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A culture model to analyze the acute biomaterial-dependent reaction of human primary neutrophils *in vitro*☆

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A B S T R A C T

Neutrophils play a pivotal role in orchestrating the immune system response to biomaterials, the onset and resolution of chronic inflammation, and macrophage polarization. However, the neutrophil response to biomaterials and the consequent impact on tissue engineering approaches is still scarcely understood. Here, we report an *in vitro* culture model that comprehensively describes the most important neutrophil functions in the light of tissue repair. We isolated human primary neutrophils from peripheral blood and exposed them to a panel of hard, soft, naturally- and synthetically-derived materials. The overall trend showed increased neutrophil survival on naturally derived constructs, together with higher oxidative burst, decreased myeloperoxidase and neutrophil elastase and decreased cytokine secretion compared to neutrophils on synthetic materials. The culture model is a step to better understand the immune modulation elicited by biomaterials. Further studies are needed to correlate the neutrophil response to tissue healing and to elucidate the mechanism triggering the cell response and their consequences in determining inflammation onset and resolution.

1. Introduction

Biomaterial-based implants are widespread in clinics, and subject to intensive research efforts in tissue engineering applications. Commercially available biomaterials are used in different clinical fields ranging from cardiovascular devices to orthopedic implants. At the time when the first biomaterials were introduced in the 50s and 60s, the emphasis was on replacing human tissues or their function without causing undesired reactions. Nowadays, biomaterials are used to repair diseased or damaged tissue by stimulating specific cellular responses at the level of molecular biology [1]. The goal of biomaterial-guided tissue healing is to design biomaterials that mobilize the body's endogenous cells and stimulate regenerative processes to drive functional healing. The biological activity of biomaterials is widely recognized, and biomaterial design parameters are functionally used to achieve successful repair. The development of biomaterials aimed at stimulating regenerative processes necessitates a thorough understanding of the biological responses to the implanted materials. The success of biomaterial-guided tissue repair closely relies on the wound healing response, which is driven by the body's innate immune system as biomaterials are initially recognized as a foreign body [2]. Today it is widely recognized that biomaterials can trigger a broad spectrum of responses, and even more importantly, there are design factors to modulate this response and foster functional tissue healing or regeneration [3]. This interaction between biomaterials and the immune system is being established as a mainstream subject in biomaterials research, and efforts dedicated to elucidating the biological mechanisms which determine the immune response to biomaterials are constantly increasing [4].

After implantation, biomaterials are coated with proteins that activate the innate immune system. The proteins that coat the materials mediate the subsequent steps and the interaction of the material with the host's immune system, particularly in attracting neutrophils [2]. Neutrophils are the most prevalent immune cell in the human blood, and the first cell to encounter a biomaterial upon implantation [5]. Neutrophils

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predominate the early inflammatory response as their numbers peak within the first 24–48 h after implantation [6]. Recruitment and differentiation of monocytes and macrophages occurs at a later stage [7]. In the field of biomaterials and tissue engineering, macrophages have attracted considerable attention, especially regarding their role in tissue healing [8]. However, the number of macrophages correlates with the levels of neutrophils present, indicating that neutrophils are important in the recruitment of macrophages and thus the consequent stages of the wound healing response [9]. Therefore, it is crucial to understand the role of neutrophils in tissue healing and their crosstalk with macrophages. In recent years, cancer research has shown that neutrophils are important in the progression and regression of tumors [10]. The occurrence of neutrophils driving disease progression is not limited to cancer. Neutrophils also play a role in myocardial infarction, systemic lupus erythematosus (SLE), rheumatoid arthritis, sepsis, psoriasis, human immunodeficiency virus (HIV) infection, asthma, antineutrophil cytoplasmic antibody associated vasculitis, malaria and Chagas' disease [11]. Furthermore, neutrophils have also been shown to be essential in bone fracture healing [12,13]. Recent reviews have acknowledged the importance of neutrophils in biomaterial-guided tissue regeneration to achieve successful repair [6,14,15]. These studies highlight the importance and potential of neutrophils for biomaterial-guided tissue regeneration.

Neutrophils unfold a spectrum of functions in response to biomaterial implantation. The first function is their capacity to survive on the material and eventually die via apoptosis. The apoptosis of neutrophils plays an important role in the transition from pro-inflammatory towards anti-inflammatory macrophages and thereby the resolution of inflammation [16]. The second function is the release of an oxidative burst upon activation. This oxidative burst consists of reactive oxygen species that are critical for killing invaders and degradation of internalized particles, but can also contribute to inflammatory damage of the tissue surrounding the implanted material [17]. The third function is the release of granules with cytokine release and formation of neutrophil extracellular traps (NETs). This function is associated with a strong pro-inflammatory activity that is important in pathogen killing, but when dysregulated it can cause tissue damage, fibrosis and prevent the neutrophils from exerting their function in the wound healing response [6]. NETs are web like structures that contain decondensed chromatin together with granular proteins such as myeloperoxidase (MPO) and neutrophil elastase. MPO and neutrophil elastase regulate the formation of NETs by digesting histones and promoting chromatin decondensation [18]. The fourth function is the production and secretion of cytokines, chemokines, and angiogenic/fibrogenic factors such as vascular endothelial growth factor (VEGF) and interleukin 8 (IL8). These factors play an important role in the foreign body response to the biomaterial; this response is crucial for the outcome of the tissue repair process [19]. Therefore the interaction between neutrophils and biomaterials is key to orchestrate inflammation at later phases. Despite the urgent need to obtain a comprehensive understanding of the neutrophil interaction with the biomaterial, there are few studies that have examined the effect of biomaterials on neutrophils *in vitro*. Chang and colleagues found that neutrophils' survival drops on rough compared to smooth surfaces [20]. Kaplan and colleagues reported that neutrophils respond with different levels of superoxide release in response to different biomaterials [21]. Szponder and colleagues showed that neutrophils release different levels of elastase on alginate biomaterials than on carbon fibers [22]. Velard and colleagues found that the addition of zinc to hyaluronic acid results in decreased levels of IL8 and matrix metallo-proteinase-9 (MMP9) [23]. All the aforementioned studies only focus on one specific neutrophil function. *In vitro* models that exhaustively characterize the neutrophil response to biomaterials are still missing.

We devised an *in vitro* model based on human primary neutrophils to enable comprehensive characterization of the acute neutrophil response to a biomaterial. To provide proof of principle that the model can discriminate the response of neutrophils to different materials, seven polymeric biomaterials were chosen including synthetic and naturally derived biomaterials as well as soft and hard materials to represent a range in physical and chemical properties. The materials investigated were: tyramine functionalized hyaluronic-acid (THA), THA mixed with collagen (THA-col), type I collagen (col), gelatin-methacryloyl (GelMA), polyvinyl alcohol (PVA), tissue culture plastic (TCP) and polycaprolactone (PCL).

We analyzed the activity and survival of neutrophils after seeding them on the biomaterials. The oxidative burst of neutrophils in response to the biomaterials was characterized by analyzing superoxide anion, the amounts of MPO and neutrophil elastase released from the granules, and a large panel of chemokines, cytokines, angiogenic, and fibrogenic factors via Olink®.

