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The activity of monocyte-derived macrophages after stimulation with platelet-rich and platelet-poor concentrates. Study on an ovine model of insertion of a tibial implant coated with silicon-doped diamond-like carbon

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Abstract

Introduction: Macrophages are crucial immune cells that play a role in tissue repair and can exhibit pro- or anti-inflammatory behaviour based on environmental stimulation. Their functional phenotype can be affected by platelet-derived products as determined by those products' composition. When the inflammatory response caused by implantation is excessive, it can lead to rejection of the implant. Therefore, a thorough evaluation of implant haemocompatibility is necessary to minimise undesirable consequences. **Material and Methods:** In an *in vitro* study, monocyte-derived macrophages (MDMs) were obtained from the whole blood of sheep after a silicon-doped diamond-like carbon–coated implant insertion. These MDMs were then exposed to autologous platelet-derived products for functional marker analysis. **Results:** Platelet-poor plasma (PPP) and pure platelet-rich plasma (P-PRP) stimulation increased arginase-1 activity, while leukocyte-rich PRP stimulation produced a mixed response involving higher O_2^- (6.49 ± 2.43 nM *vs* non-stimulated 3.51 ± 1.23 nM, P-value < 0.05) and NO (3.28 ± 1.38 µM *vs* non-stimulated 2.55 ± 0.32µM, P-value < 0.05) generation. **Conclusion:** Using PPP and P-PRP stimulation in post-implantation procedures may contribute to the polarisation of macrophages towards the M2-like pro-resolving phenotype, thereby accelerating wound healing. This would also prevent implant degradation due to an excessive inflammatory process.

Keywords: blood-derived products, macrophages, Si-DLC implant, ovine model.

Introduction

Platelets and their products are known to play an important role in the inflammatory process apart from their function in haemostasis. Therefore, blood-derived products such as platelet-rich plasma (PRP) and platelet-poor plasma (PPP), are widely used in orthopaedics, ophthalmology, neurosurgery, dentistry and the treatment of acute and chronic wounds (21). Two main products have been introduced which differ in leukocyte content, *i.e.* pure PRP (P-PRP) without leukocytes and leukocyte-rich PRP (L-PRP) (1, 8). These plasma concentrates can show different activity because of their differing contents and, therefore, can exert different effects on the tissue repair process. Platelet-poor plasma, in turn, is a by-product obtained from blood through centrifugation. It contains lower platelet concentrations compared to normal blood but is still rich in fibrinogen,

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fibronectin and thrombin. It plays a role in haemostasis and coagulation, serves as a cell attachment vector and promotes growth and survival of fibroblasts and epithelial cells. It has also been shown to promote wound healing–associated cell functions and accelerate the migration and proliferation of fibroblasts. Currently, common uses for PPP are as an alternative to bovine or other serum to culture bone marrow–derived and umbilical cord–derived mesenchymal stem cells and adipose-derived stem cells. In clinical practice, products such as PRP and PPP are widely used to promote wound healing and tissue regeneration. However, further research is needed to understand the differences between PPP and PRP in terms of composition and biological effects (33).

