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Research paper

Leukoreduction system chambers provide a valuable source of functional monocytes for the monocyte activation test by comparison with internationally validated methods

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ABSTRACT

Despite being added to the European Pharmacopoeia in 2010 and strongly supported by the European directive enforcing the "3R's" — Replace, Reduce and Refine, uptake of the monocyte activation test (MAT) in preference over the rabbit pyrogen test for the detection of pyrogens has been limited. This has been attributed to the difficulty in sourcing human monocytes due to the necessity of phlebotomy. This study has attempted to address this issue by evaluating cryopreserved peripheral blood mononuclear cells (PBMCs) isolated from leukoreduction system chambers (LRSCs), a readily available by-product of platelet apheresis, as a source of monocytes for the MAT. Validation was performed by direct comparison with the two most commonly employed primary monocyte sources: fresh whole blood (WB) and PBMCs from fresh blood, assessing their ability to detect a panel of toll-like receptor (TLR) ligands including Pam_3CSK_4, Lipoteichoic acid, Peptidoglycan, Poly(I:C) and Flagellin, as well as two different endotoxin sources, with IL-1β and IL-6 as the readouts. All three cell sources were able to detect the pyrogens included in the study with comparable sensitivities, with the exception of TLR3 ligand Poly(I:C). The WB assay produced quantifiable, but significantly lower cytokine levels with every pyrogen tested than either of the PBMCs sources used. LRSCs provided an ample and convenient source of PBMCs which were successfully cryopreserved, providing cell banks for each donor, shown to maintain stability for at least a year. The use of cryopreserved PBMCs reduced the time and effort required to set up an assay, and the availability of single donor cell banks will allow investigations into assay variables in the absence of inter-donor variability. Significantly higher sensitivity to Pam_3CSK_4 was observed with a proportion of donors. This was found to correlate to single nucleotide polymorphisms rs4833095 and rs5743618 of TLR1. This evidence, along with the wide range of other SNPs identified in TLR regions without known biological function, supports caution in the practice of pooling donor cells in order to overcome donor-to-donor variation.

1. Introduction

Pyrogens are structurally diverse substances derived from components of Gram-negative and Gram-positive bacteria, mycobacteria, fungi and viruses. Conserved motifs of these molecules are recognised by pattern recognition receptors (PRR), such as Toll-like receptors (TLRs) on the surface of monocytes and macrophages circulating in blood and resident in tissues. This leads to the activation of the receptor, signal transduction and ultimately the release of pro-inflammatory mediators including prostaglandins and cytokines such as IL-1β, IL-6 and TNFα from the cell. It is not necessary for the organism to remain intact, nor capable of replication and infection for this induction to occur. The inflammatory response readies the body's defences against the perceived microbial threat through a number of physiological changes, the most obvious of which, and perhaps therefore historically the most emphasised, is fever. More important clinically, these pro-inflammatory mediators can lead to a series of uncontrolled inflammatory events potentially resulting in multiple organ dysfunction, shock and in severe cases, death. With the potential for inadvertent contamination of parenteral products, pyrogens are of concern to regulatory bodies and the pharmaceutical industry.

Consequently, regulatory authorisation requires compliance with an established endotoxin limit, and all parenterals must be tested for pyrogenic content before being released onto the market. The current assays used to assess this include the rabbit pyrogen test (RPT); bacterial endotoxins test (BET) also known as the Limulus amebocyte lysate test (LAL); and the monocyte activation test (MAT) implemented in the

Abbreviations: BET, bacterial endotoxins test; IL, interleukin; IS, International Standard(s); LAL, Limulus amebocyte lysate; LRSC, leukoreduction system chamber; LTA, lipoteichoic acid; MAT, monocyte activation test; Pam_3CSK_4, Tripalmitoyl-S-glyceryl-L-Cys-Ser-$(Lys)_4$; PBMCs, peripheral blood mononuclear cells; RPT, rabbit pyrogen test; TLR, toll-like receptor; WB, whole blood.

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1910's, 1970's and 2010's respectively. The RPT measures the change in body temperature of rabbits resulting from the intravenous administration of test material whilst the BET allows detection of contaminating endotoxin through coagulation when brought into contact with aqueous lysate extracted from the amebocytes isolated from the Limulus crab.

The BET is relatively cheap and easy to use but suffers from the significant limitation that it is only able to detect endotoxin, a cell wall component of Gram-negative bacteria. Whilst this is the most common cause of pyrogenicity in pharmaceuticals, it is well recognised that contamination with non-endotoxin pyrogens such as those derived from Gram-positive bacteria are also capable of causing significant adverse events (Martis et al., 2005). Additionally, certain matrices commonly found in biopharmaceutical formulations are incompatible with the BET method such as high protein content, chelating agents and certain metal ions.

The RPT is capable of detecting endotoxin as well as non-endotoxin pyrogens but is expensive and the relevance of the model compared to the human system it is supposed to represent is questionable, especially considering the homogenous nature of the rabbit population used. Additionally the assay is not quantitative, is prone to being affected by ancillary parameters such as how the rabbits are handled and kept, and cannot be used with products such as radiopharmaceuticals or cancer chemotherapeutic agents.

The MAT was developed in order to address the shortcomings of the BET and RPT. The assay is based on the activation of isolated human monocytes in vitro to allow the detection of contaminating pyrogens with the cytokines released utilised as the readout. The MAT therefore provides a direct link between in vitro cytokine release and the potential in vivo adverse events which could ensue as a result of the contamination. This system allows for the detection of endotoxin as well as non-endotoxin pyrogens and if primary monocytes from a range of donors are used, is able to reflect the inter-individual differences in responses exhibited in vivo, which has been noted as particularly marked with non-endotoxin pyrogens (Hartung, 2015).

