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Safety and Efficacy of Polydioxanone Nano-Fibers as Anti-Inflammatory Agents

Sastry Gollapudi*, Chang Sok So, Michael Formica, Sudhanshu Agrawal and Anshu Agrawal*
Division of Basic and Clinical Immunology, Department of Medicine, University of California, USA

Abstract

The use of biodegradable polydioxanone (PDO) nano-fibers for various purposes is increasing but the effects of these fibers on immune responses are not well understood. We examined the potential stimulatory and inhibitory effects of PDO coated fibers on immune cell functions in vitro in humans and in vivo in mice. Short term in vitro exposure of human blood to PDO did not perturb the phagocytic function of human monocytes and neutrophils. In contrast lymphocytes exhibited an increased proliferative function in response to polyclonal T cell mitogen, PHA. However, cytokine secretion by monocytes and lymphocytes as well as NK cell cytotoxic effector cell functions were undisturbed by PDO exposure. Long term in vivo exposure to PDO had no effect on dendritic cells activation, cytokine secretion and T regulatory induction. Injection of PDO into mice with rheumatoid arthritis suggested that PDO nano-fibers tend to be anti-inflammatory as an increase in IL-10 was observed in the PDO treated groups. In spite of this the arthritic score and TNF-α and IFN-γ levels were not significantly different between PDO- treated and untreated rheumatoid arthritis induced mice. In conclusion, PDO nano-fibers have no significant adverse effect on immune functions and tend to induce anti-inflammatory responses upon long term exposure *in vivo*.

Keywords: Polydioxanone; Nanofibers; Inflammation; Immune response

Introduction

Currently, there is much interest and concern on the impact of human exposure to nano-material on health and disease. Nano-materials are defined as substances less than 100 nm in diameter and possess novel chemical and physical properties. Nano-materials have a wide range of industrial, agriculture, military and medical applications [1]. Humans are exposed to nano-materials accidentally or purposely, when introduced into the body to deliver drugs, for imaging studies or as surgical implants. Nano-materials are recognized by the host as foreign and initiate immune and inflammatory responses [2,3]. Therapeutic nano-materials that down regulate immune responses are of value in the treatment of autoimmune and inflammatory diseases.

Polydioxanone (PDO) is a colorless bioresorbable synthetic polymer which undergoes hydrolytic degradation in vivo. PDO is used in the medical field for making suture wires due to its biocompatibility, flexibility, and elasticity [4,5]. No long term cytotoxicity has been observed with PDO implanted for upto 6 months in mice [6]. The non-toxic and biodegradable nature of PDO has led to the use of PDO nano-fiber scaffolds for antimicrobial drug delivery system for regenerative endodontics [7]. PDO plates and scaffolds are also being used for cosmetic procedures such as rhinoplasty and septal surgery where they guide cartilage regeneration [8].

In alternative medicine, PDO nano-fibers are currently being used for the treatment of chronic inflammatory diseases [4,5,9,10]. According to Park [11], acupuncture with PDO needles resulted in improvements in pain and swelling in patients suffering from knee joint pain with edematous knee and other joints. More recently, PDO is being combined with other biodegradable materials such poly-lactic acid, collagen or chitosan for use in soft tissue engineering applications [12,13]. In view of the increasing use of PDO nano-fibers, it is imperative to evaluate their effect on inflammatory and immune responses.

PDO is reported to be immunosuppressive [5,10,14]. *In vitro* evaluations of the effect of PDO and PDO-elastin or PDO-collagen blends on murine immune cell mediated functions suggested that PDO blends exert immunosuppressive effects on NK, T and B cell functions, but have no significant effect on macrophage functions [5,10]. Currently, there is a paucity of information on the impact of PDO on human immune cells and on cells from organisms with immune and inflammatory disease states.

The results of this study show that PDO is not intrinsically immunotoxic to human innate and adaptive immune cell functions *in vitro*. Further PDO appears to induce anti-inflammatory responses in a chronic inflammatory disease model of rheumatoid arthritis in vivo in mice.

Materials and Methods

Blood donors

Blood samples, donated by healthy volunteers were collected. This study was approved by the Institutional Review Board of the University of California, Irvine.

PDO

PDO nano-fibers were a kind gift of Dongbang Acupuncture Ltd, Korea.

