PreImplantation factor prevents atherosclerosis via its immunomodulatory effects without affecting serum lipids

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Summary

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-cholesterolamieic ApoE-deficient murine model. Male, high-fat diet fed, ApoE-deficient (ApoE−/−) mice were administered either PBS, scrambled PIF (0.3–3 mg/kg) or PIF (0.3–3 mg/kg) for seven weeks. After treatment, PIF (3 mg/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. In conclusion, 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

Introduction

Previously considered merely as a lipid storage condition, atherosclerosis is now well established as a chronic inflammatory disease where dyslipidaemia and inflammation equally play a pivotal role. Cells from both the innate and adaptive arms of the immune system, including macrophages, neutrophils, dendritic cells, and T and B lymphocytes, have been shown to contribute to atherogenesis (1). In particular, extensive evidence has identified monocytes and macrophages as key players in various stages of lesion progression. In the early stages of atherogenesis, damaged or activated endothelium attracts the infiltration of monocytes into the subendothelial space, where they differentiate into macrophages and ingest native and modified lipoproteins to become foam cells (2).

During the intermediate and later stages, macrophages secrete cytokines that attract other leukocytes to the atheroma and amplify the inflammatory response, while apoptosis and necrosis of these macrophages leads to the formation of the ‘necrotic core’, rendering plaques vulnerable and susceptible to rupture (1, 2).

Current therapies for atherosclerosis and cardiovascular disease in general are based on targeting risk factor management, such as the lipid lowering (statins) or diabetes management (3). However, despite the extensive evidence of the role of inflammation in the pathogenesis of atherosclerosis, there are currently no effective therapies that directly target immune cells/immunomodatory pathways. Ideally, anti-inflammatory or immunomodulatory therapy should reduce inflammation without compromising normal immune function, and this still remains a clinical challenge (1, 3).
An interesting immunological paradox arises during pregnancy, – the mother (host) tolerates and accommodates the “invading” embryo (allograft) while preserving maternal immunity, the ability to fight infections and regulate autoimmune processes (4–6). Hence, the mammalian embryo can be considered a perfect immune regulatory scenario, maintaining basal immune balance and stepping-up when necessary to enhance immunity and acting as a perfect transplant. Furthermore, it is widely acknowledged that during pregnancy symptoms of autoimmune disease are alleviated, but flare up after pregnancy (7), suggesting that maternal-derived compounds may play an immunomodulatory role to improve these conditions.

Preimplantation factor (PIF) is a novel embryo-specific 15 amino acid, linear peptide, isolated and characterised by Barnea et al. (8, 9). PIF is secreted and present in maternal circulation throughout viable pregnancy but absent from non-viable embryos (4). PIF has been shown to modulate the maternal environment to create a favourable embryo implantation milieu by interacting with immune cells that play a role in decidual changes necessary for appropriate placental formation and trophoblast invasion (5, 10–13). These include antigen presenting, cytokine inducing and phagocytic maternal macrophages and dendritic cells (9, 14–17). These characteristics make PIF an ideal candidate as an immunomodulatory therapeutic.

Indeed, the therapeutic potential of the synthetic analog of PIF has been examined in several chronic inflammatory diseases in vivo. In experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis (MS), PIF-treated mice had a higher survival rate, lower pro-inflammatory interleukin (IL)-17 and IL-6 levels and advanced chronic paralysis was reversed (18). In a non-obese diabetic (NOD) mouse model of type 1 diabetes mellitus, subcutaneous administration of PIF prevented diabetes development while maintaining pancreatic function on a long-term basis, as shown by lowered plasma glucose levels, preservation of pancreatic islet architecture, a reduction in inflammatory cell infiltration and regulation of Th1/Th2 cytokine secretion (19). In the PHA-activated PBMC assay, PIF promotes anti-inflammatory IL-10 secretion and reduces pro-inflammatory interferon (IFN)-γ (20). Similarly, in semi- or allogeneic mouse bone marrow transplant models, two week administration of PIF reduced acute graft-versus-host disease while maintaining the beneficial graft-versus-leukemia, reducing liver and skin inflammation and colonic ulceration, systematically reducing circulating pro-inflammatory IL17-a levels (21), while PIF also promoted neuroprotection in a hypoxic-ischaemic brain injury model (22, 23).