Due to the inherent limitations of the RPT and the BET, as well as their reliance on animals and animal source materials respectively, the application of the MAT in favour of these tests has been strongly encouraged. This has particularly been the case in Europe where directive 2010/63/EU strengthening the legislation around the use of animal testing, enforcing the "3R's" principle (Replace, Reduce and Refine), came into force through national legislature in 2013. However, the widespread implementation of the MAT has been challenging primarily due to the difficulty in obtaining freshly drawn blood as a source of monocytes.

Several different MAT systems have been established, with the sources of monocytes used ranging from diluted whole blood (WB), isolated peripheral blood mononuclear cells (PBMCs) and monocytic cell lines including MonoMac 6 and THP-1, and readouts primarily using IL-1β and IL-6 (Hoffmann et al., 2005). Cryopreserved WB and PBMCs have also been employed successfully (Koryakina et al., 2014; Schindler et al., 2004; Solati et al., 2015). With a diverse array of assay systems, more data are needed in order to understand the differences between them, in particular in regards to sensitivity to non-endotoxin pyrogens. This would allow manufacturers to make informed decisions as to which system might be best suited to their product and most likely contaminant. Additionally, a convenient source of monocytes, with the added benefits of cryopreservation would be advantageous to improve accessibility and reproducibility of results.

To this end, the present study's aims were to evaluate the performance of mononuclear cells isolated from leukoreduction system chambers (LRSCs) when used in the MAT compared with established (and internationally validated) methods using WB and fresh isolated PBMCs (Hoffmann et al., 2005). As an easily obtainable by-product of platelet apheresis, LRSCs could serve as a valuable source of monocytes.

2. Materials and methods

2.1. Materials

2.1.1. Reagents and consumables

The 3rd WHO International Standard (IS) for endotoxin (from *Escherichia coli* O113:H10:K-), 10/178 was obtained from NIBSC (South Mimms, Hertfordshire, UK). Tripalmitoyl-S-glyceryl-L-Cys-Ser-$(Lys)_4$ (Pam_3CSK_4), Lipoteichoic acid (LTA), Peptidoglycan, Poly(I:C), and Flagellin were purchased from InvivoGen (San Diego, CA, USA) and endotoxin isolated from *Pseudomonas aeruginosa* from Sigma-Aldrich (St. Louis, MO, USA). The 1st WHO IS for IL-6 (89/548) and IL-1β (86/680), used to calibrate ELISAs, were obtained from NIBSC. IL-1β sandwich ELISA pair (mouse monoclonal MAB601 and goat polyclonal BAF201) were purchased from R&D systems (Minneapolis, MN, USA). An in-house ELISA pair described previously was used for the IL-6 ELISA (Gaines Das et al., 2004; Hoffmann et al., 2005). Reagents were reconstituted and stored in accordance with the manufacturer's instructions. All consumables used in the MAT, excluding the pyrogen panel, were purchased as sterile and pyrogen-free. Medium reagents were purchased from Sigma-Aldrich or Gibco, Life-Technologies (Carlsbad, CA, USA) unless otherwise stated.

2.1.2. Blood donations and LRSCs

Blood donations from NIBSC staff were taken under the local ethical committee approval and guidelines. All donors had to describe themselves as being in good health, not suffering from any bacterial or viral infections for a period of at least one week prior to the donation of blood, as well as not having taken any drugs known to influence cytokine production in the last 48 h. Blood was collected using a 21-gauge hypodermic needle using a 50 ml syringe and transferred immediately into a 50 ml sterile centrifuge tube (Greiner Bio-One, Nürtingen, Germany) containing sufficient heparin to give a final concentration of 10 EU/ml (Sigma-Aldrich). LRSCs were received from the Therapeutic Apheresis Unit at the John Radcliffe Hospital (Oxford, Oxfordshire, UK) through NHS Blood and Transplant (NBSBT, Bristol, UK) on the day of apheresis donation and isolated PBMCs were cryopreserved within 10 h of donation (within 4 h of receipt).

2.2. Methods

2.2.1. Preparation of pyrogen solutions and BET testing

Stock solutions of endotoxin and non-endotoxin pyrogens described in Section 2.1.1 were reconstituted under aseptic conditions using pyrogen-free water (provided by manufacturer or purchased from Charles River, Wilmington, MA, USA), aliquoted and stored below −20 °C. Stock solutions of non-endotoxin pyrogens were shown to contain minimal or no endotoxin as determined by using the BET turbidimetric method (following Chapter 2.6.14 method B (European Pharmacopoeia 8.0, 2010a), relative to endotoxin IS 10/178, with results summarised in Table 1. The equivalent units of endotoxin in the preparation from *P. aeruginosa* was determined by chromogenic-kinetic BET assay (following Chapter 2.6.14 method D, European Pharmacopoeia 8.0, 2010a), relative to endotoxin IS 10/178. Dilutions used in MAT assays were prepared by doubling serial dilution using 0.9% (*w*/*v*) saline (Freserius Kabi, Sèvres, France) for the WB and fresh PBMC assays or culture medium (described in Section 2.2.2) for the cryopreserved PBMC assay. The prepared dilutions were added to the assay plates directly after dilution.

