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Anti Inflammatory Property of PDRN—An *in Vitro* Study on Cultured Macrophages

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Abstract

Skin aging and most age-related diseases are associated with a low-grade chronic inflammation. The nucleoside adenosine, a potent endogenous anti-inflammatory agent, is deeply involved in inflammatory diseases and, by interaction with the adenosine A_2 receptor ($A_{2A}R$) it immediately promotes a mechanism of defence against the inflammatory damage. The aim of our study was to investigate whether polydeoxyribonucleotide (PDRN), a mixture of deoxyribonucleotides polymers of different lengths that like adenosine, binds the A_{2A} receptor, can reduce the inflammatory state in the macrophage cell line. RAW264.7, murine macrophage cells, were incubated with PDRN in the presence and in the absence of lipopolysaccaride (LPS), which was the major component of the outer membrane of gram-negative bacteria and which acted as a strong macrophage activator. We assessed the production of nitric oxide and the secretion of inflammatory mediators (*i.e.*, TNF-*α*, IL-10, IL-12 and VEGF-A). Our data showed that PDRN produced a significant decrease of inflammation in macrophages pre-stimulated with LPS, assessed in terms of the nitric oxide content ($p < 0.001$) and cytokines secretion ($p < 0.001$). Moreover, PDRN stimulated the release of the vascular endothelial growth factor (VEGF-A), which promoted wound healing. Our study suggested that PDRN, by binding the A_{2A} receptor, contributed to a great extent towards reducing inflammation.

Keywords

Cell Culture, Macrophages, RAW264.7, Inflammation, Skin Aging

1. Introduction

Aging is a multifactorial and progressive degenerative process defined as an accumulation of damage and is closely associated with inflammation. While the etiology of the aging process is not fully understood [1], chronic inflammation

clearly plays a major role, inextricably linking inflammation and aging. Indeed, the levels of inflammatory mediators increase typically with age even in the absence of acute infection or other types of physiologic stress [2].

Continuous up-regulation of pro-inflammatory mediators is induced during the aging process due to an age-related redox imbalance. It is likely that it is caused by the decisive effect of weakened anti-oxidative defence systems and the increased production of reactive oxygen species, such as hydrogen peroxide (H_2O_2) and reactive nitric oxide (NO) [2]. This imbalance that results in the low grade chronic pro-inflammatory state, is referred as inflammaging [3].

The process of skin aging is complex and results from a combination of intrinsic (or chronological) and extrinsic factors, *i.e.* UV radiation (or photoaging), cigarette smoking, air pollution and incorrect nutrition. Intrinsic aging is accompanied by cell loss, thinning of the dermis and fine lines. Inflammaged skin is wrinkled, is associated with dyspigmentation and shows proteolytic activation and degradation of collagen and elastic fibres in the dermis. Inflammation and the accumulation of Reactive Oxygen Species (ROS) are believed to be the main causes of skin aging [4] [5].

It has been suggested that monocytes and macrophages contribute towards inflammaging more than any other cell type. Macrophages are heterogeneous and are present in most tissues where they are responsible for numerous inflammatory, immunological and metabolic processes [6]. Monocytes change with age and contribute to inflammaging by causing a functional shift towards a pro-inflammatory phenotype and reduced function [7]; indeed these cells increase the production of inflammatory cytokines and the lengthen of the immune response [8].

One of the major pathways by which the oxidative damage produced by free radicals promotes inflammatory responses, involves the Toll-like-receptors (TLRs). TLRs are a family of ubiquitously expressed receptors that play an important role in the innate immune response, particularly in the initial interaction between the infecting microorganism and phagocytic cells, such as macrophages [9].

TLRs recognize molecules that are crucial for the integrity of the microorganism, but that are non-self molecules either due to their chemical composition or due to their cellular localisations [10]. Microbial cell-wall components such as lipopolysaccharides (LPSs), endotoxins from the outer membranes of gram-negative bacteria, lipopeptides and flagellar proteins are recognized at the plasma membrane of immune and epithelial cells by TLR4, TLR2 and TLR5, respectively [11].