2. Materials and methods

2.1. Biomaterial synthesis and hydrogel formation

A panel of seven naturally derived and synthetic materials was selected as follows: tyramine functionalized hyaluronic-acid (THA), THA mixed with collagen (THA-col), type I collagen (col), gelatin-methacryloyl (GelMA), polyvinyl alcohol (PVA), tissue culture plastic (TCP) and polycaprolactone (PCL). Prior to use, the polymer powders were UV sterilized and all solutions were sterile filtered. The materials were coated (press fit in case of PCL) on the bottom of a cell culture well plate in the desired size for each experiment. The raw materials were processed in a setup to allow cell culture in different well plates. A similar volume (150 μl/cm^2) of precursor hydrogels was casted on the bottom of a well plate using a positive displacement pipette (CP1000/CP250; Gilson, Middleton, USA) and polymerized. After casting/fitting all materials into the wells, the materials were washed with PBS 3x before seeding the neutrophils.

THA was synthesized as previously described [24]. In short, sodium hyaluronate (280–290 kDa, 5 mM carboxylic groups) (Contipro Biotech S.R.O, Dolni Dobrouc, Czech Republic) was dissolved in deionized H_2O (1% w/v) and functionalized with tyramine (Sigma-Aldrich, Buchs, Switzerland) via 4-(4,6-dimethoxy-1,3,55-triazin-2-yl)-4-mehtylmorpholinium chloride (DMTMM; TCI Europe, Zwijndrecht, Belgium) amidation by mixing at a stoichiometric ratio of 1:1:1. Functionalization was performed under mild stirring for 24 h at 37 °C. THA was precipitated by adding 16% v/v of saturated NaCl and 96% ethanol dropwise, isolated using Gooch filter no. 2, washed, and dried under vacuum. The amidation reaction was repeated to increase the degree of substitution (DoS) to 11.3% as determined by absorbance reading at 275 nm Multiskan™ GO Microplate Spectrophotometer (ThermoFisher, Waltham, USA). For each experiment the THA conjugate was reconstituted in phosphate buffered saline (PBS) at 2.5% (w/v) containing 0.6 U/ml HRP (Sigma-Aldrich) and rotated overnight at 4 °C. To initiate hydrogel formation, 1.3 mM hydrogen peroxide (H_2O_2, Sigma-Aldrich) was added and immediately mixed to provide a homogeneous distribution. The material was casted into the well and incubated at 37 °C, 5% CO_2 for 10 min to allow enzymatic gelation.

Collagen type I derived from rat tail was bought from Corning (Collagen I rat tail in 0.2 N acetic acid, 10.98 mg/ml). Collagen type I hydrogels were prepared on ice by neutralizing the collagen I solution with a mixture of 10x PBS, dH_2O and 1 N sodium hydroxide (NaOH) to a final concentration of 5 mg/ml. The hydrogel precursors were incubated at 37 °C, 5% CO_2 for 30 min until a firm gel was formed.

THA-col composites were prepared by mixing the previously described mixtures of THA and collagen in a 1:1 ratio for a final concentration of 2.5 mg/ml collagen and 2.5% (w/v) THA. Enzymatic gelation and neutralization of collagen was initiated simultaneously.

GelMA (type A, 180 g Bloom, Fluka) was synthesized by dissolving gelatin (10% w/v) in phosphate buffered saline (PBS, pH = 7.4) at 60 °C [25]. After dissolving, 0.28% methacrylic anhydride was added to the dissolved gelatin and mixed with the solution. To stop the reaction the mixture was diluted with PBS and transferred in a dialysis membrane

(12–14 kDa) to remove unreacted methacrylic anhydride and other side products. After dialysis was complete, ultrapure H_2O was added to the dialyzed solution and subsequently freeze-dried for storage. The DoS of the final GelMA was 50% as determined via NMR analysis. The GelMA macromer 15% (w/v) was fabricated into hydrogels by UV crosslinking after mixing with 0.5% 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpro piophenone (Irgacure 2959) (Sigma-Aldrich) in PBS.

PVA pellets with a molecular weight (MW) of 70,000 Da were dissolved at 90 °C in H_2O (5% w/v) under stirring until a clear solution was obtained. The PVA precursor hydrogel solution was casted in a circular well and frozen at −20 °C. To fabricate PVA hydrogels, the solution underwent 7 freeze thawing cycles ensuring complete freezing at −20 °C followed by thawing at room temperature.

PCL discs were printed in the respective diameter of the used well plate after melting PCL pellets with a MW of 45,000 Da (Sigma-Aldrich) and press fitted onto the bottom of a well plate ensuring a flat surface. An extrusion-based 3D printer RegenHu 3D Discovery (RegenHU, Villaz-Saint-Pierre, Switzerland) was used with the following parameters: inner diameter nozzle 300 μm, 1 bar, 16 revs/m, writing speed 6 mm/s, temperature tank 75 °C, temperature printer head 70 °C.

Tissue culture treated polystyrene plates (TCP) were bought from Corning.

2.2. Endotoxin analysis

The endotoxin content of each biomaterial was measured using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo-Fisher) according to the manufacturer's instructions. All polymers were diluted with endotoxin free H_2O (Sigma-Aldrich) at the polymer concentration matching those used in the neutrophil experiments. Due to high viscosity, THA and GelMA hydrogels were cast (250 μl) into a 24 well plate. All materials were incubated with endotoxin free H_2O (1 ml) for 1 h. An endotoxin standard stock solution was prepared by adding endotoxin free H_2O (Sigma-Aldrich) to the *E. coli* endotoxin standard vial to create a high standard (0.1–1.0 EU/ml) and a low standard (0.01–0.1 EU/ml). Samples harvested from THA and GelMA were diluted prior to the assay in endotoxin free water and transferred together with the standard curve into the pre-warmed plate at 37 °C. The supernatant from all other materials was directly transferred to the pre-warmed plate together with the standard curve. The amoebocyte lysate reagent was added to each well and the plates were incubated for 8 min at 37 °C. The reaction of the chromogenic substrate solution was stopped after 6 min at 37 °C by adding 25% (v/v) acetic acid to each well and the optical density was measured at 405 nm using the Infinite® 200 Pro plate reader (Tecan, Männedorf, Switzerland). The obtained values were used to calculate the concentration based on the provided standard curve for the aforementioned concentration ranges. The correlation coefficients for the standard curves were between 0.9922 and 0.9953.

2.3. Rheological characterization biomaterials

To assess the viscoelastic properties of all hydrogels, rheological measurements at 20 °C using the Anton-Paar MCR-302 rheometer equipped with 1° cone-plate geometry and a gap distance of 0.049 mm were performed (n = 4 replicates per material). Silicone oil (Sigma-Aldrich) was applied to the external border to prevent drying during the measurement. The amplitude sweep test (frequency = 1 Hz, amplitude = 0.01–100% strain) was performed to characterize the elastic storage modulus (G') and viscous loss modulus (G") of all hydrogel materials. The moduli were measured at a strain of 1%, which was within the linear viscoelastic region of each hydrogel.

2.4. Isolation and culture of human primary neutrophils

Peripheral blood was collected from 5 human healthy volunteers, 3 males and 2 females, average age 26 years, age range 24–29 years donating 24 mL of venous whole blood after written informed consent was obtained. Ethical approval was granted by the cantonal ethical committee (BASEC-Nr. 2019–02353). The blood was collected by a venipuncture into potassium EDTA-coated containers (S-Monovette®, Sarstedt, Nümbrecht, Germany). Neutrophils were isolated from whole blood by immunomagnetic negative selection using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies #19666, Vancouver, Canada) following the manufacturer's instructions. After the last separation step, the enriched suspension containing neutrophils was pipetted into a new tube, centrifuged at 300 g for 5 min and resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium [26] with HEPES (Gibco, ThermoFisher, Waltham, USA) supplemented with 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) to a concentration of 1×10^6 cells/ml. Neutrophils were counted using a hemocytometer and viability was confirmed to be >99% with trypan blue.