2.2.2. Isolation of PBMCs from LRSCs

LRSCs were sterilised externally using 70% (*v*/*v*) ethanol and handled in a class 2 laminar flow cabinet. External tubing was cut, the chamber inverted over a 50 ml sterile centrifuge tube (Greiner Bio-One) and the contents allowed to drip through. The contents (usually 5–10 ml) were then diluted to 90 ml in culture medium (RPMI supplemented

Table 1
Summary of pyrogens used in study and their endotoxin content determined by BET.

Pyrogen	Toll-like receptor (TLR)	Source	Endotoxin equivalent[b]
Endotoxin IS 10/178	4	*E. coli* O113: H10: K- (NIBSC)	8333 EU/μg[c]
Pam_3CSK_4	1/2	Synthetic (InvivoGen)	$<3 \times 10^{-7}$ EU/ng
LTA	2	*S. aureus* (InvivoGen)	6×10^{-4} EU/μg
Peptidoglycan	2[a]	*S. aureus* (InvivoGen)	6×10^{-4} EU/μg
Poly(I:C)	3	Synthetic (InvivoGen)	$<3 \times 10^{-5}$ EU/μg
Endotoxin from *P. aeruginosa*	4	*P. aeruginosa* (Sigma-Aldrich)	1660 EU/μg[d]
Flagellin	5	*S. typhimurium* (InvivoGen)	6×10^{-5} EU/ng

[a] Study with synthetically produced peptidoglycan indicates that signalling may be TLR2 independent and actually proceed through NOD1 and NOD2 (Girardin et al., 2003).
[b] Determined using turbidimetric BET unless otherwise stated.
[c] Established through international collaborative study using chromogenic-kinetic and turbidimetric LAL assay (Findlay et al., 2015).
[d] Determined by chromogenic-kinetic BET assay.

with 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, MEM non-essential amino acids, 2 mM L-glutamine and 2% (*v*/*v*) human AB serum) with 5 EU/ml heparin (Sigma-Aldrich). The PBMCs were isolated by carefully layering 30 ml fractions over 17 ml of histopaque-1077 (Sigma-Aldrich) which was then centrifuged at 340 g for 45 min at 20 °C. The PBMC layer was isolated and washed three times with culture medium with cells centrifuged at 340 g for 15 min for the first wash and 10 min for the subsequent two washes. Finally, the remaining cell pellet was resuspended in 40 ml of culture medium.

2.2.3. Cryopreservation of PBMCs from LRSCs

Cells isolated from the LRSCs were processed as previously described with some modifications (Koryakina et al., 2014). The isolated PBMCs were counted and viability assessed with Trypan blue using a haemocytometer or Countess™ automated cell counter (Invitrogen, Life-Technologies, Carlsbad, CA, USA) then centrifuged at 340 g for 10 min at 4 °C. The cell pellet was carefully resuspended by slowly adding chilled human AB serum (2–8 °C) to a concentration of 6×10^7 cells/ml followed by an equal volume of chilled (2–8 °C) RPMI supplemented with 2 mM L-glutamine and 16.28% DMSO (*v*/*v*) to obtain a final concentration of 3×10^7 cells/ml. The cell suspension was split into 1 ml aliquots in cryovials and frozen using a Kryo10 rate-controlled freezer (Planer, Sunbury-On-Thames, Surrey, UK) using the program described by (Koryakina et al., 2014). Following completion of the cryopreservation program, the vials were stored at −80 °C overnight then transferred to vapour phase nitrogen for long term storage.

2.2.4. MAT using fresh WB

Assays were carried out as previously described (Daneshian et al., 2009; Hoffmann et al., 2005). Briefly, blood from 4 donors was collected as described in Section 2.1.2 and used in the assay within 4 h. The blood was inverted slowly several times to ensure thorough mixing before use. For each donor, 20 μl of pyrogen dilution series or control was added in quadruplicate to a 96-well flat-bottomed polypropylene cell culture plate (Greiner Bio-One), followed by 100 μl of sterile 0.9% (*w*/*v*) saline (Freserius Kabi) then 20 μl of WB per well. Each well was mixed gently 5 times using a pipette. The plate was incubated at 37 °C for 16–20 h in humidified atmosphere, 5% (*v*/*v*) CO_2.

2.2.5. MAT using fresh PBMC

Assays were carried out as previously described (Hoffmann et al., 2005; Gaines Das et al., 2004). PBMCs were isolated from WB as described in Section 2.2.2 with the exception that undiluted WB was layered over histopaque-1077 (Sigma-Aldrich), and PBS subsequently used to wash cells. Isolated PBMCs were counted using Trypan blue using a Countess™ automated cell counter (Invitrogen), and diluted to 6.25×10^5 cells/ml in culture medium. 200 μl of cell suspension were added per well of 96-well round-bottomed polystyrene cell culture plate (Corning, Acton, MA, USA), followed by 50 μl of pyrogen dilution series or control added in quadruplicates. The plate was incubated at 37 °C for 16–20 h in humidified atmosphere, 5% (*v*/*v*) CO_2.

2.2.6. MAT using cryopreserved PBMCs from LRSCs

Assays were carried out as previously described with minor modifications (Hoffmann et al., 2005; Gaines Das et al., 2004). A vial of cryopreserved PBMCs prepared as described in Section 2.2.3 was thawed by warming in a water bath (37 ± 1 °C) for about 2 min until only a small piece of ice remained. The contents were then transferred to a pre-chilled 50 ml falcon tube, and 30 ml chilled (2–8 °C) wash medium added (RPMI supplemented with 1% (*v*/*v*) human AB serum) dropwise for 2 ml with gentle swirling, followed by the remaining 28 ml. The cell suspension was centrifuged at 240 g for 8 min with minimal breaking at 10 °C. The supernatant was discarded and the cell pellet resuspended in 10 ml culture medium and the viability and cell number measured using Trypan blue using Countess™ automated cell counter (Invitrogen). The cell suspension was diluted to 1×10^6 cells/ml in culture medium and 125 μl added per well of a 96-well round-bottomed polystyrene cell culture plate (Corning), followed by 125 μl of pyrogen dilution series or control added in quadruplicates. The plate was incubated at 37 °C for 16–20 h in humidified atmosphere, 5% (*v*/*v*) CO_2.

