Surgically Used Barrier Membranes Show Distinct Reaction Profiles in an Innovative Human Whole Blood Culture System

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Abstract

Background

Common *in vitro* cell culture systems for testing implant material immune-compatibility either employ immortal human leukocyte cell lines or use isolated primary cells. Compared to *in vivo* conditions, this generates an environment of substantially reduced complexity, often lacking important immune cell types, such as neutrophil granulocytes and others. This paper describes an innovative human whole blood culture model for *in vitro* testing of implant materials under *in vivo*-like conditions. The major goal of this culture model was to maintain as much of the naturally inherent complexity of immune cell interactions as possible and to avoid errors often caused by stressful conditions during cell preparation.

Methods

A closed, CO_2-independent, tube-based culture vessel was used, containing one milliliter of freshly drawn human blood for each sample. The cultures were occasionally rotated to increase immune cell contacts with the test materials. Immune cell responses were examined by multiplexed cytokine analysis.

Results

Three different types of commercially available implant materials i.e. barrier membranes, used for dental, trauma and maxillofacial surgery, were examined for their potential interactions with immune cells. The barrier membranes were either of synthetic (i.e. the polymers polytetrafluoroethylene, PTFE, and polycaprolactone, PCL), or of natural origin (porcine collagen membrane). The results identified characteristic differences in the overall activity profiles with very low immune cell responses for PTFE, intermediate ones for collagen, and strong reactions towards PCL.

Conclusions

This innovative human whole blood *in vitro* model, using a complex, organotypic matrix and all immune cells available in peripheral blood, is an excellent, easy to standardize tool to categorize immune cell responses to implant materials. Compared to *in vitro* cell culture systems used for material research, this new assay system provides a far more detailed picture of response patterns the immune system is able to develop when interacting with different types of materials and surfaces.

Background

One of the mainstays in oral and maxillofacial surgery, as well as other areas of regenerative medicine, is the use of implant materials. These are either employed to stabilize/replace broken or fragile structures (1), support the regeneration of tissues (2), or even form barriers between tissue compartments (3).

Depending on the final purpose of materials used in these situations, their characteristics need to be optimized in terms of their biological, chemical and physical properties, while biocompatibility must be regarded as the most crucial one (4, 5). Although plasma proteins and surrounding tissues are among the first to get into contact with the implant surface, the body recognizes such “foreign” materials mostly by means of its sentinels, the cells of the immune system (6). Among these, macrophages are present in all organs and tissues and play a central role in the organism’s response to implanted materials (7). Optionally they get attracted and activated also indirectly by non-cellular mechanisms like the activation of the complement system (8) or the clotting cascade triggered by the course of implantation (9). The same also applies to neutrophil granulocytes, the predominant type of immune cells in blood, which can express a wide spectrum of activities when getting in touch with foreign materials (10). Since, surgery usually causes bleeding, a functional, whole blood-based cell culture assay is a logical next step in minimizing the gap between the more traditional biocompatibility tests and the *in vivo* situation. To date, mandatory biocompatibility tests for the evaluation and approval of medical devices include topics like genotoxicity, carcinogenicity, interactions with blood, or cytotoxicity (ISO 10993), while the human immune system has not been taken into account, particularly not regarding its immanent complexity. Therefore, a plethora of *in vitro* tests exist and are being commercially offered in accordance to ISO 10993, without inclusion of immunological aspects (11).

A prominent feature of the human immune system is that it consists of a whole variety of types and subtypes of leukocytes (“white blood cells”, e.g. different kinds of monocytes/ macrophages, granulocytes, T cells, B cells, and NK cells). Each of these comes with a peculiar repertoire of not only receptors to recognize foreign materials (pattern recognition receptors, like Toll-like receptors, C-type lectin receptors, NOD-like receptors or RIG-I-like receptors), but also distinct ways of responding to such challenges, such as phagocytosis, oxygen radical release, secretion of cytokines and chemokines, low molecular weight mediators, etc. (12). However, current immuno-compatibility testing of solid materials relies mostly on employing just single types of immune cells (e.g. macrophages, (13–15)), or – when investigations aim for more complexity – mixed populations of limited diversity like peripheral blood mononuclear cells (PBMC, (16)). It would therefore be a major improvement in material testing to have an assay system available that allows a more comprehensive, more physiologic characterization of the reaction patterns of immune cells upon contact with the candidate materials. Moreover, such a system should optimally also include other physiologically relevant components. This is particularly true for blood plasma proteins, thrombocytes (17) and the complement system (18).

A static cell culture model meeting all of these criteria was developed more than two decades ago for testing drug activities in clinical studies (TruCulture®, (19, 20)). It is generally used as a static cell culture system and was primarily developed for *ex vivo* testing of pharmaceutical drug effects on the immune system. As a closed and CO_2-independent system, TruCulture formed an ideal basis to develop a new cell culture system for testing immunocompatibility of solid materials. Despite that, the setup needed to be changed, in that a static whole blood culture is suboptimal for material testing because of its inherent

sedimentation of cellular components over time. Therefore, a method with agitation needed to be implemented.