In particular, TLR4 activation by LPSs initiates an inflammatory response, whose key mediators are tumour necrosis factor-α (TNF-α) and interleukin-12 (IL-12), and secrete nitric oxide (NO), a short-lived free radical, which mediates many biological functions such as host defence, neurotransmission, neurotoxicity and vasodilation. In elderly persons, an increased level of these pro-inflammatory cytokines is often observed both in the general circulation and in tis-

sue-resident macrophages [12].

In addition to the resolution of inflammation in response to pathogens or tissue damage, macrophages are also involved in the promotion of proper wound healing, by inducing the production of VEGF and angiogenesis. These specific physiological functions derive from the plasticity of macrophages that allow them to change their form and function in response to environmental signals. Current macrophage classification recognizes polarization according to two distinct phenotypes: classically (M1) or alternatively (M2) activated macrophages [13].

M1 macrophages are activated by pathogen-associated molecular patterns, such as LPS or by the T cell-secreted cytokine interferon gamma (IFNγ). They are of the pro-inflammatory phenotype, increasing the production of pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-12 and TNF-*α*) and oxidative metabolites (e.g., NO and superoxide) to promote host defence.

M2 macrophages, on the other hand, are induced by a variety of stimuli (e.g., IL-4/IL-13 and glucocorticoids) and are of a phenotype involved in the promotion of wound healing, tissue remodelling and the resolution of inflammation [13].

Recently, Grinberg and colleagues have defined a new sub-type of M2-like macrophages, induced by the co-stimulation of macrophages with TLRs and adenosine A_{2A} receptor ($A_{2A}R$) agonists; this stimulation switches macrophages from the M1 phenotype to a M2-like phenotype, known as M2d [14] [15].

The nucleoside adenosine is an endogenous anti-inflammatory agent and is a potent physiologic and pharmacologic regulator that is produced by cells in response to stress due to the breakdown of ATP. In particular, activation of the A_{2A} receptor is one of the most fundamental and immediate mechanisms for protecting tissues against inflammatory damage, altering the cytokines network by decreasing the secretion of inflammatory cytokines by macrophages *in vitro* [16].

Several studies have indicated that adenosine is involved in the modulation of the inflammatory process [17], e.g. $A_{2A}R$ agonists inhibit cartilage damage, by diminishing IL-8 expression [18] or reduce the inflammatory response in articular chondrocytes [19].

Polydeoxyribonucleotide (PDRN) is a mixture of deoxyribonucleotides polymers of different lengths and is an A_{2A} receptor agonist. PDRN is commonly used in clinical settings for pre and post-surgical cutaneous treatments, diabetic foot ulcers and venous ulcers because it stimulates fibroblast metabolism and promotes an increase in dermal matrix component production [20]. Other clinical studies have also pointed out that PDRN promotes faster healing of autologous skin grafts at donor sites [21] [22].

The aim of this work was to investigate whether polydeoxyribonucleotide (PDRN), an A_{2A} receptor agonist, can induce an anti-inflammatory phenotype in murine macrophage cells RAW264.7, endowed with high endogenous TLR4 expression, focusing on nitric oxide production and secretion of inflammatory

mediators.

2. Materials and Methods

2.1. PDRN

Polydeoxyribonucleotide (PDRN, Mastelli srl, Sanremo, Italy) is a DNA fraction containing a mixture of deoxyribonucleotide polymers of different lengths, comprised between 50 and 2000 base pairs. It was obtained from fish for human consumption by using Mastelli's peculiar methods, by means of purification and high-temperature sterilization procedures that ensure a very high percentage of purified DNA and the absence of active proteins and peptides [23].

2.2. Cell Culture and Experimental Treatments

Murine peritoneal monocyte-macrophage cells (RAW264.7 line) were obtained from the Cell Bank of the Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna (Brescia, Italy). The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 4 mM glutamine and antibiotics (streptomycin, 100 $\mu g \cdot ml^{-1}$; penicillin, 100 $U \cdot ml^{-1}$) and kept in a humidified atmosphere of 5% CO_2 at 37˚C.