2.2.7. Determination of IL-1β and IL-6 concentration

Supernatants from the cell culture were collected and analysed for IL-1β and IL-6 content directly using sandwich ELISAs or stored at −30 °C until assayed. IL-6 antibody pair (in house) were used to quantify IL-6 in cell culture supernatants as described previously (Hoffmann et al., 2005; Gaines Das et al., 2004). IL-1β ELISA was performed as described by manufacturer. TMB was used as HRP substrate and the reaction stopped using 1 M H_2SO_4. The absorbance values were measured at 450 nm using 540 nm as a reference wavelength using SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was generated in each assay using IL-1β and IL-6 IS. Both ELISAs showed good regression over dose ranges from 31.25–4000 pg/ml with intra-assay CVs of the order 2–11%.

2.2.8. Data analysis

Dose–response curves were analysed by non-linear regression using GraphPad Prism version 5.01 for Windows (GraphPad Software) with sigmoidal dose–response (variable slope) fitting. Estimates of relative potency of non-endotoxin pyrogens to endotoxin IS could not be performed by parallel-line analysis as the fitted curves were generally not parallel. The half maximal effective concentration (EC_{50}) was therefore used instead as a measure of sensitivity and was determined by interpolation from the fitted curve at half the maximum cytokine release. The limit of detection (LOD) was calculated as described in the European Pharmacopoeia 8.0 from the mean of four replicates of the blank added to 3 standard deviations of the four replicates against the standard curve of endotoxin IS 10/178 (European Pharmacopoeia 8.0, 2010b). Significance was calculated by 2-way ANOVA and t-test, 2-tailed with equal variance, paired or unpaired as appropriate.

2.2.9. Determination of TLR1 single nucleotide polymorphism (SNP)

Genomic DNA was isolated from donor samples using QIAamp DNA Mini Kit (QIAGEN, Venlo, Limburg, Netherlands). PCR was used to amplify regions covering the SNPs rs4833095 and rs5743618, exon 4 of TLR1 on chromosome 14, Database of Single Nucleotide Polymorphisms (dbSNP) (2015), from the genomic DNA using the following primer pairs: rs4833095 Forward 5′-ATAAGTGT CTCCAACTCAGTAAGGT-3′, rs4833095 Reverse 5′-CAAGACTGTAGC AAATCTGGAACT-3′, and rs5743618 Forward 5′-CCCAGAAAGAATCG TGCCCACTATA-3′, rs5743618 Reverse 5′-CCTTCCTGGATGTGGCAG CTTTAG-3′. Sequencing of the PCR products was performed using the aforementioned primers by DBS Genomics (University of Dundee, Angus, UK) and results analysed by ContigExpress (Vector NTI Advance 11.5.3 for Windows, Invitrogen).

3. Results

3.1. Cryopreservation of PBMCs from LRSCs

Typically, 0.5–1.0 × 10^9 PBMCs were obtained per LRSC, with an average cell viability of 99% (CV 0.44%). This allowed for banks of 17 to 34 vials with 1 ml aliquots of 3 × 10^7 cells to be produced for each donor, with an average bank size of 26 vials. Cell concentrations of 1, 2 and 3 × 10^7 cells/ml were evaluated but no difference in cell activity in response to endotoxin, viability or clumping of cells following thawing was observed. The higher concentration was therefore used for convenience, as with recovery of above 70% viable cells following thawing this provided a sufficient number of cells for a typical MAT assay. In preliminary experiments, there was no difference in the IL-6 released on stimulation with endotoxin observed with cells used prior to and following cryopreservation (data not shown).

3.2. Long-term stability of cryopreserved PBMCs

The long-term stability of the cryopreserved PBMCs was established by thawing aliquots from the same 8 donors at several time-points following cryopreservation and monitoring the response to the endotoxin IS and Pam_3CSK_4 (TLR4 and TLR1/2 ligands, respectively). Each donor exhibited reproducible responses to the pyrogens with no overall trend in the sensitivity as observed by the half maximal effective concentration (EC_{50}) and maximum IL-6 level released (EC_{50} CV 11–33%, maximum IL-6 CV 11–32%), the former illustrated in Fig. 1. Additionally there was no notable difference in the viability of the cells, or the number of cells isolated per vial with time.

3.3. Comparison of fresh WB, PBMC and cryopreserved PBMCs from LRSCs as a source of monocytes for the MAT

The level of IL-1β and IL-6 produced in response a panel of pyrogens with each system was measured, the results of which are illustrated in Fig. 2. All three systems were able to detect the ligands included, displaying dose-dependent cytokine responses. For all non-endotoxin pyrogens, the response observed could not be attributed to contamination by endotoxin as evidenced by the estimated endotoxin content determined by BET assay, summarised in Table 1.

Visual inspection indicated that for the WB assay, the highest cytokine readout with regards to the LOD, EC_{50} and maximum cytokine released with all pyrogens in the panel was IL-1β. In contrast, this was found to be IL-6 with the assays employing fresh PBMC and cryopreserved PBMCs from LRSCs. This confirmed previous findings and are the most common assay setups currently employed (Daneshian et al., 2009; Hoffmann et al., 2005; Koryakina et al., 2014; Solati et al., 2015). Therefore these were the assay formats deemed most appropriate for inclusion in further analyses.