Monocyte-derived macrophages (MDM) are immune cells present in most tissues that undergo significant phenotypic and functional changes in response to growth factors and cytokines present in the local tissue microenvironment (13, 21, 31). They are involved in both innate and adaptive responses, releasing inflammatory cytokines, clearing debris, and recruiting immune cells. Following tissue injury, many inflammatory monocytes and macrophage precursors are recruited from the bone marrow *via* chemokine gradients and various adhesion molecules. Different phenotypes of monocytes and macrophages closely coordinate the subsequent stages of tissue repair (13). The pro-inflammatory macrophages are known as the M1-like phenotype, whereas the M2-like (pro-resolving) functional phenotype participates in fibroblast proliferation, extracellular matrix (ECM) remodelling and wound healing (7). These two macrophage subtypes are heavily involved in the body's response to an implant. After the first acute stage of tissue repair, pro-inflammatory mediators are released when neutrophils migrate to the implant site, promoting inflammation and attracting monocytes to diapedesis. When monocytes reach the implant site, they differentiate into macrophages. During the acute reaction to implant insertion, M1 macrophages phagocytose particles generated during the wear of biomaterial, thereby producing reactive oxygen species (ROS), nitrogen intermediates (RNI), and proteolytic enzymes. Then, the repair process moves to the chronic phase characterised by the polarisation of macrophages to the M2-like phenotype. The anti-inflammatory activity is confirmed by the release of anti-inflammatory cytokines (*e.g.* interleukin (IL)-10 and transforming growth factor beta) and higher activity of arginase-1. The hydrolysis of L-arginine to L-ornithine, mediated by arginase-1, provides substrates for ECM synthesis, which is necessary for tissue repair (6). Disturbances at any stage of this process can result in abnormal tissue repair, including uncontrolled production of pro-inflammatory mediators and growth factors, or deficiencies in the generation of pro-resolving macrophages. These imbalances can contribute to chronic wounds and fibrosis (31).

Comprehensive knowledge of the interactions between the implant and the tissues is necessary to minimise complications after orthopaedic procedures. To prevent an adverse reaction to an implant, manufacturers have developed innovative coatings that improve the biocompatibility of implants. One such coating is silicon-doped diamond-like carbon (Si-DLC), which creates a barrier around a surgical-steel implant that blocks metal ions from entering the surrounding tissues, preventing allergic reactions to the components of the implant. In addition, this layer also protects the implant from the effects of ROS and RNI, as well as proteolytic enzymes released by M1-like macrophages (5, 20, 25, 26).

Despite the large-scale use of implants, transplants and biomedical devices, adverse reactions of bone and soft tissues are a problem which has not been eliminated and one of the main causes of aseptic implant rejection. Immune responses to a foreign body can lead to strong and unwanted symptoms, such as intense pain and excessive inflammation, that create a pathological microenvironment and negatively affect the durability of the implanted material. The sheep model represents a valuable tool for testing novel medical devices, *e.g.* joint or bone implants with potential clinical applications (4).

The progress of biomedical engineering, is yet to reach the stage at which the host's immune response to the implanted material can be safely and reliably controlled. In many cases, after implantation, the material is recognised by the host's body as foreign, initiating an acute inflammatory reaction. Macrophages play a key role in the cascade of the immune response to the implant, and the response leads to the resolution of the inflammatory process and eventually to tissue remodelling (3, 14).

Creating the appropriate experimental microenvironment *in vitro* is crucial for fully determining the interaction between the implant and tissues. Different cell models can be used in order to study macrophage responses. Based on previous research, we adapted the primary MDM model for evaluation of the interactions with host tissue of Si-DLC-coated implants in ovine tibia (18).

The first aim of our study was to compare the impact of the implant coated with the innovative Si-DLC layer on the long-term host response, using MDM culture as a model for monocyte-lineage cell response. Its second aim was to evaluate the influence of some platelet-rich and platelet-poor concentrates on MDM morphological changes and functional response based on superoxide anion (O_2^-), nitric oxide (NO) generation and arginase-1 activity. The final aim was to investigate how this response is affected by previous cell contact with the implanted biomaterial.

Material and Methods

Implant insertion. The material for the research was whole blood collected from female synthetic BCP

meat- line sheep (12 months old, weight 50–60 kg) kept on the Bezek Experimental Farm of the University of Life Sciences in Lublin. Ewes were randomly selected from a large flock. The sheep were of the same genotype and age, as equal as possible in condition, maintained under identical environmental conditions and fed the same diet suited to their nutritional requirements. The experimental group consisted of sheep with an implant (n = 4), and the control group consisted of healthy sheep without an implant (n = 4). The research material was obtained with the approval of the Local Ethical Committee at the University of Life Sciences in Lublin (no. 48/2021).

