

The monocyte activation test detects potentiated cytokine release resulting from the synergistic effect of endotoxin and non-endotoxin pyrogens

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Abstract

Pyrogens are classified in two groups, endotoxin pyrogens and non-endotoxin pyrogens (NEPs). The presence of either in parenteral pharmaceuticals or medical devices can cause severe harm to subjects, and when occurring in combination, synergistic potentiation effects can occur. As the standard *in vitro* pyrogen test, the *Limulus* Amebocyte Lysate (LAL) assay can detect LPS only, an endotoxin, but not NEPs. We tested whether the Monocyte Activation Test (MAT) that measures IL-6 induction, is suited for detecting synergistic pyrogen effects. Here we show that MAT reliably detects the NEPs heat-killed *Staphylococcus aureus*, R848 and lipoteichoic acid, in addition to LPS. When combinations of these pyrogens were tested, a potentiation of IL-6 production was seen beyond an additive effect, apparently reflecting on *in-vivo* synergisms. The current study therefore demonstrates that MAT not only is a reliable and reproducible assay for the sensitive detection of both endotoxin and non-endotoxin pyrogens, but also for identifying synergistic effects when parenteral drugs are contaminated with multiple pyrogens.

Keywords

Release testing, synergistic effect, non-endotoxin pyrogens, contamination, drug-release test, Monocyte Activation Test, parenteral drugs, pharmaceuticals, medical devices

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Introduction

Pyrogens that enter the human body by parenteral administration are recognized by the human monocytes' TLR and at sufficient concentrations activate signaling pathways that launch immune and inflammatory responses and release endogenous pyrogens, e.g., IL-6. This can lead to severe adverse reactions including fever, inflammatory reactions, multiple organ failure and, in some cases, even death.^{1,2} Pyrogens are classified in two groups of endotoxin and non-endotoxin pyrogens (NEPs), both groups of substances that can induce systemic inflammation.^{3,4} Endotoxins originate from the Gram-negative bacterial cell envelope and non-endotoxin pyrogens from the Gram-positive, in addition to viruses and fungi. Though the latter can be harmful to humans, not all pyrogen detection tests currently available are capable of detecting NEPs. The most common pyrogens are endotoxins, which are LPS produced by Gram-negative bacteria such as *Escherichia coli*.⁵ Pyrogens occurring in combinations may cause synergistic biological effects and a higher induction of cytokines that may cause health risks. This study focuses on this

phenomenon because it's the most likely situation in case of contamination.

Furthermore, the study presents how the use of the Monocyte Activation Test avoids the risk of undetected contamination in parenteral pharmaceuticals. The methods currently available for pyrogen testing are the *Limulus* Amebocyte Lysate (LAL) test,^{6,7} the Recombinant Factor C (rFC) Test,⁸ the Rabbit Pyrogen Test (RPT) and the Monocyte Activation Test (MAT).⁹ For the purpose of comparison further along in this paper, a brief description of each four of these tests is presented as follows. The LAL is based on a clotting reaction of the hemolymph derived from the horseshoe crab. Three variants of LAL methodologies are gel clot, chromogenic and turbidimetric.^{10,11}

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The rFC test is a non-animal mimic of the LAL. rFC is a genetically engineered protein, which is activated by endotoxins to produce a fluorescent end product which is quantifiable.¹² The RPT method involves measuring the possible rise in temperature of three rabbits following the intravenous injection of a test solution in each rabbit.¹³ In the final method, the MAT, a sample is incubated with human monocytes which produce cytokines/IL if pyrogens are present, that are detected in an ELISA. A more elaborate explanation of the MAT is given in the following section.