For nitric oxide determination, cells were seeded in 96-well plates, at a density of 30×10^3 cells per well. For cytokine and VEGF secretion, cells were seeded in 24-well plates at a density of 15×10^4 cells per well. 24 h after seeding, the cell growth medium was replaced with fresh medium supplemented with PDRN (100 $\mu g \cdot ml^{-1}$) in the presence and in the absence of LPS (from *E. coli*, O55:B5 serotype) 1 $ng \cdot ml^{-1}$ or 10 $ng \cdot ml^{-1}$ (from 100× stock solutions in DMEM). The dose of PDRN was selected as suggested by previous research, reporting that, at the dose of 100 $\mu g \cdot ml^{-1}$, A_{2A} receptor agonists significantly suppressed LPS-induced inflammation [16]. Depending on the experimental setting, PDRN was added immediately (0 h) or after 1 h, 2 h or 3 h after the exposure to LPS and kept throughout the experiments.

2.3. Cell Viability

Cell viability was assessed with the resazurin method [24]. Resazurin is a non-fluorescent molecule which is converted by intracellular enzymes into the fluorescent compound resorufin (λ_{em} = 572 nm). After 24 h of incubation, cell viability was tested replacing the medium with a solution of resazurin (44 µM, Sigma Aldrich) in serum-free DMEM. After 20 min, fluorescence was measured at 572 nm with a multi-plate reader.

2.4. Determination of NO Production

Nitrite concentration in the culture media, as an indicator of NO production, was determined 24 h after treatments with PDRN by means of a fluorimetric approach, based on the production of the fluorescent molecule 1H-naphthotriazole from 2,3-diaminonaphthalene (DAN) in an acid environment [25]. 100 µl of medium were transferred to black 96-well plates with a clear bottom

(Corning, Cambridge, MA, USA). DAN (20 µl of a solution of 0.025 mg/ml in 0.31 M HCl) was then added. After 10 min at room temperature the reaction was stopped with 20 µl of 0.7 M NaOH. Standards were tested in the same medium from a stock solution of 1 mM sodium nitrite. Fluorescence (λ_{ex} 360 nm; λ_{em} 430 nm) was determined with a Perkin Elmer Enspire multi-plate reader (Waltham, Massachussets, USA).

2.5. Cytokine Secretion

The presence of Tumour Necrosis Factor-alpha (TNF-α), Interleukin-12 (IL-12) and IL-10 in the culture media of RAW264.7 cells was determined with ELISA RayBio® kits (Ray Biotech, Norcross, GA, USA) following the manufacturer's instructions. After the selected incubation times with PDRN (6 h for TNF-α and IL-10, 24 h for IL-12), 100 µl of medium were transferred into 96-well plates functionalized with anti-cytokine antibodies and incubated overnight at 4˚C. 100 µl of biotinylated antibody were then added to each well and incubated for 1 h at room temperature, after which 100 µl of streptavidin solution were added. After 45 min, the samples were incubated with 100 µl of the TMB One Step Reagent for 30 min. Absorbance was then immediately read at 450 nm with a plate reader. Standards were tested in the assay buffer from a stock solution (50 ng·ml^{-1}) of the recombinant cytokine.

2.6. VEGF-A Secretion

After 24 h of incubation with PDRN, the presence of Vascular Endothelia Growth Factor-A (VEGF-A) in the culture media of RAW264.7 cells was determined with ELISA RayBio® kits (Ray Biotech, Norcross, GA, USA) and performed as above described. Standards were tested in the assay buffer from a stock solution (25 ng·ml^{-1}) of the recombinant protein.

3. Materials

Unless otherwise stated, all the reagents were provided by Sigma Aldrich, Milan, Italy.

4. Statistical Analysis

The results are expressed as means ± standard deviation (mean ± SD). Statistical analyses were performed using GraphPad Prism® software version 5.00 (GraphPad Software Inc., San Diego, CA). The data were compared using one-way ANOVA tests and checked with Bonferroni's *post hoc* tests. Results were considered significant at $p < 0.05$ compared to cells treated with LPS (positive control).