For evaluating purity after negative selection, neutrophils were resuspended (1×10^6 cells) in 2 mL of 0.5% (w/v) bovine serum albumin (BSA) containing phosphate buffered saline (1X PBS) subsequent to isolation. Following the centrifugation step at 300 g for 5 min, cells were incubated with 5 μL of Fc Receptor Blocking Solution (BioLegend, USA) in 100 μL 0.5% (w/v) BSA-PBS for 15 min. The cells were stained with anti-human CD 16-APC (10 μL/1×10^6 cells) (R&D Systems, Biotechne, USA), FITC mouse anti-human CD66b (20 μL/1×10^6 cells) (BD Biosciences, USA) and PE mouse anti-human CD14 (20 μL/1×10^6 cells) (BD Biosciences, USA) antibodies for 40 min. After washing cells with 0.5% (w/v) BSA-PBS and centrifugation (300 g for 5 min), cells were analyzed to measure the intensity of CD markers via BD FACSAria™ III cytometer (BD Life Sciences, USA).

After isolation, neutrophils were seeded in complete RPMI-1640 medium on top of the materials occupying the well in a density of 1×10^6 cells/ml equal to 263,000 cells/cm^2. All plates were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. At each culture timepoint the medium was harvested from the well, centrifuged for 8 min at 300 g and stored at −80 °C until further use. Unconditioned control medium was generated by the incubation of complete RPMI-1640 medium and processed in the same way as earlier described. A complete overview of all the assays and harvesting timepoints is shown in Fig. 1. The experiment was carried out in 5 independent experiments (n = 5 donors) with two replicates each.

2.5. Metabolic activity of neutrophils

The metabolic activity, an indicator of cell viability, was measured using the resazurin-based CellTiter-Blue® Cell Viability Assay (Promega, Dübendorf, Switzerland). The CellTiter-Blue® Reagent was added at a concentration of 16.7% in RPMI-1640 medium to each sample 30 min prior to each time point (1, 3, 5, 7 and 24 h). The Fluorescence intensity was measured immediately after transferring the media to a 96 well plate using the Infinite® 200 Pro plate reader (Tecan) at an excitation wavelength (λ_{ex}) of 560 nm and emission wavelength (λ_{em}) of 590–610 nm. The final fluorescence value for each sample was calculated after subtracting the average fluorescence values of the culture medium background.

2.6. LDH release

Lactate dehydrogenase (LDH) is rapidly released into the cell culture supernatant in dying cells when the plasma membrane is damaged. The LDH release was measured using the Cytotoxicity Detection Kit^{PLUS} LDH (Roche, Sigma-Aldrich) according to the manufacturer's instructions. The low control value was determined by medium from uncultured cells at the timepoint t = 0 h and the high control value was determined by medium from cultured cells at each timepoint by adding 9% Triton X-100 15 min prior to harvest. Unconditioned control medium served as a background control. The absorbance value of the samples was measured

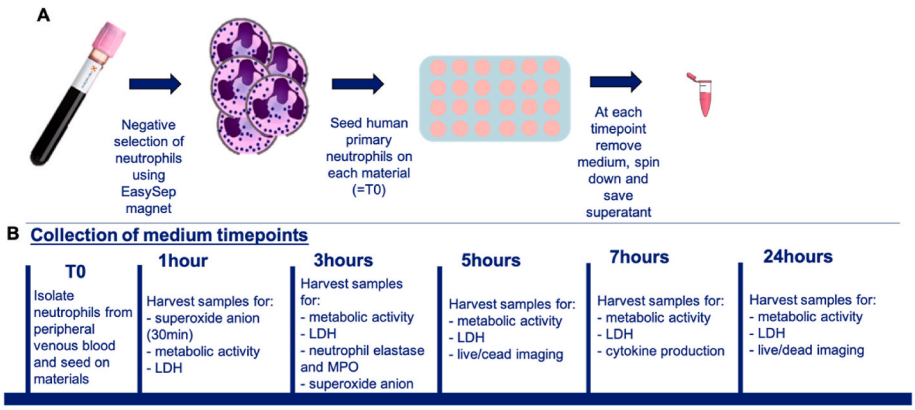

Fig. 1. Summary of the assays performed. A) Schematic experimental outline from drawing peripheral blood to collection of media from each material. B) Timeline of the assays that were carried out at each time points (n = 5 donors, n = 2 replicates).

at 490 nm using the Infinite® 200 Pro plate reader (Tecan). The percentage of cytotoxicity was calculated according to the following formula: cytotoxicity (%) $= \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}}$ x 100.

2.7. Confocal microscopy based live/dead assay

Neutrophils were stained after 5 and 24 h with 2 μM Calcein AM (Sigma-Aldrich) and 10 μM ethidium homodimer-1 (Sigma-Aldrich) in culture medium for 30 min at 37 °C and imaged using the LSM 800 confocal microscope (Zeiss, Oberkochen, Germany). Images were taken at 5 fields per sample to represent the top, lower, central, right and left zone of the material. For each image the alive and dead cells were counted using Image-J software. The percentage of living cells was calculated by the number of alive cells divided by the total number of cells per sample.

2.8. Neutrophils oxidative burst

Superoxide anion production was determined from the rate of cytochrome C reduction, measured using the Cytochrome C Assay Kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Neutrophils were seeded on the materials and cytochrome C was added. After 30 min and 3 h the medium was centrifuged, and the supernatant was transferred to a 96-well plate and the absorbance value was measured at 550 nm using the Infinite® 200 Pro plate reader (Tecan). Unconditioned, cell free control medium supplemented with cytochrome C served as a background control. After subtracting the background values, superoxide anion production was calculated with the use of the extinction coefficient of 21 mM^{-1} cm^{-1} for cytochrome *c*.

2.9. MPO and neutrophil elastase

Medium that was conditioned for 3 h by the neutrophils seeded on different materials was used to determine MPO and neutrophil elastase. The MPO content was measured using the Meso Scale Discovery (MSD) human myeloperoxidase kit and the MESO QuickPLex SQ 120 (MSD, Kenilworth, USA) according to the manufacturer's instructions. The samples were diluted to allow detection of MPO within the linear range of the standard curve (33–50,000 pg/ml). The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations using interpolation from the standard curve. Neutrophil elastase was measured using the human neutrophil elastase/ELA2 DuoSet ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions of the kit. The absorbance value of each sample was measured at 450 nm using the Infinite® 200 Pro plate reader (Tecan) and subtracted by the absorbance value at 570 nm. The samples were diluted to allow detection of neutrophil elastase within the linear range of the standard curve (47–3000 pg/ml).