From a regulatory point of view, it is found that the requirement of the European Pharmacopoeia for producers is to stop using the RPT.¹⁴ More specifically, as of June 2021, the European Pharmacopoeia has committed to a 5 yr plan to see the RPT completely replaced.¹⁵ Moreover, in tests with the absence of any alternative *in-vitro* endotoxin pyrogen and non-endotoxin pyrogen test, it expressly recommends the use of the MAT as a risk assessment where BET is used for batch release or as a batch release assay in its own right, replacing RPT.¹⁴

The information above strongly motivates the current study to use the MAT as a pyrogen detection test and in the detection of synergistic stimulation by multiple pyrogens. The focus of the current study is the importance of the assay's ability to detect all endotoxin and non-endotoxin pyrogenic contaminants, as well as synergistic stimulation when the drug is contaminated with multiple pyrogens, and how this may affect patient safety. For example, vaccines that intrinsically contain both LPS and non-endotoxin pyrogens are in a high risk of this synergistic effect, and require testing for non-intrinsic pyrogenic, endotoxin and non-endotoxin contaminants.¹⁶

The hypothesis of the current study is that MAT with cryopreserved pooled PBMC is suitable for detecting synergistic stimulation, due to its ability to detect both endotoxin and non-endotoxin pyrogens, and the ability to detect synergistically mixed pyrogen-induced cytokines.

Materials and methods

Detection of synergistic effect is essential in the process of drug release testing. To investigate the synergistic effect, PBMC were stimulated with the individual and mixed pyrogens and IL-6 induction was measured for different conditions. The assay that is used for serial testing and release testing must also provide reproducible results and ensure that the contamination in the drug will be detected accurately, always. Therefore, eight different concentrations of endotoxin (LPS) and seven different concentrations of multiple non-endotoxin pyrogens (HKSA, R848 and lipoteichoic acid (LTA)) were tested in duplicate, with three independent batches of cryopreserved pooled PBMC, and the reproducibility of the results were determined using the two-way ANOVA-based analysis.

Performing MAT

The MAT is used to detect and quantify endotoxin and non-endotoxin pyrogenic contaminations, which activate human monocytes to release endogenous mediators.¹⁷ CTL-MAT cryopreserved human pooled PBMC was used as the source of monocytes and the test was performed by thawing cryopreserved pooled PBMC, co-culture overnight with a test substance in a humidified incubator at 37°C in the presence of 5% CO₂ for 19 h. Measuring IL-6 production was used as the readout. CTL-MAT kits have been used for this study.

Cryopreserved pooled PBMC

Human PBMC were isolated from the blood of healthy donors and pre-screened according to the Ph. Eur. 2.6.30 guidelines. Isolated PBMC were processed, pooled, and cryopreserved in culture medium within 4 h of collection. To perform MAT, cryovials containing cryopreserved pooled PBMC were taken out of the transfer liquid nitrogen tank (or dry ice), placed immediately in a water bath at 37°C to thaw until the last ice crystal is remaining. Thawed PBMC were immediately transferred from the vial and taken up in culture medium to make a homogeneous cell suspension (400.000 PBMC/ml). Finally, 100 µl cell suspension was added to 100 µl of the sample being tested, in 96-well flat-bottom microtiter plates.

Endotoxin standard

The European Directorate for the Quality of Medicines (EDQM) LPS *E. coli*, calibrated in International Endotoxin Units, was analysed using the four parameters logistic curve according to chapter 2.6.30 of the European Pharmacopoeia, MAT protocol, as a standard curve for endotoxin pyrogens.⁹ A 2000 EU/ml LPS master standard was prepared according to the Ph. Eur. Reference Standard leaflet, aliquoted (50 µl) in cryovials and stored at -80°C. To prepare the LPS standard curve, first, 950 µl culture medium was added to the thawed, 50 µl aliquoted LPS and vortexed to prepare 100 EU/ml LPS working standard. This was followed by diluting 100 EU/ml LPS in culture medium to generate a twofold dilution of LPS to the final concentrations ranging from 0.004-0.5 EU/ml in the plate.

Non-endotoxin pyrogens

To demonstrate the test's ability to detect NEPs reproducibly, three independent batches of cryopreserved pooled PBMC were used. MAT is carried out for different NEPs (HKSA, R848 and LTA) which can activate monocytes via TLR2, TLR7/8 and TLR2, respectively.

To show that the NEPs are pure, the batches used in this study were tested for the presence of endotoxin. HKSA, at the dilution of 30 million times, and R848, at the concentration of 10,000 ng/ml, were tested in a kinetic chromogenic LAL assay and were found negative for endotoxin (< 0.005 EU/ml). LTA was tested in MAT with 40 $\mu\text{g/ml}$ PMB (Polymyxin B Sulfate - Calbiochem, United States of America) with no significant difference in the LTA-induced IL-6 production (10 $\mu\text{g/ml}$ PMB is sufficient to eliminate the stimulation with 1 EU/ml LPS).