5. Results

5.1. Cell Viability

The effects of PDRN, alone or in combination with LPS (1 - 10 ng·ml^{-1}), on the

viability of RAW264.7 cells were tested using the resazurin assay after exposure for 24 h. PDRN, both alone and with LPS (1 - 10 ng·ml^{-1}), did not significantly reduce cell viability, as shown in Figure 1.

5.2. Nitric Oxide Production

Figure 2 shows the data on the production of nitric oxide (NO), a major inflammatory mediator produced by activated macrophages, assessed on the basis of the nitrite concentration in the medium after 24 h of treatment of RAW264.7 cells with LPS (1 - 10 ng·ml^{-1}) or with PDRN (100 μg·ml^{-1}). The effects of PDRN were more evident in the presence of LPS 10 ng·ml^{-1} than that of 1 ng·ml^{-1} and

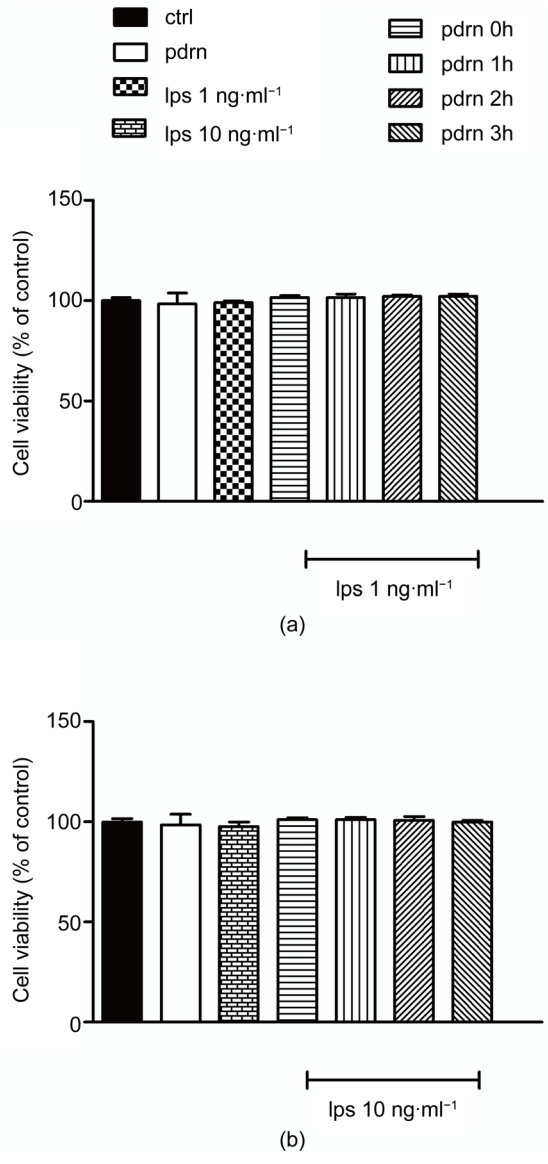

Figure 1. Effects of PDRN on cell viability in RAW264.7 cells. Cells, grown for 24 h in complete culture medium, were treated with 1 ng·ml^{-1} (a) or with 10 ng·ml^{-1} (b) of LPS plus PDRN (100 μg·ml^{-1}), added either simultaneously or 1 - 2 or 3 h after stimulation with LPS. 24 h after the treatment, cell viability was determined using the resazurin assay. The results are expressed as the means of three independent determinations ± S.D.