Phagocytosis assay

Phagocytosis was assessed by flow cytometry using pHrodo

***Corresponding author:** Anshu Agrawal, Division of Basic and Clinical Immunology, Department of Medicine, University of California, USA, Tel: 949-824-7706; Fax: 949-824-4362; E-mail: aagrawal@uci.edu

Sastry Gollapudi, Division of Basic and Clinical Immunology, Department of Medicine, University of California, USA, E-mail: svgollap@uci.edu

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Escherichia coli or Staphylococcus aureus Bioparticles phagocytosis assay Kit as per manufacturer's instructions (Invitrogen, Carlsbad, USA). pHrodo™ BioParticles are non- or weakly fluorogenic when attached to the outer surface of the phagocyte, but are highly fluorescent in the acidic environment of the phagosome upon internalization. Briefly, whole blood (5ml) was incubated with or without different concentration PDO at 37°C. After overnight incubation, 100 l aliquots of PDO treated and untreated whole blood samples added into flow cytometry analysis tubes. The pHrodo labeled Eshchericia coli (gram negative bacteria) or Staphylococcus aureus (gram positive bacteria) bio particles were added and incubated for 15 minutes at 37°C. Whole blood exposed to bio particles and incubated at 4°C served as a negative control. After incubation the red blood cells were lysed with lysing buffer. The samples were centrifuged washed, suspended in wash buffer and analyzed on a FACS Calibur. Forward and side scatter properties were used to gate PMNs (Polymorphonuclear cells) and monocytes. The percentage of active phagocytes was determined by collecting pHrodo fluorescence signals using 540 nm-606 nm emission filter.

NK cell cytotoxic activity

Whole blood was exposed overnight to PDO fibers and peripheral blood mononuclear cells (MNCs) were isolated. MNCs (effectors) from PDO exposed and unexposed MNCs were incubated with CFSE-labeled target cells (K562) at effector to target cell ratio of 1:50. After 4 hours 5 ul of 7 AAD was added and the extent of CFSE labelled target cells death was measured by flow cytometry as described [15]. NK activity of MNCs is expressed as Lytic Units (LU)/10, cells and is calculated as the ratio of percent killing two percent NK cells present in the sample.

Lymphocyte proliferation

Whole blood was exposed to different amounts of PDO (1, 2 or 3fiber) for 24 h and subsequently cultured with Phytohaemagglutinin (PHA, 10 ug/ml), a polyclonal T cell mitogen and Pokeweed Mitogen (PWM, 10 ug/ml), a polyclonal B cell mitogen, for 72 h. Proliferation was determined by ^{3}H thymidine incorporation as previously described [16].

Human Cytokine assay

Peripheral blood was exposed to PDO fibers overnight and MNCs were isolated. MNCs from PDO exposed and unexposed peripheral blood cells were incubated with Lipopolysaccharide (LPS, a known activator of monocytes and dendritic cells) or anti-CD3 and anti-CD28 beads (Activator of T cells). Cell culture supernatants were collected and production of IL-1β, TNF-α, IL-6 and IL-10 was assayed in the supernatants using specific ELISAs (BD Biosciences) as per the manufacturer's instructions. Briefly, Nunc Maxisorp plates were coated overnight with the capture antibody at 4°C. Next day, the plates were washed using an automatic plate washer and blocked for 1 h using PBS containing 10%FBS at room temperature. Subsequently, the plates were washed and the samples were added and incubated for 2 h. After washing, biotinylated second antibody and streptavidin HRPO was added and plates were incubated for another 1 h. The color was developed with TMB substrate and the reaction was stopped with 2N H_2SO_4.Theplates was read at 450 nm on a spectrophotometer (Biotek).

PDO injection in mice

PDO (3 fibers) was injected in the left leg and right leg was used as control. Mice were sacrificed 4 weeks after the PDO injection. The inguinal lymph nodes were collected and assessed for dendritic cells (DC) using CD11c, CD11b, MHC-II and CD86 antibodies via flow cytometry. T regulatory cells in the lymph node were detected by staining with CD4, CD25 and FoxP3 antibodies.

Lymph node cells were also stimulated with PMA and ionomycin *in vitro* for 24 h. Supernatant collected was assayed for TNF-α, IFN-γ and IL-10 by specific ELISAs.

