F4/80⁺CD11b⁺ monocytes/macrophages in the peritoneal cavity (Fig. 3C). Lastly, we examined whether PIF modulates the oxidative burst of ROS by macrophages. PIF had no effect on PMA-induced ROS production or H₂O₂ production in human and murine macrophages (Supplementary Fig. 2), suggesting that PIF does not prevent the respiratory oxidative burst, an important innate immune function against bacterial and viral infections.

Kv1.3 and IDE are potential binding partners of PIF

For further insight into the mechanism/s by which PIF affords its protection against atherosclerotic lesion progression, we set out to examine PIF's binding targets. Several intracellular targets, including the voltage-gated potassium channel Kv1.3 and the Insulin-degrading enzyme (IDE) have previously been identified as potential binding partners of PIF in a ProtoArray ¹¹. More recently, a microarray assay showed that biotin-PIF significantly binds to anti-IDE positive embryo lysate fractions and to Kv1.3-positive fractions, albeit to a smaller extent, further implicating their potential as PIF-binding targets ²⁶. To determine whether the observed inhibition by PIF on leukocyte transmigration is exerted thorough the Kv1.3 channel, we used the specific Kv1.3 antagonists margatoxin in the thioglycollate-induced peritonitis model; Margatoxin is a scorpion-derived peptide venom that blocks T-cell proliferation and mixed lymphocyte reaction (MLR) ²⁷. Compared with the PBS control, margatoxin (1pmol/g) significantly decreased thioglycollate-induced leukocyte transmigration in the peritoneal cavity to a similar extent as observed with PIF administration (Fig. 4A). In addition, margatoxin has no synergistic effect on PIF treated mice, indicating that inhibiting Kv1.3 is one of the key mechanisms responsible for PIF's anti-atherosclerotic effects.

In order to demonstrate the involvement of IDE in PIF's immune-modulatory effects, a siRNA based approach was used. Transfection of THP-1 cells using gene-specific siRNA

resulted in knockdown of IDE mRNA expression, as assessed by quantitative real-time PCR (Fig. 4B). Transfected THP-1 human monocytes were incubated with PIF (10 μ M), scPIF (10 μ M) or PBS prior to stimulation with TNF- α . At 48hrs, PIF significantly reduced TNF- α induced VCAM-1 mRNA expression (Fig. 4C). Following knockdown of IDE mRNA, the PIF inhibitory effect on VCAM-1 or IL-12b expression was blocked (Fig. 4C). However, PIF's inhibitory effect on TNF- α -induced IL-12b expression was not significant (Fig. 4C). Thus PIF-antagonist data generated supports interaction of PIF with these critical cellular targets.

Our final experiment using a Kv1.3 potassium efflux assay further confirmed that PIF at least in parts exerts its effects through the inhibition of the Kv1.3 channel. The effect seen with PIF was comparable to cortisone. The experiment was carried out in Jurkat cell line, known to express high ratio of slow potassium vs calcium channels. PIF had higher suppressor potency compared to the non-selective Kv1.3 blocker 4-Aminopyridine. ScPIF had no significant effect of suppression of Kv1.3 flux when compared to non-specific stimulus buffer (that opened up the channels), suggesting PIF specific action upon this target

(Fig. 5A). We further screened over 111 crystallographic structure models for PIF binding using an *in silico* "blind" predictive algorithm implemented in the PepSite server (Russell lab) (Fig. 5B). The predictive score of binding, the binding residue pattern and the probability of binding to different potassium channel families were further fed to the data mining suite Orange and analyzed in order to test whether PIF prefers a specific family of potassium channels over another. A hierarchical clustering dendrogram showed that potassium channel regulatory subunits (KCNA) had different binding characteristics compared to all other families (Fig 5C). The voltage gated potassium channels group was the largest and comprised of different channels from Kv1, 2, etc. families. When all significant top scores (scores ranged from 80 to 100, where p-value was less than 0.005) were linked to their potassium families Groups in parallel plots, only two groups of PDB

(Protein Data Bank) models were found to be good potential PIF binders (KCNAB and Voltage-dependent K⁺ channel beta subunit) (Fig. 5D). The prediction of PIF binding to only KCNAB corroborates to our experimental data on Kv1.3, since the potassium channel flux is controlled by its regulatory subunit, that happens to be KCNA3B (Kv1.3b). To further confirm this specificity of binding we used an unsupervised learning artificial neural network (self-organizing map, SOM) to blindly classify the predictions data based on the score and PIF amino-acids binding patterns. SOM showed that KCNAB were systematized closely together to have highest score of PIF binding suggesting that PIF is most specific to this group of potassium channel regulators (Fig. 5E).

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394 Discussion

395 It is well established that atherosclerosis involves chronic inflammation of the
396 arterial wall, and that cells from the innate and adaptive immune systems play important
397 contributory roles. However, current therapies primarily target inciting risk factors for
398 atherosclerosis, and not the underlying inflammatory process. Primary and secondary
399 prevention interventions have greatly reduced the risk of cardiovascular events.
400 Nevertheless, residual risk remains high despite intense management of cardiovascular risk
401 factors, with cumulative risk reductions of only 30%-40% despite intensive risk factor
402 modification ²⁸. Recent trials (focusing primarily on non-statin lipid lowering therapies)
403 have been disappointing, with either neutral or harmful effects ²⁹⁻³³. Hence, there is a clear
404 need for alternative therapies that can modulate the immune process contributing to
405 atherogenesis.

406 There has been far less investigation of anti-inflammatory agents in atherosclerotic
407 cardiovascular disease. However, the limited clinical data available suggests a benefit of
408 targeting the underlying inflammatory process. Colchicine (an agent effective in multiple
409 inflammatory disorders) has been shown in small trials to prevent both intracoronary stent
410 restenosis and cardiovascular events, in conjunction with a reduction in C reactive protein
411 (CRP) levels ³⁴⁻³⁷. Similarly, patients with rheumatoid arthritis have an increased risk of
412 cardiovascular events, which appears to be attenuated by the use of tumor necrosis factor
413 alpha (TNF-alpha) inhibitors and methotrexate ³⁸⁻⁴¹. Interestingly, methotrexate may provide
414 its protective effect via inhibition of macrophage foam cell formation, in a manner similar to
415 PIF⁴². Given such observations, two large clinical trials are currently investigating the use of
416 anti-inflammatory agents (methotrexate and canakinumab, a IL-1 β neutralizing monoclonal
417 antibody) in patients with coronary artery disease ⁴³. Of obvious concern is the potential
418 toxicity of such potent, immune modulating agents.