Heat-killed *Staphylococcus aureus* (HKSA) (heat-killed, formalin-fixed *S. aureus* cells; Calbiochem, United States of America) was diluted in the culture medium to generate a three-fold dilution of HKSA with the final dilution ranging from 0.03 million-fold diluted to 21.87 million-fold diluted in the plate.

One mg/ml R848 (Resiquimod) was prepared by resuspending lyophilized R484 (tlr-r848-5; Invivogen) with sterile endotoxin-free water. This was followed by mixing R848 (1 mg/ml) in culture medium to generate a threefold dilution of R848 with the final concentrations ranging from 14- 10,000 ng/ml in the plate.

One mg/ml LTA was prepared by re-suspending purified LTA from *S. aureus* (tlrl-pslta; Invivogen) with sterile endotoxin-free water. Next, LTA (1 mg/ml) was mixed in culture medium to generate a threefold dilution of LTA with the final concentrations ranging from 3–2000 ng/ml in the plate.

Testing synergistic effect

Cryopreserved pooled PBMC were stimulated with the defined amount of LPS (0.031 EU/ml), HKSA

(0.5 million-fold diluted) and R848 (200 ng/ml R848), separately. The pyrogen spiked amount was equal to or close to the middle dose of the individual pyrogen dose-response curve. In the same experiment, the PBMC were stimulated with mixed LPS and HKSA of the same amount, and mixed LPS and R848 of the same amount.

Synergistic effect in a biological model drug

Human albumin (5%) was spiked with LPS (0.031 EU/ml), HKSA (0.5 million-fold diluted) and R848 (200 ng/ml R848), separately. In the same experiment, human albumin was spiked with mixed LPS and HKSA of the same amount, and mixed LPS and R848 of the same amount. Next, human albumin spiked with individual and mixed pyrogen, was tested in quadruplicate in the MAT.

To study the shift in the response curve of contaminated human albumin 5% with mixed LPS and NEP compared to contaminated drug with LPS, an undiluted human albumin 5% was spiked with LPS (0.25 EU/ml), HKSA (0.1 million-fold diluted) separately and mixed. Next, four different dilutions of spiked human albumin with individual and mixed pyrogens were tested in triplicate in MAT.

Cytokine measurement

IL-6 concentration in the harvested supernatant was determined after 19 h of incubation in a humidified incubator at 37°C in the presence of 5% CO₂, using the CTL-MAT IL-6 ELISA kit, with the lower limit of quantitation (LLQ) of $< 1\text{pg/ml}$.

Statistical analysis

The IL-6 concentration was identified by two categorical predictor variables, different concentrations of LPS and NEPs, and three independent PBMC batches to conduct a two-way ANOVA test

The IL-6 concentrations were identified for mixed LPS, and NEPs compared to the theoretical additive values of individual LPS and NEPs using an independent two-samples t-test assuming unequal variances, 95% confidence interval.

Results

Inter-batch reproducibility of cryopreserved pooled PBMC upon testing endotoxin LPS

Three independent batches of cryopreserved pooled PBMC showed comparable reactivity upon different concentrations of LPS (two-way ANOVA, multiple comparisons; $F(2, 27) = 1.797$, $P = 0.185$, not significant), and a consistent limit of detection (LOD) < 0.004 EU/ml as presented in

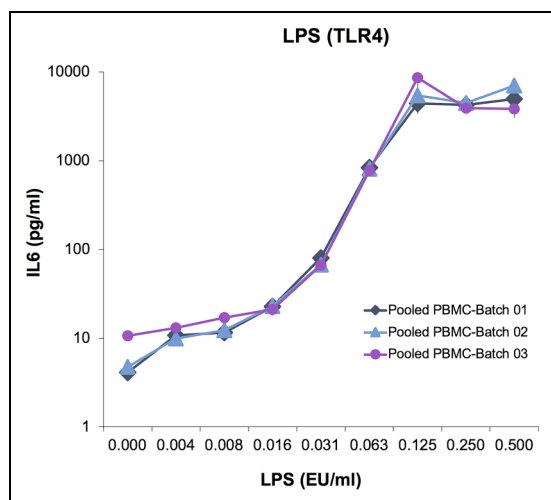


Figure 1. Inter-batch reproducibility of cryopreserved pooled PBMC upon testing endotoxin LPS. Three independent batches of cryopreserved 4-donor pooled PBMC were diluted in the culture medium to a final concentration of 400,000 PBMC/ml. PBMC were added to the LPS standard, with the final concentration ranging from 0.004 to 0.5 EU/ml.