The main goal during the development of this innovative cell culture model was to define the optimal incubation conditions for such material testing in whole blood cultures. One of the most important features of whole blood, when incubated over periods of more than a few minutes, is an inevitable, moderately rapid sedimentation of its cellular elements (erythrocytes, leukocytes and thrombocytes) (21, 22). These exhibit different density and sedimentation speed, causing the formation of two layers: a thick bed of red blood cells in the bottom of the culture vessel, on top of which the white blood cells (i.e.: the immune cells) settle much slower, forming a second, very thin layer called the “buffy coat” (23). Hence, the use of whole blood cultures generates a peculiar problem when testing solid or semi-solid materials: Static cultures, as normally used for functional immune cell assays, would require a very precise positioning of the test piece in relation to the buffy coat to make sure that the material is in sufficient contact with the thin layer of white blood cells. Moreover, the position of the buffy coat is different for every individual blood donor since the amount of red blood cells (i.e.: their hematocrit) varies. Alternatively, vertically inserted test bodies in such whole blood cultures, would pierce the buffy coat resulting in a very reduced contact area between the material surface and the immune cells forming a thin line that would minimize the sensitivity of such an assay. A possible remedy to this would be agitated cultures, preventing cell sedimentation and redistributing immune cells from adhesion to suspension (and back). This also increases the number of cells getting in touch with the test material during incubation. On the other hand, agitation introduces shear forces, possibly leading to a premature detachment of immune cells, again impairing the translational value of such test results. In terms of hemocompatibility testing, Chandler loops and their modifications, are the most prominent *in vitro* systems to test solid materials with whole blood (24, 25). Their biggest disadvantage are large volumes of blood needed, comparatively large contact area of the loop with regard to the specimen and short incubation times of only a couple of hours. Alternative *in vitro* whole blood assays to investigate immunocompatibility of implant materials are highly demanded.

The experiments presented here aimed at establishing such an assay system and optimizing basic assay parameters. In addition, three materials commonly used for dental implantation were examined for their immunobiological activities.

Methods

Blood donors: All healthy blood donors provided written informed consent before phlebotomy, as approved by the Ethics Committee of the University of Tübingen. Heparinized blood (50 IU/ml) was obtained and used in the cultures not later than 60 minutes after drawing in order to avoid storage-related changes in viability and the activation state of the leukocytes. Exclusion criteria for blood donation were as follows: Symptoms of systemic or local inflammatory reactions (except for single small and superficial skin lesions), last symptoms of systemic or local inflammatory reactions of an inflammatory disease (or first symptoms of a new episode) within the last 14 days before blood donation, vaccination

within the last six weeks, surgery within the last three months, chronic diseases with inflammatory components (even during symptom-free intervals), drug intake within the last 14 days (except for contraceptives) or consumption of alcohol (e.g. >0.5 L of wine or 1 L of beer on the evening prior to blood donation), or strenuous exercise performed within three hours before blood donation.

Test materials

Three different types of commercially available barrier membranes made of polytetrafluoroethylene (PTFE; Cytoplast TXT, Osteogenics Biomedical), natural porcine collagen membrane (collagen; Bio-Gide®, Geistlich Pharma AG), or polycaprolactone (PCL; Osteoguide, Genoss Co.) were tested in these whole blood cultures. Nelfilcon A (NelA) contact lenses (Dailies AquaComfort Plus, Alcon), consisting of polyvinyl alcohol, hydroxypropylmethyl-cellulose, polyethylene glycol and N-formylmethyl acrylamide and High-density polyethylene (HDPE; Food and Drug Safety Center, Hatano Research Institute) served as biological inert materials.

Cell culture system

All specimens were trimmed to fit into the 3 ml tubes (used for TruCulture) and two milliliter CO_2-indepenent proprietary medium (TruCulture) was added. One milliliter of freshly drawn, heparinized human whole blood was transferred into these tubes and incubated at 37°C for 48h. Tubes were cultured either in a block thermostat (static culture) or periodically resuspended in a sample mixer to prevent sedimentation of cellular components. Unstimulated cultures were used as negative control, while lipopolysaccharide (LPS) in combination with Staphylococcal Enterotoxin B (SEB) were used at suboptimal concentrations in order to induce a pronounced, but still not maximal cytokine response. After incubation cell cultures were centrifuged (500g; 10 minutes) and supernatants were stored (< -20°C) until cytokine detection.

Cytokine detection: The release of cytokines characteristic for leukocyte responses were chosen to evaluate functional immune cell responses to the different materials tested. Mediator release was measured using bead-based multiplexed sandwich immunoassays (Luminex™ technology) on a Luminex 200™ analyser system. Data was interpreted using proprietary analysis software developed by Myriad RBM (Austin, USA). The following endpoints were measured: IFNγ, IL-1β, IL-1RA, IL-6, IL-8, IL-10, IL-12p40, GM-CSF, TNFα, MCP-1, and MIP-1β.

Evaluation software: Data processing and evaluation was performed using Microsoft Excel 2019 or Prism (version 9.0.0; GraphPad). Principal component analysis (PCA) was created with ClustVis software (https://biit.cs.ut.ee/clustvis/).

Results

Establishing the agitated human whole blood assay to test solid materials

In accordance with static whole blood cultures, agitated cultures showed similar kinetics for the release of cytokines (data not shown). A few mediators (such as TNFα or IL-1β) are being released already within the first 24 hours, while many other cytokines need more time to be synthesized and released (e.g.: IFNγ, IL-2, or IL-10). Therefore, the number of measurable endpoints in in the cultures presented below was optimized by choosing an incubation time of 48 hours. Incubation times of 72 hours or more are not recommended, since nutrients are gradually getting used up, culture conditions deteriorate and cell viability decreases substantially.

