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Mitigation of monocyte driven thrombosis on cobalt chrome surfaces in contact with whole blood by thin film polar/hydrophobic/ionic polyurethane coatings

Audrey Gossart[a,b,c], Didier Letourneur[a], Adeline Gand[b], Véronique Regnault[d], Mohamed Amine Ben Mlouka[e], Pascal Cosette[e], Emmanuel Pauthe[b], Véronique Ollivier[a,**], J. Paul Santerre[c,*]

[a] Laboratory for Vascular Translational Science (LVTS), Inserm U1148, Université Paris Diderot, Université Paris 13, Hôpital Bichat, Paris, France
[b] Equipe de Recherche sur les Relations Matrice Extracellulaire-Cellules (ERRMECe), Biomaterial for Health Research Group, Institut des Matériaux, Maison International de la Recherche, Université de Cergy-Pontoise, 95000 Neuville sur Oise, France
[c] Translational Biology and Engineering Program, Ted Rodgers Centre for Heart Research, Institute of Biomaterials and Biomedical Engineering (IBBME) and the Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada
[d] Université de Lorraine, Inserm, DCAC, F-54000 Nancy, France
[e] Polymers, Biopolymers, Surface Laboratory, UMR 6270 CNRS, PISSARO Proteomic Facility, IRIB, 76821 Mont-Saint-Aignan, France

HIGHLIGHTS

- Confluent coatings of multifunctional polyurethane thin films (MPTF) (5 μm) on CoCr.
- MPTF coating prevents whole blood coagulation activation and fibrin formation.
- MPTF coating reduces platelet, monocyte and macrophage activation.
- MPTF coating reduces clotting by mitigating tissue factor expression from monocytes.
- MPTF coating reduces secretions of monocytes and macrophages inflammatory cytokines.

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ABSTRACT

Monocytes are active at the crossroads between inflammation and coagulation processes since they can secrete pro-inflammatory cytokines and express tissue factor (TF), a major initiator of coagulation. Cobalt-chrome (CoCr), a metal alloy, used as a biomaterial for vascular stents, has been shown to be potentially pro-thrombotic and pro-inflammatory. Research work with a polymer from a family of degradable-polar hydrophobic ionic polyurethanes (D-PHI), called HHHI, has been shown to exhibit anti-inflammatory responses from human monocytes. We have generated multifunctional polyurethane thin films (MPTF) based on the HHHI chemistry, as a thin coating for CoCr and have evaluated the reactivity of blood with MPTF-coated CoCr. The results showed that the coating of CoCr with MPTF derived from HHHI prevents thrombin generation, reduces coagulation activation, and suppresses fibrin formation in whole blood. Activation of monocytes was also suppressed at the surface of MPTF-coated CoCr and specifically the decrease in thrombin generation was accompanied by a significant decrease in TF and pro-inflammatory cytokine levels. Mass spectroscopy of the adsorbed proteins showed lower levels of fibrinogen, fibronectin and complement C3, C4, and C8 when compared to CoCr. We can conclude that MPTFs reduce the pro-thrombotic and pro-inflammatory phenotype of monocytes and macrophages on CoCr, and prevent clotting in whole blood.

* Corresponding author. IBBME, Faculty of Dentistry, 661 University Ave, 14th floor, Room 1435, Toronto, ON, M5G 1M1, Canada.
** Corresponding author. LVTS, UMR_S1148, Hôpital Bichat, 46 rue Henri Huchard, 75877 Paris cedex 18 France.
E-mail addresses: veronique.ollivier@inserm.fr (V. Ollivier), paul.santerre@utoronto.ca (J.P. Santerre).

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1. Introduction

A dominant limitation of cardiovascular related biomaterial implants in whole blood is the challenge of thrombosis. Specifically, stent thrombosis remains an important issue because of its serious adverse effects on patient care [1]. Studies have focused largely on minimizing coagulation through the adhesion of biomolecules that reduce clotting protein activation and the adhesion/activation of platelets. In the context of thrombosis related to biomaterials, the multi-faceted nature of all the main blood components renders a biological approach quite challenging, since the factors associated with the complement system and leukocytes have also been implicated in coagulation protein and platelet activation pathways. Unfortunately, many biomaterial studies have been limited in their assessment of leukocytes in their analysis of clot formation. These latter cells are more often studied in the context of inflammation and tissue integration. Yet, over the last decade the hematology field has clearly accentuated and delineated their role in thrombosis [2–4]. Hence, related clotting and pro-inflammatory states have to be considered simultaneously in biomaterial design [5].

Monocytes reside at the crossroads of inflammation and coagulation processes [2–4]. They are among the first cells to arrive at the site of injury post implantation [6]. In blood, human monocytes are the major source of tissue factor (TF). In the resting condition, monocytes express low levels of intracellular TF. However, pathological activation leads to a rapid and transient expression of TF by monocytes. For example, bacterial lipopolysaccharide induces monocytic TF expression *in vitro* and *in vivo* [7,8]. This TF expression is under the control of the transcription factor NF-κB [9]. TF is a major initiator of blood coagulation [10]. Blood contact with biomaterials represents a potential exogenous stimulus to induce TF expression by monocytes. For example, TF expression by monocytes has been observed *in vivo* during or after cardiopulmonary bypass [11,12]. TF is a high affinity receptor for factor VII and activated FVII (FVIIa). The formation of the TF-FVIIa complex is responsible for the first traces of thrombin generated *via* the activation of factor X and IX [13,14]. Moreover, monocytes are of particular importance in the remodeling of tissues around long-term implanted biomaterials. After recruitment to the site of inflammation, monocytes will differentiate into macrophages that can have a pro- or an anti-inflammatory phenotype and influence the outcome of tissue regeneration and wound healing. Therefore, the effect of macrophages can be beneficial or deleterious. Post implantation, a biomaterial can be encapsulated and rejected *via* a classical foreign body response (FBR) generated by the immune system [15,16], which involves the fusion of macrophages to form foreign body multinucleated giant cells (FBGC). The presence of the latter cells are characteristic of chronic inflammation [17].

Cobalt chrome (CoCr) is a metal alloy used in the fabrication of vascular stents [18,19]. It is non-magnetic, resistant to wear, corrosion and heat, and is often described as being “biocompatible” [20,21]. However, this material has severe limitations with respect to blood compatibility. Previous studies have shown that bare CoCr is pro-thrombotic and pro-inflammatory due to its ability to activate platelets and coagulation factors, and to induce leukocyte adhesion and activation [22,23]. A strategic approach to improve this material is to generate thin film coatings of biomaterials using polymers and/or bioactive molecules. For example, previous studies have shown that CoCr can be surface modified using phosphoric acid in order to alter its wettability, change the surface chemistry, and reduce the roughness features [23]. Modification of these properties influences the adsorption of fibrinogen (Fg) and albumin resulting in a decrease of platelet adhesion and activation [23]. Another candidate for coating includes the class of polymers defined as polyurethanes (PUs). PUs have excellent mechanical properties and depending on the selected chemistry, have exhibited moderate to good blood compatibility, in part because of the phase domain structure [24]. Further, they are generally easy to prepare in various forms. For example, the use of polymers consisting of 1,4 diisocyanatobutane (BDI)-based poly(ester-urethane) ureas and poly (ether-ester-urethane) ureas have been shown to be of interest for the development of cardiovascular devices [25,26].