Rheumatoid arthritis in mice

Susceptible mice strain- DBA/1 was injected intradermally with chicken collagen emulsified in Complete Freunds Adjuvant (Hooke Laboratories Inc., MA) [17]. Mice developed visual signs of arthritis such as paw swelling, abnormal gait by 3 weeks post injection. The severity of arthritis was scored by subjective evaluation using a scale of 0-4 with 4 representing maximum severity. 0 No evidence of erythema and swelling; 1 Erythema and mild swelling confined to the tarsals or ankle joint; 2 Erythema and mild swelling extending from the ankle to the tarsals; 3 Erythema and moderate swelling extending from the ankle to metatarsal joints;4 Erythema and severe swelling encompass the ankle, foot and digits.

At day 21, PDO (3 fibers) was injected in the left leg and right leg was used as control. Mice were scored every 5-6 days and sacrificed 4 weeks after the PDO injection. The inguinal lymph nodes were collected and DC, macrophage phenotype, T regulatory cells and cytokines were detected as described above.

Statistical analysis

Data were analyzed and figures were generated using GraphPad Prism™ 5.00 software (Graph Pad Software, San Diego, USA). Significant differences between groups were determined by Mann-Whitney test at 90% confidence interval (p value <0.05 was considered significant).

Results

Innate Immune responses are the first line of defense against any foreign material (antigen) and involve a variety of immune cells which work in tandem to perform different functions to eliminate and neutralize the threat. This includes the uptake of antigens by neutrophils and monocytes via phagocytosis [18]. We examined whether presence of PDO affected the phagocytic function of neutrophils and monocytes. There were no statistically significant differences between the PDO treated and untreated control group in their ability to phagocytose gram positive-*E.coli* (Figure 1A and B) and gram negative- *S. aureus* (Figure 1C and D) bacteria, suggesting that exposure to PDO does not impair phagocytic function of PMNs and monocytes.

Besides uptake of antigens, innate immune cells also secrete pro-inflammatory cytokines in response to a threat [19]. LPS stimulation induced significantly increased levels of TNF-α, IL-1β IL-6 and IL-10 (Figure 1E, F, G, H) in both PDO treated and untreated groups. However, the levels of all these cytokines were comparable between the two groups.

NK cells display natural cytotoxicity against several tumors [20]. We determined the effect of PDO on NK cell cytotoxic function. Exposure to PDO had no significant effect on NK cell cytotoxic activity (Figure 1I).

Together, this data demonstrates that short term *in vitro* exposure of human innate immune cells to PDO does not adversely affect the physiological functions of PMNs, monocytes and NK cells.