Figure 1. Results are shown as mean \pm SD of duplicate measurements for the pooled PBMC.

Inter-batch reproducibility of cryopreserved pooled PBMC upon testing non-endotoxin pyrogens

Three independent batches of cryopreserved pooled PBMC showed comparable reactivity upon different dilutions of HKSA and a consistent limit of detection (LOD) $<$ 21 million-fold (0.005% V/V) as presented in Figure 2 (two-way ANOVA, multiple comparisons; $F(2, 24) = 2.360$, $P = 0.116$, not significant). Results are shown as mean \pm SD of duplicate measurements for the pooled PBMC.

As presented in Figure 3, three independent batches of cryopreserved pooled PBMC showed comparable reactivity upon different concentrations of R848 and a consistent limit of detection (LOD) $<$ 14 ng/ml (two-way ANOVA, multiple comparisons; $F(2, 24) = 0.2804$, $P = 0.75779$, not significant). Results are shown as mean \pm SD of duplicate measurements for the pooled PBMC.

As presented in Figure 4, three independent batches of cryopreserved pooled PBMC showed comparable reactivity upon different concentrations of LTA and a consistent limit of detection (LOD) $<$ 3 ng/ml (two-way ANOVA, multiple comparisons; $F(2, 24) = 3.035$, $P = 0.0668$, not significant). Results are shown as mean \pm SD of duplicate measurements for the pooled PBMC.

In summary, these results present a highly reproducible MAT which is of great importance for further investigation of the synergistic effect of mixed pyrogens.

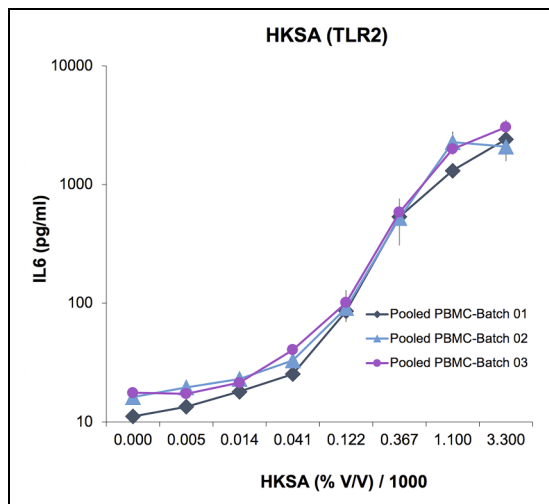


Figure 2. Inter-batch reproducibility of cryopreserved pooled PBMC upon testing HKSA. Three independent batches of cryopreserved 4-donor pooled PBMC were diluted in the culture medium to a final concentration of 400,000 PBMC/ml. PBMC were added to HKSA, with the final dilution ranging from 0.03 million-fold diluted to 21.87 million-fold diluted.

Synergistic effect by mixed pyrogens in a model biological drug

Cryopreserved pooled PBMC were stimulated in quadruple with the defined amount of LPS, HKSA and R848 separately and released cytokine (Figure 5). In the same experiment, the PBMC were stimulated with mixed LPS and