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In this study, we investigated whether PIF (a novel, synthetic analog of the embryo-derived peptide PreImplantation Factor) can prevent atherosclerosis. The rationale for such investigation is PIF's natural biological function during pregnancy: exerting immunomodulatory properties that prevent the maternal immune system (serving as a host) from rejecting the embryo (serving as a semi-allograft), while otherwise allowing for maintenance of normal immune function ⁴⁻⁶. More importantly, PIF has been shown to reduce inflammation, reverse associated adverse effects and prolong survival in other murine models of chronic inflammatory diseases such as EAE ¹⁸ and Type 1 Diabetes (NOD) ¹⁹. Our investigation of PIF administration in the HFD-fed ApoE^{-/-} atherosclerosis mouse model shows for the first time that: 1) PIF administration prevents atherosclerosis progression, pro-inflammatory marker expression and macrophage infiltration; 2) PIF exerts such effects despite minimal impact on plasma lipid profile; 3) PIF modulates monocyte function *in vitro* and *ex vivo*; 4) Kv1.3 and IDE are potential binding partners that may contribute to PIF's immunomodulatory function. Thus, this study supports the premise that PIF may serve as an effective immunomodulatory drug with beneficial effects on atherosclerosis.

The decrease in atherosclerotic plaque burden after seven weeks of treatment was mostly noted at the higher (3mg/kg) PIF dose, it was specific, and not replicated by the corresponding dose of scrambled PIF used as a control. This range of concentrations is in line with levels that were found to be present in maternal circulation during pregnancy⁴. To better delineate PIF's protective effects on plaque size, we conducted histological analysis of the aortic arch and sinus. We observed a significant reduction in MCP-1 (CCL2), a chemokine that recruits activated monocytes to the plaque. Second, expression of VCAM-1 (a main mediator of monocyte recruitment and adhesion to the vascular endothelium) was also decreased within atherosclerotic plaques. Finally, PIF-treated mice had lower lipid content (demonstrated with oil red O staining) and lower CD68⁺ cells within plaques,

indicating reduced numbers of macrophage-derived foam cells. Taken together, these findings indicate that PIF reduces atherosclerotic lesion burden by preventing monocyte recruitment to the atherosclerotic plaque.

PIF's regulatory effects were also observed at the systemic level. There was a significant decrease in pro-inflammatory circulating cytokine IFN- γ . IFN- γ is secreted by several cell types including T_H1 cells and macrophages, is prominent in atherosclerotic lesions, and plays an important role promoting expression of adhesion molecules, activation of macrophages and induction of foam cell formation⁴⁴. This is consistent with our previous finding on the PHA-activated PBMC, where PIF reduced IFN- γ secretion *ex vivo*²⁰. Indeed, double IFN- $\gamma^{-/-}$ /ApoE $^{-/-}$ knockout mice have fewer macrophages within plaques⁴⁵. The specific effects of PIF on IFN- γ are beyond the scope of this study, but it is tempting to speculate that a reduction in IFN- γ levels may contribute to PIF's anti-atherosclerotic effect. Furthermore, PIF has recently been demonstrated to decrease Let-7 expression, leading to an increase in anti-inflammatory cytokine IL-10, providing the potential mechanism by which PIF induces neuroprotection after hypoxic-ischemic brain injury²².

To further assess PIF's modulatory effect on monocyte function, three different *ex vivo* and *in vitro* assays were utilized. Together, the MCP-1 induced chemotaxis assay, the peritonitis model and intra-vital microscopy demonstrated that PIF prevented monocyte migration induced by several different stimuli, further substantiating our findings *in vivo*. Overall, this data is consistent with recent observations that PIF primarily targets monocytes following activation, and that PIF reduces macrophage migration and leukocyte extravasation^{17, 21, 22}. In addition, microarray data implicate Kv1.3 and IDE proteins as potential PIF binding partners, and may provide a likely mechanism for the peptide's effect¹¹. Thus, we examined *in vitro* the effect of Kv1.3 and IDE siRNA knockdown on PIF activity in monocytes. PIF blocked TNF- α induced VCAM-1 and IL-12b expression in

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THP-1 monocytes, further supporting our *in vivo* observations in atherosclerotic plaques. Importantly, knockdown of Kv1.3 and IDE mRNA in THP-1 cells reversed PIF's anti-inflammatory effect. These findings, while preliminary, provide some evidence that PIF may act via these targets. Overall, these findings provide further evidence that PIF's anti-atherosclerotic effects may be due to modulation of monocyte function via targeting of Kv1.3 and IDE proteins⁴⁶. However, this does not rule out a role for other potential binding targets. Indeed, single-step PIF-based affinity columns followed by peptide mass spectrometry have identified several PIF binding targets in addition to Kv1.3 and IDE such as PDI and heat shock proteins, and further investigations would be of interest to determine if these targets also act downstream of PIF binding²⁶.

 An additional clinically relevant finding is that PIF had minimal effects on plasma lipid profile, despite the evident reduction in inflammation and in atherosclerotic lesion burden. This highlights that PIF's anti-inflammatory effects are unlikely to be due to cholesterol lowering. Interestingly, we recently defined another immunomodulatory anti-atherosclerotic approach via CpG oligodeoxynucleotides mediated TLR9 stimulation that was not caused by lipid changes⁴⁷. As noted earlier, despite potent reductions in serum cholesterol, currently available lipid lowering agents are limited in their ability to prevent recurrent cardiovascular events. Statins (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors) are currently the most effective and clinically useful lipid lowering agents. However, a significant proportion of the statin benefit may be due to their pleiotropic, anti-inflammatory effects. Statins have been shown to reduce CRP levels independent of cholesterol lowering effects; in addition, the statin mediated reduction in cardiovascular events has been correlated with a reduction in CRP (reflecting an anti-inflammatory effect) rather than a reduction in cholesterol⁴⁸. In addition, statins may also have beneficial effects on distinct inflammatory disorders such as rheumatoid arthritis⁴⁹. These observations, along

with the observation that alternative lipid lowering therapies have minimal impact on outcomes, together suggest that the statins (the best currently available risk-modifying agents, which were developed for lipid modulation) exert benefit largely via anti-inflammatory mechanisms. Also, significant residual risk persists despite effective control of cholesterol. Statins are very well tolerated, but are still limited by systemic side effects (myalgias, potential for liver toxicity, memory loss and potential for development of diabetes). PIF has potential to serve as an ideal immune modulating, anti-inflammatory agent that inhibits atherosclerosis, independent of lipid lowering and with minimal toxicity.