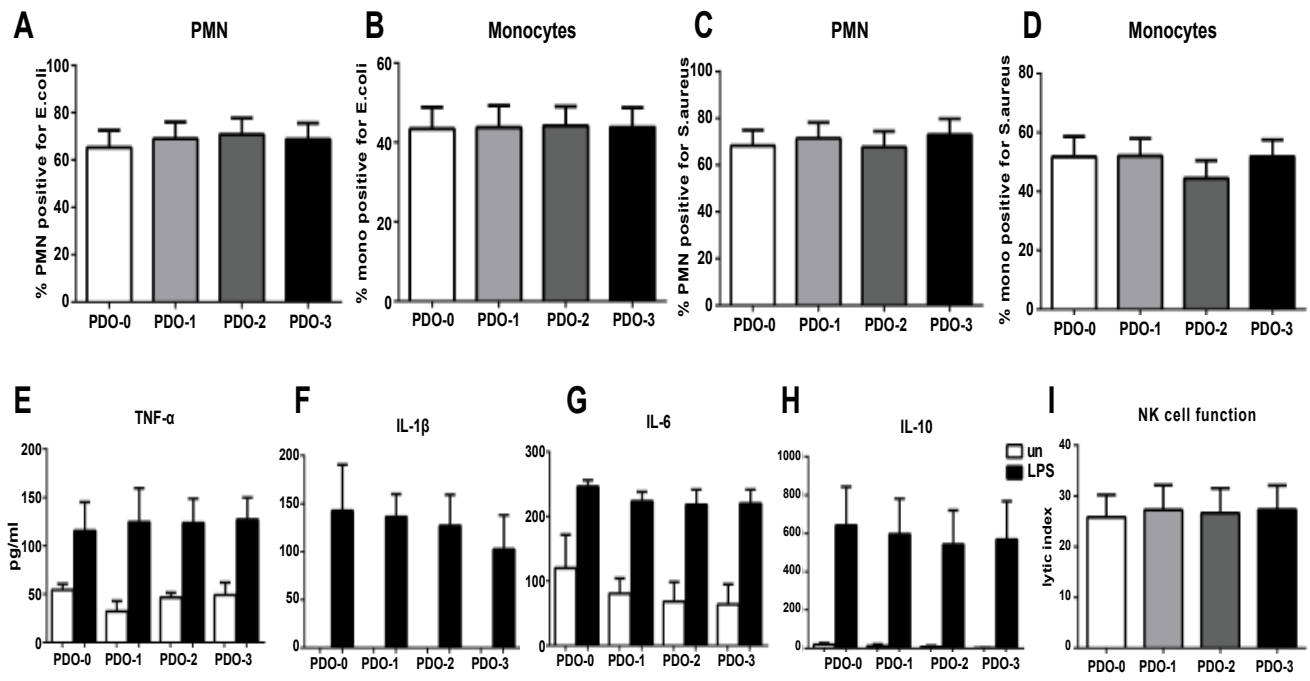

Figure 1: PDO has no effect on the innate immune responses in vitro in human cells. Blood was exposed to 1, 2 or 3 PDO nano-fibers. The phagocytic capacity, cytokine secretion and NK cell cytotoxicity was determined. Bar graphs depict the phagocytic uptake of A & B. fluorescent E.coli bacteria C & D. fluorescent S. aureus bacteria by PMNs and monocytes respectively. E. F. G. & H. MNCs were stimulated with LPS in the presence or absence of PDO. Bar graphs depict the secretion of cytokines, TNF-α, IL-1β, IL-10 and IL-10. I. Bar graph depicts the lytic activity of NK cells. PDO-1, PDO-2, PDO-3 depict the number of fibers added/well. Data is mean +/- S.E. of 10 different subjects.

Effect of PDO on adaptive immune cells functions *in vitro*

Lymphocyte proliferation is an important early event in the initiation of an effective immune response. Any agent that interferes with this early cellular process might therefore dampen the immune response and reduce the ability of the host to eliminate infectious agents. We investigated the effect of PDO on mitogen-stimulated proliferation of lymphocytes. The proliferative response of PHA-stimulated lymphocytes exposed to PDO was substantially higher than the control group (Figure 2A). The magnitude of this increase in proliferation was dose dependent and statistically significant stimulatory effect of PDO was seen at higher dose of exposure (PDO 3 fibers, p=0.015). These results suggest that short term exposure to PDO may enhance T cell function.

Exposure of blood to PDO did not appreciably alter the ability of lymphocytes to respond to PWM, a polyclonal B cell mitogenic agent (Figure 2B) suggesting that PDO does not interfere with B lymphocyte function. MNCs were also stimulated with anti-CD3 and anti-CD28 beads to activate T cells. Exposure to PDO had no significant effect on T cell TNF-α IFN-γ and IL-10 secretion (Figure 2C, D and E). In summary, PDO enhances the proliferation of T cells but has no adverse effect on B cell proliferation and T cell cytokine secretion.

In vivo effects of PDO after injection in mice

Next, we determined the effect of long term exposure of PDO fibers injected in thigh of mice on immune responses in vivo. PDO injection had no significant effect on the size as well as number of cells recovered from lymph nodes (data not shown). To determine if PDO activated the immune system we measured the expression of activation markers CD86 and MHC-II on DCs in the lymph nodes. DCs are the initiators and regulators of the immune responses and any change in their function would have a profound effect on downstream adaptive immune responses [21]. Figure 3A demonstrates that no significant difference in the expression of CD86 and MHC-II between the PDO injected and control group existed, suggesting that PDO does not activate DCs. Similar results were observed for macrophages (data not shown).