A particular polyurethane of interest to our work is a polyfunctional polyurethane (referred to as D-PHI, or Degradable Polar/Hydrophobic/Ionic). D-PHI is a platform of multiple polymers with multifunctional chemistry. In their original formulations, these were developed to contain a divinyl polycarbonate-lysine urethane oligomer –DVO-, methacrylic acid –MAA- and methyl methacrylate –MMA- (Supplementary Fig. S1). D-PHI has been shown to be generally quite compatible and non-toxic to multiple cell types when used in the engineering of vascular tissues with vascular smooth muscle cells (VSMCs) [27], endothelial cells [28] and fibroblasts [29]. Furthermore, the D-PHI platform has been shown to reduce the pro-inflammatory activation of monocytes when compared to tissue culture polystyrene (TCPS) and polylactic glycolic acid (PLGA) [30,31], two very prominent materials reported on in the biomedical literature. The block structure chemistry of D-PHI has been formulated by varying the hydrophobic and anionic monomer content relative to the polar divinyl oligomer content in order to obtain a range of formulations that differentially interact with human monocytes. For example, a specific DVO/MAA/MMA ratio of 1: 20: 39 (referred to as HHHI, high hydrophobic and high ionic) has been reported to promote a significant reduction in inflammatory biomarkers released from human blood monocytes relative to that of original D-PHI formulation with a 1: 5: 15 ratio [31,32]. Battiston *et al.* showed that the surface of HHHI exhibited less presentation of the Fab-IgG binding site than the 1:5:15 ratio of monomers. Since previous studies have implicated the Fab domain as being critical for monocyte–biomaterial interactions [33], the selection of the HHHI material is of inherent interest to the current work. None of the physical properties which have been measured and previously reported on to compare HHHI *vs.* the original D-PHI formulation, such as contact angle, surface chemical group composition *via* X-ray photoelectron spectroscopy, or gel content (monomer conversion) were shown to explain the differences in protein (IgG) or cellular (monocyte) interactions observed with the change in chemistry introduced by HHHI. This suggests that it may be more the distribution of the different chemical functional groups associated with the specific change in chemistry that is possibly implicated as the primary mediator resulting in different protein binding and presentation of Fab activating groups to the cells, followed by the subsequent cellular responses observed with this material [31,32].

We have generated multifunctional polyurethane thin films (MPTF) (5 μm thick) of HHHI in order to study their use as thin coatings for CoCr [34]. The HHHI formulation was characterized in a previous study and the surface morphology, wettability and thickness was reported by Gossart *et al.* [34]. Note that at no time in our studies at 37 °C did we see any sign of delamination. We have shown that the exposure of specific sequences of fibronectin (Fn) and immunoglobulin G (IgG) (Fab-IgG, and the cell binding domain (CBD) and N terminal domain of Fn) were significantly lower on materials coated with this PU when compared to bare metal CoCr. Further, in other work by Battiston *et al.* [31] it was specifically shown when using defined inhibitors of the IgG Fab domains that this domain, in preference to the Fc domain of IgG, was highly correlated to reduced monocyte activation upon exposure to different biomaterials. This process was highly dependent on the relative anionic content in the polymer. In the work of Gossart *et al.,* [32] it was further shown to be beneficial with respect to regulating monocyte activation. Given the prevalence of tissue factor in activated monocytes in vascular systems, it was of interest to investigate the use of the HHHI material with respect to addressing monocyte activated pro-thrombotic events.

The objective of the current study was therefore to evaluate the reactivity of blood on MPTF coated onto CoCr, and in particular to determine if thin films of the HHHI materials on the order of a few micrometers would be able to mask the pro-thrombotic and pro-inflammatory effects of CoCr from whole blood elements. In the first

phase of this work, we studied the activation of coagulation from whole blood in contact with these biomaterials, whereas in the second phase of the work, we assessed the molecular and blood element behavior of individual cells, specifically monocytes and macrophages, in order to elucidate key mechanistic pathways involved for the HHHI coated CoCr substrates in comparison to non-coated CoCr.

2. Material and methods

2.1. Cobalt-chrome discs

Electropolished CoCr discs (L605, 6 mm diameter, 0.25 mm thickness) were purchased from Goodfellow (Lille, France). These discs were designed to fit into the wells of 96-well plates. Before using them in the experiments, the CoCr discs were incubated with 70% ethanol for 1 h at room temperature and rinsed with PBS. Some CoCr discs were incubated with 1% human serum albumin (HSA) endotoxin free (Biopure, Clinisciences, Nanterre, France) for 2 h at 37 °C, and were used as a negative control with respect to blood thrombus formation. All experiments were performed in 96-well plates.

2.2. HHHI coating preparations

HHHI synthesis was carried out using previously established methods [27]. Divinyl oligomer (DVO), methacrylic acid (MAA) and methyl methacrylate (MMA) at a stoichiometry of 1: 20: 39 [31] were mixed with the initiator benzoyl peroxide (BPO, 0.032 moL/mol vinyl group). The resin mixture was diluted in tetrahydrofuran (THF) (250 µL of the resin mixture with 3.750 mL of THF) and cast onto CoCr discs, to generate thin films as previously described [34]. Subsequent to adding the resin/THF solution onto the CoCr substrates, the polymerization was then performed at 65 °C for 1 h, and completed at 110 °C for 24 h under nitrogen gas purge [30,31].

2.3. Blood collection

Whole human blood was obtained from healthy volunteers who were exempt of medication for at least 10 days, following full informed consent, according to the Declaration of Helsinki. Legal and ethical authorization for the use of collected blood for research was obtained through a national convention between the French National Institutes of Health and Medical research (INSERM) and the French Blood Institute (EFS; convention number A18ZPA07000011). Blood was collected *via* venipuncture into siliconized Vacutainer™ tubes (Becton Dickinson, Le Pont de Claix, France) containing different anticoagulants as specified in the following procedures.

2.4. Monocytes and macrophages

Ethylene diamine tetra-acetic acid (EDTA) anticoagulated whole blood was centrifuged at 120 g for 15 min. The buffy coat was collected and monocytes were further purified using anti-CD14-coated microbeads according to the manufacturer's instructions (Myltenyi Biotec, Paris, France). Pure monocytes (> 98%) were resuspended in RPMI medium without fetal calf serum. For the different experiments, the monocyte concentration was adjusted to either 100 000 or 300 000 monocytes/mL, as specified in the following studies.

Monocytes were incubated 8 days with 5% fetal calf serum RPMI at 37 °C and 5% CO_2 to allow for the differentiation towards macrophages. The latter population was used to perform macrophage related experiments.

2.5. Preparation of platelet-free plasma (PFP)

Platelet-free plasma (PFP) was prepared from citrated blood (sodium citrate 0.129 M) using a double centrifugation protocol. Whole blood was centrifuged at 1200 g for 15 min. The PFP was then collected and centrifuged at 13000 g for 3 min.

2.6. Scanning electron microscopy (SEM)

SEM was performed on samples incubated in whole blood (anticoagulated with lithium heparinate) after 24 h of incubation at 37 °C, and for monocytes/macrophages prepared from EDTA anticoagulated blood after 24 h of incubation at 37 °C for monocytes, and 8 days of incubation at 37 °C for macrophages. All samples were rinsed with PBS and fixed with a 3% glutaraldehyde solution overnight at 4 °C, subjected to ethanol dehydration, and then underwent gold metallization (2 × 1 min). Samples were analyzed using a JEOL It 100 scanning electron microscope, at an operating voltage of 10 kV. The semi-quantification of monocyte surface area was obtained using Image J software.

2.7. Immunofluorescence of whole blood components

Surfaces were rinsed with PBS, fixed with a 4% para-formaldehyde solution for 20 min at room temperature and then rinsed with PBS. The permeabilization of the cells was performed with PBS-Triton 0.1%-Tween 20 0.05% for 15 min at room temperature. Non-specific binding sites were blocked by incubating the substrates in PBS-BSA 3% for 1 h at room temperature. Nuclei were labelled using NucBlue (Invitrogen). Specific antibodies were used to label fibrin (Clone 59D8, generous gift from Pr. CT. Esmon [35]), leukocytes (CD45, clone YTH24,5, ABD Serotec) and platelets (CD42b, clone SZ2, Beckman Coulter). Images were recorded with a LSM710 confocal microscope (Carl Zeiss).

2.8. Thrombin generation

Thrombin generation on the different surfaces was measured using citrated whole blood or in the presence of pre-seeded purified monocytes with PFP, by means of the Calibrated Automated Thrombogram (CAT) method [36] (Thrombinoscope BV, Maastricht, Netherlands).