All animals from the experimental group had a Si-DLC-coated implant (fabricated by Medgal, Białystok, Poland) inserted into the bone. Both tibia of each experimental sheep had six cylindrical implants placed in them, which were 4 mm in diameter and 12 mm in length as stipulated in the ISO 10093 standard for local effect testing after biomaterial implantation. The implant was manufactured according to the ISO 5832 standard. Before the surgical procedure, the sheep were anaesthetised with intramuscular injections of xylazine (Sedazin, 0.1 mg/kg; Biowet, Puławy, Poland) and butorphanol (Torbugesic Vet, 0.1 mg/kg; Zoetis, Poland). The surgical area was aseptically cleaned, and the standard surgical approach for the proximal tibia was prepared, then after periosteal elevation, the implants were inserted. Postoperative medication included subcutaneous injections of meloxicam (Melovem 5%, 0.1 mg/kg subcutaneously; Dopharma Research, Raamsdonksveer, the Netherlands) as an analgesic and Combi-ject (200, 000 IU/mL penicillin and 200 mg/mL streptomycin, 10,000 IU/kg, 10 mg/kg; Dopharma Research) to prevent infection. All sheep from the experimental group were monitored postoperatively for ten days in terms of the condition of the animal, respiration, heart rate, and body temperature. Additionally, the area of the postoperative wound was inspected and the skeletal system was examined to exclude motor disorders of the operated limb. During the experiment, the animals were kept in an outdoor environment and exposed to natural periods of light and darkness. The blood for the experiment was drawn from the sheep as follows: before implantation (T0), four weeks after implantation (T1), and four months after implantation (T2).

***In vitro* culture.** A 36 mL sample of whole blood was collected from the jugular vein into tubes containing ethylenediaminetetraacetic acid as an anticoagulant (Vacuette K3EDTA; Greiner Bio-One, Kremsmünster, Austria). Gradisol L (Aqua-Med, Łódź, Poland) was used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood. In the first step, the collected blood was mixed 1:1 with phosphate buffered saline (PBS), and then the diluted blood was layered on 4 mL of Gradisol L and centrifuged for 25 min at a speed of 2,800 rpm. The ring formed after centrifugation was collected, transferred to new tubes, and then washed twice in PBS. The pellet was suspended in 1 mL of Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA). Cell number and viability were determined using an R1 Automated Cell Counter (Olympus, Tokyo, Japan). Thereafter, the culture was transferred to a 96-well plate (MicroWell 96-Well, Nunclon Delta-Treated; Nunc, Roskilde, Denmark) at a concentration of 1.0×10^6 cells/mL and incubated at 37°C in a 5% CO_2 concentration in DMEM with 10% bovine calf serum (Biomed, Lublin, Poland). After 24 h of incubation, the culture medium was changed, and adherent cells were left for differentiation into MDM. A 20-µL volume of L-PRP, P-PRP, PPP or DMEM was added to appointed wells after 48 h of incubation. Wells with added DMEM were described as non-stimulated (NS). The stimulated and non-stimulated cultures were then incubated under the same conditions. Two different PRP products were obtained using two commercial kits as previously described by Zdziennicka *et al.* (32). The first system, based on the technology of curasan AG (Frankfurt, Germany), obtained L-PRP, and the second one (Xerthra; Biovico, Gdynia, Poland) obtained P-PRP. Platelet-poor plasma was prepared as a byproduct of the PRP systems. Blood was collected from the sheep for the preparation of blood-derived products at T0 (before implantation), and then the preparations were lyophilised and stored at −80°C for stimulation at T0, T1, and T2. The culture medium was collected 96 h after isolation of PBMCs to determine the NO and O_2^- generation as well as the arginase-1 activity (Fig. 1). Morphometric analysis of cells was performed every 24 h using an inverted microscope (Olympus) at 40× magnification.

Evaluation of functional phenotype. All spectrophotometric measurement was carried out with an Elx800 plate reader (BioTek, Winooski, VT, USA). The Griess reaction was used to determine the NO generation based on the nitrite concentration in the culture medium. The Griess reagent comprising 10 mg of sulphanilamide (Sigma-Aldrich), 1 mg of N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich) and 2.5% H_2PO_4 (Aventor Performance Materials, formerly POCH, Gliwice, Poland) was prepared and mixed 1:1 with the sample. The mixture was then incubated for 10 min at room temperature, and the absorbance was measured. The $NaNO_2$ standard curve was used to convert the absorbance to micromoles of nitrite.