Since previous reports have indicated that PDO may be anti-inflammatory [14], we compared the number of T regulatory cells (Treg) between PDO and control group. CD4+ and CD25+ T cells expressing the transcription factor FoxP3 are defined as T regulatory cells [22]. These cells are important regulators of immune and inflammatory response and play a major role in suppressing these responses when they are no longer required. Increased percentage of these cells is therefore indicative of immunosuppression. The percentage of FoxP3 expressing T reg cells was higher in PDO injected group; however, the difference was not significant (Figure 3B, p=0.66).

To further determine the effect of PDO on inflammation, we measured the secretion of pro-inflammatory cytokines, TNF-α, IFN-γ and anti-inflammatory cytokine, IL-10 secretion by PMA, ionomycin stimulated lymph node cells. There was a slight, though insignificant, increase in IL-10 production in the PDO group (Figure 3C). IFN-γ and TNF-α also displayed a slight decrease in PDO treated group compared to controls (Figure 3D, 3E). These data suggest that PDO may be anti-inflammatory in nature.

Altogether, the data suggests that long term exposure to PDO does

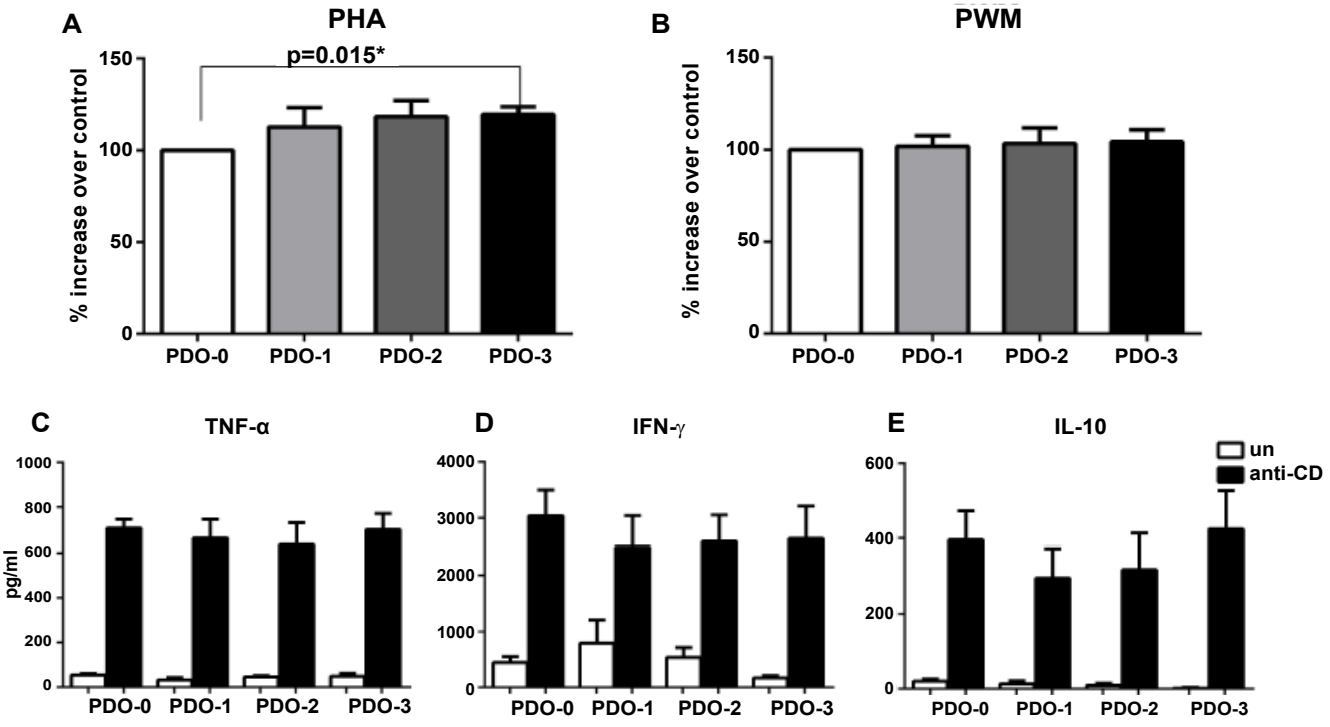

Figure 2: Effect of PDO on adaptive immune responses in vitro. Blood was exposed to PDO nano-fibers and stimulated with PHA or PWM. The proliferation of lymphocytes was determined by thymidine [H]3 incorporation. Bar graph depicts percent increase after A. PHA stimulation. B. PWM stimulation. C, D, E. MNCs were stimulated with anti-CD3 and anti-CD28 in the presence or absence of PDO. TNF-α (C), IFN-γ (D) and IL-10 (E) secretion was measured by ELISA. Data is mean +/- S.E. of 10 different subjects.