2.10. Quantification of neutrophil cytokine secretion

A panel of proteins associated with inflammation was measured in the media supernatant using the Olink® inflammation multiplex immunoassay (Olink® Proteomics, Uppsala, Sweden). This panel covers 92 established and validated inflammation related markers including pro- and anti-inflammatory cytokines, chemokines, growth factors, receptors and factors involved in acute inflammatory and immune responses. The Olink® immunoassay panel is based on the proximity extension assay technology that include oligonucleotide-labeled antibody probe pairs that can bind to their respective protein targets in the sample and can be detected and quantified using standard real-time PCR. Data of the 7 h timepoint is presented as normalized protein expression values (NPX) which are comparable in their distribution to log2-transformed protein concentrations. The full description of the inflammatory markers is given in Supplementary Material Table S1. The data analysis was done using the Olink®'s Normalized Protein eXpression Manager software. In this Olink® inflammation panel matrix metallopeptidase 9 (MMP9), an important regulatory factor in neutrophil migration, angiogenesis and wound repair [27], was not present. For this reason, MMP9 was quantified in samples of the 7 h timepoint by the human MMP9 DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. The absorbance value of each sample was measured at 450 nm using the Infinite® 200 Pro plate reader (Tecan) and subtracted by the absorbance value at 570 nm. The samples were diluted to allow detection of neutrophil elastase within the linear range of the standard curve (31–2000 pg/ml).

2.11. Statistical analysis

All samples were measured in technical duplicates per each donor (n = 10 samples of 5 independent donors in total), group and timepoint. Statistical analysis was performed with Graph Pad Prism (Prism 9, Graphpad Software, San Diego, USA). A one-way analysis of variation (ANOVA) with a post hoc Tukey honestly significant difference analysis was performed to analyze differences between materials and to correct for multiple testing. In case of large donor variability and gaussian distribution of the data, a linear mixed model (LMM) was used. For the LMM, the different materials were considered a fixed parameter and the donors (experiments) as a random factor. For the Olink® data, the Olink® Insights Stat Analysis app was used to analyze differences between groups within a protein. All statistical analysis included two tailed tests. A *P* value of <0.05 was considered as statistically significant. In the manuscript statistical differences are indicated as follows: a = statistically significant to THA, b = statistically significant to THA-collagen, c

= statistically significant to collagen, d = statistically significant to GelMA, e = statistically significant to PVA, f = statistically significant to TCP, g = statistically significant to PCL. All graphs with the exact *P* values are shown in the supplementary materials to allow for better visualization of the graphs in the main paper.

3. Results

3.1. Materials characterization

Bacterial endotoxins or lipopolysaccharides (LPS) are cell wall constituents of gram negative bacteria which can induce a strong immune response [28]. Therefore, materials were characterized for their endotoxin levels. For collagen type I, PVA, PCL and TCP endotoxins were on the lower detection limit (Fig. 2B). The endotoxin levels of THA and GelMA were significantly higher than the other materials ($P < 0.001$), but still relatively low (Fig. 2B). The other reagents used for the crosslinking displayed values below the lower limit of detection (data not shown). Concentrations of polymers and crosslinkers were selected to achieve a similar shear storage modulus for all hydrogels except collagen, as measured by rheological amplitude sweep. THA, THA-col, GelMA and PVA showed similar shear storage moduli around 4 kPa (Fig. 2C). The storage modulus of collagen hydrogel was significantly lower than the moduli of all other hydrogels ($P < 0.001$, Fig. 2C).

3.2. Purity, metabolic activity and survival of neutrophils cultured on different biomaterials

Neutrophil purity after negative selection was evaluated via cytometry by assessing the presence of cell surface markers CD66b and CD16 and the absence of CD14 [29]. The isolated neutrophils were characterized by 99.7% CD66b, 99% CD16 and 0.2% CD14, confirming a high purity, as expected from immunomagnetic selection (Fig. 3A). Neutrophil survival and metabolic activity on different materials was evaluated with cell-titer blue assay, LDH and calcein AM/ethidium homodimer staining. Overall, neutrophils showed the highest level of metabolic activity after 1 h with a gradual decrease over time. After 24 h, most neutrophils were no longer metabolically active independent of the material. Clear differences between materials were observed. Neutrophils cultured on TCP and PCL were significantly more metabolically active after 1 and 3 h than neutrophils cultured on other materials. The difference in activity diminished after 5 h of culture (Fig. 3B, Suppl. Figure 1A). This indicates that neutrophils cultured on hard hydrophobic surfaces have a different activity than neutrophils cultured on softer hydrated substrates.

A similar trend was seen for release of LDH in the medium. After 1 h, LDH was only released by neutrophils cultured on PCL, which was approximately 20% of the maximum amount (Triton x-100 treated high control) and significantly higher than from neutrophils cultured on other materials. The LDH release increased over time. After 24 h neutrophils cultured on synthetic materials PVA, TCP and PCL released significantly more LDH than neutrophils that were cultured on naturally derived materials like THA, THA-collagen, collagen and GelMA, that released almost no LDH up to 24 h (Fig. 3C, Suppl. Figure 1B).

To visualize the survival of the neutrophils cultured on different materials we performed a live and dead assay, with representative images after 5 and 24 h shown in Fig. 3D. This assay also showed clear differences between materials. After 5 h, 44% of the neutrophils cultured on PCL and 88% of the neutrophils on TCP were viable. This was significantly lower than neutrophils cultured on the other hydrogel materials with a viability of more than 96% after 5 h (Fig. 3E, Suppl. Figure 1C). After 24 h, more than 98% of the neutrophils cultured on THA, collagen, and THA-collagen were still viable. Neutrophils were significantly less viable on GelMA, PVA, TCP and PCL ranging from 30 to 75% (Fig. 3F, Suppl. Figure 1D).

3.3. Release of oxidative burst by means of reactive oxygen species production

To better understand the effect of biomaterials on the ROS production by neutrophils, we measured the amount of superoxide anion released in the culture media. Significantly more superoxide anion was produced by neutrophils cultured on THA, THA-collagen, collagen, GelMA than by neutrophils cultured on PVA, TCP, PCL after 30 min (Fig. 4A, Suppl. Figure 2A). After 3 h, the superoxide anion production increased in all groups. The level of superoxide anion was significantly higher for all naturally derived materials (THA, THA-collagen, collagen, GelMA) than for all synthetic (PVA, TCP, PCL) materials (Fig. 4B, Suppl.

A

Material	Source	Mw (kDa)	w/v (%)	d.o.s.	Crosslinking
THA	cell wall of bacteria streptococcus zooepidemicus	280-290	2.5	11.3	0.6 U/ml HRP 1.3mM H2O2
THA-collagen	see THA and collagen	n/a	THA 2.5 col 0.25	n/a,	see THA and collagen
collagen type I	rat tail	n/a	0.5	n/a	10x PBS, dH2O 1N NaOH
GelMA	porcine skin	40	15	50	0.5% irgacure
PVA	synthetic	70 000	5		7 freeze thaw cycles
TCP	n/a	n/a	n/a	n/a	n/a
PCL	synthetic	45 000	n/a	n/a	n/a

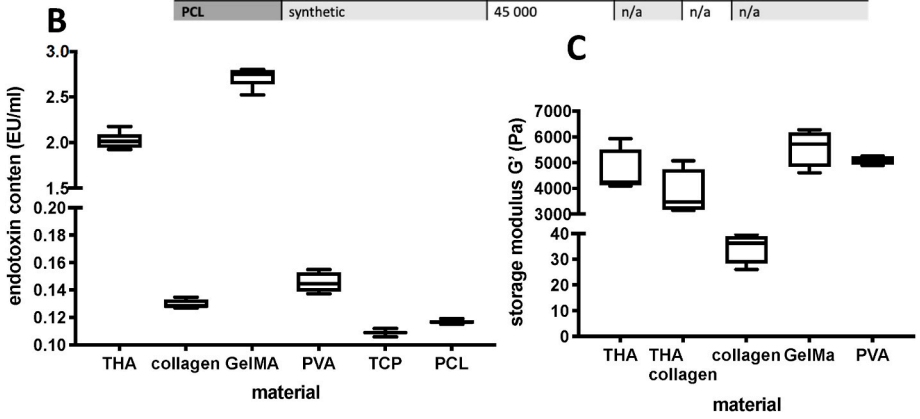

Fig. 2. Composition and characterization of the biomaterials
A) Summary of the characteristics of the materials used in this study, including the source, molecular weight, polymer concentration, degree of substitution, and materials used for crosslinking. B) Endotoxin content of different materials (n = 2–6) with endotoxins present between 2 and 3 EU/mL in THA and GelMA. C) Storage moduli of different hydrogels (n = 4) ranging between 4-5k Pa for THA, THA-collagen, GelMA and PVA and 35 Pa for collagen. Box plots represent the 25th and 75th percentile with the median, the whiskers indicate the maximum and minimum. Abbreviations: Mw, molecular weight; kDa, kilo Dalton; w/v, weight per volume; d.o.s., degree of substitution.