The ApoE^{-/-} model combined with a HFD is a powerful clinically relevant tool to examine the combination of individual susceptibility to atherogenesis further impacted by an unhealthy diet and life style. It appears, based on the current study, that PIF has a favorable influence on development of atherosclerosis due to this lethal combination. Whether PIF could also reverse atherosclerosis via plaque regression remains to be seen. Analogous benefits have already been demonstrated in both neuroinflammation and neuro-trauma models^{18, 22}. This is of direct clinical interest, as plaque stabilization and regression may be difficult to achieve with currently available agents.

In conclusion, intermittent PIF administration effectively prevents the development of atherosclerosis by modulating immune responses independent of any effects on circulating lipids. This has immediate clinical relevance, given evidence that currently available agents may have benefits attributed largely to lipid-lowering effects, and there is substantial residual cardiovascular risk despite the use of such agents. PIF's immunomodulatory effect is at least partially due to an ability to modulate monocyte function, such that it reduces monocyte migration and their subsequent contribution to the inflammatory milieu present within athero-sclerotic plaques. While further studies will be required to elucidate the biological mechanisms involved in PIF's actions, preliminary findings indicate that Kv1.3

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519 and IDE are potential PIF binding partners. Overall, PIF potentially represents a novel
520 immunomodulatory drug that can selectively target inflammation and atherosclerosis
521 independent of lipid lowering and without compromising immune function, and thus
522 warrants further investigation in animal models and human trials.

For Peer Review

523 **Disclosures/Conflict of Interest**

524 PIF is a patented compound owned by BioIncept, LLC. Dr. Eytan R. Barnea is its
525 (uncompensated) Chief Scientist. All other authors declare no conflict of interest.

526

527 **Acknowledgements:** This study was partially funded by a Grant-in-Aid of the National
528 Heart Foundation of Australia and the National Health and Medical Research Council of
529 Australia. [Y.C. Chen is supported by Heart Foundation Postdoctoral Fellowship \(100513\).](#)

530 We thank Andrew P. Zinn, MD, FACC Interventional and Consultative Cardiology,
531 Cardiovascular Assoc. Delaware Valley Director of Interventional Cardiology, Inspira
532 Medical Center Vineland for his valuable comments for the manuscript.

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Figure legends

Figure 1. PIF inhibits plaque development in the aortic sinus and aortic arch of hypercholesterolemic ApoE^{-/-} mice. ApoE^{-/-} mice were maintained on a high fat diet for 7 weeks and received either PBS, or a low and high dose of scPIF and PIF three times weekly via i.p. injection. (A) Quantification of lesion burden in the aortic sinus, expressed as a % of total lesion area (left panel). Representative sections stained with H & E (right panel). (B) Quantification of lesion burden in the aortic arch, expressed as a % of total lesion area (left panel). Representative sections stained with H & E (right panel). Data presented are mean ± SEM of at least 8 animals per group. Statistical significance was calculated using one-way ANOVA, followed by Dunn's post test. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. PIF reduces lipid content, pro-inflammatory protein expression and macrophage infiltration within atherosclerotic plaques of ApoE^{-/-} mice. ApoE^{-/-} mice were maintained on a high fat diet for 7 weeks, and received either PBS, a low (0.3mg/kg) or a high dose (3mg/kg) of scPIF and PIF three times weekly via i.p. injection. Cross-sections of the aortic sinus and aortic arch were analyzed using histology and immunohistochemistry. (A) Oil red O staining for lipids, (B) VCAM-1 expression, (C) MCP-1 expression, and (D) CD68⁺ macrophage infiltration. (E) Plasma levels of IFN-γ were measured using a commercially available ELISA kit. Data presented are mean ± SEM of at least 6 animals per group. Statistical significance was calculated by unpaired, student's T-test between the indicated groups. *P<0.05, **P<0.01, ***P<0.001.

Figure 3. PIF inhibits monocyte migration. (A) MCP-1 (10 ng/ml)-induced migration of THP-1 cells pre-treated with one of two doses of PIF (1 μM, 10 μM), scPIF (1 μM, 10 μM) or PBS. Transmigrated cells were collected and quantified using the hemocytometer. (B) C57BL6/J mice were administered PBS, scPIF or PIF, followed by LPS (80 μg). After 16

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hours, rhodamine was administered via the jugular vein and the mesentery arteries were exteriorized to visualize (right panel) and quantify (left panel) leukocyte rolling and adhesion. (C) C57BL6/J mice were administered scPIF, PIF (0.38 mg/kg i.p.) or PBS as a vehicle control and peritonitis was induced using 4% thioglycollate. After 20 hours, peritoneal fluid was collected and flow cytometry was used to count the percentage of F4/80⁺CD11b⁺ cells in the peritoneal fluid. All data represent mean ± SEM of at least 6 independent experiments per group. Statistical significance was calculated using one-way ANOVA, followed by Dunn's post test. *P<0.05.

Figure 4. Kv1.3 and IDE are potential binding targets of PIF. (A) Kv1.3 inhibitors margatoxin (1pmol/g) reduced monocyte influx in the thioglycollate-induced model of peritonitis to a similar extent of PIF (0.3mg/kg). Margatoxin did not show any synergistic effect in the PIF treated mice. (B) Validation of IDE mRNA knock-down using gene-specific siRNA. (C) TNF- α -induced VCAM-1 mRNA expression was reduced by PIF and this effect was negated after IDE knockdown using siRNA. A similar trend was observed when IL-12b mRNA levels were examined. All data represent mean ± SEM of at least 10 independent experiments per group. Statistical significance was calculated using one-way ANOVA, followed by Dunn's post test, *P<0.05, **P<0.01.

Figure 5. FluxORTM assessment of PIF effects on slow potassium channels in Jurkat T cells. A. PIF suppresses potassium flux similar to cortisone as assessed by FluxORTM. Jurkat T cells were treated with potassium channel activating buffer after no treatment or treatment using any of the compounds: PIF (10 μ M, 33 μ M, 77 μ M), scPIF (10 μ M, 33 μ M, 77 μ M), Cortisone (2 μ M), or 4-Aminopyridine (4-AP, 100 μ M, 250 μ M, 500 μ M). Raw pre-stimulus peak and baseline values were averaged and normalized. Data shown demonstrate the time-dependent fold increase in fluorescence over baseline (F/F₀, n=3). B.