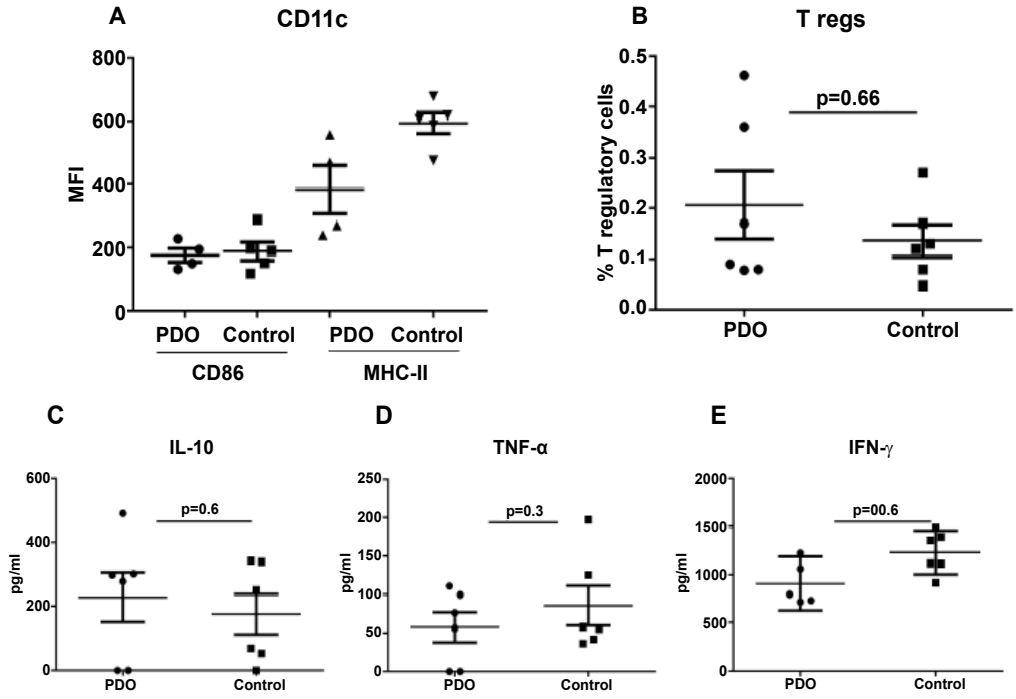

Figure 3: In vivo effects of PDO after injection in mice. The effect of PDO on DC activation, T reg generation and cytokine secretion was measured in inguinal lymph node cells using flow cytometry and ELISA. A. Dot Plot depicts the MFI of expression of activation markers, CD86 and MHC-II on CD11c gated DCs. B. The percentage of CD4+, CD25+, FoxP3+ T reg cells were measured by flow cytometry and are depicted in the dot plot. C, D & E. Secretion of cytokines, IL-10, TNF-α and IFN-γ was determined by ELISA. Data is mean +/- S.E. of 6 mice. Each dot corresponds to one mouse.

not adversely affect the functions of innate and adaptive immune cells. PDO injection leads to minor decreases in inflammation which is not substantial enough to compromise the functions of immune cells.

Effect of PDO on inflammation in a murine model of rheumatoid arthritis

Since the experiments described above suggest that PDO may be anti-inflammatory, we used a rheumatoid arthritis (RA) model to determine the effect of PDO on inflammation. RA is an autoimmune disease where an over reactive immune system enhances inflammation and leads to the destruction of joints [23]. The injection of collagen in CFA induces the production of inflammatory cytokines, TNF-α, IL-6, etc. that contribute to joint destruction along with autoimmune reactions against collagen. The body tries to counteract the inflammation via induction of anti-inflammatory cytokine, IL-10 and Tregs. However, in this model, the inflammation is not suppressed successfully and mice develop RA. Therefore, it serves as a good model to determine the effects of PDO on inflammatory responses. RA was induced by injection of collagen. PDO was injected in the right leg when the disease was at its peak around score 3. Left leg was used as control. PDO injection resulted in increased swelling of ankles for up to a week to ten days after injection but after that the swelling was reduced in the PDO injected group and did not come back up till the end of the experiment (Figure 4A, 4B). The arthritic score was lower in the PDO treated group compared to non-treated group but the overall difference was not significant [24].