Various types of materials were considered appropriate controls, during the developmental process. Just like for static cultures (TruCulture®) non-stimulated cultures and LPS/SEB activated cultures could be used as negative and stimulation controls, respectively. HDPE, classified as negative control for cytotoxicity testing according to DIN EN ISO 10993-5, showed very low activation of immune cells and was used as negative material control. NelA exhibited moderate, inter-individually different response patterns and was used as positive material control.

The final design of this assay as employed to generate the results presented in this study was as follows: Test materials were introduced into the TruCulture tubes and two milliliters of medium were added. Control tubes were supplemented with either LPS (bacterial lipo-polysaccharide, HyCult) plus SEB (staphylococcal enterotoxin B, Bernhard Nocht Institute, Germany; stimulation control), or left without stimulation (negative control). Thereafter, one milliliter of freshly drawn blood was transferred into these tubes and incubated for a total of 48 h at 37°C with intermittent rotation. At the end of incubation culture vessels were centrifuged (500 g; 10 minutes) and cell culture supernatants stored (< -20°C) until testing for cytokine release.

The positive and negative controls were used to compare the agitated test system side-by-side to the static assay. In addition, two different types of materials were analyzed: 1) HDPE, positioned in the tubes at approximately the level where the buffy coat was expected to form during the static culture, 2) NelA, a daily disposable hydrogel contact lens that is not meant to get into direct contact with whole blood and is generally classified as medical device class II.

In general, there were only minor differences between the results of the static and the agitated cell culture model. Negative controls (non-stimulated and HDPE-containing cultures) were very low for both systems, with stronger effects for the positive control (NelA) and very strong effects for the stimulation control (LPS/SEB; see Figure 1 and 2). These results were promising, since the static whole blood culture served as benchmark for the agitated system. A closer look at the cytokine levels revealed, that both, negative as well as positive controls behaved as expected in the agitated system, while showing an overall lower background for most cytokines for the unstimulated negative control and HDPE, for example for IL-1β, IL-6, MCP-1, and MIP-1β. In addition, some cytokines showed slightly higher levels for NelA, when tested in the agitated system (e.g.: MCP-1, MIP-1β, and IL-10; with the same tendency for IL-1β and IFNγ). Moreover, NelA elicited strong inter-individual differences in the response profiles of the cultures of the three different healthy volunteers.

Proof of concept experiments with dental barrier membranes

After completing the development of this agitated human whole blood assay, three different types of commercially available barrier membranes for dental, trauma and maxillofacial surgery, were tested. These differed especially in terms of their basic composition, namely: PTFE, collagen, and PCL. Barrier membranes were ideal specimens for this type of test system, since they get into direct contact with whole blood *in vivo*, as will be the case for most other implantable materials. The whole blood tests were performed using the blood of three different healthy volunteers to be able to identify inter-donor variations. Cytokines were quantified in the culture supernatants. Figure 3 shows nine representative analytes, that can be grouped into chemokines (Figure 3A), cytokines mainly produced by myeloid cells (Figure 3B) and/or by lymphocytes (Figure 3C). Negative controls consistently showed low to undetectable values, while stimulation controls (LPS/SEB) induced high values for these analytes in the cultures of all three donors.

Among the materials tested, NelA mediated the biggest inter-donor variations. In general, immune cells of donor A were highly activated for several cytokines (see figure 3: IL-8, MCP-1, TNAα, IL-1β, and IL-10), whereas those of donors B and C secreted comparatively low concentrations of these mediators. However, when looking at the results of the barrier membranes, the strongest average responses were triggered by PCL, while the opposite was true for PTFE for which no significant immune cell activation could be observed (see Figure 3). The collagen membrane, on the other hand, presented with moderately induced cytokine releases (see Figure 3). Some cytokine levels induced by the barrier membranes even exceeded the levels of the stimulation control, like IL-8, TNFα, and IL-1β for PCL and MCP-1 for collagen.

Characterization of immune cell responses measured by means of this whole blood system

Based on the results presented in Figure 3, additional methods were used for data deconvolution. For better visualization data was transformed and normalized and a heatmap was created. The heatmap underlined barrier membrane specific clusters, that could also be seen in each graph of Figure 3 (see Figure 4). The very low values induced by PTFE resembled quite well the heatmap pattern observed for the unstimulated control, whereas also this type of evaluation differentiated clearly the responses to collagen, on the one hand, and PCL on the other. For a couple of mediators, PCL even looked quite similar to LPS/SEB. Due to the subject-specific reaction patterns, NelA seemed to confirm its intermediate position in this ranking.

Upon interaction with immune cells in these agitated whole blood cultures, the negative, positive and stimulation controls, as well as the three different materials tested in this first series of experiments generated clearly distinct activity profiles. This could also be demonstrated nicely by principal component analysis (PCA), as shown in Figure 5. The unstimulated negative controls from all three donors and the

results for cultures with PTFE clustered in the same area of the PCA (see Figure 5). The stimulation control LPS/SEB and PCL also formed separate, distant clusters compared to the negative control and PTFE. A separate, well-defined cluster was formed by the collagen results. PCA was also able to identify the inter-individual differences observed with NelA (see Figure 5).