Thrombin generation studies for whole blood experiments were carried out as follows. Paper discs (5 mm diameter and 180 µm thickness) (Whatman 589/1, Whatman GmbH) were placed in flat-bottom wells of a 96-well polystyrene microplate (Thermo Electron Corporation). Separately, 30 µL of citrated whole blood was incubated in the different condition (CoCr and CoCr coated with HSA or HHHI) and was then mixed with 10 µL of P2Rho, a rhodamine-based thrombin substrate (1.8 mmoL/L) and 20 µL $CaCl_2$ (50 mmoL/L). Immediately after mixing, 5 µL of the mixture were loaded onto the paper discs and covered with 40 µL of mineral oil (USB Corporation). In parallel, a calibration experiment was carried out where the $CaCl_2$ containing solution was replaced with 20 µL of a thrombin calibrator (300 nmoL/L thrombin activity, Diagnostica Stago, Asnières, France). The fluorescence signal from the instrument was recorded with a Fluoroskan Ascent microplate fluorometer at an excitation of 485 nm and emission of 538 nm (Thermolabsystems). Samples were run at least in triplicate and the experiment repeated a minimum of three times. The calibrated thrombin generation curves were calculated as previously described [37]. All procedures were performed at 37 °C in the absence of exogenous TF.

For monocyte coated substrates, measurements were conducted with 80 µL PFP in a total volume of 120 µL. Thrombin generation was performed in the absence of TF. Samples that were spiked with 20 µL thrombin calibrator (Diagnostica Stago, Asnières, France) were run in parallel with each cycle of test sample. Thrombin generation was triggered by the addition of 20 µL FluCa (2.5 mM fluorogenic substrate Z-Gly-Gly-Arg-AMC, 87 mM calcium chloride). The fluorescence level was read using a Fluoroskan Ascent reader (Thermo Labsystems) equipped with a 390/460 nm filter set, and thrombin generation curves were calculated using the thrombinoscope software. The velocity index (nM/

min) values for the latter protocol was calculated as the ratio [Peak/(time to Peak-LagTime)]. LagTime (LT) is related to the initiation phase of coagulation on the thrombinoscope. The velocity index is related to the initiation and amplification phase of coagulation. The endogenous thrombin potential (ETP) represented the total activity of thrombin generated.

2.9. Tissue factor activity assay

TF activity at the surface of adherent monocytes was determined as the ability of monocytes to promote factor X activation in the presence of activated factor VII (factor VIIa, *Novoseven*, 10 nM) using a chromogenic activity assay [38]. The specificity of the assay was ascertained by using a blocking *anti*-TF MoAb (CD 142, clone HTF-1, BD Pharmingen, Le Pont de Claix, France). A standard curve was constructed using relipidated recombinant human TF (PPP reagent low, Diagnostica Stago, Asnieres, France). TF quantitation was expressed as pM of TF for 10 000 monocytes/well.

2.10. TNF-α, IL-10 and thrombin-antithrombin complex levels

TNF-α and IL-10 concentrations after 24 h incubation were measured in the supernatants by ELISA (Invitrogen), according to the manufacturer's instructions, in conditioned media for monocytes or macrophages after 24h or 8 days of incubation, respectively. Thrombin-antithrombin (TAT) complexes were measured by ELISA in the plasma after incubation of whole blood on the different surfaces for 1 or 5 h, and according to the manufacturer's instructions (Siemens Healthcare diagnostics, Enzygnost TAT micro).

2.11. Mass spectrometry

The bicinchoninic acid (BCA) assay was used to quantify the adsorbed protein on the material samples according to the manufacturer's protocol (Thermo Scientific), then a protein digestion was carried out with the following protocol: after incubation with plasma for 2 h at 37 °C, surfaces were briefly rinsed with 1% PBS solution. Adsorbed proteins were submitted to a proteolysis stage by applying a trypsin solution onto the material. The digestion was carried out overnight at 37 °C with trypsin (Promega, Madison, WI), at a ratio of 1:10, trypsin to protein. To stop the reaction with trypsin, the sample was treated with 50 µL of 10% formic acid (FA). This step was followed by two washing steps with 100% acetonitrile (ACN) to collect peptides in separate Eppendorf tubes for the different materials. After evaporation, peptide solutions were then concentrated using ZipTips (Agilent Technologies, OMIX 96C18 10 µL). Briefly, ZipTips were first equilibrated with 100% ACN, and then conditioned in 0.1% FA before sample loading. The unbound fraction was eluted by several washing steps (0.1% formic acid FA) followed by centrifugation. The bound peptides were then eluted using a 50% ACN, 50% FA 0.1% solution and centrifugation (2500 g for 5 min). This step was repeated 3 times. Peptide samples were concentrated by evaporation using a Speedvac, and finally dissolved in 10 µL of 0.1% FA for nanochromatography. All experiments were carried out with a hybrid linear ion trap–orbitrap mass spectrometer (LTQ Orbitrap Elite, Thermo Scientific) equipped with a nano-ESI source and coupled to a liquid nano-chromatography system (Easy-nLC II, Thermo Scientific). Mass spectrometry Analysis, protein identification and quantification were performed according Obry A. *et al,* 2014 [39].

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, CA) and using an analysis of variance (ANOVA) or an independent samples *t*-test. When appropriate, the statistical significance was reported for $p < 0.05$. All experiments were repeated in triplicate with three technical replicates for each experimental value.

3. Results and discussion

3.1. Thin films significantly reduce biomolecular and inflammatory cell activation, thrombin generation, and fibrin formation induced by CoCr in whole blood

Cardiovascular medical devices that are used in direct contact with blood should have good blood compatibility and avoid activation of protein and platelet complexes, as well as minimize activation of immune sensitive processes that could catalyze the former. Foreign surfaces such as metallic substrates may activate blood cells leading to uncontrolled thrombotic and inflammatory responses that ultimately result in the failure of the implant [5]. It is therefore essential to limit the thrombogenicity of bare metal surfaces used as cardiovascular devices. One option involves the coating of bare metal surfaces with multi-functional polymers that are capable of minimizing the denaturation of key proteins that activate cellular elements and more specifically monocytes [30,32]. In this study, we have investigated the use of a novel polymer derived from a platform system, HHHI, for such a purpose as it can be formed into thin crosslinked films which mask the direct contact between the metal and the blood. These materials have been specifically shown to reduce the activation of monocytes. The endpoint objective was to reduce the probability of monocyte activated thrombosis.

To evaluate the biological effect of HHHI thin films on CoCr, well defined surfaces (CoCr (positive control), CoCr-HSA (negative control) and CoCr-HHHI) were incubated for 24 h at 37 °C with whole blood. Hemolysis is a detrimental event to the outcome of biomaterial implantation as it destroys erythrocytes that are abundant blood cells and are important for the transport of oxygen (O_2) from the lung to all tissues and cells, as well as carbon dioxide (CO_2) from tissues back to the lung. They also play a pivotal role in enabling platelets to interact with surfaces in flow systems. We assessed hemolysis on the 3 different surfaces. No induction of hemolysis was observed for any of the experiments after the contact of whole blood (Supplementary Fig. S2). When examining the whole blood contacted surfaces by SEM, the images (Fig. 1) showed that an intense fibrin network was formed on the bare CoCr surface, with a large presence of platelets, leucocytes and

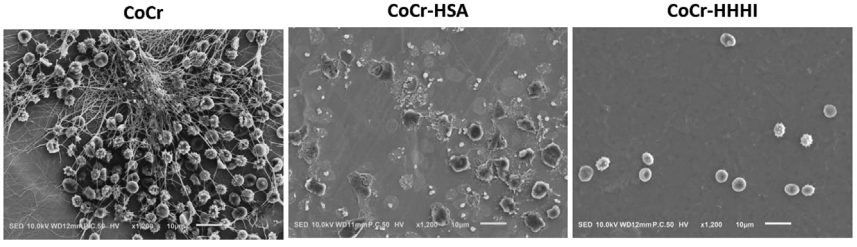

Fig. 1. Cell morphology after 24 h incubation at 37 °C of whole blood on bare CoCr, CoCr coated with HSA or HHHI. SEM images are representative of 3 different experiments performed in triplicate with 3 different blood donors. Scale bar = 10 µm.