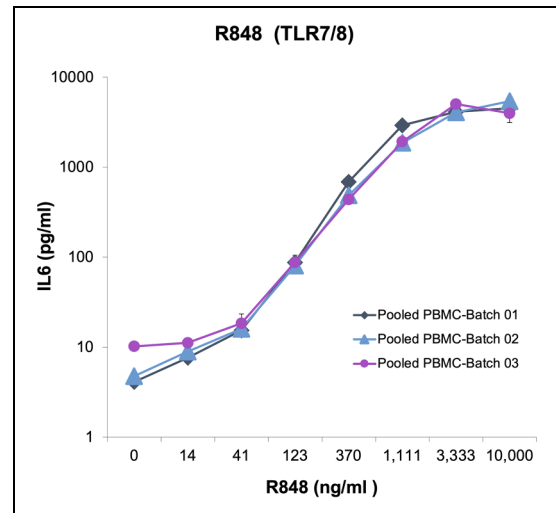


Figure 3. Inter-batch reproducibility of cryopreserved pooled PBMC upon testing R848. Three independent batches of cryopreserved 4-donor pooled PBMC were diluted in the culture medium to a final concentration of 400,000 PBMC/ml. PBMC were added to R848, with the final concentration ranging from 14 to 10,000 ng/ml.

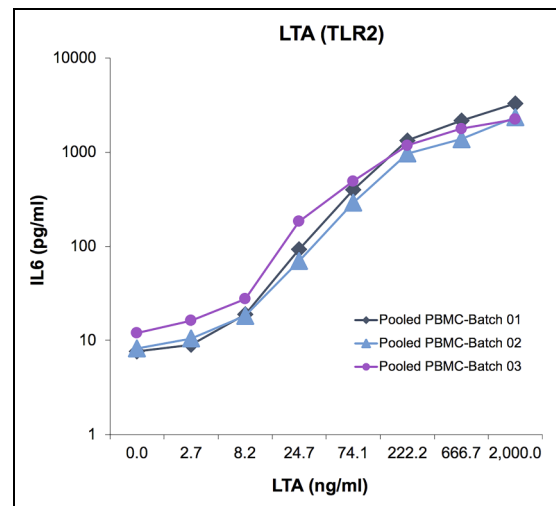


Figure 4. Inter-batch reproducibility of cryopreserved pooled PBMC upon testing purified LTA from *S. aureus*. Three independent batches of cryopreserved 4-donor pooled PBMC were diluted in the culture medium to a final concentration of 400,000 PBMC/ml. PBMC were added to LTA, with the final concentration ranging from 3 to 2000 ng/ml.

HKSA of the same amount. In case the IL-6 production by this mixed LPS and HKSA would have an additive nature, this would mean that the combination of pyrogens would result in the sum of individuals ($70 + 104 = 174$ pg/ml IL-6). But a much higher IL-6 production was observed of about four times the amount of what simply an additive nature would show, reflecting the synergistic stimulation of the cells by multiple pyrogens (828 pg/ml IL-6). There was a significant difference between the expected additive value of mixed LPS and HKSA induced IL-6 production ($M = 173.07$, $SD = 21.65$) and the measured, mixed LPS and HKSA induced IL-6 production ($M = 828.73$, $SD = 129.18$), $t(3) = 10.01$, $P = 0.002$. A similar synergistic effect was observed for mixed LPS and R848, which induced an IL-6 production of two times higher than an additive amount. There was a significant difference between the expected additive value of mixed LPS and R848 induced IL-6 production ($M = 135.98$, $SD = 11.59$) and the measured, mixed LPS and R848 induced IL-6 production ($M = 297.89$, $SD = 29.10$), $t(3) = 10.34$, $P = 0.0004$ (presented with the blue indicator in Figure 5). Seeing this synergistic effect of multiple pyrogens when tested in medium, lead to further investigation to find out the effect when a biological drug is contaminated with multiple pyrogens. Therefore, the same amount of LPS, HKSA, and R848 was spiked in human albumin 5% with a defined dilution (f1). There was a significant difference between the additive value of mixed LPS and HKSA induced IL-6

production ($M = 247.98$, $SD = 30.96$) and the measured, mixed LPS and HKSA induced IL-6 production ($M = 1040.91$, $SD = 406.61$), $t(3) = 3.89$, $P = 0.03$. Moreover, there was a significant difference between the expected additive value of mixed LPS and R848 induced IL-6 production ($M = 221.31$, $SD = 16.48$) and the measured, mixed LPS and R848 induced IL-6 production ($M = 666.99$, $SD = 96.00$), $t(3) = 9.15$, $P = 0.003$. The results present that the synergistic effect of mixed LPS with NEPs in the drug was even higher and produced more IL-6, compared to the medium (presented with the green indicator in Figure 5).

