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PreImplantation factor (PIF*) regulates systemic immunity and targets protective regulatory and cytoskeleton proteins

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ABSTRACT

Secreted by viable embryos, PIF is expressed by the placenta and found in maternal circulation. It promotes implantation and trophoblast invasion, achieving systemic immune homeostasis. Synthetic PIF successfully transposes endogenous PIF features to non-pregnant immune and transplant models. PIF affects innate and activated PBMC cytokines and genes expression. We report that PIF targets similar proteins in CD14+, CD4+ and CD8+ cells instigating integrated immune regulation. PIF-affinity chromatography followed by mass-spectrometry, pathway and heatmap analysis reveals that SET-apoptosis inhibitor, vimentin, myosin-9 and calmodulin are pivotal for immune regulation. PIF acts on macrophages down-stream of LPS (lipopolysaccharide-bacterial antigen) CD14/TLR4/MD2 complex, targeting myosin-9, thymosin-α1 and 14-3-3eta. PIF mainly targets platelet aggregation in CD4+, and skeletal proteins in CD8+ cells. Pathway analysis demonstrates that PIF targets and regulates SET, tubulin, actin-b, and S100 genes expression. PIF targets systemic immunity and has a short circulating half-life. Collectively, PIF targets identified; protective, immune regulatory and cytoskeleton proteins reveal mechanisms involved in the observed efficacy against immune disorders.

1. Introduction

Immune homeostasis is essential for survival. Mammalian pregnancy is the prime example of an effective and nearly optimal immune environment. It is perceived to be the best natural graft–host interaction. Our goal has been to decipher this highly effective embryo/maternal signaling and understand its therapeutic potential.

The earliest events leading to and required for achieving maternal recognition of pregnancy post-fertilization were investigated. Specific signals derived from the embryo (and not from the mother) are essential for maternal recognition; Preimplantation factor (PIF) is a peptide which plays a determining role in that respect (Barnea, 2004, 2007a; Stamatkin et al., 2011a, 2011b; Barnea et al., 1999; Than et al., 2007a). PIF is secreted by viable embryos, and absent in non-viable ones; the peptide's presence in maternal circulation correlates significantly with live birth (Barnea, 2004, 2007a; Stamatkin et al., 2011a, 2011b; Barnea et al., 1999; Than et al., 2007b; Roussev et al., 1996; Ramu et al., 2013). Beyond promoting implantation and trophoblast invasion, PIF also has autotrophic and protective effects on the embryo (Stamatkin et al., 2011a, 2011b; Paidas et al., 2010; Barnea et al., 2012a; Duzyj et al., 2010; Moindjie et al., 2014). Specifically, PIF targets proteins in the embryo protecting against oxidative stress and protein misfolding: protein-disulfide isomerase (PDI) and heat shock proteins (HSPs). Furthermore, PIF targets proteins that support nerve and visceral development (tubulins and actins) (Stamatkin et al., 2011a, 2011b; Duzyj et al., 2014; Barnea et al., 2014).

Development of tolerance without concomitant immune suppression is essential for effective embryo/maternal interaction. To

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this end, PIF was shown to regulate global immunity by exerting only a minimal effect on the innate portion, while exerting a concurrent robust effect on adaptive immunity (Barnea et al., 2012b, 2015c; Roussev et al., 2013). Preclinical studies of autoimmunity, transplantation and reversal of brain injury models confirmed PIF's immune protective properties in non-pregnant models (Weiss et al., 2011, 2012; Shainer et al., 2013; Mueller et al., 2014, 2015; Azar et al., 2013). The FDA awarded Fast-Track designation, and University-sponsored clinical trials for immune disorder in non-pregnant subjects are ongoing (Clinicaltrials.gov).

PIF directly targets immune cells, specifically macrophages and neutrophils. Following activation it targets both CD4+ and CD8+ cells reducing mixed lymphocyte reaction, anti-CD3 antibody and phytohemagglutinin (PHA) induced proliferation. Following anti-CD3/CD28 antibody activation of PBMC, PIF promotes cytokines secretion and associated genes expression leading to Th2 cytokine bias while preserving the Th1 response required for anti-pathogen protection. PIF action is mostly intracellular and independent of Ca^{2+} mobilization, affecting downstream the calcineurin pathway (Barnea et al., 2012b, 2015c; Roussev et al., 2013). Data in LPS activated macrophages demonstrated that PIF reduced iNOS (NOS2) as well as nitric oxide secretion, confirming the protection against graft *vs.* host disease development (Azar et al., 2013). The effect of PIF on microglia culture revealed novel mechanisms involved in PIF action, namely the reduction in let-7microRNA dependent pathway involving PKC/PKA kinases, confirming the neuroprotective effects observed against brain injury (Mueller et al., 2014, 2015). However, specific protein targets involved in PIF's regulatory action in the immune system thus far have not been characterized. Identification of targets involved would provide valuable mechanistic insight into PIF's immune regulatory action.