A list of 111 PDB (Protein Data Bank) crystallographic structure models of different potassium channels family members (Group) probed for putative PIF binding using PepSite ver 2 with putative PIF binding (score) and prediction probability (p-val), and PIF residues binding matrix (showing in red residues participating in the predicted interface). **C.** The distances matrix between individual potassium channels binding scores was measured using *Euclidian distance* metrics and clustered using a *hierarchical* algorithm with “*Complete linkage*” that defined the distance between two clusters as the distance between their most distant elements. Leafs were annotated with potassium channel Group type. **D.** Parallel Coordinates multidimensional visualization of PIF binding *score* vs. potassium channels *Group* vs. PIF binding probability p-val. The score and p-values were filtered for values between 100 and 80, and 0 and 0.005, respectively. The filtered links that become irrelevant are depicted in light colours. **E.** Self-Organizing Maps artificial neural network in unsupervised learning mode on PIF binding to potassium channels predicting data using predicted score, p-value and channel Group. Nodes (neurons) are hexagonally arranged, background is score colour coded, while nodes are coloured based on the Majority value of the Group, thus presenting “*grouping*” of most significant by score PIF binding predictions and their target Group of potassium channels, while considering the PIF binding pattern.

Supplementary 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 $\pm$ SD; p=0.0074 in VCAM-1 and p=0.049 in IFN- γ).

Supplementary figure 2: PIF does not affect H₂O₂ 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 H₂O₂ 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.

Supplementary 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 (TCRb+ CD4+), CD8 (TCRb+ CD8+), NK (TCRb- NK1.1+), and NKT (TCRb+ NK1.1+) lymphocytes remain unchanged between two groups.

Supplementary 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.

Supplementary methods

H₂O₂ production in leukocytes

Whole blood (100 μ l) of mice was pretreated with PIF (1 μ M, 10 μ M), scPIF (1 μ M, 10 μ M) 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 μ M PIF, 10 μ M 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 μ g/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, 700 μ l 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

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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.

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877 Flow cytometry for immune cell subsets

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

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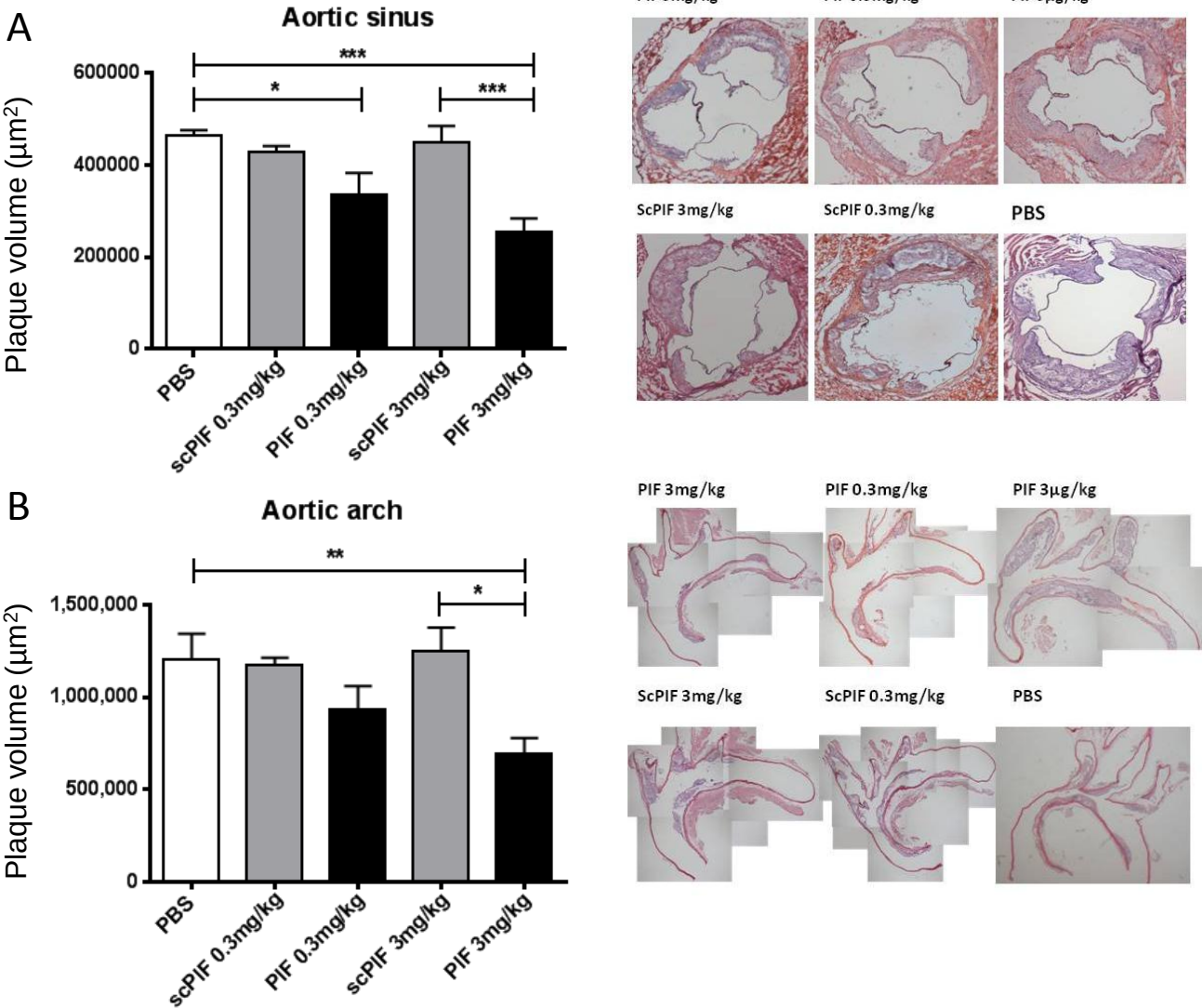
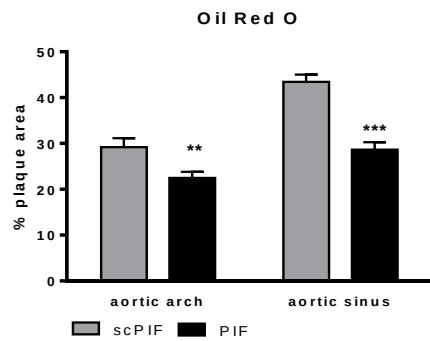
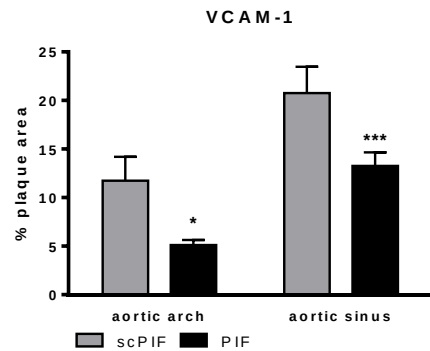