The LOD of endotoxin IS 10/178 with all systems were comparable, with the WB/IL-1β system the most sensitive at 0.007 EU/ml compared to fresh PBMC and cryopreserved PBMC from LRSCs with IL-6 at 0.022 and 0.013 EU/ml respectively. The EC_{50} and maximum cytokine release were determined to allow a comparison of the performance of the three assay systems in more detail (summarised in Fig. 3 and Table 2) providing indications of both the sensitivity and magnitude of the cytokine release.

3.4. Inter-individual differences and system sensitivities

Considerable donor-to-donor variation was observed in each of the three assay formats in regards to EC_{50} and the maximum cytokine released with every pyrogen tested as illustrated in Fig. 3 and summarised in Table 2. The ligands, Poly(I:C) and Flagellin were excluded from this analysis due to the plateau of the dose–response curve not having been reached with the WB and fresh PBMC systems with Flagellin, and the generally low cytokine release with Poly(I:C) (Fig. 2E and G). The variation observed was generally in a similar range for each of the different systems for both the EC_{50} and maximum cytokine released as summarised in Table 2. This ranged from an inter-donor CV of 10 to 65% with no discernible pattern to the degree of variation. Each of the systems ranked the pyrogenicity of the ligands by EC_{50} as follows: Endotoxin IS > Endotoxin from *P. aeruginosa* > Pam_3CSK_4 > Peptidoglycan > LTA, apart from the cryopreserved PBMCs where LTA was marginally more pyrogenic than peptidoglycan ($P = 0.0354$), Fig. 3A and Table 2A.

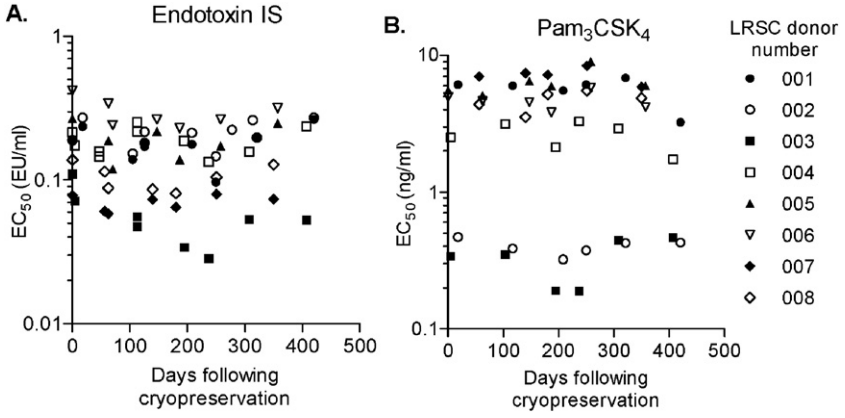

Fig. 1. Stability of cryopreserved PBMCs from LRSCs with time. EC_{50} of PBMCs with Endotoxin IS (A) and Pam_3CSK_4 (B) from 8 different LRSCs, up to 420 days following cryopreservation. Illustrates that no overall trend in the EC_{50} values was observed on stimulation with either pyrogens as would have been expected if the quality of the cells had been compromised.