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

Materials and methods
PIF peptide synthesis

Synthetic PIF, a fifteen-amino-acid peptide (MVRIKP-GSANKPSDD), scrambled PIF (control) (scPIF; GRVDPKSNMP-KDIA) were obtained from Biosynthesis Inc. (Lewisville, TX, USA).

Animals

Male, ApoE−/− mice on a C57BL6/J background were originally obtained from the Animal Resource Centre (Canning Vale, WA, Australia). At six weeks of age, mice were placed on a high fat diet (HFD; 22% fat, 0.15% cholesterol; SF00–219, Specialty Feeds, Glen Forrest, WA, Australia) and tap water ad libitum throughout the experimental period. Mice were randomly assigned to receive one of the following treatments: PIF (0.3 mg/kg or 3 mg/kg), PBS, or scrambled PIF (0.3 mg/kg or 3 mg/kg). The mice received intraperitoneal injections three times per week for seven weeks. All animal procedures were reviewed and approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in compliance with the National Health and Medical Research Council of Australia’s (NHMRC) Guidelines for the Ethical and Humane Use of Animals in research.

Tissue processing and analysis

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

Histology and immunohistochemistry

For histology and immunohistochemistry analysis of atherosclerotic lesions, the aortic arch and aortic sinus were sectioned at 6 μm thickness. To allow for quantification of lesion area, lipid content and collagen sections were stained with either standard Mayer's haematoxylin/eosin (Sigma-Aldrich, St. Louis, MO, USA) 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 Biotechnologies, 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 visualised using 3,3’- diaminobenzidine (DAB) and

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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 60 µm 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

Plasma IFN-γ and tumour necrosis factor (TNF)-α levels were measured using enzyme linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) according to manufacturer’s instructions.

Intravital microscopy

For intravital microscopy studies, male C57BL6/J mice, 8–12 weeks old were administered PIF (0.3 mg/kg), scPIF (0.3 mg/kg) or PBS i.p., followed by administration of LPS (1mg/ml; i.p.). At 16 hours after LPS administration, mice were anaesthetised with ketamine/zylazine (100 mg/kg and 10 mg/kg body weight, respectively). The small intestine and supporting mesentery were exteriorised via the incision and placed onto a specially designed transparent viewing stage. The jugular vein was catheterized to administer the fluorescence dye rhodamine 123 (0.05 %; Molecular Probes, Mulgrave, VIC, Australia) to allow for visualisation of leukocytes. Two to four vessels were selected for each experiment, and the same section of each vessel was observed throughout the experiment. Leukocyte migration rolling and adhesion was captured on video for up to 5 min per vessel.

Thioglycollate-induced peritonitis leukocyte visualisation

Peritonitis was induced as previously described (25). Briefly, eight- to 12-week-old C57BL6/J mice were administered PIF (0.3 mg/kg), scPIF (0.3 mg/kg), margatoxin (MTX, 1 pmol/g. Sigma Aldrich) or PBS i.p. followed by 1 ml of 4% thioglycollate (Thioglycollate-Bouillon, Merck, Darmstadt, Germany) to induce peritonitis. At 20 hours (h) after thioglycollate administration, mice were euthanized with (Ketamine/Zylazine). The total peritoneal lavage leukocyte content was quantified using a hemocytometer and monocyte numbers were quantified by flow cytometry.