The O_2^- generation assay used 0.1% tetrazolium nitroblue solution (Sigma-Aldrich). The reagent and samples were mixed in equal parts and incubated for 10 min at room temperature. The absorbance was then determined. The extinction coefficient of 21.1 nM was used to determine the production of O_2^-.

Arginase activity was defined as the concentration of urea produced after arginase-dependent L-arginine hydrolysis. For this purpose, 50 µL of 0.1% Triton X solution (Sigma-Aldrich) was added to each well and the plates were incubated at room temperature for 30 min. After this time, 50 µL of 25 mM tris-HCl (Sigma-Aldrich) and 10 µL of $MnCl_2$ (Standard, Lublin, Poland) were added, and the contents of each well were transferred to glass tubes and incubated in a water bath

for 10 min at 55°C. Then, 100 µL of 0.5 M L-arginine (Sigma-Aldrich) was added, and the samples were kept for 60 min at 37°C. The reaction was stopped by adding 400 µL of a solution containing H_2SO_4 (Aventor Performance Materials), H_3PO_4 and H_2O in the proportions 1:3:7. In the last step, 40 µL of an ethanolic α-isonitrosopropiophenone (Sigma-Aldrich) solution was added. The urea level was measured spectrophotometrically after heating the samples for 30 min at 100°C.

The statistical analysis for this study was conducted using Statistica 13.3 software (TIBCO, Palo Alto, CA, USA). The results of the experiments were presented as the mean ± standard error. Student's *t*-test was used to compare multiple groups, and a P-value of less than 0.05 was considered statistically significant.

Results

All sheep were examined for possible complications in the course of the experiment and no adverse effects were observed up to four months after the procedure. During the postoperative period, the clinical examination of animals revealed no abnormalities, and no complications were observed at the site of implantation.

Three functional markers were used to compare the effect of stimulation with platelet-derived products on the activity of macrophages obtained from sheep after biomaterial implantation. The rising concentrations of NO and O_2^- in the medium were markers of pro-inflammatory activity, whereas the increase of arginase-1 activity indicated anti-inflammatory and pro-resolving action. Stimulation of macrophages with PPP at each time point resulted in a lower generation of NO and O_2^- compared to healthy sheep without implants (Fig. 2). In contrast, arginase-1 activity at each time point increased significantly (P-value < 0.05) compared to the control sheep.

Treatment of macrophage cultures with P-PRP caused insignificant changes in NO and O_2^- generation compared to untreated cells at each time point and a significant (P-value < 0.05) rise in arginase-1 activity (Fig. 2).

In vitro culture with L-PRP addition was characterised by a significant increase of NO and O_2^- generation as well as arginase-1 activity compared to the unstimulated group at each time point (P-value < 0.05). These results were confirmed by the urea/nitrite generation ratio. No meaningful differences were detected between the control and experimental groups four weeks or four months after implantation. Therefore, according to the results of this long-term study, the new Si-DCL implant coating did not evoke any response from monocytes which could create adverse MDM reactions (Fig. 2).

Morphological changes suggested mixed pro- and anti-inflammatory phenotypes of MDM after stimulation with blood-derived products. Extended pseudopodia were clearly visible on one or two sides of the cells, especially in cultures stimulated with L-PRP (Fig. 3).