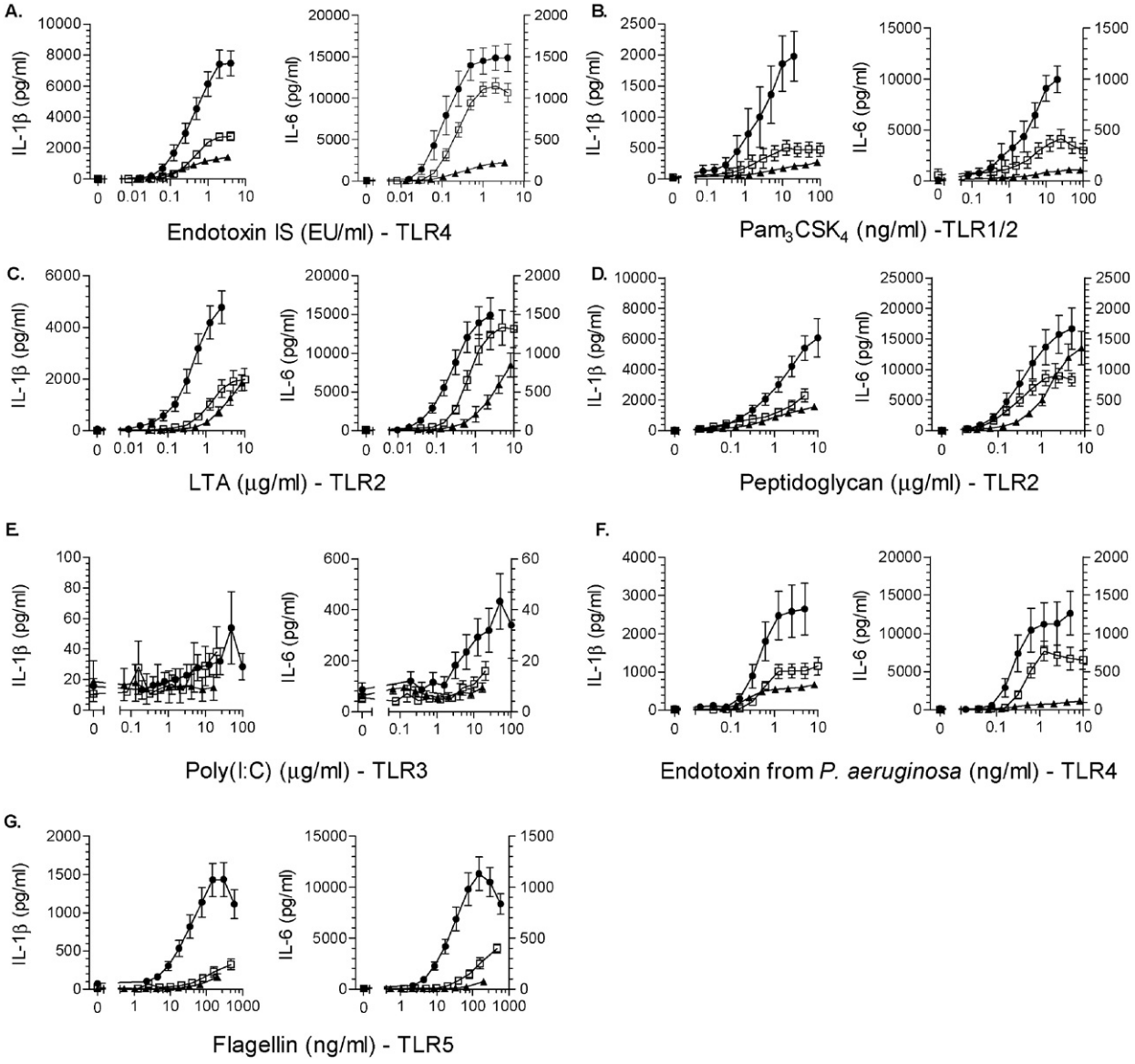

Fig. 2. Production of IL-1β and IL-6 after stimulation of fresh WB (▲), PBMC (□) and cryopreserved PBMCs from LRSCs (•) with pyrogen panel: A. Endotoxin IS 10/178 from *E. coli* O113:H10:K-; B. synthetic Pam_3CSK_4; C. LTA from *S. aureus*; D. Peptidoglycan from *S. aureus*; E. synthetic Poly(I:C); F. Endotoxin from *P. aeruginosa*; G. Flagellin from *S. typhimurium*. WB/IL-6 plotted on right hand axis. Data presented as means ±SEM (n = 8, from 2 independent experiments).

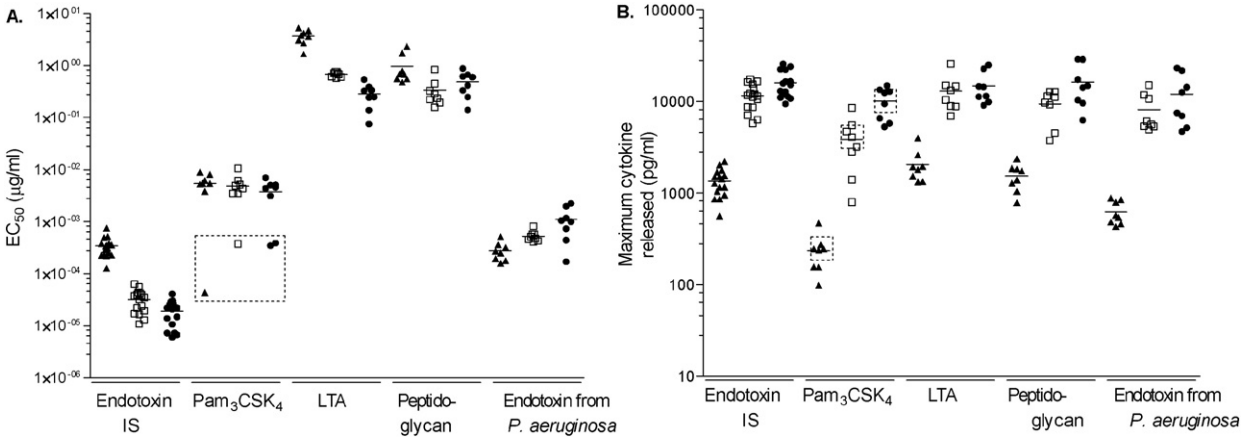

Fig. 3. EC_{50} (A) and maximum cytokine release (B) following stimulation of fresh WB/IL-1β (▲), PBMC/IL-6 (□) and cryopreserved PBMCs from LRSC/IL-6 (•) with pyrogen panel. The same 8 donors were used with WB and PBMC assay. Poly(I:C) and flagellin were not included in this analysis as cytokine levels were too low or the plateau had not been reached. Dashed boxes highlight high responders to Pam_3CSK_4. Averages, CV% and significance levels calculated for each pyrogen and assay system summarised in Table 2 for clarity.

Table 2
Summary of average EC_{50} (A) and maximum cytokine released (B) and corresponding inter-donor CVs and significance levels for each MAT system and pyrogen displayed in Fig. 3.