Flow cytometry

For quantification of monocytes in the peritoneal fluid, fluorochrome-labelled antibodies F4/80 (rat anti-Mouse PE, eBioscience) and CD11b (Anti-Mouse APC, eBioscience, San Diego, CA, USA) 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 analysed 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, VA, 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 (1×10⁶/ml) were pre-treated with either PBS, scPIF (10 µM) or PIF (10 µM) for 40 min at 37°C, then placed in the upper chamber of a cell culture insert with PET membrane (BD; 8 µm pore). The insert was placed into individual wells of a 24-well plate containing MCP-1 (10 ng/ml) (R&D systems). Samples were incubated with MCP-1 for 4 h 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 (100 nM) or human IDE siRNA (50 nM) using DharmaFECT Transfection reagent (all from Dharmacon). At 24 h after transfection, cells were pre-treated with one of PBS, scrambled PIF (10 µM) or PIF (10 µM) for a further 16 h, followed by stimulation with TNF-α (20 ng/ml) for 6 h before cells were harvested for quantitative real-time PCR analysis.

FluxOR™ Potassium Ion Channel Assay

The FluxOR™ Potassium Ion Channel Assay (LifeSciences, Marlborough, MA, USA) 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, Manassas, VA, USA) was loaded with FluxOR reagent and challenged according manufacturer protocol using one of the following compounds in

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Figure 1: PIF inhibits plaque development in the aortic sinus and aortic arch of hypercholesterolaemic ApoE<sup>-/-</sup> mice. ApoE<sup>-/-</sup> mice were maintained on a high fat diet for seven 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 eight 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.

Table 1: Serum cholesterol, triglycerides, VLDL/LDL and HDL. All data represent mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>PBS (n=12)</th>
<th>scPIF 0.3mg/kg (n=8)</th>
<th>PIF 0.3mg/kg (n=8)</th>
<th>scPIF 3mg/kg (n=8)</th>
<th>PIF 3mg/kg (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cholesterol (mmol/L)</strong></td>
<td>3.53 ± 0.57</td>
<td>2.96 ± 0.54</td>
<td>2.49 ± 0.4</td>
<td>3.13 ± 0.65</td>
<td>3.43 ± 0.293</td>
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<tr>
<td><strong>VLDL/LDL (mmol/L)</strong></td>
<td>2.10 ± 0.3</td>
<td>1.50 ± 0.33</td>
<td>1.47 ± 0.28</td>
<td>1.74 ± 0.44</td>
<td>2.10 ± 0.24</td>
</tr>
<tr>
<td><strong>HDL (mmol/L)</strong></td>
<td>1.29 ± 0.31</td>
<td>1.33 ± 0.21</td>
<td>0.97 ± 0.18</td>
<td>1.26 ± 0.23</td>
<td>1.19 ± 0.16</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/L)</strong></td>
<td>0.31 ± 0.03</td>
<td>0.26 ± 0.09</td>
<td>0.14 ± 0.03</td>
<td>0.27 ± 0.16</td>
<td>0.29 ± 0.07</td>
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</table>
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+ Ly6C low) macrophages, B1 (CD5+ CD19+), B2 (CD5- CD19+), CD4 (TCRβ+ CD4+), CD8 (TCRβ+ CD8+), NK (TCRβ- NK1.1+), and NKT (TCRβ+ NK1.1+) lymphocytes. PIF treatment significantly reduced CD4 T cells in blood samples.

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, very low-density lipoprotein (VLDL)/low-density lipoprotein (LDL), high-density lipoprotein (HDL) were measured using a commercially available kit. Our results demonstrate that PIF (0.3 mg/kg - 3 mg/kg) had no significant effect on the lipid profile (Table 1).