Similar experiments as described in Figure 3 were performed. No significant difference in the expression of either the numbers

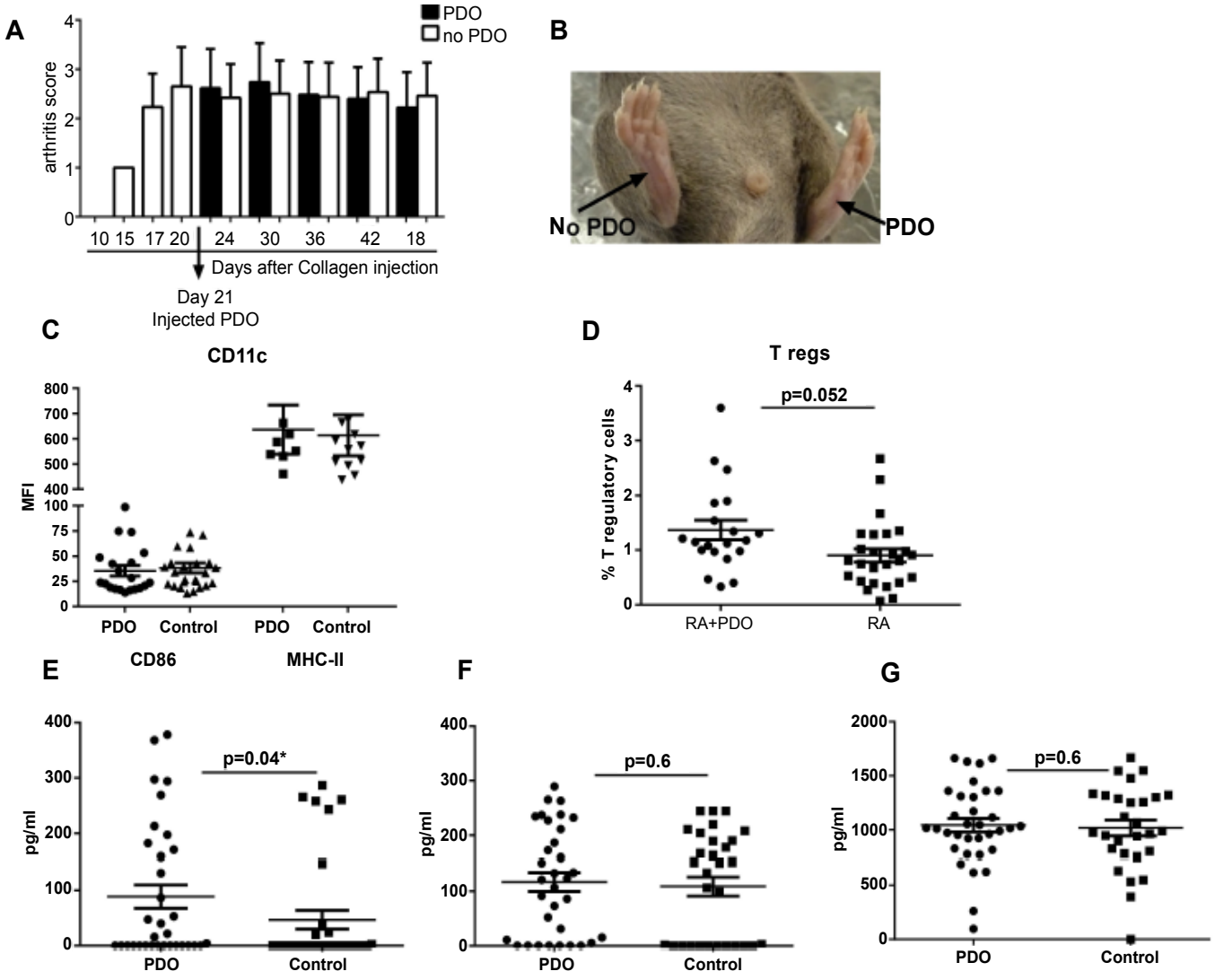

Figure 4: Effect of PDO on inflammation in a murine model of rheumatoid arthritis. RA was induced in mice by injecting with collagen in CFA. 21 days later, 3 fibers of PDO were injected in the right thigh of mice. 4 weeks after the injection of PDO, the inguinal lymph nodes were removed and the effect of PDO on DC activation, T reg generation and cytokine secretion was measured using flow cytometry and ELISA. Left thigh lymph node from the same mice was used as control. A. Bar graph depicts the arthritic score of mice in PDO- treated and untreated legs. B. Picture shows arthritic paw from PDO treated and non-treated mice at day 42 after collagen injection. C. Dot Plot depicts the MFI of expression of activation markers, CD86 and MHC-II on CD11c gated DCs. D. The percentage of CD4+, CD25+, FoxP3+ T regulatory cells were measured by flow cytometry and are depicted in the dot plot. E, F & G. Collected lymph nodes were stimulated overnight with PMA and ionomycin and secretion of cytokines, IL-10, TNF-α and IFN-γ was determined by ELISA. Data is mean +/- S.E. of 28 mice. Each dot corresponds to one mouse.

(data not shown) or the expression of CD86 and MHC-II on DCs (Figure 4C) was observed. The percentage of FoxP3 expressing T reg cells was higher in PDO treated RA lymph nodes nearly approaching significance (p=0.052, Figure 4D) suggesting that PDO tends to be anti-inflammatory.

Next, we determined the difference in secretion of TNF-α, IFN-γ and IL-10 between PDO-treated and untreated RA lymph nodes. IL-10 was significantly higher (p=0.04, Figure 4E) in PDO-treated lymph nodes compared RA alone lymph nodes. The secretion of TNF-α and IFN-γ was comparable between the two groups (Figures 4F and G). IL-1RA levels were below the detection limits of the assay (data not shown). Enhanced IL-10 secretion also suggests that PDO is anti-inflammatory.

In summary, injection of PDO in RA induced lymph nodes does not enhance the inflammation or worsens the disease. Instead it leads to slightly increased T regs and significantly increased IL- 10 induction that can dampen the inflammation this result is beneficial for the treatment of RA. However, PDO had no significant effect on arthritic score or the secretion of TNF-α and IFN-γ suggesting that PDO is only mildly anti-inflammatory and cannot be used by itself for treatment of inflammatory disorders.