Dose response curve of contaminated drug

As presented in Table 1, the triplicate measured values for IL-6 production induced by human albumin spiked with mixed LPS (0.25 EU/ml) and HKSA (0.1 million-fold diluted), compared to the triplicate additive values for IL6 production induced by human albumin spiked with LPS (0.25 EU/ml), and HKSA (0.1 million-fold diluted), demonstrated a significantly higher value for each of the four different tested dilutions of human albumin. Furthermore, there is a significant difference in the dose-response curve of tested human albumin when contaminated with mixed LPS and HKSA, compared to LPS or HKSA alone, and also theoretical additive values of individual pyrogens as shown in Figure 6 (two-sample paired

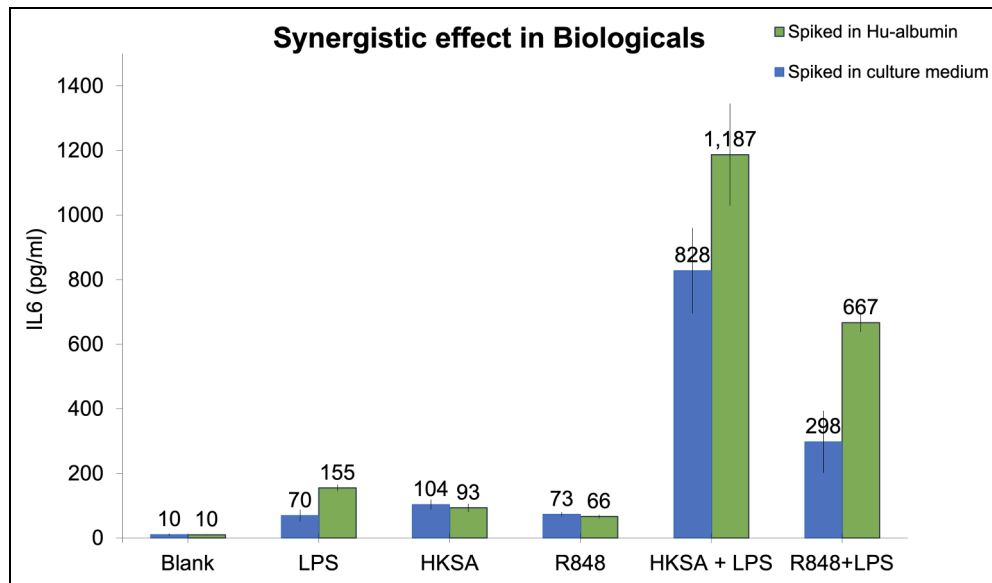


Figure 5. To study the synergistic effect by mixed pyrogens, cryopreserved pooled PBMC were stimulated with LPS (0.031 EU/ml), HKSA (0.5 million-fold diluted) and R848 (200 ng/ml R848) separately. In the same experiment, the PBMC were stimulated with mixed LPS and HKSA of the same amount and with mixed LPS and R848 of the same amount. IL-6 concentration in the harvested supernatant was determined (presented with the blue indicator). Additionally, to study the synergistic stimulation due to possible contamination by mixed pyrogens in human albumin 5%, a defined dilution (f1) of this drug is spiked by the same amount of LPS, HKSA and R848 individually and mixed. Spiked albumin is incubated with PBMC and IL-6 concentration in the harvested supernatant is determined (presented with the green indicator). Results are shown as mean \pm SD of quadruplicate measurements for the pooled PBMC.