PIF attenuates atherosclerotic plaque progression

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

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

Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4 T cells</th>
<th>CD4/CD8 ratio</th>
<th>CD11b+ Ly6C+ M1 Macrophages</th>
<th>CD11b+ Ly6C- M2 Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>500 ± 50</td>
<td>0.8</td>
<td>10 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>scPIF 0.3 mg/kg</td>
<td>400 ± 40</td>
<td>0.7</td>
<td>12 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>PIF 0.3 mg/kg</td>
<td>300 ± 30</td>
<td>0.6</td>
<td>15 ± 3</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>scPIF 3 mg/kg</td>
<td>250 ± 25</td>
<td>0.5</td>
<td>18 ± 4</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>PIF 3 mg/kg</td>
<td>200 ± 20</td>
<td>0.4</td>
<td>20 ± 5</td>
<td>30 ± 6</td>
</tr>
</tbody>
</table>

PIF treatment significantly reduced CD4 T cells in blood samples (Suppl. Figure 4A-B, available online at www.thrombosis-online.com). 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 (Suppl. Figure 4C, available online at www.thrombosis-online.com).
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 characterised. Lipid content of atherosclerotic plaques was assessed using oil Red O staining. PIF (3 mg/kg) significantly reduced plaque lipid content in both the aortic sinus and aortic arch when compared to scPIF 3 mg/kg (p<0.0001, p = 0.0093, respectively; ▶ Figure 2 A).

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 (3 mg/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; ▶ Figure 2 B). 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; ▶ Figure 2C). Expression of the macrophage marker CD68 in the aortic sinus decreased (43%) when compared to scPIF 3 mg/kg (p = 0.0017; ▶ Figure 2D, and a significant reduction in CD68 expression was also evident in the aortic arch (p = 0.0035; ▶ Figure 2D; Suppl. Figure 4A, available online at www.thrombosis-online.com).

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 (3 mg/kg) treated mice as compared to scPIF (3 mg/kg) or PBS (▶ Figure 2E). PIF had no effect on plasma TNF-α levels.

![Image of 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 haemocytometer. B) C57BL/6J mice were administered PBS, scPIF or PIF, followed by LPS (80 µg). After 16 h, rhodamine was administered via the jugular vein and the mesentery arteries were exteriorised to visualise (right panel) and quantify (left panel) leukocyte rolling and adhesion. C) C57BL/6J 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 h, 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 six independent experiments per group. Statistical significance was calculated using one-way ANOVA, followed by Dunn’s post test. *P<0.05.](image-url)
Chen, Rivera et al. PreImplantation factor prevents atherosclerosis 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; Suppl. Figure 1, available online at www.thrombosis-online.com). The adhesion molecule VCAM-1 was also downregulated by PIF treatment compared to scramble siRNA. C) TNF-α-induced VCAM-1 mRNA expression (normalized to 1) was reduced by PIF and this effect was negated after IDE knockdown using siRNA. A similar trend but no statistical significance of PIF’s effect 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.

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 lymphocytes (17). 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 µM & 10 µM) significantly reduced MCP-1 (10 ng/ml)-induced chemotaxis in vitro when compared to PBS or corresponding doses of scPIF (p<0.01; Figure 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 (Figure 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 h after induction of peritonitis and F4/80+CD11b+ monocytes/macrophages were quantified via flow cytometry. In line with our intravital microscopy findings, both doses of PIF used (0.3 mg/kg and 3 mg/kg) caused a significant reduction in influx of F4/80+CD11b+ monocytes/macrophages in the peritoneal cavity (Figure 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 (Suppl. Figure 2, available online at www.thrombosis-online.com), 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 ProteArray (11). More recently, a microarray assay showed that biotin-PIF significantly binds to anti-IDE positive embryonic 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 thiglycollate-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 (1 pmol/g) significantly decreased thiglycollate-induced leukocyte transmigration in the peritoneal cavity to a similar extent as observed with PIF administration (Figure 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 (Figure 4B). Transfected THP-1 human monocytes were incubated with PIF (10 µM), scPIF (10 µM) or PBS prior to stimulation with TNF-α. At 48 h, TNF-α induced upregulation of VCAM-1 mRNA expression, whereas PIF significantly reduced TNF-α induced VCAM-1 mRNA expression (Figure 4C). Following knockdown of IDE mRNA, the PIF inhibitory effect on VCAM-1 expression was blocked (Figure 4C). IL-12 mRNA expression was investigated in parallel to VCAM-1. However, although there was a trend towards an inhibitory effect of PIF on IL-12b expression, none of the changes in expression level of IL-12 mRNA reached statistical significance (Figure 4C). Overall, the data obtained confirm IDE as a potential target for PIF’s inhibitory effects.