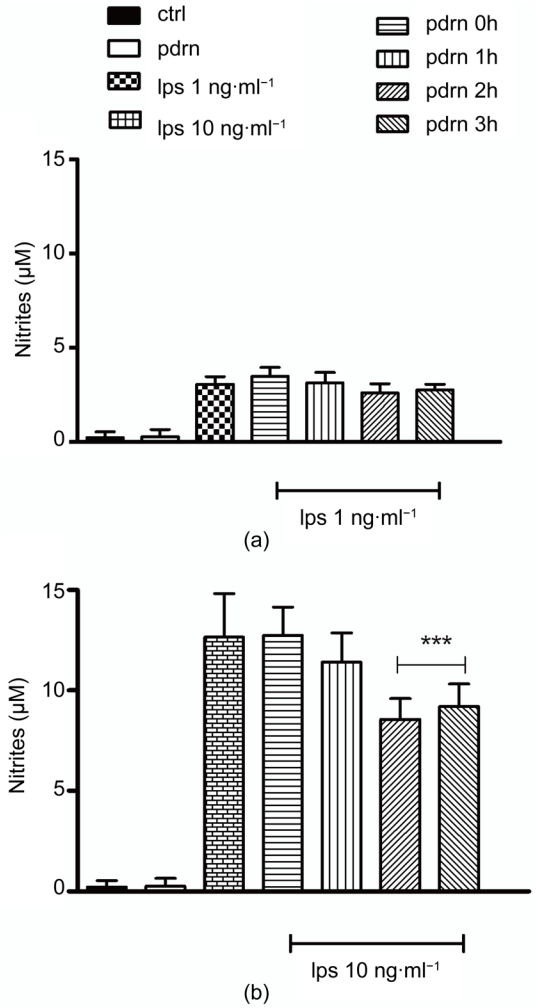

Figure 2. Effects of PDRN on NO production in RAW264.7 cells. Cells grown for 24 h in complete culture medium, were treated with 1 ng·ml^{-1} (a) or with 10 ng·ml^{-1} (b) of LPS plus PDRN (100 μg·ml^{-1}), added either simultaneously or 1 - 2 or 3 h after stimulation with LPS. 24 h after the treatment, the nitrite concentration was determined in the culture medium of the cells. The experiment was performed three times with comparable results. The results are expressed as the means of three independent determinations ± S.D. ***$p < 0.001$ vs. positive control, cells treated with LPS.

PDRN, added 2 and 3 h after stimulation with LPS, induce in a decrease in NO production of 30% compared with cells treated only with LPS ($p < 0.001$).

5.3. Secretion of Pro- and Anti-Inflammatory Cytokines

The secretion of the pro-inflammatory cytokines TNF-*α*, IL-12 and anti-inflammatory cytokine IL-10 was quantified after the treatment of RAW264.7 cells with LPS (10 ng·ml^{-1}) plus PDRN (100 μg·ml^{-1}), added either simultaneously or 2 h after stimulation with LPS, depending on the NO production results.

TNF-*α* and IL-10 were determined 6 h after treatments while the production of IL-12 was assessed after 24 h after treatments with LPS and PDRN.

As shown in **Figure 3**, the exposure to PDRN added after 2 h of stimulation with LPS produced a significant and marked decrease both of TNF-*α* and of