Discussion

The whole blood-based test system described above was developed particularly for a more comprehensive and *in vivo*-like characterization of potential immune cell interactions with solid and semi-solid materials. The major goal of this project was to use un-touched immune cells to avoid any kind of artificial loss or gain in activities, due to handling. Such undesired activities can easily be caused by storing, shipping, or manipulations during immune cell preparation. Therefore, the cultures should be started not later than 1 h after drawing the blood (26, 27). In addition to this, shear forces and temperature shifts, often being the result of blood sample shipping, means additional stress to immune cells (28, 29). However, the most crucial point is the preparation of the immune cells prior cultivation, including centrifugation and resuspension of the cells, or exposure to buffers, different to their natural matrix, etc. (27, 30, 31).

Moreover, whole blood cultures mimic excellently the complexity of the human immune system *in vivo* (32–34). All types of immune cells are still available in their native composition, such as monocytes, macrophages, T cells, B cells, NK cells, or granulocytes, as well as platelets, soluble factors like complement proteins, antibodies, etc. Each of these elements do have the capacity of either triggering or at least modulating the response of immune cells to materials (35). Maintaining an *in vivo*-like complexity in test models enhances the translational value of results obtained with these considerably (30, 33, 36).

The most decisive component in these cultures is human whole blood. In order to obtain reliable and reproducible results, strict inclusion and exclusion criteria must be met when selecting the donors (see material and methods). These must ensure that immune cell activities have not been triggered (or suppressed) already *in vivo*, short before the blood is drawn, e.g. as a consequence of immunological illnesses, medication, surgery, vaccination or similar (37–41).

The easiest and also most sensitive endpoints to be measured in whole blood culture models are cytokines secreted by the different types of immune cells into the culture fluid (42). This enables a simple and quick processing of even larger series of samples by means of standard immunoassays, such as single ELISAs, or the more informative multiplexed assays like Luminex® (43). It is self-evident that, while providing a far more comprehensive overview on the activities of a whole variety of cell types, especially multiplexed assays provide the best match to the complexity represented by such organotypic cell culture systems. Besides, other parameters, such as mRNA expression, surface activation-markers, cell viability, morphology, intra-cellular cytokine levels, etc. can be determined by recovery of the cellular components from these cultures as well (33, 44, 45).

A very important aspect when developing new test models is the definition of controls and reference samples. Here, the non-stimulated negative control defined the basal level of immune cell activity. This was of great importance for further data analysis and interpretation. The stimulation control illustrated, whether immune cells of each donor could be activated properly. Both, negative control and stimulation control, were well established for static whole blood culture model (TruCulture®) (33, 33, 46). HDPE was found the most suitable negative control material for this test model with very low, almost basal cytokine levels. HDPE was also selected as negative control for cytotoxicity testing according to DIN EN ISO 10993-5. More difficult was the search for an appropriate positive control, since it was meant to activate immune cells without causing hemolysis or cytotoxicity. NelA fulfilled these criteria, although its immune cells activation was donor dependent with inter-individual variations. Only by taking into account the results of negative and stimulation control cultures, as well as those containing negative and positive material controls, will allow a reliable processing of such data.

As proof of concept, three different types of commercially available barrier membranes, used for maxillofacial surgery, were examined for their potential interactions with immune cells in this new whole blood test system. The materials, namely PTFE, collagen, and PCL, can be regarded as excellent examples for this test, representing not only different strengths of responses (low – medium – high), but also reaction patterns of different, yet reproducible complexity.

On the other hand, some results obtained with collagen indicated another interesting feature, which will surely not be limited to this material: While inducing moderate to high concentrations of several mediators, unexpectedly low values occurred for others, like MIP-1β (see Figure 3) or MMP3 (data not shown). This was likely caused by an adsorption of these proteins to the collagens, which has been shown for MIP-β (47), as well as for MMP3 (48). Besides the fact that such properties will interfere with a reliable quantitation of these mediators, properties like these will also have the potential to contribute to modulate the integration process of implants into the surrounding tissues. The whole blood test system presented in this paper is also able to detect such additional features of implant materials not only of natural origin, but also of synthetic composition. Further investigations will be needed to characterize the influence of the adhesion of specific mediators.

Follow-up experiments will focus on immune activating properties of materials used frequently for medicinal purposes and try to establish correlations between reaction patterns observed in this novel whole blood culture system and clinical outcome. Integration of this information into a database will ease the characterization of the analyzed materials. In addition, future tests will also address additional culture conditions, such as co-activation of immune cells by bacterial stimuli and other inflammatory signals, but also use of a wider spectrum of endpoints in order to characterize material properties more comprehensively.

Conclusions

Our results show that this innovative whole blood assay for testing immunocompatibility of implantable materials is able to safely differentiate between a) materials that do not elicit much activity in immune cells, b) others triggering weaker (and/or less variable) responses, c) those generating strong and more extensive responses, as well as d) characteristic, but subject-specific differences in reaction patterns.

The new *in vitro* model employed in these experiments provided a reliable means to sensitively detect complex reaction profiles of native human immune cells while avoiding cell culture artifacts, such as stress-induced false positive or false negative results when using immune cells that need to be isolated from whole blood before testing.

Abbreviations

GM-CSF
granulocyte/monocyte colony-stimulating factor
IL-X
interleukin X
LPS
lipopolysaccharide
IFNγ
interferon gamma
MIP-1β
macrophage inflammatory protein beta
NelA
Nelfilcon A
PBMC
peripheral blood mononuclear cells
PCA
principal component analysis
PCL
polycaprolactone
PTFE
polytetrafluoroethylene
TNFα
tumor necrosis factor alpha

Declarations

Ethics approval and consent to participate

All healthy provided written informed consent before phlebotomy, as approved by the Ethics Committee of the University of Tübingen (Project No. 457/2021BO2).