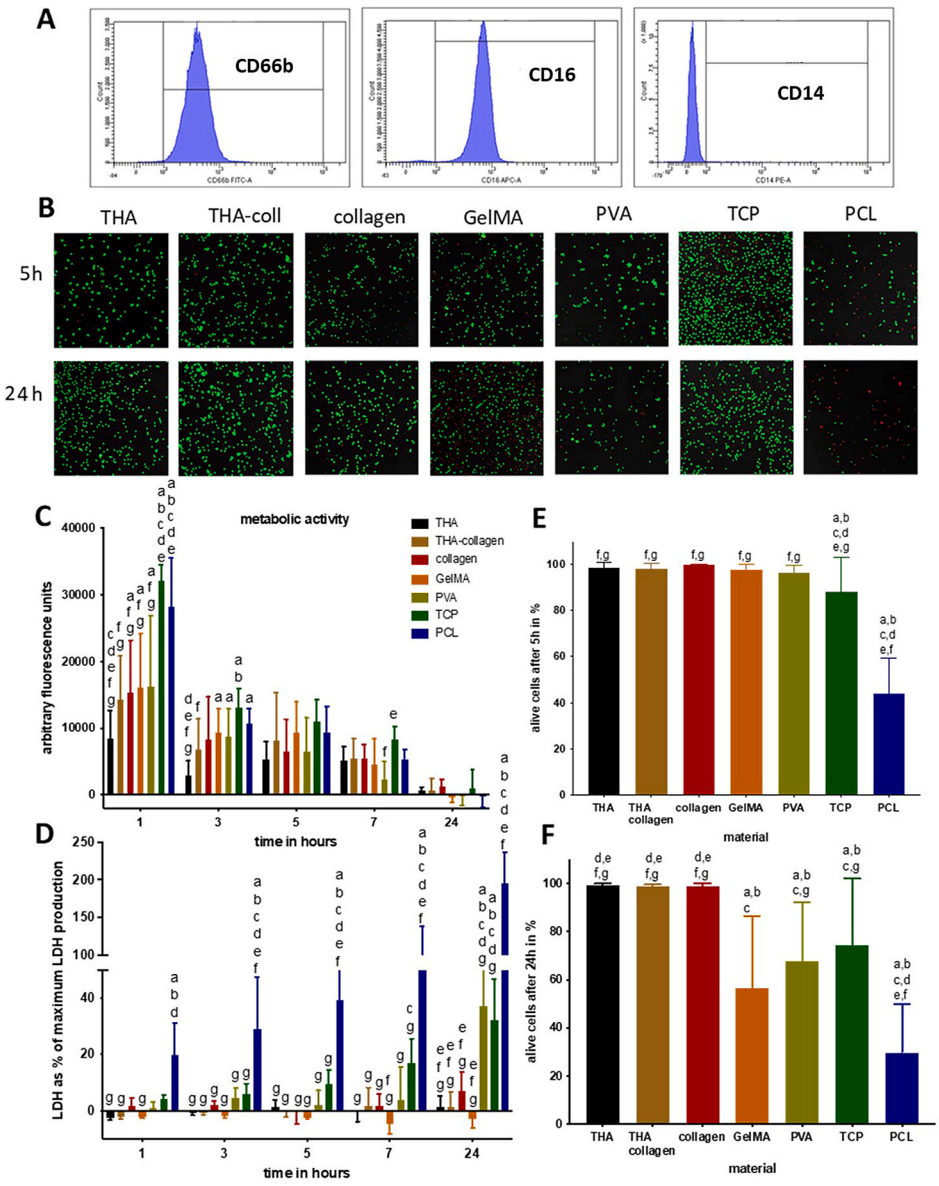

Fig. 3. Neutrophil purity assessment, activity and cell survival on different material.
A) Flow cytometry histograms showing CD66b and CD16, and CD14 expression on neutrophils. B) Representative images of live/dead staining after 5 and 24 h showing living cells in green with dead cells in red. C) Metabolic activity of neutrophils measured via cell titer blue assay and expressed as fluorescence units over time for each material. D) LDH release by neutrophils cultured on different materials expressed as a percentage of the maximum amount of LDH release after cell lysis. E) The percentage of living cells after 5 h. F) The percentage of alive cells after 24 h. Each bar represents the mean of 5 donors + SD. Abbreviations: LDH, lactate dehydrogenase. a = statistically significant to THA, b = statistically significant to THA-col, c = statistically significant to col, d = statistically significant to GelMA, e = statistically significant to PVA, f = statistically significant to TCP, g = statistically significant to PCL.

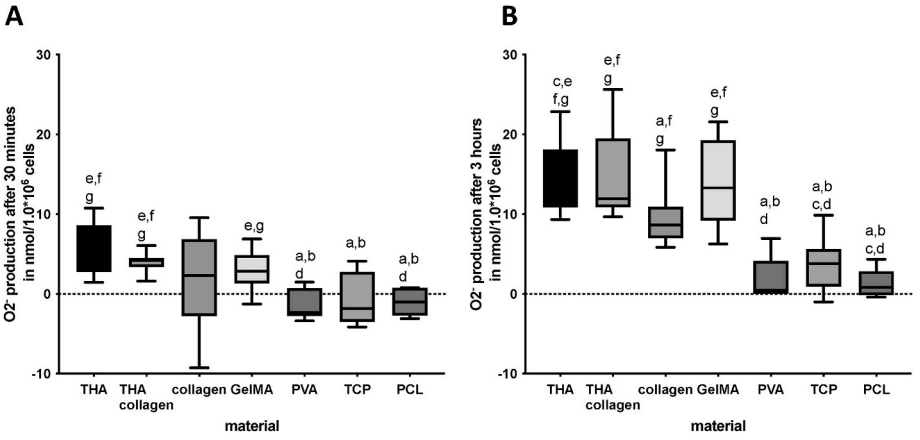

Fig. 4. Superoxide anion production by neutrophils cultured on different materials
Superoxide anion production after A) 30 min and B) 3 h of culture. Box plots represent the 25th and 75th percentile with the median, the whiskers indicate the maximum and minimum. a = statistically significant to THA, b = statistically significant to THA-col, c = statistically significant to col, d = statistically significant to GelMA, e = statistically significant to PVA, f = statistically significant to TCP, g = statistically significant to PCL.

Figure 2B) at both time points.