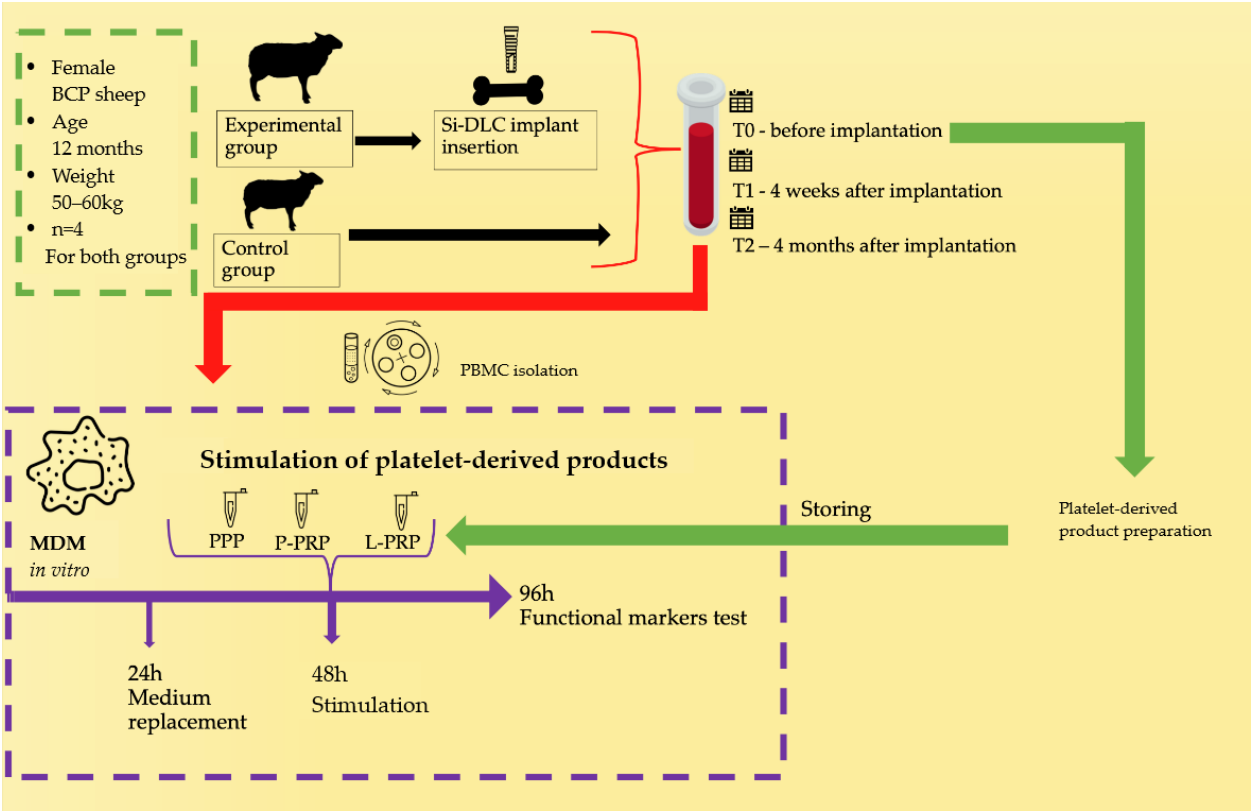

Fig. 1. Diagram of the experimental design for evaluating monocyte-derived macrophage (MDM) activity in blood drawn from animals in an ovine model of silicon-doped diamond-like carbon–coated (Si-DLC) implantation in tibia. T – time point; PBMC – peripheral blood mononuclear cells; PPP – platelet-poor plasma; P-PRP – pure platelet-rich plasma with no or a low number of leukocytes; L-PRP –platelet-rich plasma with a high number of leukocytes