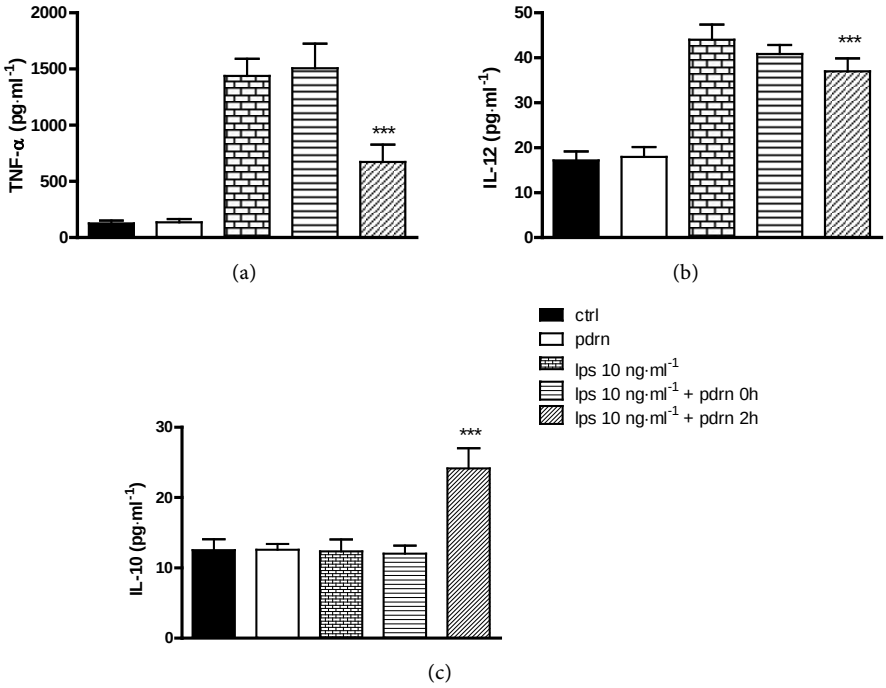

Figure 3. TNF-*α*, IL-12 and IL-10 secretion in RAW264.7 cells. Cells grown for 24 h in complete culture medium, were treated with 10 ng·ml^{-1} of LPS plus PDRN (100 μg·ml^{-1}), added either simultaneously or 2 h after stimulation with LPS. The cytokines indicated above were measured after 6 h for TNF-*α* (a) and IL-10 (c) and after 24 h of treatment for IL-12 (b). They were measured in the extracellular medium, as described in Materials and Methods. The results are expressed as the means of three independent determinations ± S.D. ***$p < 0.001$ vs. positive control, cells treated with LPS.

IL-12, by 47% and 16% respectively ($p < 0.001$).

Our results showing that PDRN inhibit TNF-*α* and IL-12 production suggest that the beneficial effects of PDRN in inflammatory processes or injury may be attributed partly to this inhibition.

Moreover, PDRN produced a pronounced increase of IL-10 compared with stimulation with LPS (2-fold induction).

Anti-inflammatory properties of IL-10 are well documented and include the control of TNF-*α* release *in vitro* and *in vivo* [26]. The present study showed that PDRN is able to increase IL-10 production after stimulation with LPS and this phenomenon contributed to TNF-*α* inhibition.

5.4. Secretion of VEGF-A

The effects of PDRN on VEGF-A production by RAW264.7 cells was evaluated 24 h after treatment with LPS (10 ng·ml^{-1}) plus PDRN (100 μg·ml^{-1}), added either simultaneously or 2 h after stimulation with LPS. As shown in **Figure 4**, PDRN markedly increases the secretion of VEGF-A by 42%.

6. Discussion

Age-associated low grade inflammation (inflammaging) is now recognized to be the driving force of many age-associated diseases [27].

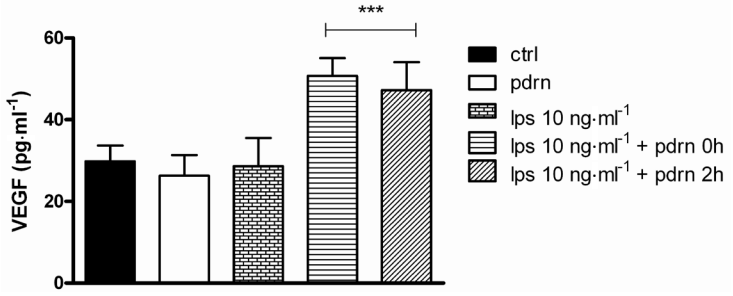

Figure 4. VEGF-A secretion in RAW264.7 cells. Cells grown for 24 h in complete culture medium were treated with 10 ng·ml^{-1} of LPS plus PDRN (100 μg·ml^{-1}). After 24 h of treatment, VEGF-A was measured in the extracellular medium, as described under Materials and Methods. The results are expressed as the means of three independent determinations ± S.D. ***$p < 0.001$ vs. positive control, cells treated with LPS.

As we age, the innate immune system becomes dysregulated and is characterized by persistent inflammatory responses that involve multiple immune and non-immune cell types. This dysregulation involves both elevated levels of basal inflammation and an associated impaired ability to mount efficient innate and adaptive immune responses [28].