A.	EC_{50} (ug/ml)								
	WB/IL–1β			PBMC/IL–6			LRSC/IL–6		
	Average	CV (%)	Significance	Average	CV (%)	Significance	Average	CV (%)	Significance
Endotoxin IS	0.00026	29		0.000028	61		0.000018	49	
Pam_3CSK_4	0.0054	47		0.0048	56		0.0037	58	
LTA	3.6	30		0.68	10		0.29	47	
Peptidoglycan	0.98	65		0.34	61		0.49	47	
Endotoxin *P. aeruginosa*	0.00028	39		0.00053	23		0.0011	60	

B.	Maximum cytokine release (pg/ml) WB/IL–1β ANOVA $P = 1.48 \times 10^{-8}$			PBMC/IL -6			LRSC/IL–6		
	Average	CV (%)	Significance	Average	CV (%)	Significance	Average	CV (%)	Significance
Endotoxin IS	1594	25	ANOVA $P = 1.48 \times 10^{-8}$	11457	32	ns, ANOVA P = 0.0798	16429	33	ns, ANOVA P = 0.268
Pam_3CSK_4	230	48		4227	59		10093	35	
LTA	2058	40		12984	43		14821	38	
Peptidoglycan	1537	31		9452	35		16271	49	
Endotoxin *P. aeruginosa*	624	28		8127	45		12035	57	

Significance levels: ns $P > 0.05$, * $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$, **** $P \le 0.0001$.

3.4.1. Detection of different endotoxin forms

Using the chromogenic-kinetic BET assay the potency of the *P. aeruginosa* endotoxin was estimated at 1660 EU/μg, compared to the assigned 8333.3 EU/μg of the IS, a five-fold difference in potency. In accordance with previous findings, the difference in potency observed with the fresh and cryopreserved PBMC was even more marked with an average difference of 25 and 65 fold in EC_{50} values respectively ($P = 6.31 \times 10^{-6}$ and 0.0034 respectively). In contrast, this difference in sensitivity was not observed with the WB assay where there was no significant difference in the EC_{50} observed for each donor with the two different endotoxin forms ($P = 0.7179$).

3.5. Inter-individual difference in response to Pam_3CSK_4

When comparing the EC_{50}'s observed with the TLR1/2 ligand Pam_3CSK_4, one particular donor when used in the WB and fresh PBMC assay and two donors used in the cryopreserved PBMC assay showed a markedly lower EC_{50} compared to the other donors (highlighted in Fig. 3A), indicating a higher sensitivity to this pyrogen. This difference between the donors was not observed with the maximum cytokine levels released, illustrated in Fig. 3B. This phenomenon was explored further by assessing an additional 2 new donors with both the WB and fresh PBMC system with Pam_3CSK_4. Of these, one donor was found to show the higher level of sensitivity while the other produced similar activity to the majority of donors. This was the case with the WB and fresh PBMC assay systems and with both IL-1β and IL-6 used as the readout.

In order to assess if this effect was transitory or an inherent property of the donors' cells, fresh blood donations were taken from the two high and four of the low responders and their reactivity to Pam_3CSK_4 reassessed. Their response to endotoxin was monitored in parallel to exclude a change in the overall activity of the cells. All donors maintained the response to Pam_3CSK_4 with very little change in the sensitivity as represented by the EC_{50}'s presented in Fig. 4. The response to endotoxin was also unchanged from the original donation.

3.5.1. Sequencing of SNP of TLR1

The maintenance of sensitivity of the donors to Pam_3CSK_4 supported the conclusion that the striking difference in reactivity was due to an inherent property of the cells, not a transient activation. Pam_3CSK_4 is detected by a heterodimer of TLR1 and 2 (Jin et al., 2007). As there was no difference in activity observed with these donors compared to the others when probed with TLR2 ligands, LTA and Peptidoglycan, it was hypothesised that the difference in responsiveness to Pam_3CSK_4 was most likely due to variation of the TLR1 protein. This was further investigated by sequencing two commonly observed SNPs in the sequence of TLR1 of the donors used in this study. Namely, SNPs rs4833095 and rs5743618 resulting in non-conserved amino acid changes N248S and

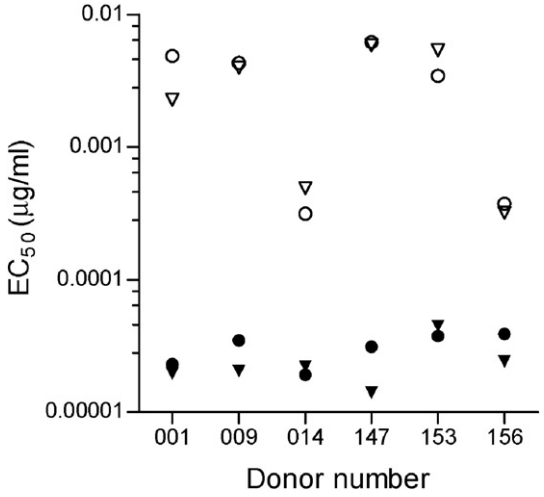

Fig. 4. EC_{50}'s from first (circle) and second (triangle) assay with endotoxin (filled symbol) and Pam_3CSK_4 (open symbol). Maintenance of sensitivity to Pam_3CSK_4 with repeated assay indicates a difference in inherent property of the donor cells of 014 and 156 compared to other donors.

I602S respectively (Johnson et al., 2007; Mikacenic et al., 2013). The results of the sequencing confirmed that the difference observed in sensitivity towards Pam_3CSK_4 correlated with a heterozygous phenotype of both of these SNPs. All low responders had a homozygous phenotype (Asn 248, Ser 602).

4. Discussion

This study set out with the aim to investigate if PBMCs isolated from LRSCs could serve as a source of monocytes to be utilised in the MAT. The isolation of PBMCs from the chambers proved trivial, with only gravity required to decant the cell suspension contained within. The cryopreservation procedure itself did not appear to alter the cells response to pyrogens, also recently observed by groups using PBMCs isolated from WB and lymphocyte filters (Solati et al., 2015; Koryakina et al., 2014). LRSCs therefore provide the distinct advantage over other currently used monocyte cell sources of delivering an ample, convenient, stable source of cells without the requirement of phlebotomy. This considerably reduces the time and effort required to set up an assay, and the improved assay reproducibility as a consequence of single donor cell banks should allow for more in-depth investigations into factors contributing to activity in the MAT, otherwise potentially masked by donor-to-donor variability. The PBMCs used in this study were isolated from the LRSCs within 10 h of the platelet apheresis procedure; however preliminary results indicate that PBMCs processed the following day are equally responsive (data not shown).