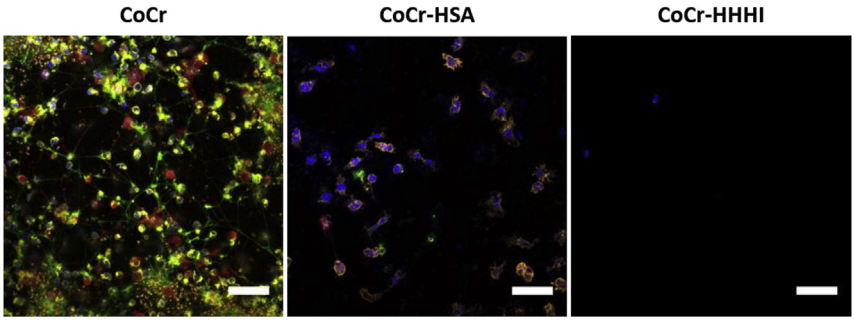

Fig. 2. Immunostaining after 24 h of incubation of whole blood at 37 °C on bare CoCr, CoCr coated with HSA or HHHI. Confocal microscopy images are representative of 2 different experiments performed in triplicate with 2 different blood donors. Cell nuclei (blue), fibrin (green), platelets (red) and leukocytes (yellow). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

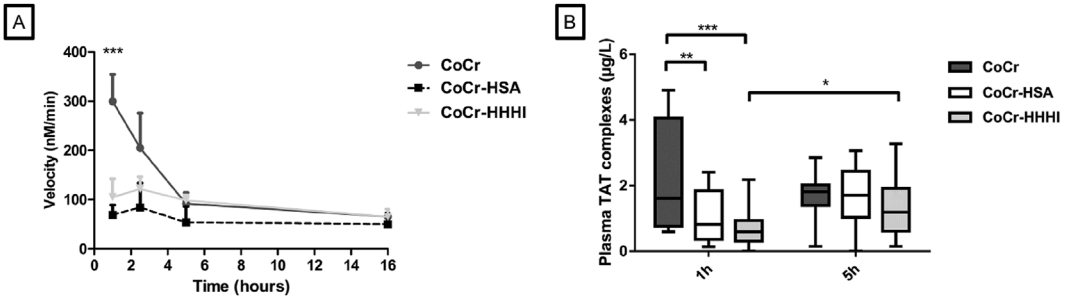

Fig. 3. Thrombin generation and TAT complexes in whole blood on bare CoCr, CoCr coated with HSA or HHHI. A) Kinetic of thrombin generation (CAT) at 1 h, 2.5 h, 5 h and 16 h of incubation at 37 °C. n = 2–4, triplicates with 2–4 different blood donors, Mean ± SD, ***p < 0.001. B) Quantification (ELISA) of Thrombin/Antithrombin complexes in plasma after 1 h or 5 h of whole blood incubation at 37 °C. n = 6, triplicates with 6 different blood donors, Medians and quartiles, ***p < 0.001, **p < 0.01 and *p < 0.05.

red blood cells trapped within it. On the HSA passivated surface, no fibrin network was detected but the presence of what were likely activated platelets and leukocytes was observed. On HHHI-coated CoCr surfaces, very few cells were adherent, and among those found, the cells were mostly red blood cells.

The nature of the deposited blood elements was confirmed by immunostaining with specific antibodies against fibrin, platelets and leukocytes (Fig. 2 and Supplementary Fig. S3). The CoCr showed an extensive amount of fibrin, platelets and leukocytes whereas when these surfaces were coated with HSA (CoCr-HSA) the number of leukocytes and platelets were substantially reduced and only showed a few isolated fibrin domains. When CoCr was treated with thin films of HHHI, the adhesion of platelets was eliminated and no fibrin formation was seen. There were a few adherent leukocytes however their activated state was much less noticeable than either of the other two surfaces. Previous studies have shown that platelets and neutrophils were activated after CoCr contact [22,23]. However, the results of our current study show a global cell activation with extensive leukocyte mediated events, suggesting that anti-platelet therapy, which is often proposed after cardiovascular device implantation may not be sufficient to prevent all pathways that lead to clot formation, and specifically those occurring *via* leukocyte cell activation. The importance of this hypothesis is substantiated by a recent a multicenter European study [1], where it was shown that thrombi from patients with stent thrombosis were heterogenous in composition. One feature of these thrombi was the presence of numerous leukocytes suggesting an important role for inflammatory activity in the pathogenesis of stent thrombosis events.

The formation of a fibrin network on bare CoCr indicates that coagulation is activated. This activation of coagulation may be partially mitigated with HSA coating of CoCr; however, it is clear that the presence of HSA has a limited ability to remain at the surface and block leukocyte activation and the formation of fibrin networks, as these complexes are still clearly evident on the CoCr-HSA substrates. In contrast, the HHHI coatings appear to completely prevent the activation of coagulation and fibrin networks, as it mitigates cell adhesion and subsequently cell activation. This is an impressive outcome when considering that the material does not inherently possess any pharmaceutical anticoagulant function, andit solely achieves this outcome based on the unique multi-function chemistry and its interaction with the blood elements. Previous studies have shown that HHHI based materials are potent disruptors of monocyte activation. This feature was previously described to be achieved by enabling reduced denaturation of key proteins known to activate these cells, *e.g.* fibronectin and immuno-globulin G [30,31].

In order to quantify the activation of coagulation in whole blood induced by the different surfaces, we have measured thrombin generation. Thrombin is the key enzyme that allows for the conversion of fibrinogen to fibrin in order to form a clot, but it also plays a role in inflammation [40] and in the activation of complement factors [41]. In this study, we have measured thrombin generation in whole blood using an adapted global test: the Calibrated Automated Thrombogram (CAT). This technique allowed for thrombin detection despite the presence of red blood cells [37]. As shown on Fig. 3A, the velocity of thrombin generation at 1 h was significantly ($p < 0.001$) higher on CoCr (299 ± 54 nM/min) as compared to CoCr-HHHI (105 ± 37 nM/min). With respect to the kinetics of thrombin generation, the velocity on CoCr was decreased to a value similar to the two other surfaces only after 5 h. Similar results were obtained when Endogenous Thrombin Potential (ETP) was assessed (Supplementary Figs. S4A and B).

Importantly, the activation of coagulation in whole blood was substantially different among the different surfaces (Fig. 3B) as evaluated by the amount of thrombin/antithrombin (TAT) complex using the enzyme immunoassay. In agreement with the thrombin generation observed in whole blood, the level of TAT complex, reflecting activation of the coagulation, was higher on bare CoCr (2.2 ± 0.1 μg/L) after 1 h of whole blood incubation when compared to the HSA coated surfaces with a significant (at $p < 0.05$) 2-times reduction (1.1 ± 0.1 μg/L). The level of TAT complexes was reduced by almost 3-times in the presence of the HHHI coatings (0.7 ± 0.1 μg/L). After 5 h of whole blood incubation, the level of TAT complex was similar for all surfaces. This may be explained by an enzymatic degradation or dissociation of

the complexes in this static test.

Under healthy physiological conditions, endothelial cells provide an anticoagulant and antithrombotic barrier which is in direct contact with blood [42]. Vascular homeostasis is maintained through the release of numerous vasodilatory and vasoconstrictive substances from the endothelium, and by surface proteins expressed from endothelial cells, such as thrombomodulin and heparin-like molecules, which exhibit antithrombotic actions through defined pathways. In particular, heparin-like molecules act as cofactors for antithrombin which contributes to the antithrombotic properties of the endothelium [43]. Bare CoCr is a foreign surface that does not have the ability to exhibit the properties of the endothelium as demonstrated by the observed thrombin generation, confirmed from the increased level of TAT complexes, both reflecting the activation of coagulation. The HHHI coatings prevents the burst in coagulation activation observed with CoCr. The presence of activated platelets and leukocytes and the formation of an important fibrin network at the surface of bare CoCr confirm the observation that this metal is procoagulant and that blood contact with this metallic substrate can initiate thrombus formation, whereas the HHHI coating on CoCr prevents thrombin generation and fibrin formation.