No clinical data were obtained.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due proprietary rights in the assay procedure, but are available from the corresponding author on reasonable request.

Competing interests

HOT Screen GmbH is a private contract research organization.

- During the development of this assay procedure, SK was employed as scientist at HOT Screen GmbH
- MS is chief executive officer and chief scientific officer at HOT Screen GmbH

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Authors' contributions

- SK performed all cell culture experiments and wrote major parts of this manuscript
- MS provided major contributions to the design of this assay model and to writing this publication
- TK was responsible for cytokine testing
- BS contributed to the scientific rationale of the experiments and reviewed the manuscript
- JC-S provided the samples of biomaterials and reviewed the manuscript
- AM-F reviewed the manuscript

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Figures

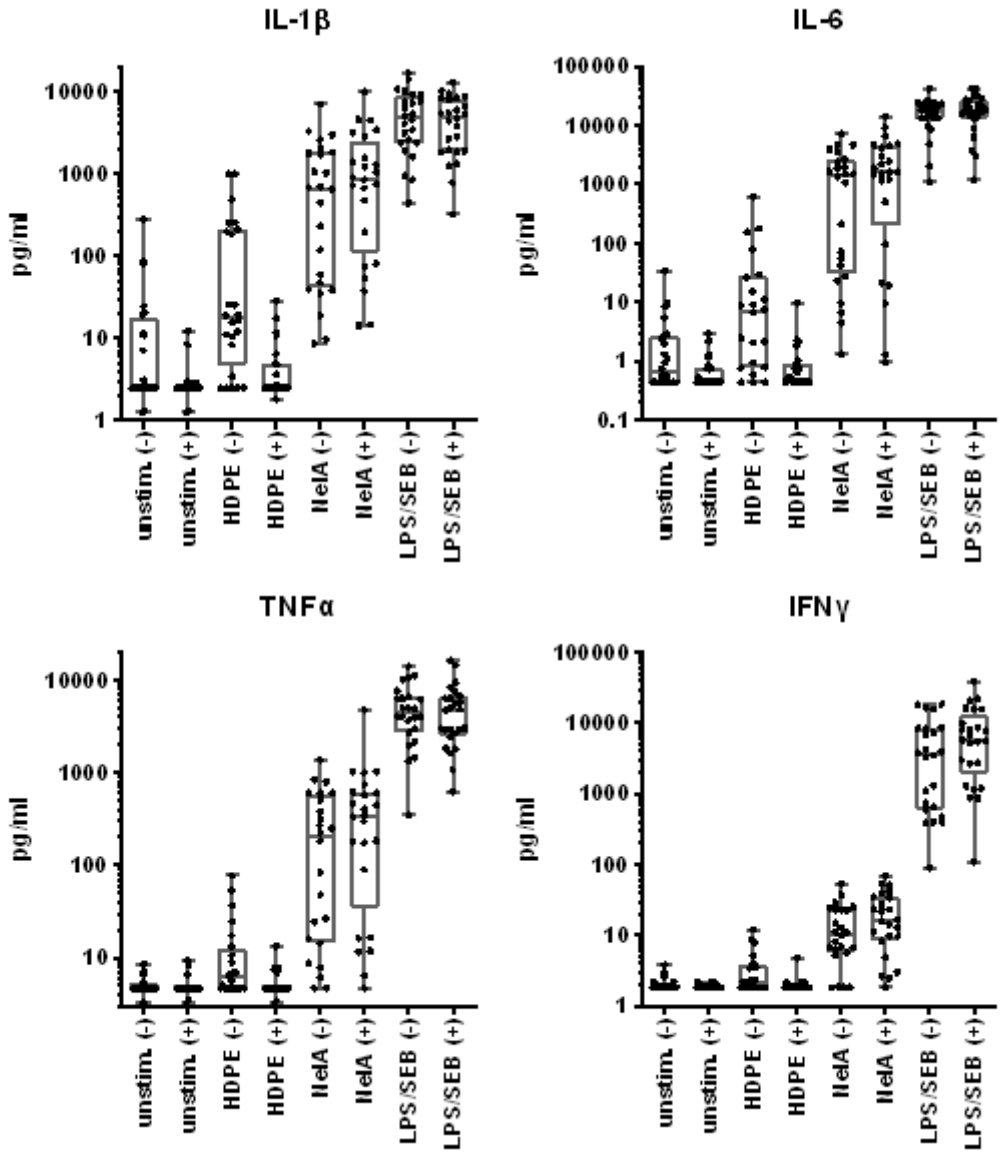

Figure 1

Comparison of agitated versus static whole blood cultures. Human whole blood was cultured without stimulation (non-stim.), or in the presence of either high-density polyethylene (HDPE; material control), as negative controls, NelA (positive control), or LPS/SEB as stimulation control. Samples were cultured either in the static manner ("-") or under agitation ("+"). Supernatants were examined by multiplex immunoassays for the concentrations of different cytokines. Box-plots with single values of eight independent runs, using the blood of three varying healthy donors for each run (N = 24).