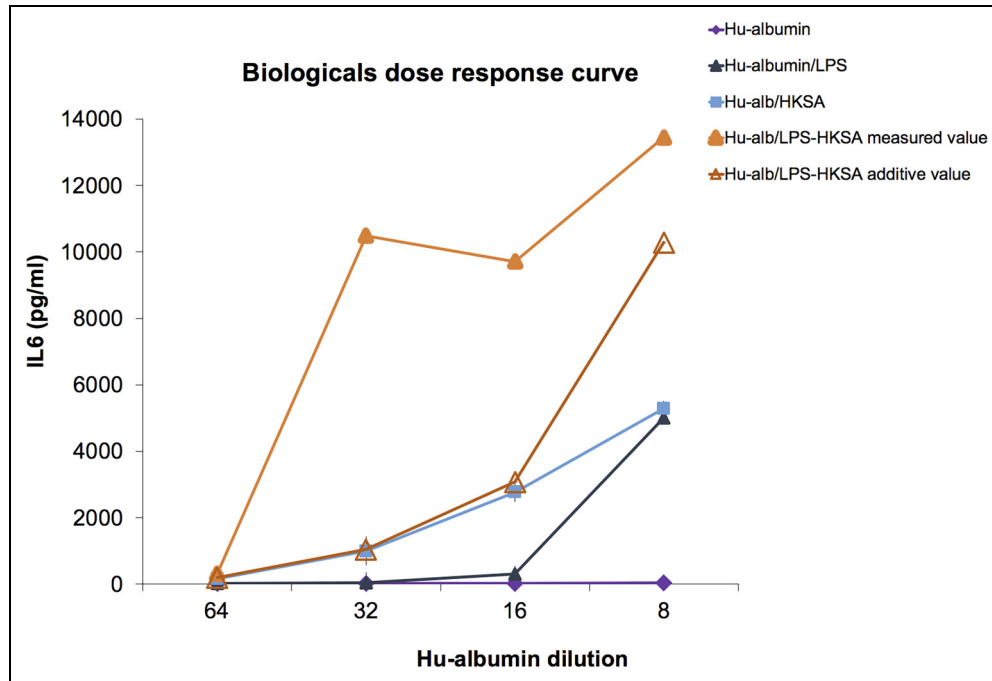


Figure 6. To study the shift in dose-response curves of contaminated human albumin 5% with LPS and HKSA alone compared to contaminated drug with mixed LPS and NEP, an undiluted human albumin 5% is spiked with LPS (0.25 EU/ml), HKSA (0.1 million-fold diluted) separately and mixed. Cryopreserved pooled PBMC is incubated with four different dilutions of spiked human albumin with individual and mixed pyrogens, and IL-6 concentration in the harvested supernatant was determined. As shown in this Figure, the observed dose response curve of contaminated human albumin 5% with mixed LPS and HKSA is significantly different from theoretical additive values, presenting the synergistic effect of mixed pyrogen in this drug. Results are shown as mean \pm SD of triplicate measurements for the pooled PBMC.

Table 1. Shift in the dose-response curve of contaminated human albumin with mixed LPS and HKSA.

Human albumin dilution	IL-6 production (pg/ml)				Significant different
	Measured value		Additive value		
	M	SD	M	SD	
8	13447	236	10284	135	$t(3) = 20.2,$ $P = 0.0001 < 0.05$
16	9709	111	3077	52	$t(3) = 94.0,$ $P = 0.0000001 < 0.05$
32	10489	3447	1043	42	$t(3) = 4.75,$ $P = 0.020 < 0.05$
64	358	18	178	23	$t(3) = 8.8,$ $P = 0.006 < 0.05$

t-test for means, significant, $t(3) = -2.383$, $P = 0.049$, theoretical additive values and measured values for mixed pyrogens.

Discussion

In this study, we incubated endotoxin LPS and non-endotoxin pyrogens such as HKSA, R848 and LTA with cryopreserved pooled PBMC. The IL-6 concentration measured in the harvested supernatant showed a high reactivity and sensitivity

of the assay to detect these pyrogens, which confirms the ability of MAT to detect endotoxin as well as non-endotoxin pyrogens. In addition, all these pyrogens have been tested with three independent batches of cryopreserved pooled PBMC and provided highly reproducible results.

To investigate the synergistic effects of mixed pyrogens, cryopreserved pooled PBMC were stimulated with LPS, HKSA and R848 separately, and released cytokine measured in the harvested supernatant. In the same experiment,

the PBMC were stimulated with different combinations of mixed pyrogens such as LPS with HKSA and LPS with R848. As it was shown in the results, IL-6 production upon mixed pyrogens was much higher than the sum of individuals. The IL-6 induction with mixed LPS and HKSA was four times higher than expected, and for mixed LPS and R848 was two times higher. These results confirm the dynamic interaction between different pyrogens that cause synergistic stimulation of monocytes, resulting in the significantly greater release of cytokines. This study has shown that the observed dose-response curve of contaminated human albumin 5% with mixed LPS and HKSA is significantly different than the individual and theoretical additive values, suggesting the synergistic effect of mixed pyrogen in this drug.