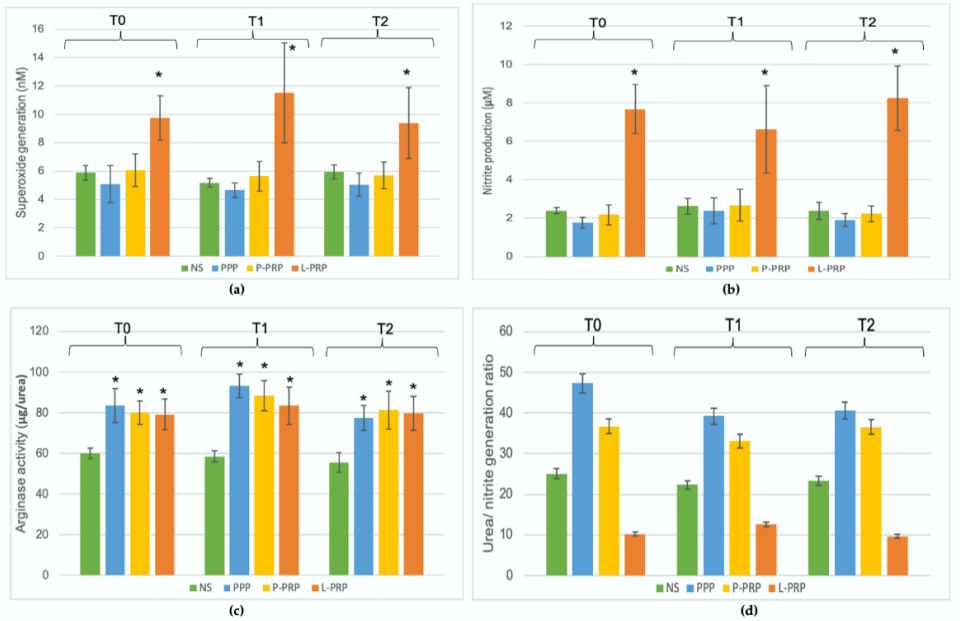

Fig. 2. Comparison of the effect of blood-derived product stimulation on macrophage response in blood drawn from animals at three time points in an ovine model of silicon-doped diamond-like carbon–coated implantation in tibia. (a) superoxide generation; (b) NO generation; (c) arginase-1 activity; (d) urea/NO ratio. NS – non-stimulated macrophages; PPP – platelet-poor plasma–stimulated macrophages; P-PRP – macrophages stimulated with pure platelet-rich plasma with no or a low number of leukocytes; L-PRP – macrophages stimulated with platelet-rich plasma with a high number of leukocytes; T0 – time point before implantation; T1 – four weeks after implantation; T2 – four months after implantation; * – significant difference to NS at each time point (P-value < 0.05)