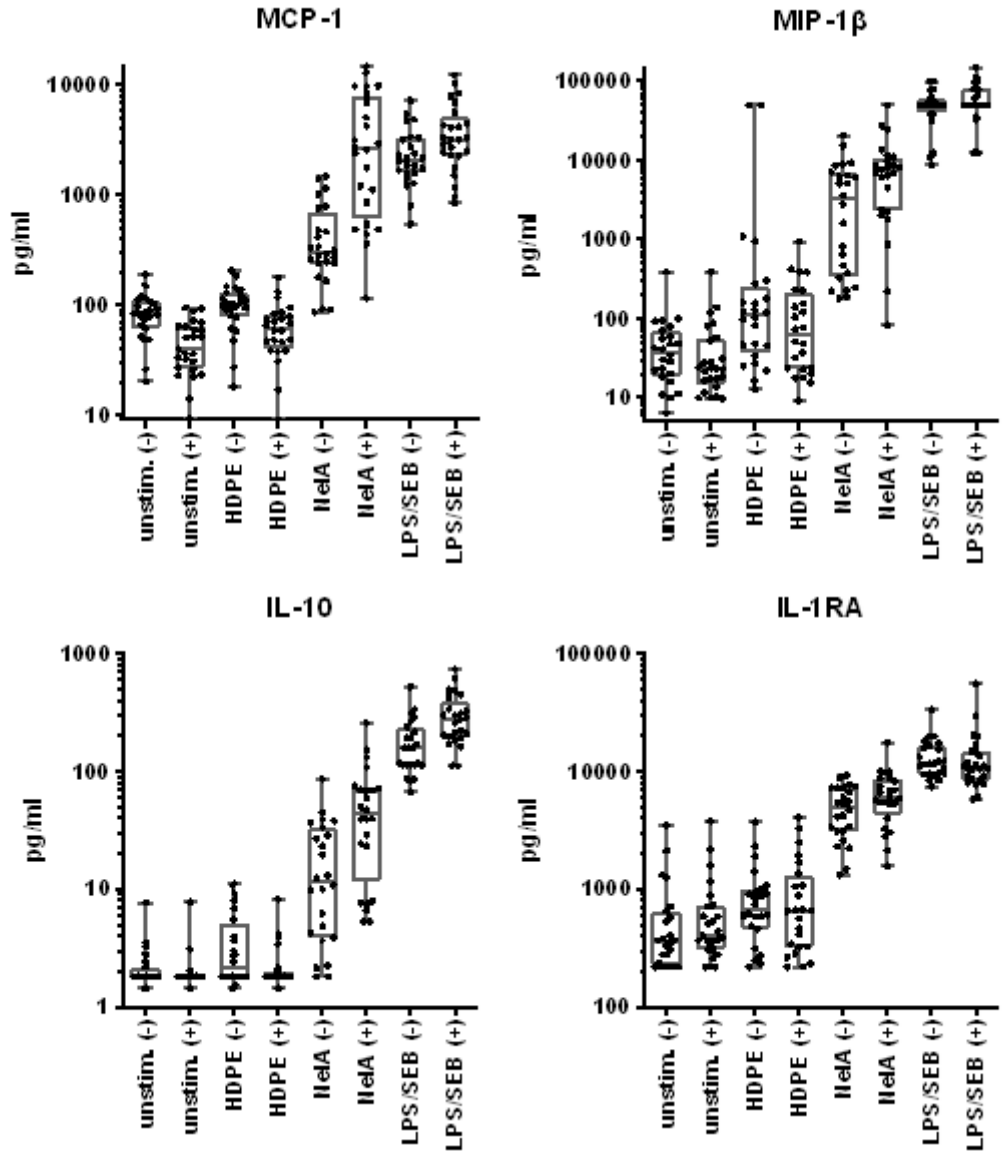

Figure 2

Comparison of agitated versus static whole blood cultures. Shown are the immunoregulatory cytokines MCP-1, MIP-1β, IL-10 and IL-1RA. According to figure 1.