The isolation of PBMCs prior to cryopreservation allows for the removal of DMSO following thawing by washing before use in the assay, thereby minimising any activation or cytotoxicity related to DMSO (Xing and Remick, 2005; Schindler et al., 2004). This is not possible when using cryopreserved WB in the MAT (Daneshian et al., 2009; Schindler et al., 2004). Additionally, removal of red blood cells reduces any interference from haemolysis products (Figueiredo et al., 2007; Schindler et al., 2004).

With direct comparison to two internationally validated MAT systems using WB and freshly isolated PBMCs against a panel of pyrogens, the cryopreserved PBMCs isolated from LRSCs performed well. As expected, all systems tested were able to detect the TLR ligands included in the study with broadly comparable sensitivities in terms of EC_{50}'s. The only exception was the synthetic double stranded RNA viral mimetic and TLR3 ligand, Poly(I:C), which was undetectable using WB and only produced modest cytokine release with the fresh and cryopreserved PBMCs (Fig. 2E). Poly(I:C) has been used previously with a WB assay with substantial IL-6 release (Nakagawa et al., 2002). However, in other studies the response has been more moderate (Solati et al., 2015). This could be due to the high variation in the structure of Poly(I:C) preparations and its susceptibility to degradation which has been shown to affect TLR activation (Naumann et al., 2013).

Whilst using fresh PBMCs and cryopreserved PBMCs from LRSCs produced similar results overall, some differences were observed. The PBMCs from LRSCs released higher levels of cytokine, particularly IL-1β, with all the pyrogens tested compared to the fresh PBMCs. This may have been due to the inherent susceptibility of the donors used. However, it is more likely to originate from the environment experienced by the cells during the platelet apheresis procedure or in the LRSC. The cells would have been at high cell density for a period of time before PBMC isolation (more than 1×10^9 cells in about 10 ml of plasma) which may have affected protein expression (Hussain et al., 2015). Nonetheless, the sensitivity as revealed by the LOD and EC_{50} was highly comparable with the two monocyte cell sources. Additionally, neither system gave any suggestion that the cells had been pre-activated as indicated by the low background cytokine levels.

There were more apparent differences identified when using the WB in the assay. Although releasing quantifiable levels of cytokine, the WB system produced distinctly lower levels than using the PBMCs with all pyrogens tested, especially considering the IL-6 released. This is perhaps not unexpected as the 1/12 dilution required in order to achieve an optimal balance of cytokine release and inflammatory suppressor factors such as α-1-antitrypsin, will have resulted in approximately 6 fold fewer mononuclear cells present compared to the PBMC assay (Pott et al., 2009; Daneshian et al., 2009). Furthermore, whilst the PBMCs showed higher sensitivity towards the endotoxin isolated from *E. coli* compared to *P. aeruginosa* as has been observed previously (Koryakina et al., 2014; Dehus et al., 2006), this was not the case with WB. These differences cannot be explained through variation in the donors as the WB and fresh PBMCs used for the two assays were from the same blood donations.

Whilst the assays perform differently in terms of cytokine readouts, it is not possible to conclude which most accurately reflects the in vivo situation that the MAT is used to model. However, as a convenient and reliable source of monocytes, this study demonstrates that cryopreserved PBMC from LRSCs are able to detect both endotoxin and non-endotoxin pyrogens equally well to both the WB and fresh PBMC systems.

It might be tempting to pool cells from multiple donors in order to overcome the high degree of donor-to-donor variation observed with all monocyte cell sources and produce larger cell banks. However, caution should be exercised with this approach. As was observed with the response to the TLR1/2 ligand Pam_3CSK_4, some donors are much more sensitive than others to potential contaminants due to inherent genetic predisposition, which could be masked by pooling donations. The TLR1 polymorphism observed in this study associated with higher sensitivity affects the minority of the European population (up to 20%) whilst being at moderate to high frequency in the rest of the world (Heffelfinger et al., 2014; Hawn et al., 2007; Johnson et al., 2007). There have been several other polymorphisms identified within TLR protein coding regions resulting in nonsynonymous mutations, as well as base changes in the promoter regions controlling TLRs and cytokines with significant geographical variation in incidence, but several above 10% representation in the population (Barreiro et al., 2009). The vast majority of these have not had the functional consequence elucidated, but with many present in regions crucial to protein structure and function, or introducing stop codons, several are likely to have a significant impact on pyrogen sensing (Barreiro et al., 2009). It may therefore be prudent to maintain donor cells separately until the product and likely contaminants are understood in relation to the donor population, as well as the intended recipients of the therapeutic, minimising the risk of overlooking particularly susceptible individuals.

5. Conclusion

The LRSCs have been shown to be a valuable source of PBMCs which can be successfully employed in the MAT to detect endotoxin and non-endotoxin pyrogens. The cells performed well when compared to established (and internationally validated) methods using WB and fresh isolated PBMCs (Hoffmann et al., 2005). The creation of stable cryopreserved cell banks from individual donors, without the need for phlebotomy, should significantly improve the ease of use and reproducibility of the MAT, thereby facilitating its more widespread implementation over the RPT and BET.

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