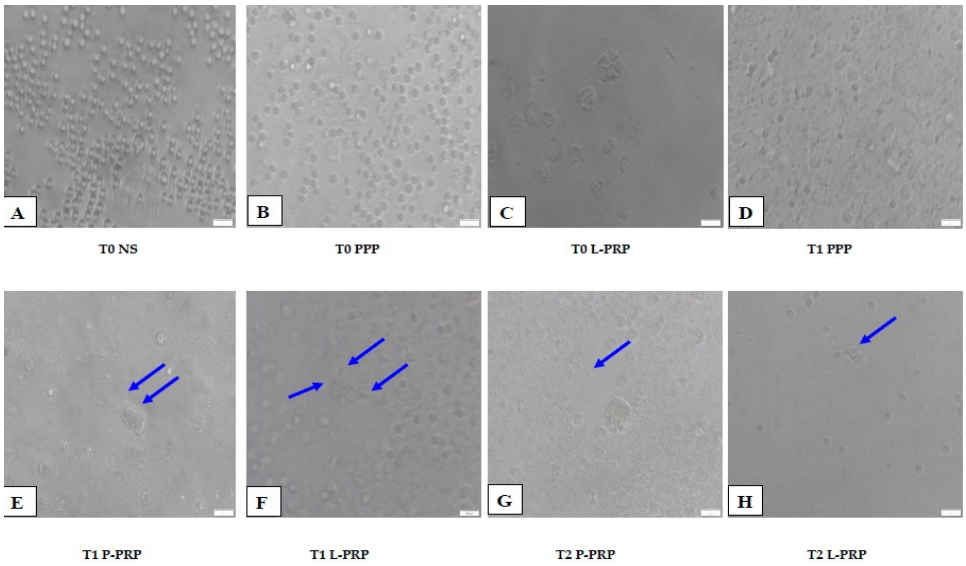

Fig. 3. Representative microphotographs of morphological changes in macrophages in blood drawn from animals in an ovine model of silicon-doped diamond-like carbon–coated (Si-DLC) implantation in tibia. Optical microscopy ×40 carried out 48 h after stimulation with different blood-derived products at three time points. A – naïve macrophages after DMEM and 10% BCF supplementation; B, D – rounded cells visible in all cultures after platelet-poor plasma (PPP) stimulation; C, E–H – pseudopodia (blue arrows) induced after stimulation by platelet-rich plasma with a high number of leukocytes (L-PRP) or with platelet-rich plasma with no or a low number of leukocytes (P-PRP); T0 – time point before implantation; T1 – four weeks after implantation; T2 – four months after implantation

Discussion

Our study compared the influence of platelet-rich and platelet-poor concentrates on the function of macrophages differentiated from circulating monocytes after biomaterial implantation in the ovine model. In human *ex vivo* studies, mononuclear cells obtained from healthy donor peripheral blood are a key source of monocytes. Furthermore, MDMs from this source are the main cells for *in vitro* studies on human macrophages (19).

Platelets are important regulators of inflammation because they express toll-like receptors and can enhance leukocyte effector functions including pro-inflammatory

activity (10). We showed that platelet-derived products influence the changes in macrophage functional phenotype. Stimulation with PPP and P-PRP resulted in a shift in macrophage activity towards an M2-like functional phenotype, evidenced by the high activity of arginase-1. In turn, macrophage stimulation with L-PRP caused mixed responses involving increases in both pro- and anti-inflammatory functional markers. This mixed response could be provoked by the high number of leukocytes in this type of platelet product and by previous stimulation of platelets by neutrophils. There is no standardised method to obtain PRP, and different methods may yield products which are unalike. Platelet-rich plasma may include erythrocytes, leukocytes and a small fraction of stem cells. The presence of leukocytes, especially neutrophils, in PRP is a source of concern and a matter provoking debate in the medical field. This is because neutrophils release pro-inflammatory cytokines and metalloproteinase, which can exacerbate the initial inflammatory response to tissue injury (15). Some studies highlighted the role of PRP in modulating inflammation during tissue repair. This concentrate has been shown to release anti-inflammatory growth factors and inhibit the release of pro-inflammatory cytokines. However, the presence of leukocytes in PRP may counteract these effects by increasing inflammation. This has been demonstrated in experiments where their presence significantly increased the gene and protein expression of pro-inflammatory cytokines such as IL-1β, IL-6 and tumour necrosis factor alpha (TNF-α) in tendons. This suggests that while PRP alone may have anti-inflammatory properties, the presence of leukocytes in the mixture may exacerbate inflammation (34).

In our experiment, the high activity of arginase-1 after L-PRP stimulation of cell culture suggests the presence of pro-resolving M2-like macrophages but this effect is masked by leukocyte activity. In turn, treating MDM cultures with PPP or P-PRP to obtain a pro-resolving phenotype can improve tissue repair. This assumption is confirmed by the study conducted by Uchiyama *et al.* (29), who observed a reduction in M1 macrophage markers after the addition of purified PRP and confirmed promotion of M1 macrophage polarisation to the M2 and inhibition of MDM polarisation to the M1 phenotype. The authors indicated that the effect of PRP was also observed *in vivo*, leading to the recruitment of macrophages. Previous research suggested that the polarising activity of L-PRP and P-PRP has different impacts on the healing process. Pure platelet-rich plasma stimulates anabolic processes by hastening the proliferation and remodelling phases during healing (22).

However, it should be borne in mind that based on current knowledge, there is no simple distinction of macrophages into two extreme phenotypes, M1 and M2. There is a broad spectrum of different types, from highly pro-inflammatory to pro-fibrotic, pro-tumoral, anti-inflammatory, and many others. These findings were confirmed by several transcriptome analysis studies in which the response of macrophages to a wide range of stimuli led to the induction of a plethora of transcriptional phenotypes. This *in vitro* model of a spectrum of responses reflects the *in vivo* changes in humans (16, 27). Our study confirmed the presence of sub-types of MDM with mixed activity profiles induced in response to blood-derived product stimulation. These findings broaden our understanding of how blood-derived products can control the inflammatory response.