3.4. Neutrophil elastase and MPO release induced by biomaterials

Neutrophils cultured on PVA and PCL released significantly more MPO and elastase than neutrophils cultured on THA, THA-collagen, collagen and GelMA (Fig. 5). Neutrophils cultured on TCP displayed the same trend, albeit non-significant, possibly due to the high variability between donors (Fig. 5, Suppl. Figure 3).

3.5. Cytokine secretion by neutrophils cultured on different biomaterials

To further understand the response of neutrophils to different biomaterials, proteins which are differentially regulated can be identified. We used the Olink® technique to analyze a panel of 92 cytokines, chemokines, growth factors and receptors all being related to inflammation (Fig. 6). In total, 19 proteins were detected in more than 75% of the samples (Suppl. Table 2). Further analysis showed that most proteins are related to biological processes such as cellular response to cytokine stimulus, chemotaxis, inflammatory response, cell adhesion and secretion (Fig. 6).

In a second step we specifically investigated the top-6 most abundant proteins released by the neutrophils: vascular endothelial growth factor A (VEGF-A), hepatocyte growth factor (HGF) urokinase-type plasminogen activator (uPA), extracellular newly identified receptor for advanced glycation end-products binding protein (EN-RAGE), oncostatin M (OSM), and interleukin 8 (IL-8) (Fig. 6C–H, Suppl. Figure 4). Neutrophils cultured on PCL and PVA secreted significantly more of the selected proteins than neutrophils that were cultured on the naturally derived materials THA, THA-col, collagen and GelMA. Interestingly, neutrophils cultured on THA tended to secrete less than the neutrophils cultured on other natural materials, reaching statistical significance for OSM. Secretion of IL-8 was not different between neutrophils cultured on the different materials. Since the Olink® panel did not include the important neutrophil regulator MMP9, we separately analyzed this enzyme by ELISA. The secretion of MMP9 increased 5-17-fold depending on the material where the neutrophils were cultured, with an overall higher secretion in PVA, TCP and PCL (Suppl. Figure 5).

4. Discussion

Although neutrophils are the most prevalent immune cells in the human body and the first responders to invaders, their role in triggering and resolving inflammation after biomaterial implantation is still scarcely understood. A first step in unraveling these mechanisms, consists of introducing methods to characterize the interaction of neutrophils with biomaterials. Therefore, we devised an *in vitro* model describing multiple functions of human primary neutrophils and investigated their variation after exposure to biomaterials.

To our knowledge, this is the first *in vitro* study comprehensively evaluating early neutrophil functions in response to a biomaterial. We selected a broad panel of assays to represent different neutrophil functions relevant for tissue repair [1]: survival (live-dead staining, metabolic activity, LDH release) [2], release of oxidative burst (ROS production) [3], MPO and elastase release and [4] the release of granules and inflammation related proteins (Olink®). Each of these assays could distinguish specific differences in the response of neutrophils between biomaterials.

The proposed approach analyzing multiple neutrophil functions is of benefit towards understanding the possible consequences of biomaterial properties for tissue repair. Based on our results, metabolic activity of neutrophils was highest in PCL and TCP after 1 and 3 h, in conjunction with elevated levels of LDH release in PCL, TCP and PVA and lower cell viability after 24 h. Serum was not added to cell culture media to prevent possible effects of serum proteins on neutrophil activation, that could mask the specific biomaterials effect. The role of serum in influencing the interaction between neutrophils and biomaterials is a crucial point requiring further investigation. Previous studies investigated the effects of physicochemical properties like size, shape, surface topography, wettability and surface charge on neutrophils, suggesting that these features might play a role in neutrophil survival and activation [14]. The low viability of neutrophils on PCL might be due to low adhesion on the hydrophobic surface because of the serum-free conditions, implying lower levels of protein coating on all substrates. Interestingly, in presence of serum hydrophobic surfaces are prone to marked protein absorption, fostering survival or activation of neutrophils [14,30]. Although TCP is also intrinsically hydrophobic, plates for tissue culture are surface treated to increase hydrophilicity and thereby cell adhesion. Unlike for other cell types, identifying a standard substrate for neutrophil cell culture to study their interaction with materials is not straightforward, because every material will inevitably influence neutrophils activation. Consequently, our experiments and data analysis compared the response on different materials rather than against a chosen standard. After 24 h the survival of neutrophils on a hydrophilic PVA (hydrogel) surface was lower than on hydrogels consisting of naturally derived macromolecules with matched mechanical properties. Therefore, chemical composition of materials also plays a role in neutrophil survival. This difference in response is further supported by the elevated levels of LDH that were found in the supernatant of neutrophils cultured on the aforementioned synthetic materials. After 24 h, LDH levels were significantly increased indicating higher levels of cell membrane damage or cell death for neutrophils cultured on synthetic materials in comparison to naturally derived materials. The high levels of LDH in PCL, PVA and TCP indicate that the prevalent form of cell death is necrosis rather than apoptosis or other types of programmed cell death. In apoptosis the cell-membrane is still intact in contrast with necrosis where a damaged cell-membrane results in the release of LDH [31]. Whether a cell will undergo necrosis, or the more regulated

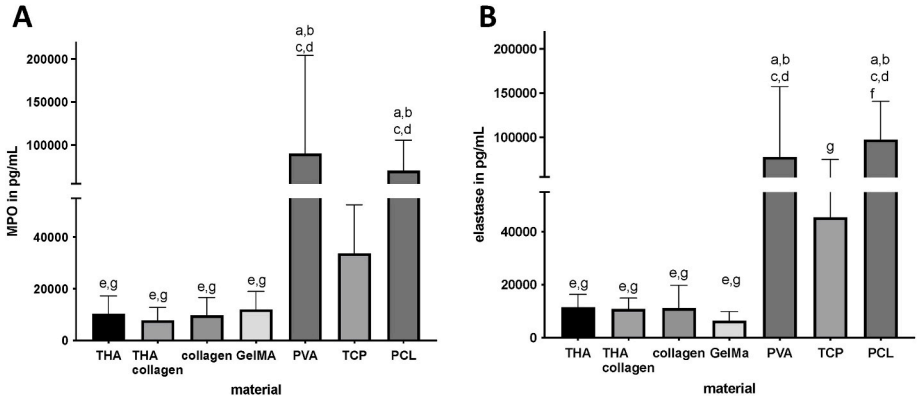

Fig. 5. Release of MPO and neutrophil elastase on different materials
The release of A) MPO in pg/ml and B) neutrophil elastase in pg/ml by neutrophils cultured on different materials. Each bar represents the mean level + SD. Abbreviations: MPO, myeloperoxidase. a = statistically significant to THA, b = statistically significant to THA-col, c = statistically significant to col, d = statistically significant to GelMA, e = statistically significant to PVA, f = statistically significant to TCP, g = statistically significant to PCL.