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 (Figure 5A).

We further screened over 111 crystallographic structure models for PIF binding using an in silico “blind” predictive algorithm implemented in the PepSite server (Russel lab) (Figure 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 analysed in order to test whether PIF prefer a specific family of potassium channels over another. A hierarchical clustering dendrogram showed that potassium channel regulatory subunits (KCNAB) had different binding characteristics compared to all other families (Figure 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 channel 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) (Figure 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 KCNAB3B (Kv1.3b). To further confirm this specificity of binding we used an unsupervised learning artificial neural network (self-organising map, SOM) to blindly classify the predictions data based on the score and PIF amino-acids binding patterns. SOM showed that KCNAB were systematised closely together to have highest score of PIF binding suggesting that PIF is most specific to this group of potassium channel regulators (Figure 5E).
Discussion

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

There has been far less investigation of anti-inflammatory agents in atherosclerotic cardiovascular disease. However, the limited clinical data available suggests a benefit of targeting the underlying inflammatory process. Colchicine (an agent effective in multiple inflammatory disorders) has been shown in small trials to prevent both intracoronary stent restenosis and cardiovascular events, in conjunction with a reduction in C reactive protein (CRP) levels (34–37). Similarly, patients with rheumatoid arthritis have an increased risk of cardiovascular events, which appears to be attenuated by the use of TNF-α inhibitors and methotrexate (38–41). Interestingly, methotrexate may provide its protective effect via inhibition of macrophage foam cell formation, in a manner similar to PIF (42). Given such observations, two large clinical trials are currently investigating the use of anti-inflammatory agents (methotrexate and canakinumab, a IL-1β neutralising monoclonal antibody) in patients with coronary artery disease (43). Of obvious concern is the potential toxicity of such potent, immune modulating agents.

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 immune-modulatory 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 (3 mg/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 pregnancy (4). 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 T₃/₃ 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 formation (44). This is consistent with our previous finding on the PHA-activated PBMC, where PIF reduced IFN-γ secretion ex vivo (20). Indeed, double IFN-γ⁻⁻/ApoE⁻⁻ knockout mice have fewer macrophages within plaques (45). 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-ischaemic brain injury (22).

To further assess PIF’s modulatory effect on monocyte function, three different ex vivo and in vitro assays were utilised. Together, the MCP-1 induced chemotaxis assay, the peritonitis model and the MCP-1 induced chemotaxis assay, the peritonitis model

Figure 5: FluxORMT assessment of PIF effects on slow potassium channels in Jurkat T cells. 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. Leaves were annotated with potassium channel Group type. D) Parallel coordinates multidimensional visualisation 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-Organising 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.

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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 effect (11). Thus, we examined in vitro the effect of Kv1.3 and IDE siRNA knockdown on PIF activity in monocytes. PIF blocked TNF-a induced VCAM-1 and IL-12b expression in 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 proteins (46). 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 binding (26).

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 changes (47). 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 statins mediated reduction in cardiovascular events has been correlated with a reduction in CRP (reflecting an anti-inflammatory effect) rather than a reduction in cholesterol (48). In addition, statins may also have beneficial effects on distinct inflammatory disorders such as rheumatoid arthritis (49). 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 favourable 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 stabilisation 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 atherosclerotic plaques. While further studies will be required to elucidate the biological mechanisms involved in PIF's actions, preliminary findings indicate that Kv1.3 and IDE are potential PIF binding partners. Overall, PIF potentially represents a novel immunomodulatory drug that can selectively target inflammation and atherosclerosis independent of lipid lowering and without compromising immune function, and thus warrants further investigation in animal models and human trials.

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

References