Discussion

The increasing use of PDO nano-fibers for cosmetic and other purposes [4-10] makes it imperative to understand its effect on the immune system. Our data suggests that PDO has no adverse effect on the functions of PMNs, monocytes and NK cells in humans (Figure 1). It enhances T cell proliferation (Figure 2A) but has no effect on B cell proliferation as determined by PWM stimulation (Figure 2B). In contrast to our observations, Smith et al. [5,10] reported significant suppression of murine NK, T and B cell functions by PDO. This discrepancy in experimental results may be in part due to differences in susceptibility of human versus murine lymphoid cells to the inhibitory effects of PDO or due to differences in experimental conditions.

Our finding that PDO did not decrease the production of pro-inflammatory cytokines but caused significant increase in IL-10 production and enhanced T regs in mice with RA suggests that PDO alone is not sufficient to cure inflammatory diseases. However, it may dampen inflammation which may be one of the reasons PDO acupuncture is effective in reducing pain associated with lumbago. In summary, PDO does not adversely affect the function of immune cells *in vitro* and in vivo. PDO does not elicit inflammatory response in vivo and does not interfere with an ongoing immune response; it was not extremely immunosuppressive as it did not show an effect in the absence of inflammation. Therefore, when used for cosmetic purposes, PDO will not prevent the immune system from responding to infections. PDO fibers may be used in treatment of inflammatory diseases in conjunction with other treatments. PDO can also be used for delivery of drugs in inflammatory diseases where it can help in suppressing the inflammation as well as aid in providing slow and sustained release of drugs. PDO is thus a safe and well tolerated bioresorbale polymer that has a number of potentially useful biological applications.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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