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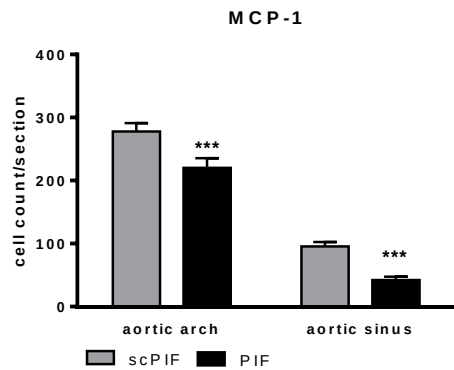
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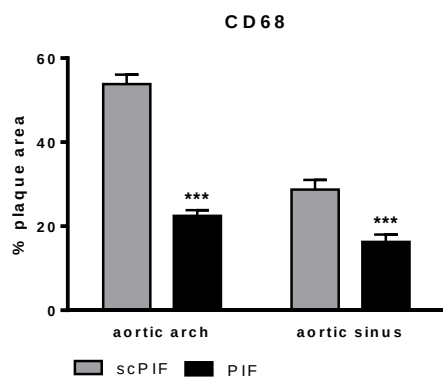
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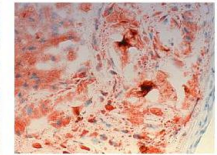
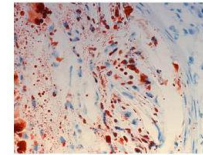


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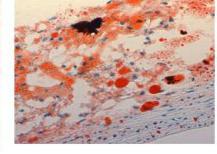
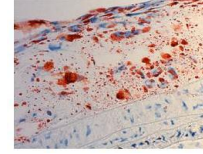
PIF

scPIF

Aortic
sinus

PIF

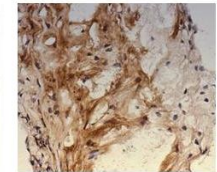
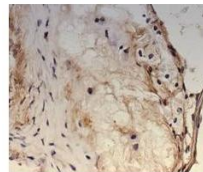
scPIF

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VCAM-1

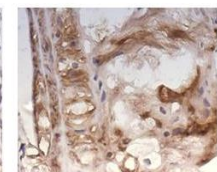
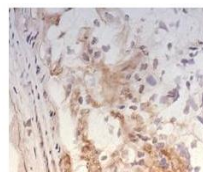
PIF

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PIF

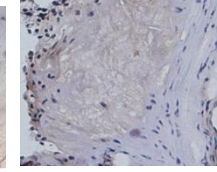
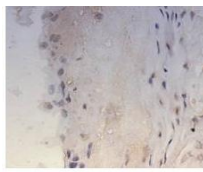
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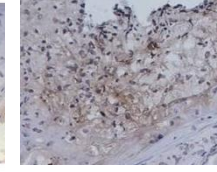
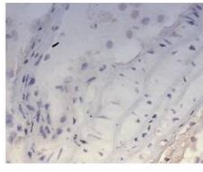
PIF

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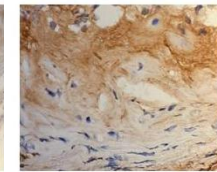
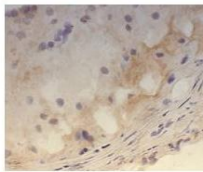
scPIF

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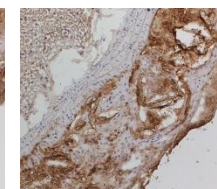
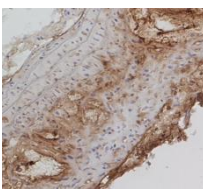
Aortic
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Figure 2.

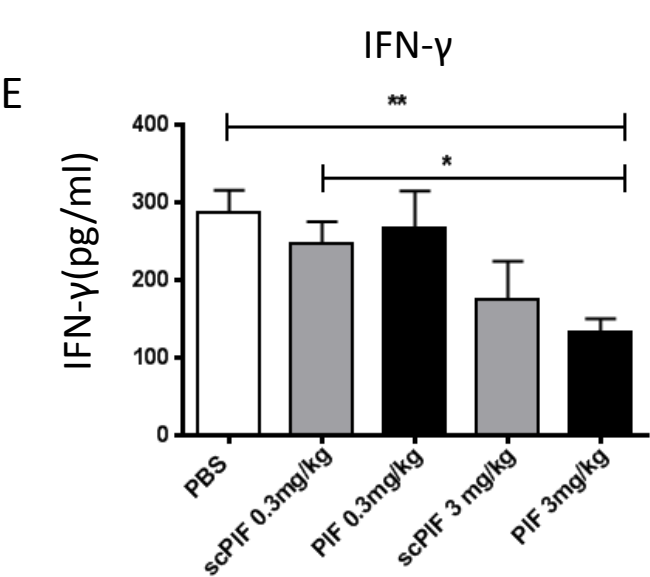
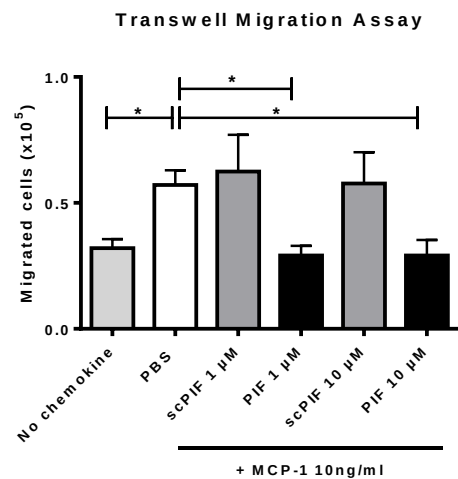
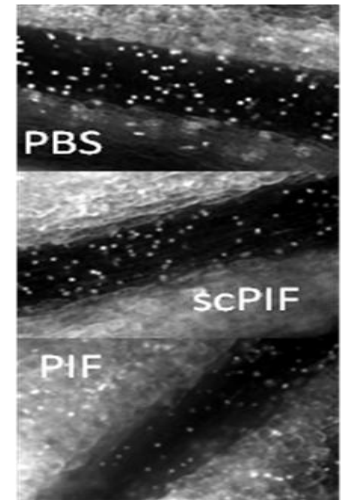
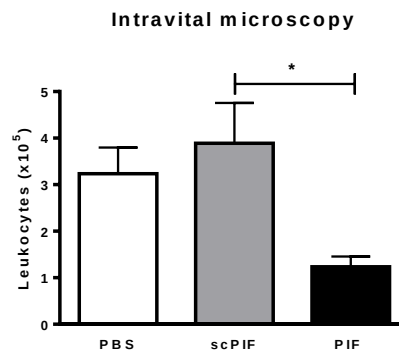