PIF blocks LPS-induced activation in macrophages which may be transduced by TLR4 dependent or independent pathways (Mueller et al., 2014; Azar et al., 2013; Hoebe et al., 2003; Kayagaki et al., 2013; Hagar et al., 2013). PIF's inhibitory effect is abolished after TLR4 siRNA-mediated silencing. Further PIF regulatory effect on the PKC/PKA phosphorylation pathways was abolished in the presence of TLR4 siRNA (Mueller et al., 2015).Whether PIF directly targets TLR4 has not yet been elucidated. Further, the inhibitor itself can alter protein expression (Vabulas et al., 2002; Ueno et al., 2005). Information whether PIF is directly or indirectly involved in TLR4 pathways would provide important information on PIF interaction with macrophages.

In order to determine which targets are involved in PIF's immune regulatory action, protein targets in unstimulated CD14+ and CD4+ or CD8+ cells were identified. Since the TLR4 pathway is important for activated macrophages, we examined PIF-TLR4 interaction and associated pathways. To confirm PIF's systemic immune system interaction, the *in vivo* binding to macrophages and lymphocytes was examined.

The PIF protein targets, identified exert an integrated protective, immune and cytoskeleton regulatory role. This targeting is coupled by changes in associated genes that are modulated by PIF in PBMCs, PIF's action is independent of TLR4 binding but involves TLR4-regulated proteins. Finally, PIF shortly after administration directly targets the immune system *in vivo*. Collectively, PIF's effective interaction in systemic immunity as well as related preclinical data support clinical translation for the treatment of immune disorders.

2. Methods

Synthetic PIF_{15} (MVRIKPGSANKPSDD) and a random peptide, designated RP (GMRELQRSANK) were synthesized by solid-phase peptide synthesis (Peptide Synthesizer, Applied Biosystems) employing Fmoc (9-fluorenylmethoxycarbonyl) chemistry at Bio-Synthesis, Inc. (Lewisville, TX). Final purification was carried out by reversed-phase HPLC and identity was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and amino acid analysis at >95% purity.

2.1. PBMC isolation

Whole blood units were obtained from three different non-pregnant healthy donors after obtaining consent (Sanguine BioSciences, Inc., CA). Sanguine BioSciences's policies and procedures protect the subjects from whom we collect specimens and medical data. These policies and procedures were in accordance with current regulations and guidelines and approved by the Office of Human Research Protections (OHRP), an office within the U.S. Department of Health & Human Services (HHS) (www.Sanguinebio.com). Following separation using Ficoll-Hypaque, isolated PBMC were passed separately over CD14+, CD4+ or CD8+ affinity columns. The isolated cells were washed with PBS, frozen in serum-free media, and shipped at −80 °C to Eprogen for further processing.

2.2. CD14+, CD8+ & CD4+ cells lysis and PIF binding extraction protocol

A PIF-resin affinity column was specifically designed for this study to replace the multistep method (Barnea et al., 2014). Briefly, a carbon spacer (C6) was affixed to PIF (15AA) at the N-terminus with a terminal cysteine moiety thiol group which was then conjugated to an agarose resin (Biosynthesis, Lewisville, TX). A 15 mL centrifuge tube containing 8–10 million cells was lysed with 1.5 mL of a non-detergent lysing buffer (NDLB) (Eprogen, Downer's Grove, IL) by two freeze–thaw cycles from room temperature to −80 °C; the resulting lysate was centrifuged at ~6000 × *g*. The general protocol for extraction of cells was as follows: 50 μL mL of PIF resin was centrifuged for 1 min (~6,000 × *g*) and washed twice with 150 μL of NDLB. We added 450 μL of the lysate supernatant to the washed resin and then incubated 1 h. at 4 °C, with intermittent vortexing to ensure good PIF resin–protein contact. The tubes were then centrifuged for 1 min (~6000 × *g*) and washed twice with 100 μL NDLB. Filtrates were combined and diluted to 400 μL total volume with NDLB for ProteoSep® RP HPLC runs. The lysate-treated resin containing the proteins bound to PIF was extracted twice with 150 μL of 0.1 M glycine-HCl solution by vortexing for 10 min and then centrifuging or 1 min. The resulting filtrates containing the PIF extracted proteins were combined and frozen at − 80 °C prior to MS analysis.

2.3. Proteomic MS analysis

2.3.1. Trypsin digestion

Trypsin digestion of the protein extracts was conducted using the Filter-Assisted Sample Preparation digestion kit (FASP) per manufacturer procedure (Protein Discovery, Expedeon, San Diego, CA). Briefly, 40 μL of protein lysate extract, described above, was reduced with 4 μmol dithiothreitol (DTT) at room temperature for 1 h. The sample was then mixed with 200 μL of urea sample solution in the spin filter and centrifuged at 14,000 × *g* for 15 min. Sample flow-through was discarded after washing with another 200 μL of urea sample solution. Proteins on the spin filter were then alkylated with iodoacetamide in 90 μL urea sample solution for 20 min in the dark. The proteins in the filter were next washed twice with 100 μL urea sample solution and centrifuged at 14,000 × *g* for 10 min. Then, 100 μL of 50 mM ammonium bicarbonate (NH4HCO3) was added to the spin filter, centrifuged at 14,000 × *g* for 10 min, and repeated twice. Trypsin digestion was conducted overnight at 37 °C using a trypsin:protein ratio of 1:100. After this incubation, the spin filter