3.2. Reduction of tissue factor generated by monocytes activation is a key biomolecular factor in the reduction of thrombin generation on CoCr coated with HHHI thin films

The initiation of clotting involves a highly interconnected mechanism of activated molecular sequences, where the intrinsic pathway of coagulation, initiated by the contact of blood proteins, and the extrinsic pathway, initiated by TF, converge into the common pathway resulting in platelet activation that accelerates thrombin formation and allows the formation of a fibrin rich clot [5]. Activation of the extrinsic pathway is TF-dependent and monocytes can provide a major source of TF in blood [10]. They are able, after activation, to express TF at their membrane. Membrane-associated TF is fully active and able to initiate the coagulation cascade. Moreover, its activity is largely increased in the presence of negatively charged phospholipids such as phosphatidyl serine [44]. Thus, given the high level of adherent leukocytes shown in Fig. 2 on CoCr surfaces, it was hypothesized that TF expression by monocytes is likely to be implicated in some manner with respect to the activation of coagulation observed on CoCr in whole blood. Moreover, a previous study has shown the importance of leukocytes and more particularly of monocytes in triggering coagulation, in addition to the more traditional studied pathways involving platelets on biomaterial surfaces [45]. These studies showed that the quantity of TAT complex formation was almost negligible in platelet poor plasma (PPP) or platelet rich plasma (PRP) when compared to whole blood, therefore potentially implicating the importance of leukocytes [45]. To support this hypothesis, we purified monocytes and then incubated them on the different surfaces and tested for cell adhesion, thrombin generation in PFP, TF activity and cytokine release. Macrophage adhesion and their cytokine release were also investigated. Monocytes were first incubated at 37 °C for 24 h on different surfaces (CoCr, CoCr-HSA and CoCr-HHHI). No differences in cell mortality were observed on the different surfaces as shown by the LDH data (Supplementary Fig. S5A). Monocyte morphology was observed by SEM (Fig. 4A). Monocyte activation after adhesion can be evaluated, in part, by the morphology of these cells *i.e.* increase of the cell size, cytoplasmic propagation and presence of pseudopodia which are markers of activation [46]. On the CoCr surface, monocytes were very spread with a strong presence of pseudopodia suggesting activation. Spreading and pseudopodia were less pronounced on CoCr-HSA, whereas monocytes on CoCr coated with HHHI were very small in size, round with a low presence or absence of pseudopodia suggesting low monocyte activation on CoCr coated with thin films as compared to bare CoCr. By measuring monocyte surface area (Fig. 4B) we have observed a significant ($p < 0.001$) increase (4x) of the cell surface area on CoCr as compared to HHHI-coated CoCr ($232 \pm 123\,\mu m^2$ for CoCr *vs* $40 \pm 23\,\mu m^2$ for HHHI). In the presence of HSA, the surface area of monocytes was also significantly reduced as compared to CoCr but was still significantly increased, almost twice, when compared to HHHI-coated CoCr. According to their morphology, monocytes were therefore much less activated on the PU-coated CoCr as compared to CoCr and even CoCr-HSA.

To confirm that the changes in cell morphology were related to cell activation, and to determine the implication of monocytes in the activation of coagulation observed in whole blood, the pro-coagulant activity of monocytes was investigated through the measurement of thrombin generation at the cell surface. As shown on Fig. 5A, the velocity index, reflecting the initiation and amplification phases of coagulation, was significantly ($p < 0.05$) increased 2-times on CoCr as compared to the HHHI-coated CoCr (12.2 ± 3.4 nM/min for CoCr *vs* 6.3 ± 2.1 nM/min for HHHI). In the presence of HSA, the velocity index had an intermediate value to the above two states (8.2 ± 2.3 nM/min), with no significant difference compared to either CoCr or CoCr-HHHI. The time required to produce the first traces of thrombin (LagTime) that would allow for the amplification of the coagulation was significantly ($p < 0.05$) lower for the CoCr surface as compared to HHHI-coated CoCr (2.1 ± 0.6 min *vs* 4.3 ± 1.3 min respectively) (Fig. 5B). Again, the HSA coated CoCr displayed a LagTime value that was intermediate and not significantly different from those of CoCr or CoCr-HHHI. These results suggest that thin films of HHHI delay the initiation of thrombin generation and reduce the level of thrombin generated and thus globally reduce the CoCr-induced pro-thrombotic phenotype of monocytes. Supporting this hypothesize was the observation that the correlation coefficients between monocyte surface area, LagTime and Velocity index were highly significant ($r^2 = 0.999$ for monocyte surface *vs* LagTime and $r^2 = 0.989$ for monocyte surface *vs* velocity index). The observed effect on thrombin generation is mainly due to an effect of the monocyte, as no significant difference was observed between the different surfaces when only PPP was used in the assay (Supplementary Fig. S6).

As the LagTime parameter for thrombin generation is highly dependent on the level of TF [47], monocyte TF activity was evaluated (Fig. 5C and D). TF activity was measured at the surface of intact cells as a function of time. As early as the 1 h time point (Fig. 5C), TF activity was significantly ($p < 0.01$) increased for monocytes in contact with the CoCr surface (0.2 ± 0.1 pM) as compared to the HHHI-coated CoCr (0.7 ± 0.02 pM) or HSA (0.7 ± 0.03 pM). Over time (5 and 24 h) (Fig. 5D), CoCr-induced TF activity is still induced at 2-times higher when compared to PUs-coated CoCr or HSA.

As NF-κB activation has been shown to be involved in TF gene transcription, we have looked at NF-κB translocation into the monocyte's nuclei (Supplementary Fig. 7). We observed a high NF-κB activation after 1 h for monocyte contact with CoCr or CoCr-HSA. In contrast, only a faint NF-κB activation was observed after contact with CoCr-HHHI. These results could explain the differences that were observed in TF expression induced by the different surfaces at later time points (5 and 24 h). It was interesting to see that even after a short period of 1 h of contact with CoCr, monocytes were able to display a significant level of TF activity. This activity could not be explained by the generation of new protein synthesis as the latter would require at least 4 h or more, even if the TF gene is an early response gene (mRNA synthesized in 1–2 h) [7]. It has been reported that approximately 1.5% of human monocytes express low levels of intracellular [48] or membrane encrypted TF [49] in resting conditions. We can hypothesize that monocyte TF activity from cells on the surface of CoCr is then likely due in large part to the early activation of the already present, but encrypted TF immediately following contact with CoCr, and/or that TF activity may be increased by the translocation of negatively charged phospholipids on the external cell membrane of activated monocytes [50]. CoCr is known to oxidize at its surface, and the oxide by-products carry a negative charge at physiological conditions (neutral pH). Such

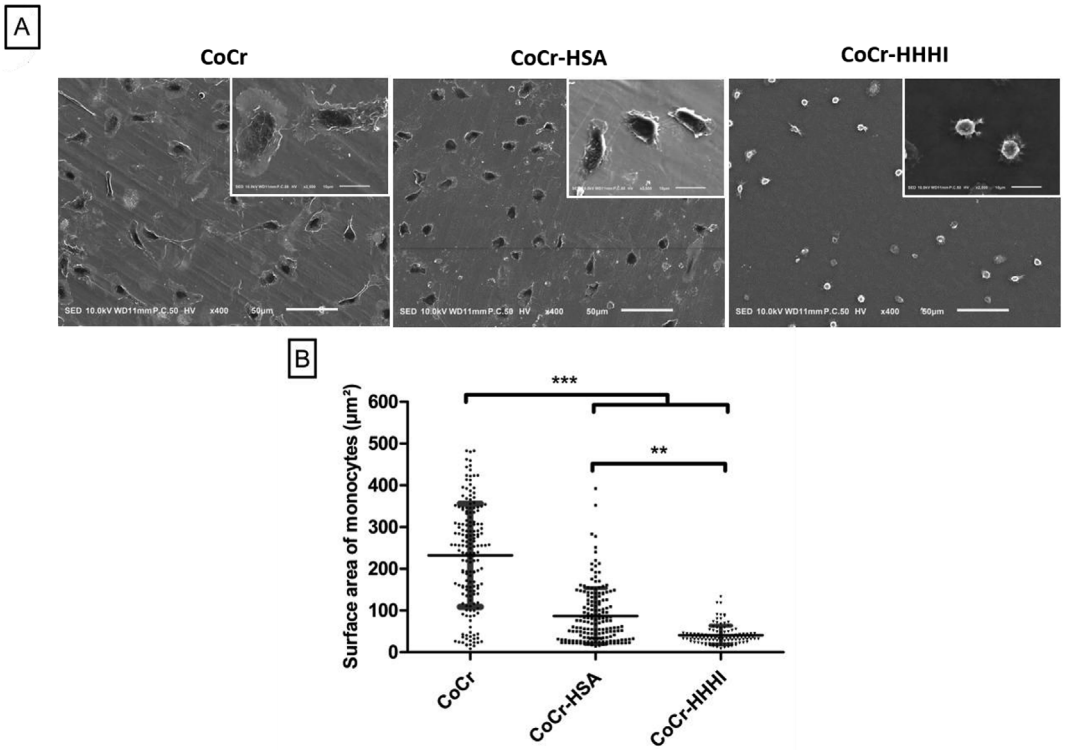

Fig. 4. Morphology of isolated monocytes after 24 h of incubation at 37 °C on bare CoCr, CoCr coated with HSA or HHHI. A) SEM images are representative of 3 different experiments performed in triplicate with 3 different blood donors. Scale bars = 50 µm and 10 µm (inset). B) Measurement of surface area from SEM images. Each point represents the surface area of one monocyte. 18 images were measured per conditions. Mean ± SD. ***p < 0.001 and **p < 0.01.