Figure 2. (cont)

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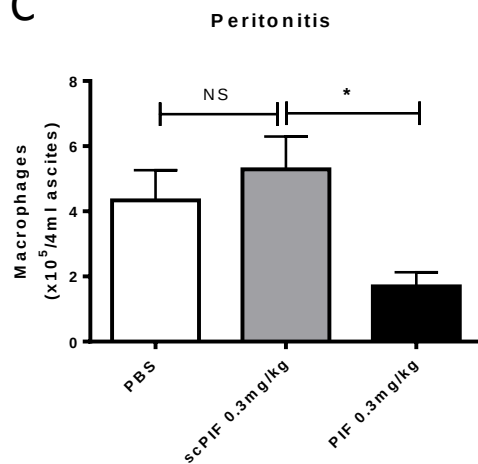


Figure 3.

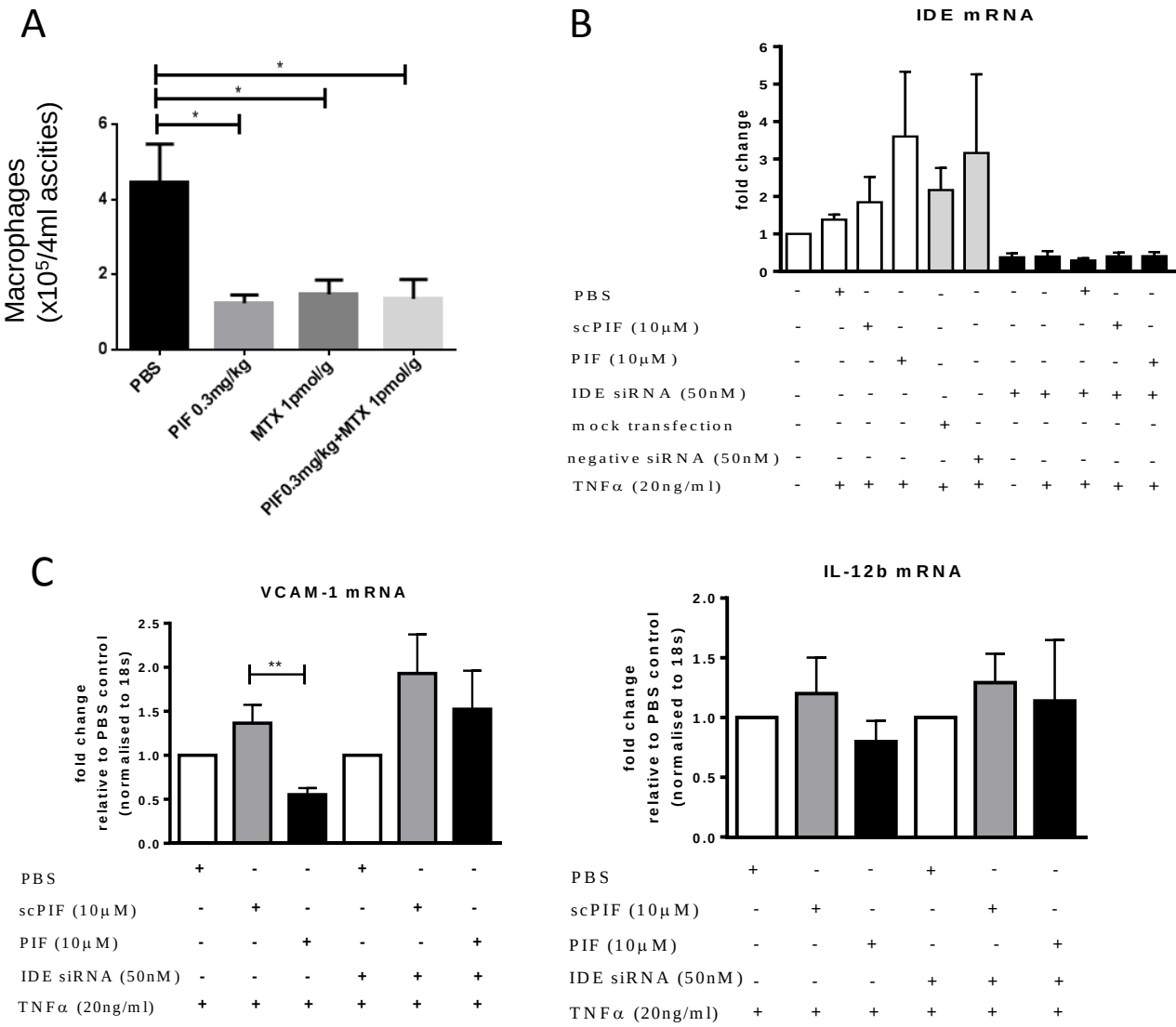
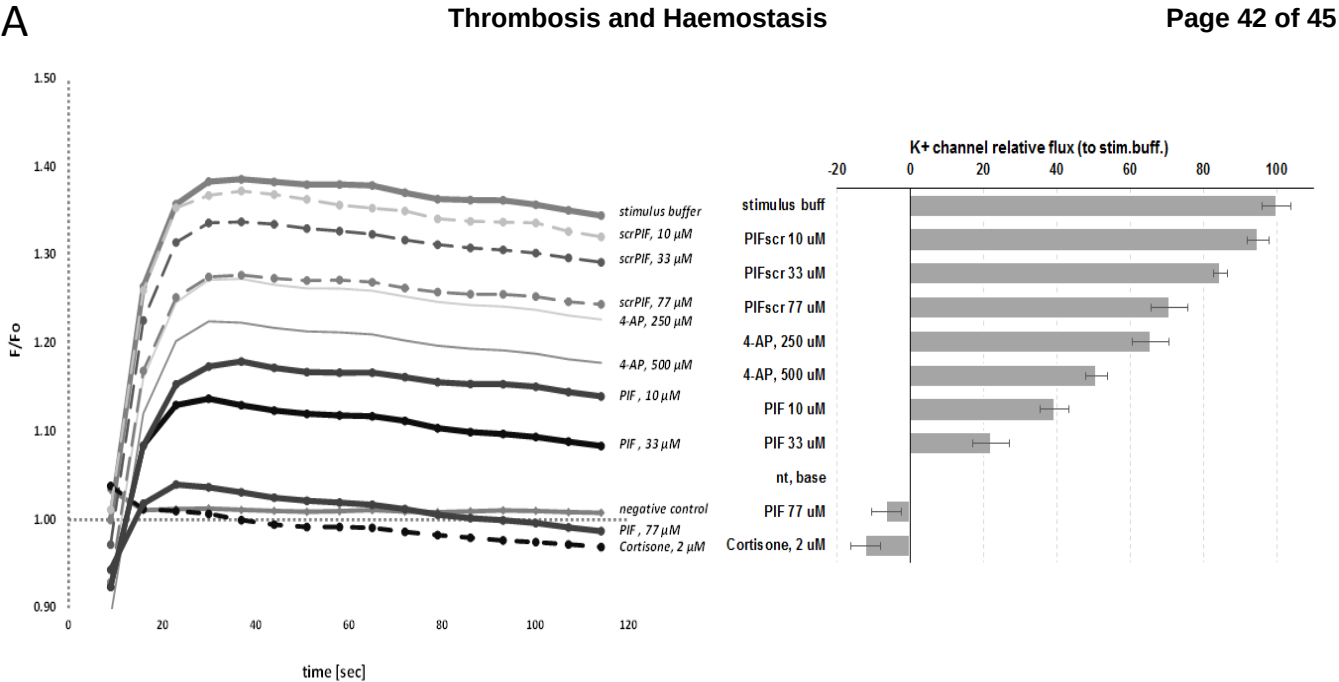