It has recently been established that inflammation and innate immunity play roles in skin aging process. The immune system protects the skin from infection, removes damaged cells and prevents undesirable autoimmune reactions; it may, however, promote the aging process, due to intrinsic (or chronological) and extrinsic factors, such as exposure to the sun, air pollution, cigarette smoking, bad nutrition and through the generation of Reactive Oxygen Species (ROS) generation released by macrophages [29].

Not only inflammatory states, but also ischaemia and tissue injury are pathological conditions in which intracellular ATP metabolism is accelerated, leading to the accumulation of intracellular adenosine.

Adenosine is a purine nucleoside that is released in response to metabolic stress and is a potent endogenous regulator, acting via four cell surface receptor subtypes (A_1, A_{2A}, A_{2B} and A_3).

In particular, adenosine A_{2A} receptors are present in the monocyte-macrophage cell line [30] and, as a result of interaction with adenosine or agonists, these agents have been shown to be able to modulate macrophage functions, such as nitric oxide (NO) production, and to regulate cytokine production both *in vitro* and *in vivo* [16] [31].

In this paper, we study the effects of polydeoxyribonucleotide (PDRN), an adenosine A_2 receptor (A_{2A}R) agonist, on RAW264.7, a murine macrophage cell line.

Our results showed that PDRN did not affect cell viability or cause a decrease in nitric oxide production when added to a macrophages pre-stimulated with relatively high doses of LPS (10 ng·ml^{-1}). Our data suggest that in an on-going state of inflammation PDRN has as anti-inflammatory effect.

Our results were consistent with other studies [26] [32] [33] [34] [35], since it

was observed that PDRN markedly potentiated IL-10 production and induced a significant decrease of TNF-α and IL-12 secretion in LPS-stimulated macrophages.

In addition to their anti-inflammatory effects, A_{2A} receptor agonists promote faster wound closure in murine models [36]. During wound healing, macrophages play a key role in inducing angiogenesis, by producing the vascular endothelial growth factor-A (VEGF-A).

Regulation of the expression of VEGF-A via adenosine receptors has been demonstrated in several cell types, including endothelial cells and smooth muscle cells [37] [38].

Leibovich and colleagues have shown that VEGF-A expression by murine macrophages was synergistically up-regulated by LPS together with adenosine or A_{2A}R agonists [39]. Moreover, the synergistic up-regulation of VEGF-A expression did not occur in macrophages from mice that lacked the adenosine A_{2A} but not the A_3 receptor and the response did not occur in macrophages from mice that lack functional TLR4 receptors, indicating a critical role of the TLR4 receptor in the signalling pathway.

In view of the promising role of adenosine and adenosine receptors in improving blood supply and wound healing [40] [41], we assumed that PDRN might exert an important role in angiogenesis.

In an experimental model of ischaemic skin flaps, Polito and colleagues demonstrated that PDRN improved blood flow by stimulating VEGF expression and restored tissue architecture: flaps treated with PDRN showed a complete re-epithelialization and well-formed granulation tissue rich in fibroblasts [42].

Moreover, PDRN significantly reduced the wound surface area and improved the condition of the pressure ulcers, healed graft donor site and diabetic foot ulcers by stimulating neoangiogenesis [21] [43] [44].

Our observations indicated that treatment of LPS-stimulated macrophages with PDRN strongly increase VEGF-A secretion to well above the level induced by PDRN or LPS alone.

In view of this, binding of the A_{2A} receptor is one of the most important mechanisms for the protecting tissue from inflammatory damage. An important finding of our study was, therefore, that triggering of the adenosine A_{2A} receptor by PDRN may constitute a therapeutic strategy for counteracting and reducing inflammation and reactive species.

7. Conclusion

To conclude, PDRN may constitute a new, active and safe anti-inflammaging treatment, acting as an anti-inflammatory agent that can avoid or heal damage to the cellular and extracellular components.

Conflict of Interest

The authors have no conflict of interest to declare.

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