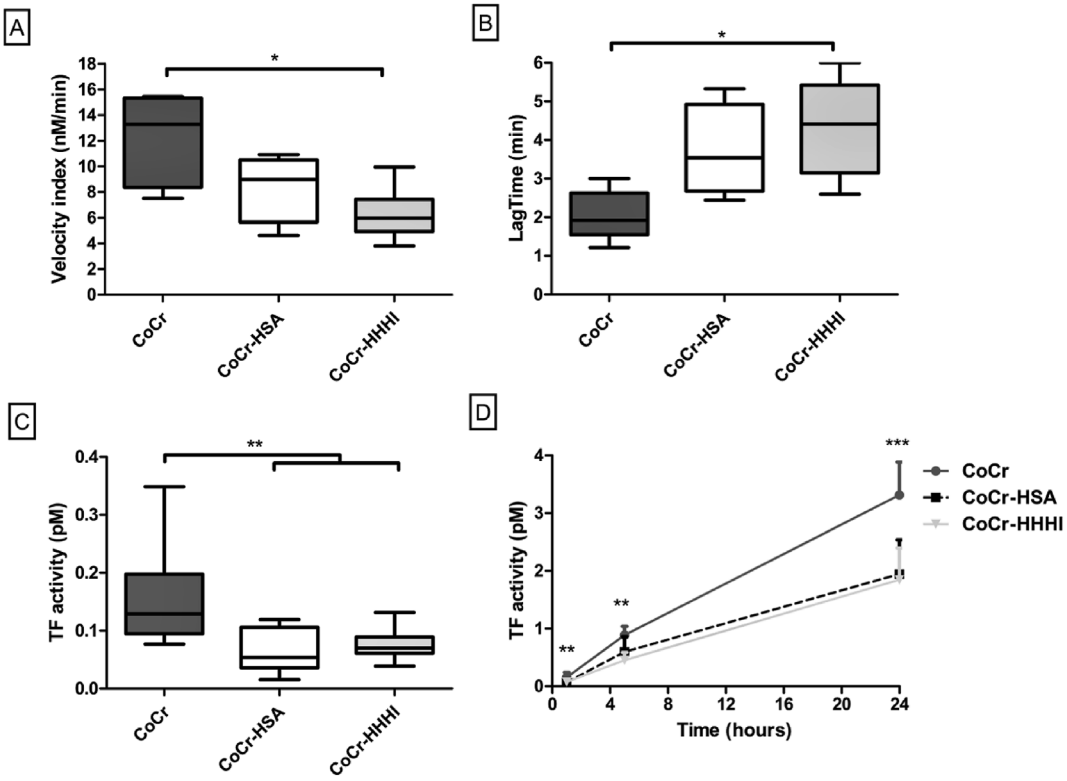

Fig. 5. Thrombin generation and TF activity of isolated monocytes incubated on bare CoCr, CoCr coated with HSA or HHHI. A - B) Thrombin generation (CAT): Velocity index (A) and Lagtime (B) after 24 h of incubation at 37 °C. n = 6, triplicates with 6 different blood donors. C-D) TF activity after 1 h of incubation (C) and Kinetic at 1 h, 5 h and 24 h of incubation at 37 °C (D). n = 5, triplicates with 5 different blood donors, Medians and quartiles (A, B and C) and Mean ± SD (D). ***p < 0.001, **p < 0.01 and *p < 0.05.

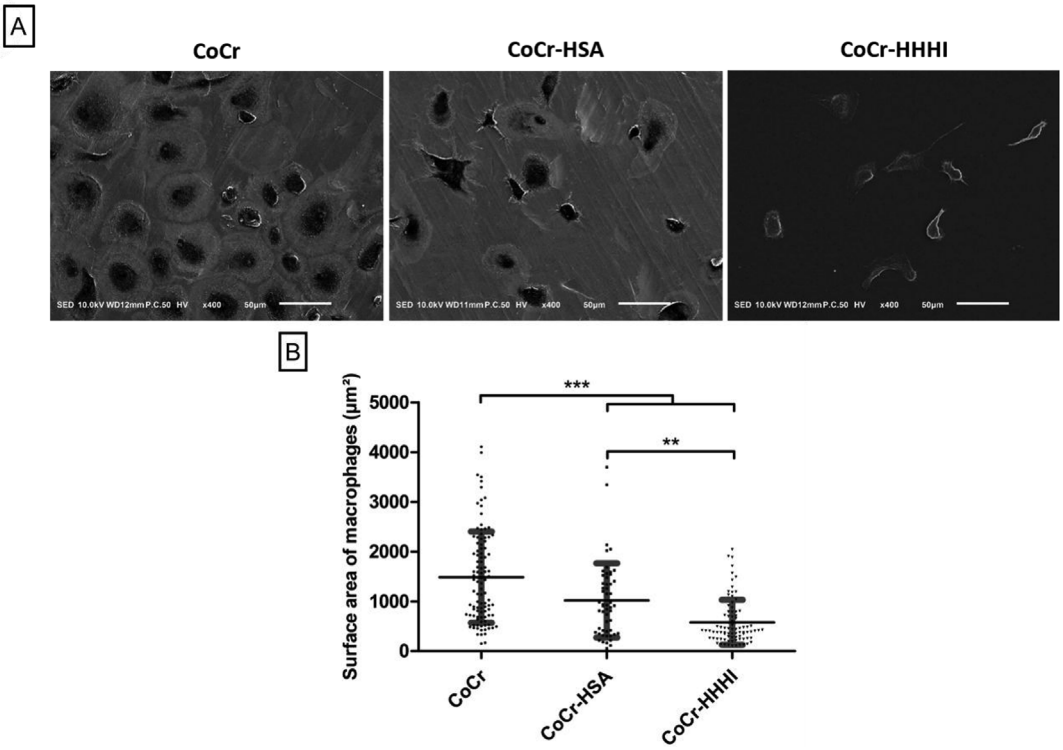

Fig. 6. A) Morphology of isolated macrophages after 8 days of incubation at 37 °C on bare CoCr, CoCr coated with HSA or HHHI. A) SEM were representative images of 3 different experiments performed in triplicate with 3 different blood donors. Scale bar = 50 µm. B) Measurement of surface area from SEM images. Each point represents the surface area of one monocyte. 18 images were measured per conditions. Mean ± SD. ***$p < 0.001$ and **$p < 0.01$.

negative charged species have been reported to contribute which to TF activation [51–53]. When monocytes are recruited to the site of biomaterial implantation, they will evolve into macrophages that could secrete pro-inflammatory and/or pro-wound-healing cytokines and growth factors that influence subsequent healing events, and can contribute to the eventual fate of the implanted biomaterial [54]. To study macrophage behavior, monocytes were incubated for 8 days on the different surfaces, allowing them to reach the supposed state of differentiation in macrophages [55]. The morphology of macrophages was observed by SEM (Fig. 6A). On a CoCr surface, the macrophages had the appearance of a fried egg (about 10 times the size of the monocytes on the same surfaces), highly spread with a strong presence of pseudopodia. This was not observed on HHHI-coated CoCr. An intermediate phenotype was found on the HSA coated CoCr. Macrophage surface area was significantly ($p < 0.001$) increased 3-times on CoCr as compared to the HHHI-coated CoCr (1519 ± 978 µm^2 for CoCr *vs* 578 ± 451 µm^2 for HHHI) (Fig. 6B). For HSA the surface area was 1020 ± 738 µm^2. According to their morphology, macrophages were therefore much less activated on PU-coated CoCr as compared to CoCr and even CoCr-HSA. Looking at the morphology of macrophages, it seems that they tended to fuse with each other. One hypothesis was that the phenomenon of foreign body giant cells (FBGC) could occur with a longer incubation time. FBGC are formed when the foreign body exceeds the capacity of a single macrophage to internalize foreign objects. Macrophages adhering to the biomaterials will fuse and form a large multi-nuclei cell to try to remove the foreign body [56,57].