Figure 4.

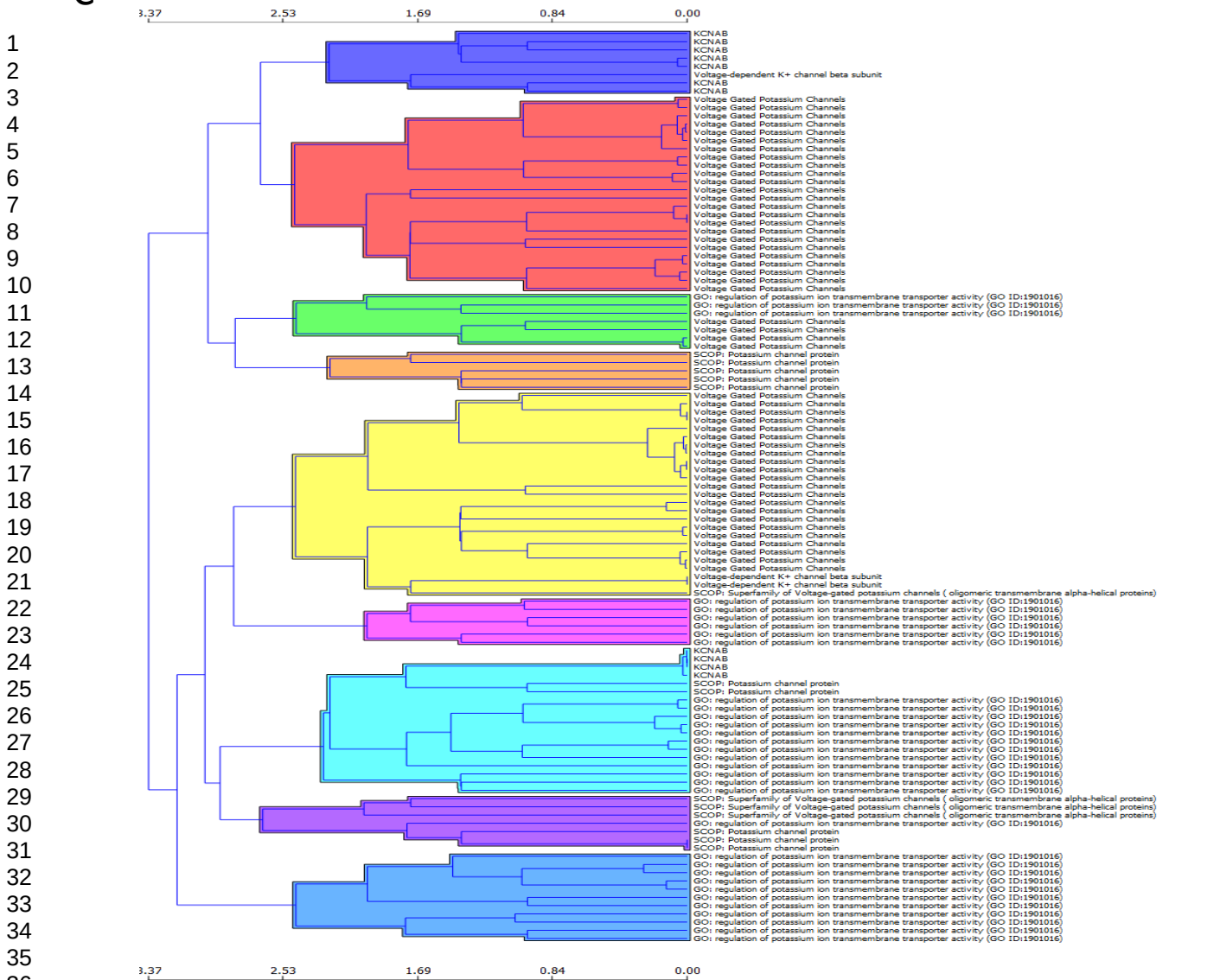


B

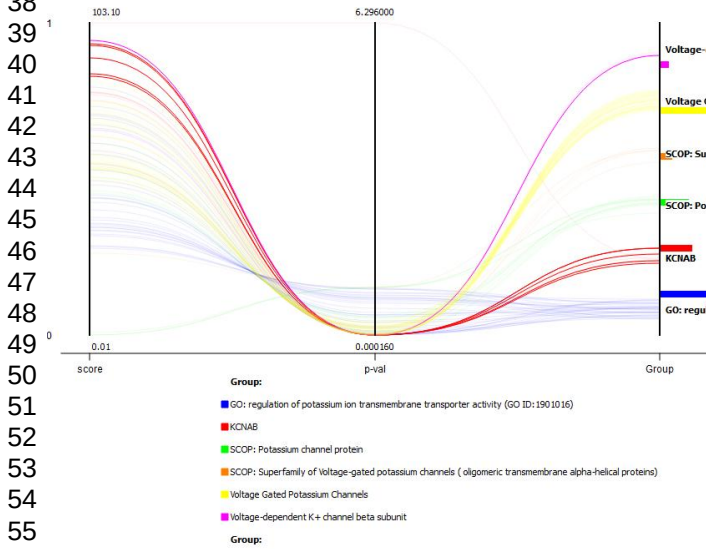
Group	PDB ID	score	p-val	M	V	R	I	K	P	G	S	A	N
Voltage-dependent K+ channel beta subunit	KCNAB 3EAU	103.1	6.30E+00										
	1EXB	97.31	0.00016										
	KCNAB 2A79	96.21	0.0002										
	KCNAB 3LUT	95.78	0.000216										
	KCNAB 3EB4	91.41	0.000451										
	KCNAB 4JTA	86.35	0.00105										
	KCNAB 1ZSX	85.47	0.00122										
Voltage Gated Potassium Channels	3STZ	83.79	0.00162										
Voltage Gated Potassium Channels	2HVJ	83.45	0.00172										
Voltage Gated Potassium Channels	2IH3	81.93	0.00222										
GO: regulation of potassium ion transmembrane transporter activity (GO ID:1901016)	3N23	81.85	0.00225										
	KCNAB 2R9R	80.3	0.00292										
	KCNAB 4JTC	80.14	0.003										
	KCNAB 4JTD	80.05	0.00305										
	KCNAB 3EB3	79.28	0.00347										
Voltage Gated Potassium Channels	2NLJ	77.86	0.0044										
	KCNAB 3LNM	77.54	0.0046										
Voltage Gated Potassium Channels	2ATK	77.3	0.00484										
Voltage Gated Potassium Channels	2IH1	77.08	0.00502										
Voltage Gated Potassium Channels	2A0L	76.48	0.00555										
Voltage Gated Potassium Channels	2P7T	76.03	0.00598										
GO: regulation of potassium ion transmembrane transporter activity (GO ID:1901016)	2M0Q	75.45	0.006										
Voltage Gated Potassium Channels	3OR6	74.37	0.0079										
Voltage Gated Potassium Channels	1ORQ	74.18	0.00816										
Voltage Gated Potassium Channels	2ITC	73.35	0.00937										
GO: regulation of potassium ion transmembrane transporter activity (GO ID:1901016)	3WGU	72.95	0.01										
Voltage Gated Potassium Channels	4LBE	72.7	0.0104										
GO: regulation of potassium ion transmembrane transporter activity (GO ID:1901016)	4HQJ	72.59	0.0106										
Voltage Gated Potassium Channels	3OR7	72.55	0.0107										
GO: regulation of potassium ion transmembrane transporter activity (GO ID:1901016)	3WGV	71.57	0.0126										
Voltage Gated Potassium Channels	4MSW	71.5	0.0127										