Seeing this synergistic effect of multiple pyrogens when tested in medium led to further investigation to find out the effect when a biological drug is contaminated with multiple pyrogens. Therefore, the same amount of LPS, HKSA and R848 were spiked in human albumin 5% with a defined dilution (f1). The synergistic effect of mixed LPS with NEPs in the drug also occurred and was even higher, producing more IL-6, compared to medium. This is an indication of possible additional effects of the drug that can specifically enhance the synergistic effect, and consequently may pose a higher risk to the recipient of the drug.

In future experimental work, more products with different formulations should be tested by spiking them with various individuals and combinations of endotoxin and non-endotoxin pyrogens. The aim of the following study is to investigate the synergistic behavior of individual drugs and to find out whether there is any categorization of drugs when it comes to synergistic effects. Furthermore, it must be investigated whether this phenomenon is pyrogen-specific and whether different combinations have a different effect.

The MAT, using cryopreserved PBMC as cell-source, was selected as the test method for this study. The synergistic stimulation of multiple pyrogens has been observed in the current study, using the MAT. The experiments in the current study also showed that mixed pyrogens, when spiked in a biological drug, show a higher synergistic effect compared to when spiked in medium, and demonstrate the consequences of possible contamination by endotoxin and NEPs. There are manufacturers using BET/LAL or rFC as a stand-alone batch release test. Those assays can detect endotoxins, but not NEPs, nor the synergistic stimulation of multiple pyrogens and the resulting high IL-6 production that is significant. In addition, in case the endotoxin contamination in the drug would be lower than the CLC (limit concentration of contaminants) of the drug, this would allow the drug to be released to the market without detecting the entirety of the contamination. This can be a serious health risk for patients. Furthermore, previous studies reported that the MAT response to endotoxins did not always correlate

with LAL assay results and the *Limulus* test may indicate false negative values.^{18,19} In such cases, patients are at serious health risk and administration of this drug can cause severe inflammatory reactions. This advocates for the necessity of using MAT as a final batch release test, given its capabilities to prevent these situations. The use of this assay allows for the detection of all endotoxin and non-endotoxin pyrogenic contaminants and the synergistic stimulation if the drug is contaminated with multiple pyrogens, minimizing the risk of undetected pyrogens that are harmful for patients.

Due to the great efforts of international validation studies comparing the RPT and the MAT, this test has been proven to be not only comparable to RPT, but also more sensitive and specific.²⁰ and in contrast to RPT, applicable to all parenteral products.²¹ Given the fact that EP recently committed to replacing RPT within the next five years, the statement of the General Chapter on the Rabbit Pyrogen Test (2.6.8.) to replace the [rabbit] pyrogen test by the MAT wherever possible and after product-specific validation,⁶ and the aforementioned benefits of this study, MAT proves to be a qualified replacement for RPT.

MAT based on fresh and cryopreserved whole blood assay and fresh PBMC has been fully and successfully validated for endotoxin and NEPs.²⁰⁻²² Though cryopreserved PBMC was not involved as a cell-source in these international validations, advantages of MAT based on cryopreserved PBMC have been demonstrated in recent years in research and development.¹⁷

Conclusions

The MAT does not only detect NEP contaminants in addition to endotoxin, but also the eventual synergistic stimulation if the parenteral drug is contaminated with multiple pyrogens. MAT using cryopreserved pooled PBMC has proven to be an effective, reliable, and reproducible test with a consistent sensitivity for the tested biological in this study. This allows for the detection and quantification of endotoxin and non-endotoxin pyrogens and their dynamic interaction which cause unexpected high production of cytokine. MAT therefore is a reliable pyrogen test that supports manufacturers to release pyrogen-free products and protects the patient from the consequences of undetected non-endotoxin pyrogens and the synergistic stimulation that can occur.

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Declaration of conflicting interests

CTL-MAT produces MAT kits.

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