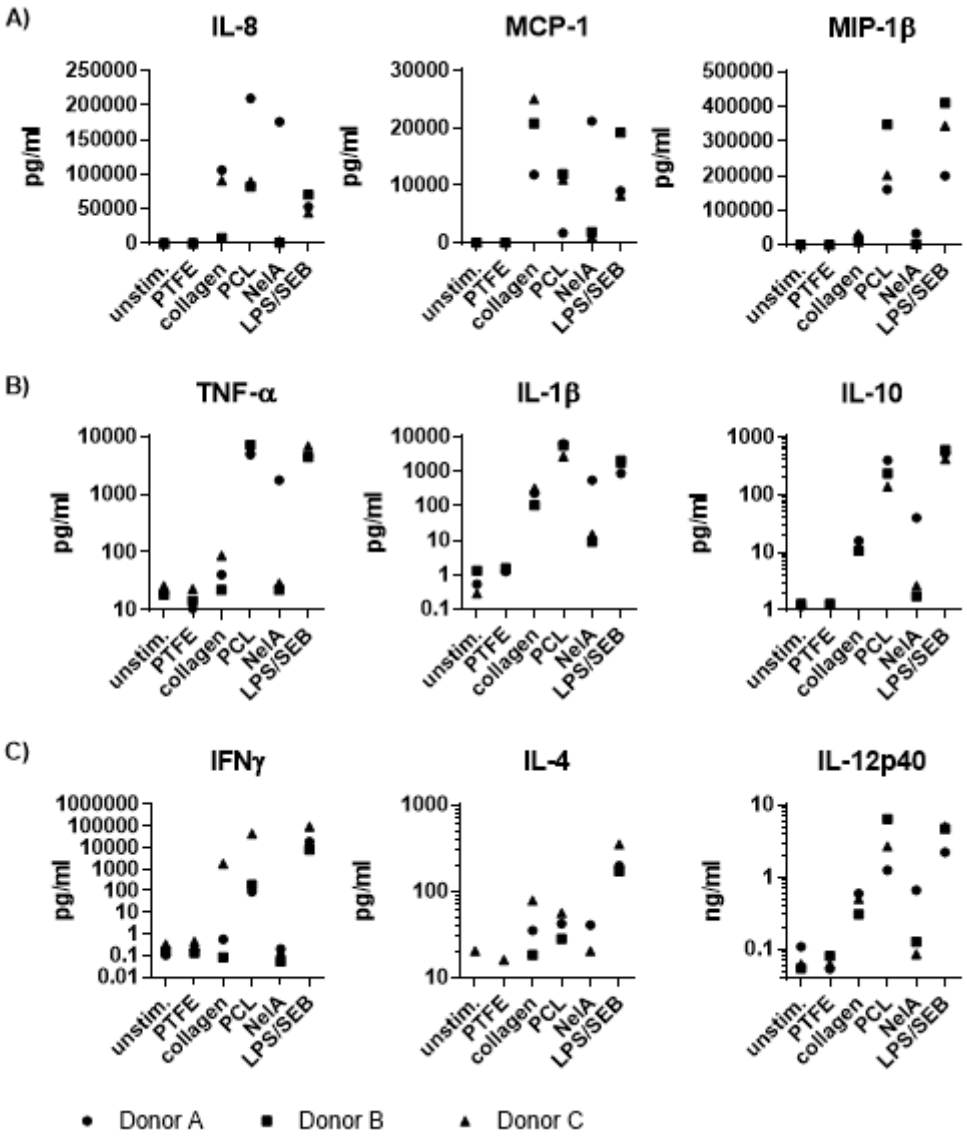

Figure 3

The agitated whole blood assay revealed distinct cytokine responses for different barrier membrane materials. Human whole blood from three healthy donors was cultured in the new agitated test system without stimulation (unstim.; negative control), with barrier membranes (made of PTFE, collagen, or PCL), and NelA (positive control) or LPS/SEB (stimulation control). Subsequently, culture supernatants were analysed by multiplex immuno-assays. The graphs above show a representative selection of cytokines that can be grouped into A) chemokines, B) cytokines, mainly produced by myeloid cells and C) cytokines also produced by lymphocytes.