Biomaterials implanted for long periods cannot be considered inert and should meet different criteria in order to minimise the risks of adverse events. Nurdin *et al.* (23) used human blood for the *in vitro* assessment of the haemocompatibility of Si-DLC-coated surfaces based on the observation of platelet adhesion and activation, thrombin generation, and complement convertase production induced by the sample surface. Several studies showed that covering the implant with an Si-DLC layer protects the surrounding tissues from metal ions that the implant may release (5, 25). Some authors showed that DLC coating increased the implant's resistance to corrosion, reduced friction, and was highly smooth and durable (24, 28). Previous studies on the biocompatibility of biomaterials indicated that implant debris could significantly affect macrophages by inducing an immune response in which pro-inflammatory cytokines and ROS are released. The exacerbated inflammatory response at the implant site can evoke adverse reactions which may even be as powerful as implant rejection (9). However, previous *in vitro* studies indicated that even 12 weeks after implantation of a DLC-coated implant, there was no cytotoxicity, and that macrophages, fibroblasts and osteocytes tolerated it well. Furthermore, exposure of macrophages to DLC-coating particles did not increase TNF-α generation or messenger RNA levels of pro-inflammatory factors such as inducible nitric oxide synthase or IL-6 (2). A previous *in vivo* study on a mouse model conducted by Wachesk *et al.* (30) requires extension to non-rodent mammals to afford a better understanding of the behaviour of DLC-coated implants. Our experiment on an ovine model proved that coating the implant with Si-DLC did not meaningfully affect the body's response.

The sheep is commonly used as a model for biomedical research, despite certain limitations such as high maintenance costs and limited availability of antibodies, as well as the lack of an atlas as detailed as that of rodents. Nevertheless, using sheep in basic science, applied technologies and translational medicine can help bridge the knowledge gaps between what has been learned from studies conducted in smaller models and what is still necessary to learn for success in human trials. *In vitro* models such as MDMs or cell lines provide alternatives to studying macrophages *ex vivo* from humans, although they may be limited in terms of heterogeneity compared to *in vivo* models. Our study on the sheep model not only expands existing knowledge

about the response of mammalian macrophages to Si-DLC coatings but also investigates the effect of *in vitro* MDM stimulation with various autologous blood-derived products (17, 30). We assessed the response of macrophages obtained from circulating blood after long-term contact with the implant and confirmed the good compatibility of this coating in terms of MDM response.

In the course of this research, we found that the use of blood-derived products may have different effects on macrophages. A mixed pro- and anti-inflammatory response was observed after stimulation with L-PRP, while P-PRP and PPP caused an anti-inflammatory effect. However, cultured macrophages were unaffected in their response to the studied blood-derived products by any previous contact of circulating monocytes with a Si-DLC-coated implant. These results enhance our comprehension of the interactions between biomaterials and the host and the application of blood-derived products. However, further in-depth analysis is required to fully comprehend the spectrum of macrophage response to implants and the potential of blood-derived products to modulate their activity.

Conclusion

The study found no significant differences in the responses of MDM between the control group and those obtained from sheep after four weeks and four months of Si-DLC coated implant insertion, indicating the potential biocompatibility of the Si-DLC coating. The use of platelet concentrates, such as L-PRP, P-PRP, and PPP, showed varying effects on macrophages, with a mixed pro- and anti-inflammatory response observed with L-PRP and an anti-inflammatory effect with P-PRP and PPP. While the Si-DLC-coated implant did not affect the response of monocytes, further in-depth analysis is needed to fully understand the spectrum of macrophage response to the implant and the modulating potential of platelet-derived products on their activity.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The study was approved by the Local Ethics Committee in Lublin (approval no. 48/2021). All procedures involving animals were carried out in accordance with the Polish legislation on animal welfare.

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