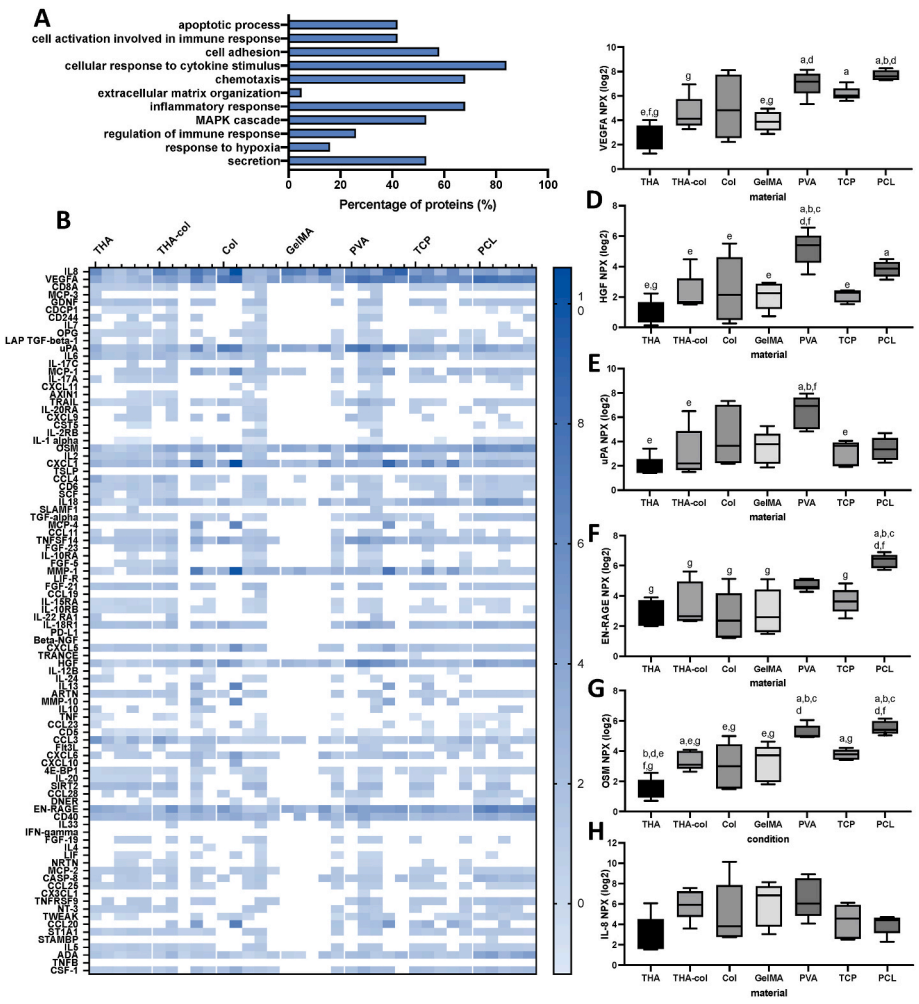

Fig. 6. Analysis of cytokine secretion by neutrophils using the Olink® technology.
A) Heatmap of 92 inflammation related proteins that were quantified in NPX on a Log2 scale. Each column within a material represents one donor (5 Donors). The darker the color the higher the protein level. B) Biological analysis to which biological process the detected proteins contribute. C–H) Box plots showing the level of C) VEGF-A, D) HGF, E) uPA, F) EN-RAGE, G) OSM, and H) IL-8. Box plots represent the 25th and 75th percentile with the median, the whiskers indicate the maximum and minimum. a = statistically significant to THA, b = statistically significant to THA-col, c = statistically significant to col, d = statistically significant to GelMA, e = statistically significant to PVA, f = statistically significant to TCP, g = statistically significant to PCL.

apoptosis depends on what stimulus the neutrophil receives [32,33]. Neutrophil death by necrosis is usually a trigger for inflammation and a major cause of tissue damage [33,34]. This may fuel an unwanted inflammatory response after the implantation of a biomaterial. After 24 h on the natural materials, almost all neutrophils were alive and released little LDH indicating that they might be in an activated but still living state.

The present study was limited to investigating the early response to biomaterials because, *in vivo*, at later time points, neutrophils recruit and activate additional leukocytes, such as macrophages by releasing pro-inflammatory mediators and thereby promote tissue repair [35]. Therefore, neutrophils that are cultured on substrates from naturally derived materials might still be secreting factors to attract cells needed for the repair to the material and thereby eventually promote tissue repair. The aforementioned examples highlight the importance to compare different assays related to viability, only the entire panel of assays will allow to understand the neutrophil response over time.

Once activated, neutrophils do not necessarily directly undergo cell death. Instead, they may respond with other effector functions such as cytokine release, degranulation and pathogen destruction by the release of ROS [35]. ROS production, i.e. oxidative burst, is an important and powerful mechanism in the defense against bacterial and fungal infections [36]. Besides their role in antimicrobial clearing, ROS are also important in the regulation of inflammation [37]. The oxidative burst released by neutrophils can also help in the degradation of particles previously internalized. In our model, we observed clear differences between materials in the production of superoxide anion, a prominent specie within the ROS family. Neutrophils seeded on the naturally derived materials showed higher ROS secretion compared to synthetic ones, especially after 3 h. If the oxidative burst is sustained for an extended period of time, it can also contribute to inflammatory damage of the tissue surrounding the implanted material [17]. It has also been shown that ROS production leads to neutrophil apoptosis, indicating that neutrophils releasing high amounts of ROS might undergo this form of programmed cell-death [38]. Neutrophil death by apoptosis in turn is critical for the resolution of inflammation by the polarization of pro-inflammatory macrophages into anti-inflammatory macrophages [14,39]. This process is essential for the final outcome of tissue healing and biomaterial integration. Future studies should determine whether the high levels of ROS produced by neutrophils on natural materials indeed lead to this change in polarization state. One limitation of our study is that superoxide anion is only one of several ROS, and future studies should investigate a broader spectrum of ROS production. Given the inherent ROS instability and the lack of off-the-shelf assays, this might prove non-trivial.

Besides cell survival and producing an oxidative burst, neutrophils can release NETs in response to a variety of stimuli that can activate them. NETs are composed of a network of chromatin fibers that are covered with proteins such as neutrophil elastase and myeloperoxidase (MPO) [40]. NET formation is one of the most interesting and unique aspects of neutrophil behavior. In our setup, neutrophils that were cultured on the synthetic materials PCL and PVA secreted 8–10 times higher levels of both MPO and neutrophil elastase. This might be indicative of increased neutrophils activation and NET formation on synthetic in comparison to natural materials, although other techniques such as direct imaging of the genetic material should be carried out to

confirm NETosis. This finding is in line with a recent study that reported that coating PCL fibers with a HA based hydrogel alleviated the neutrophil elastase response compared to PCL fibers without coating [41]. At the same time, this highlights the potential to modulate the neutrophil response by selection of the biomaterials, therefore, it is important to understand the consequences of the response to different immune cells to biomaterials before we can actively modulate the neutrophil response. Besides being important as an antimicrobial defense, neutrophil elastase also acts as a protease and can cleave components of the extracellular matrix such as elastin [42]. High levels of neutrophil elastase can lead to degradation of the extracellular matrix of tissues surrounding the biomaterial and thereby compromise the integration and final outcome when a biomaterial is used. Next to neutrophil elastase MPO is also an important molecule involved in the formation of NETS. MPO is known as an important neutrophil attractant [43]. This means that MPO secretion attracts more neutrophils to the implant site eventually leading to chronic inflammation that might impair tissue repair or stimulate the foreign body reaction. Furthermore, MPO has been demonstrated to be involved in tissue damage, and as such it plays a role in many inflammatory diseases [44]. For this reason, MPO is even considered as a new biomarker of inflammation in chronic inflammatory diseases like rheumatoid arthritis and ulcerative colitis [45]. Regarding the synthetic materials that demonstrated a higher release of MPO in our study, this could mean more inflammation and eventually even a risk of tissue damage at the implantation site. Given the clear difference between the two classes of materials in the neutrophil elastase and MPO response, these assays can be used to distinguish between the response a biomaterial elicits and eventually be helpful in choosing the biomaterial with the preferred immune response.