3.3. Thin films of multi-functional HHHI coatings significantly reduce the CoCr-induced release of the pro-inflammatory cytokine TNF-α by monocytes/macrophages

The extent of the inflammatory response induced by the biomaterial can be evaluated in terms of specific pro-inflammatory cytokines released from adherent monocytes/macrophages such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6) or IL-12 and anti-inflammatory cytokines such as IL-10 [58,59]. In particular, TNF-α release is an important indicator of monocyte activation because it is a potent inflammatory cytokine that can activate virtually all cells [60] and is implicated in inflammation and tissue destruction in chronic inflammatory diseases [61].

To evaluate the pro- or anti-inflammatory state of monocytes and macrophages after contact with the different biomaterials, the secretion of the pro-inflammatory cytokine, TNF-α, and of the anti-inflammatory cytokine IL-10 were measured in the 24 h culture supernatant after 8 days of monocyte incubation (Fig. 7). The results were normalized by cell quantification performed using the LDH assay (Supplementary Figs. S5A and B). On CoCr, the level of TNF-α secreted by monocytes (Fig. 7A) was 5 times higher ($p < 0.01$) than on the HHHI-coated CoCr (5229 ± 138 µg/mL for CoCr *vs* 1435 ± 111 µg/mL for HHHI). A significant difference ($p < 0.05$) between HHHI-coated CoCr and HSA in TNF-α secretion was also observed but there was no statistical difference between HSA and CoCr. The secretion of IL-10 (Fig. 7B) was also largely enhanced after CoCr or HSA contact when compared to HHHI-coated CoCr (10.6 ± 0.7 µg/mL for CoCr *vs* 2.3 ± 0.1 µg/mL for HHHI). On CoCr or CoCr-HSA, monocytes secreted a large amount of pro- and anti-inflammatory cytokines. These findings suggest a strong influence from the unique chemistry of the HHHI thin films coatings, as in this condition the level of secreted cytokines (TNF-α and IL-10) was low in comparison to the other two material surfaces. These results were aligned with our observations on cell morphology and procoagulant activity. Macrophages on CoCr also exhibited more than 2-times increase ($p < 0.001$) in TNF-α secretion (Fig. 7C) when compared to HHHI-coated CoCr (167 ± 8.5 µg/mL for CoCr *vs* 66 ± 0.8 µg/mL for HHHI). On HSA, TNF-α secretion was not different when compared to PU-coated CoCr. IL-10 secretion was similar on all surfaces (Fig. 7D). The overall conclusion is that the interaction of monocytes/macrophages with thin films of HHHI limits their activation resulting in a modification of their inflammatory phenotype.

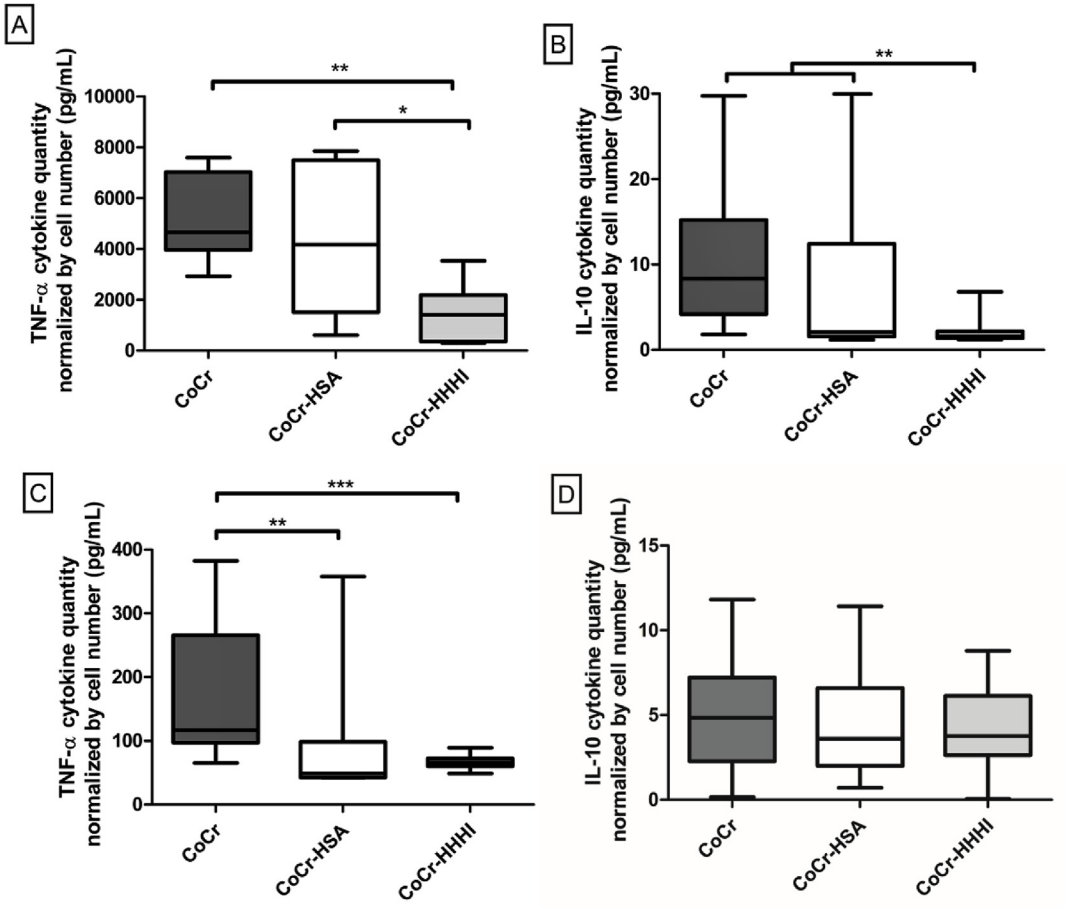

Fig. 7. Quantification (ELISA) of TNF-α and IL-10 in the 24 h supernatant of monocytes (A-B) and macrophages (C-D) incubated on bare CoCr, CoCr coated with HSA or HHHI. Results are normalized by cells quantity. n = 6, duplicates with 6 different blood donors, Medians and quartiles, ***p < 0.001, **p < 0.01 and *p < 0.05.

Previous studies have shown that activated monocytes (endotoxins, immune complexes, monocyte chemotactic protein (MCP-1) or TNF-α) are able to induce the expression of TF [62,63]. Moreover, plasmatic TF antigen has been shown to be correlated with the expression of TNF-α and MCP-1 in chronic thromboembolic pulmonary hypertension [64]. The observed secretion of TNF-α by monocytes or macrophages in contact with CoCr may explain the TF activity measured at later times (5 h and 24 h), values that remain 2-times higher than that measured on the other surfaces.

Many recent reports have focused on the controlled release of bioactive molecules to reduce inflammation and improve tissue repair. For example, to treat local inflammation, a study has used a chitosan hydrogel loaded with anti-TNF-α antibodies [65]. Matrices formed on the biomaterial surface were reported to be rich in cytokines, growth factors and chemoattractants. This favors the recruitment of innate immune system cells to the site of injury [55]. Therefore, it is important to minimize TNF-α secretion. HHHI therefore has a considerable advantage with regards to the low level of TNF-α secreted by monocytes on this surface.