Voltage Gated Potassium Channels	4LCU	71.23	0.0133										
Voltage Gated Potassium Channels	2DWE	70.1	0.016										
Voltage Gated Potassium Channels	1S5H	69.97	0.0164										
Voltage Gated Potassium Channels	2HVK	69.78	0.0169										
Voltage Gated Potassium Channels	2BOB	69.67	0.0172										
GO: regulation of potassium ion transmembrane transporter activity (GO ID:1901016)	2K21	69.4	0.018										

Figure 5.

C



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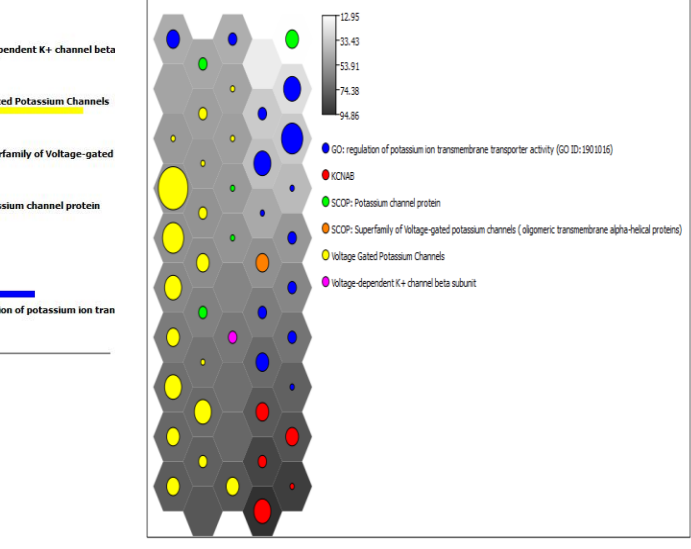


Figure 5. (cont)

Table 1. Serum cholesterol, triglycerides, VLDL/LDL and HDL

	PBS (n=12)	scPIF 0.3mg/kg (n=8)	PIF 0.3mg/kg (n=8)	scPIF 3mg/kg (n=8)	PIF 3mg/kg (n=8)
Total Cholesterol (mmol/L)	3.53 ± 0.57	2.96 ± 0.54	2.49 ± 0.4	3.13 ± 0.65	3.43 ± 0.293
VLDL/LDL (mmol/L)	2.10 ± 0.3	1.50 ± 0.33	1.47 ± 0.28	1.74 ± 0.44	2.10 ± 0.24
HDL (mmol/L)	1.29 ± 0.31	1.33 ± 0.21	0.97 ± 0.18	1.26 ± 0.23	1.19 ± 0.16
Triglycerides (mmol/L)	0.31 ± 0.03	0.26 ± 0.09	0.14 ± 0.03	0.27 ± 0.16	0.29 ± 0.07

(Note: All data represent mean ± SEM)

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What is known about this topic?

- Preimplantation Factor (PIF) in the maternal circulation is essential to ensure the embryo's/fetus's viability throughout pregnancy.
- PIF interacts with immune cells and thereby exerts immunomodulatory effects.
- PIF has been fast-track approved by the FDA for a clinical trial in autoimmune hepatitis
- PIF has beneficial effects in several disease models such as experimental autoimmune Encephalitis (EAE), type 1 diabetes mellitus and graft-versus-host disease.
- Effective anti-atherosclerotic therapies are highly sought-after.

What does this paper add?

- PIF reduces atherosclerosis in the Western diet-fed ApoE-deficient mouse model.
- PIF preferentially binds to monocytes and inhibits their function.
- PIF exerts its function by targeting potassium channel KCNAB3 (Kv1.3) and insulin degrading enzyme (IDE).
- PIF holds strong promise as a side-effect poor (free) treatment for atherosclerosis.

For Peer Review