Neutrophils are increasingly acknowledged for their role in the regulation and development of inflammatory and immune responses. Besides the release of an oxidative burst, release of enzymes and the formation of NETs, neutrophils also secrete a large variety of cytokines and play a significant role in the development of inflammatory diseases and the foreign body reaction [19]. It was, however, unclear if all these cytokines would also be involved in the response of neutrophils exposed to different biomaterials. For this reason, we screened a panel of 92 cytokines related to inflammation. In total, we found 19 cytokines to be secreted by neutrophils that were cultured on different materials. Amongst them common neutrophil cytokines such as VEGF-A, MMP9 and IL-8, but also less commonly studied proteins such as HGF, EN-RAGE, and uPA. Interestingly, further cytokine analysis showed a distinctive response between neutrophils cultured on synthetic materials and on natural materials. Four out of the six cytokines that were overall highest expressed in our set-up follow the trend in neutrophil response discussed before. Neutrophils on synthetic materials secrete higher amounts of VEGF-A, HGF, EN-RAGE, OSM compared to natural materials. The higher release of cytokines is accompanied by reduced cell survival and the release of MPO and elastase on these synthetic materials compared to natural materials. The difference in cytokine release on synthetic compared to natural materials might be even more pronounced considering the increase in dead cells in the synthetic materials. The lower cell viability in the synthetic materials (PCL and TCP) supports this assumption.

The high level of VEGF-A released by neutrophils indicate that neutrophils contribute to angiogenesis often related to inflammation and tissue injury. In a mouse model it was shown that VEGF-A expressing neutrophils are actors in inflammatory angiogenesis. In an *in vitro* experiment the authors demonstrated that the release of VEGF from murine neutrophils was only present upon activation with phorbol-12-myristate 13-acetate [46]. VEGF-A released by activated neutrophils might recruit monocytes, stimulate macrophage polarization and thus contributing to the inflammatory response. Depending on the cytokines released by the neutrophils during the initial response, the foreign body reaction might be modulated. The mechanistic reasons for this behavior and the implications of these findings in tissue repair are still unclear, and should be subject of further investigations. Based on our results, cytokines commonly associated with neutrophils such as IL-6, IL-10, IL-17A, MCP-1, TNF [19,47] were low or absent in the conditioned media, including the groups with the highest endotoxin level. This might suggest that under our experimental conditions the materials did not significantly activate neutrophils resulting in release of pro- or anti-inflammatory cytokines. More knowledge is needed on the role of the specific cytokines in tissue healing, as well as a potential synergistic or antagonistic effect they could have. A thorough understanding of these mechanisms could lead to a classification system for neutrophil function analog to what is commonly recognized for macrophages, whose phenotype can be classified as classically activated M1 (pro-inflammatory) or alternatively activated M2 (anti-inflammatory/repair) [48]. Attempts to introduce neutrophils classification in “N1 and N2 phenotype” have already been described, underlining a spectrum of specific neutrophil functions. Most of this research has been undertaken in the field of oncology identifying N1 as anti-tumoral and N2 as pro-tumoral neutrophils. However, this classification is still debated, together with the set of parameters that could be used to indicate neutrophil [11,49]. A classification system to discriminate between a more pro- or anti-inflammatory response of neutrophils would help in the development and selection of biomaterials for specific applications. The prolonged release of pro-inflammatory cytokines and chemokines might stimulate chronic inflammation, and trigger macrophage recruitment and fusion on materials, which in turn may lead to an increased foreign body reaction. The extent to which *in vitro* response of neutrophils can predict the foreign body reaction can only be speculated and needs more studies *in vitro* and *in vivo* in future. One additional aspect to consider is the preservation of neutrophils phagocytic activity, and the role which could be played.

One possible bias in evaluating neutrophil behavior *in vitro* is the endotoxin content of the substrates analyzed. Our endotoxin assay revealed higher endotoxins for GelMa and THA. This is to be expected since their natural origin. Collagen is also of natural origin, however the strong acidic environment and thorough dialysis used for its extraction might have deactivated or removed the endotoxins. Despite the comparative higher level, the endotoxins in GelMa and THA are still at a low level according to FDA guidelines [50]. GelMA, which was the material displaying the highest endotoxins amount, could be safely administered up to 0.36 ml/kg with parenteral administration, corresponding to 25 ml for an individual weighing 70 kg in a single administration. Taking as a reference other recent FDA regulations for endotoxin levels in medical devices, the endotoxin level of the products should be below 0.5 EU/mL when the device is rinsed with 40 mL of non-pyrogenic water. Based on this, the endotoxin concentration of GelMA is approximately 0.067 EU/mL, which is less than the acceptable level [51]. Also, the maximum level in our experiments of 2.7 EU/ml (equal to 0.27 ng/ml) was lower than the 100 ng/ml described in literature for potential activation of neutrophils [52]. Additionally, GelMa and THA did not trigger a higher inflammatory response compared to the other materials, indicating that the results in our study should not have been biased by the endotoxin level.

In summary, there is a gap of knowledge concerning neutrophils interaction with biomaterials, what this means for foreign body response and tissue regeneration, and how to design biomaterials to modulate this process. This study is a step towards further investigations in this direction, and introduces a series of experimental methods to assess initial response of neutrophils to different biomaterials. This method was applied to a selection of naturally derived and synthetic polymeric biomaterials, identifying clear differences between the substances tested. The overall trend showed increased neutrophil survival on naturally derived constructs, together with higher oxidative burst, decreased MPO and elastase release, and cytokine secretion compared to neutrophils on synthetic materials. Neutrophils have the potential to be exploited in foreign body reaction biomaterial guided tissue repair strategies with the ability to modulate inflammation based on their

response to biomaterials. Neutrophils are an under-investigated target in this context, and further studies are necessary to unravel mechanistic aspects of their response to materials and the biological implications for tissue healing. Future studies should address the complex interaction of neutrophils with other immune cells in innate and adaptive immunity. Unraveling these mechanisms, has the potential to open new avenues in biomaterial design.

CRediT authorship contribution statement

Marinus A. Wesdorp: Conceptualization, Methodology, Formal analysis, Investigation, experimental work, data Formal analysis, interpretation, Visualization, Writing – original draft. **Andrea Schwab:** Conceptualization, Investigation, Supervision, analyzing and interpreting the data and reviewed and edited the manuscript. **Ezgi Irem Bektas:** Investigation, Writing – review & editing, interpreting the data and reviewed and edited the manuscript. **Roberto Narcisi:** Supervision, analyzing and interpreting the data and reviewed and edited the manuscript. **David Eglin:** Conceptualization, Supervision, Project administration, Funding acquisition. **Martin J. Stoddart:** Supervision, Project administration, Funding acquisition, manuscript writing-review and editing. **Gerjo J.V.M. Van Osch:** Supervision, analyzing and interpreting the data and reviewed and edited the manuscript. **Matteo D'Este:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing, All the authors approved the final version of the manuscript.

Declaration of competing interest

Hereby, on behalf al all authors I declare that none of the authors has any actual or potential conflict of interest to declare concerning the manuscript entitled "A culture model to analyze the acute biomaterial-dependent reaction of human primary neutrophils *in vitro*" submitted for consideration in Bioactive Materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.05.036.

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