Surface chemistry appears to potentially play a contributing role in the activation of adherent leukocytes [66]. More specifically, the behavior of monocytes/macrophages and whole blood is differentiated according to the surface chemistry, as in this study we controlled for surface roughness [34]. In this work, we observed that 5 μm thick thin films on the surface of CoCr could modulate the cellular response. We recently have shown [34] that the differential adsorption of two plasma proteins, IgG and fibronectin, were observed on the CoCr *vs* CoCr-HHHI surfaces. The exposure of the Fab fragment from IgG was significantly higher on CoCr when compared CoCr coated with the HHHI. Exposure of IgG is a critical factor shown to increase monocyte adhesion and propagation [31,33]. Regarding human plasma fibronectin, exposure of the cell binding domain (CBD) and N-terminal domain was significantly increased by 4-times on CoCr as compared to CoCr surfaces coated with HHHI. The CBD contains the amino acid sequences RGD and PHSRN. The potential synergy between these two sequences favors the fusion of macrophages to form foreign body giant cells (FBGC) [67] and activates monocytes through the VLA-5 receptor, inducing the production of proinflammatory cytokines such as IL-1, IL-6 and TNF-α [68]. The N-terminal domain and more particularly the 70 kDa fragments of Fn play an important role in coagulation. This latter domain contributes to cross-linking activity within fibrin clots by the formation of ε-(γ-glutamyl)-lysyl bonds, and is catalyzed by the thrombin-activated coagulation factor XIII (FXIIIa, plasma transglutaminase) [69]. This cross-linking increases both thrombus size and platelet adhesion [70]. All these observations suggest that the surface chemistry of the HHHI coatings may be controlling thrombin formation indirectly *via* multiple pathways that are mediated, in part, by monocytes, and that harnessing this relatively simple multifunctional polymer chemistry in a manner that reduces monocyte activation appears to be in part influenced by the nature of protein adsorption [71], which in turn has been shown to influence the degree of adhesion and activation of monocytes and macrophages, as well as the secretion of cytokines. The latter in turn could propagate the generation of TF, and thus produce TAT and clot formation.

It is well known that the adsorbed protein layer onto a biomaterial surface is of particular importance for subsequent events and may

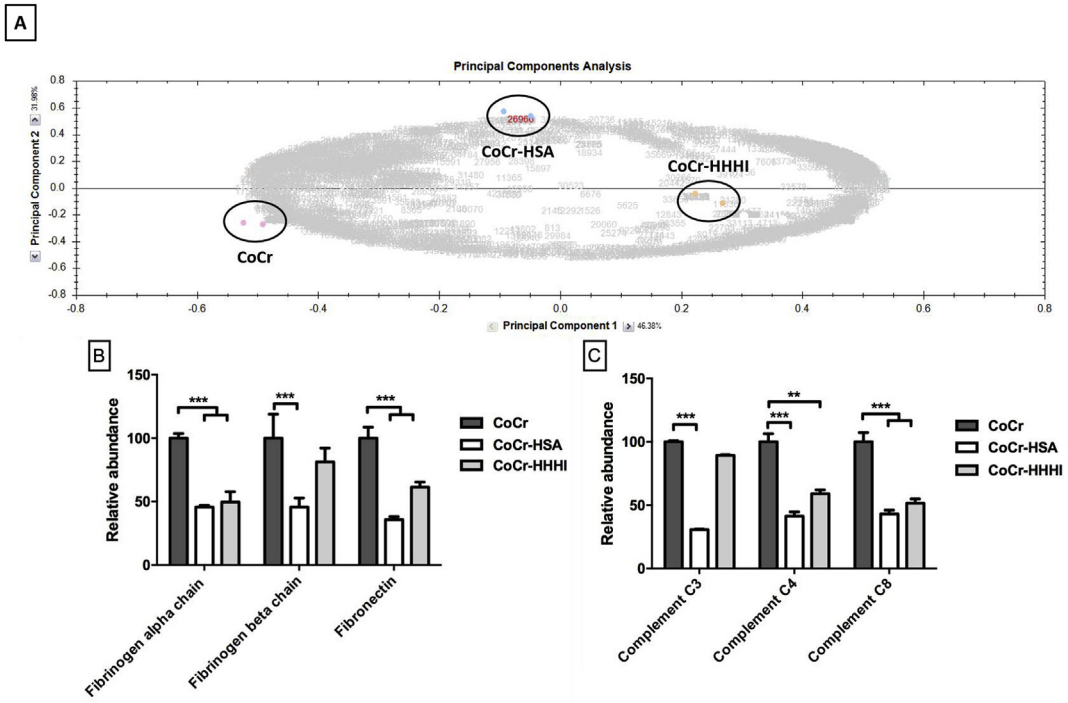

Fig. 8. Principal components analysis (PCA) and compared relative abundance of specific proteins by mass spectrometry on the different surfaces (bare CoCr, CoCr coated with HSA or HHHI) after 2 h of incubation of PPP. A) Graphical representations of the PCA results (principal components 1 and 2). B) Illustration of relative quantification for proteins fibrinogen alpha chain, beta chain and fibronectin and C) complement C3, complement C4, complement C8. CoCr was used as a reference material and considered as a 100%. n = 3, duplicate with pool of 18 different blood donors. Mean ± SEM, ***p < 0.001, **p < 0.01 and *p < 0.05.

condition cell behavior [55]. Therefore, in order to gain a further understanding with respect to the mechanisms occurring on the surface of these different materials, an analysis of the adsorbed proteins was carried out by mass spectrometry after adsorption of PPP for 2 h at 37 °C. Principal component analysis (PCA) was used to evaluate the capacity for the different surfaces to show a relative abundance of differentially adsorbed proteins (Fig. 8A). In this analysis, the two first components of the PCA explain nearly 80% of the total variability. Principal component 1, which accounts for 46% of the total diversity, allows one to discriminate between bare CoCr and CoCr coated with HHHI. The finding of differences on these surfaces would signal the extent of distinctiveness in terms of protein adsorption. Not surprisingly, the CoCr-HSA surface is discriminated from the other surfaces in the second component because of the presence of HSA which accounts for another 32% of the analyzed proteins. At a more focused level, the relative abundances of two subsets of proteins are presented (Fig. 8B and C). Fig. 8B shows that fibrinogen (Fg) alpha chain/beta chain and Fn proteins are presented at a higher relative abundance on CoCr surfaces, while a rather low level was present on CoCr-HSA surface, and an intermediate level was found for HHHI. The low level of adsorbed proteins on the CoCr-HSA may be in part explained by the presence of HSA protein that prevented access of other proteins from interacting with the surface. With respect to complement system proteins, *e.g.* C3, C4 and C8, the profiles are highly comparable to what was displayed in Fig. 8C. Note that many other proteins have been identified but only a few differentially detected proteins were reported on here, as they are known to have a role in coagulation and/or inflammation. Specifically, Fg was shown to be actively involved in neutrophil adhesion [72], to lead to activation of the procoagulant state in platelets [73] and to promote the monocytes adhesion [74]. Fn is known to have a role in coagulation by consolidating the fibrin clot [69] and inflammation by activating monocytes [68]. Fn, as is Fg, is also known to activate platelets [73]. C3 complement protein is known to have a role in monocyte adhesion [75]. Monocytes and neutrophils have receptors for complement products (C3a, C4a, C5a) which leads to their activation [76,77]. These latter results related to the complement system were very interesting and provide an alternative perspective in regards to studying the reactivity of the blood cells in contact with the PU investigated here.

4. Conclusion

There is a need for establishing effective biomaterials in the area of medical cardiovascular devices, and this is driving the scientific community to improve the blood and tissue compatibility of these biomaterials. The strategy identified in this study focused on applying multifunctional thin film polyurethane coatings onto metal surfaces in order to neutralize its pro-thrombotic and inflammatory properties. This study highlighted that thin films of HHHI coated on CoCr circumvent the fibrin network formation associated with whole blood interactions on CoCr substrates, without the need for any anticoagulant adjuvants. In particular, the expression of TF by monocytes after contact with CoCr could be an important agent for the initiation of coagulation and this phenomenon should become a more mainstream consideration in the evaluation of other blood contacting biomaterials. In the current work this pathway is impaired by the HHHI coatings that reduce the pro-thrombotic and pro-inflammatory phenotype of monocytes and macrophages induced by CoCr.

Data availability

The raw data and the processed data required to reproduce these findings are available upon request to the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2019.119306.

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