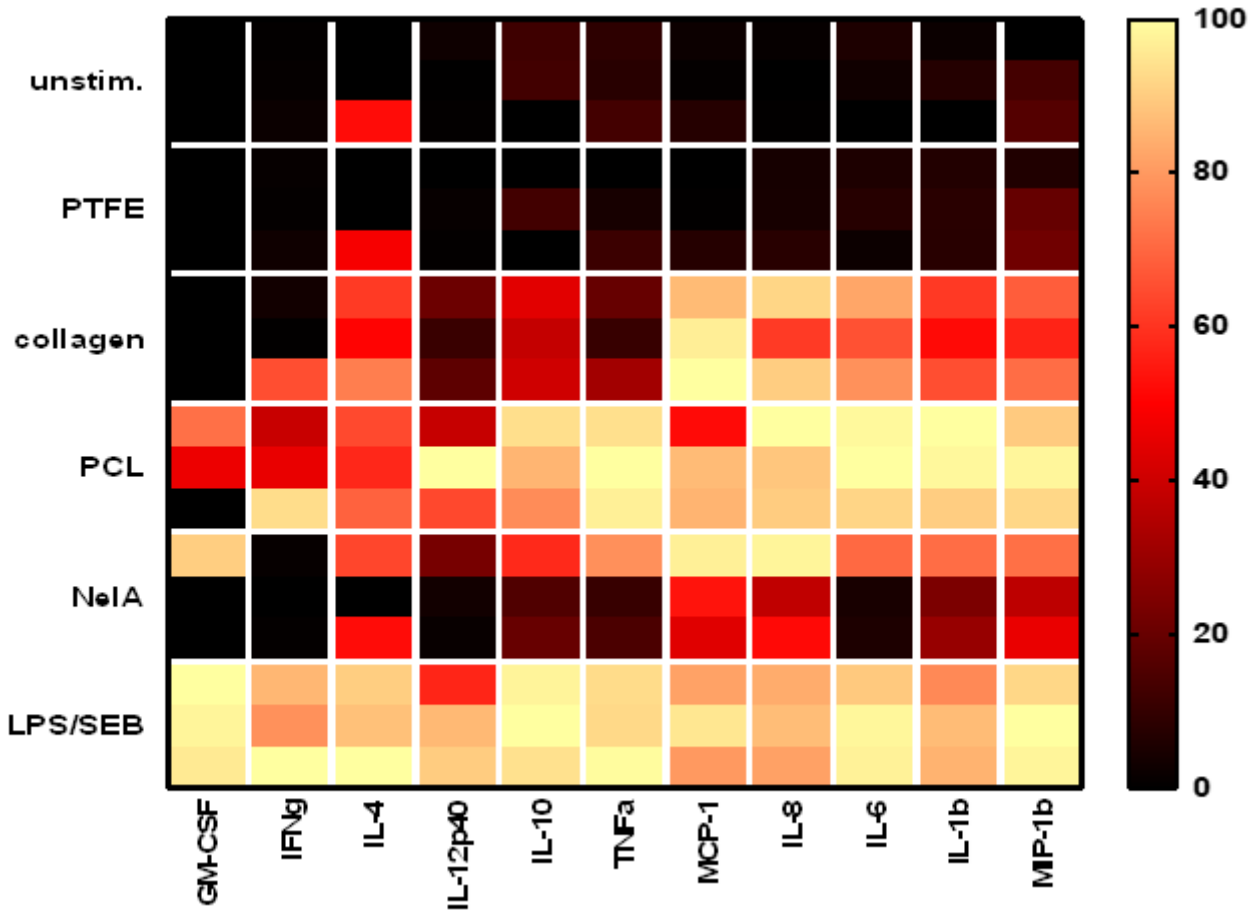

Figure 4

Heatmap analysis shows different cytokine-patterns for different materials. The assay was performed as described above. Rows of the heatmap show different conditions: unstim. (negative control), barrier membranes (PTFE, collagen, or PCL), NelA (positive control) or LPS/SEB (stimulation control) of three different donors (stacked in each row). Columns indicate the according analyte. Data of individual values was transformed (y = ln(x + 1)), subcolumns were normalized (smallest value = 0%; largest value = 100%) and colour coded.

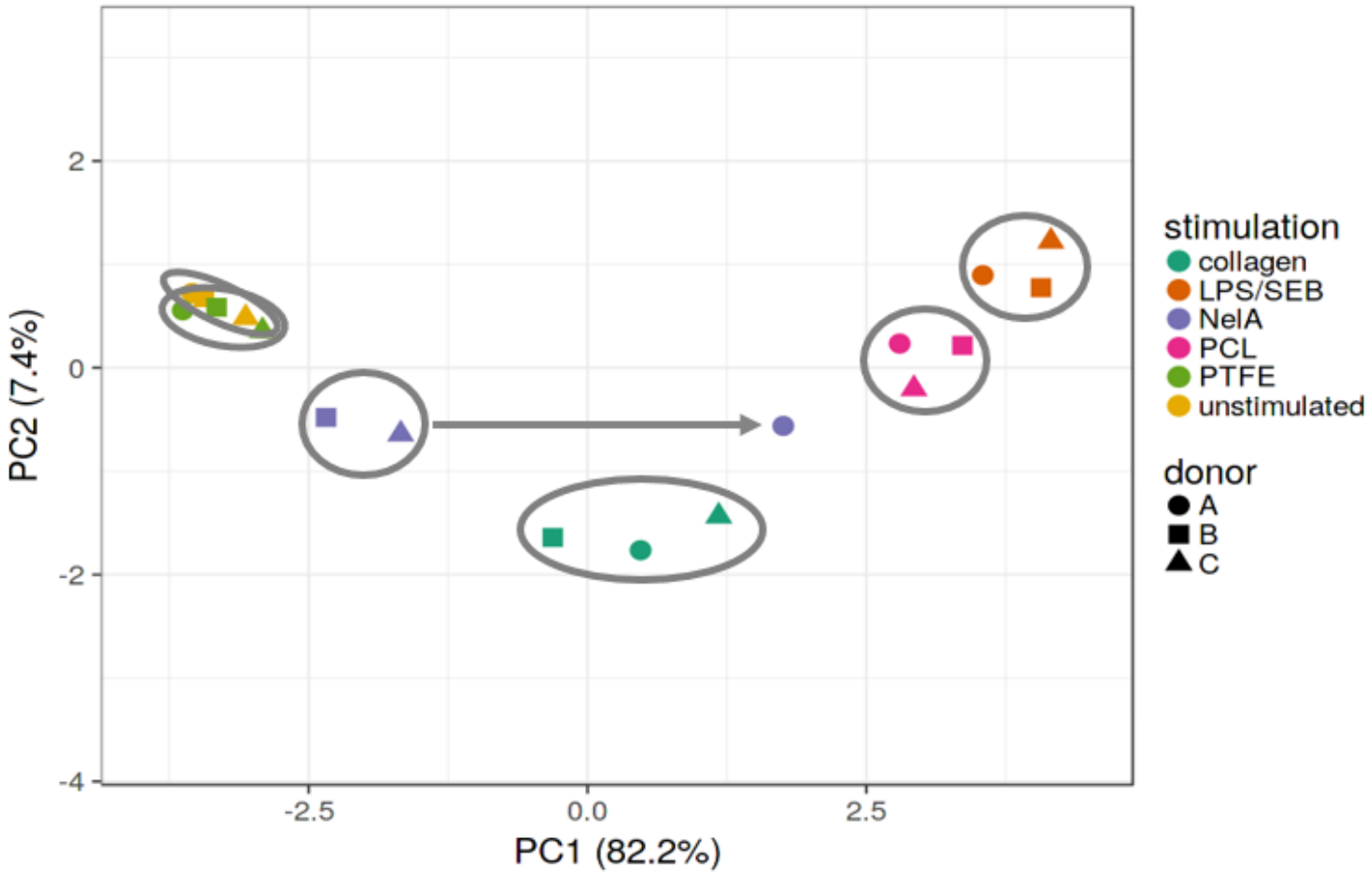

Figure 5

Clustering immune cell responses to different stimulation conditions and materials via PCA. The assay was performed as described above. Culture supernatants were analysed for cytokines and used for principal component analysis. Original values were ln(x + 1)-transformed. Unit variance scaling was applied to rows and singular value decomposition with imputation was used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 82.2% and 7.4% of the total variance, respectively. N = 18 data points.