Pyrogen detection in pharmaceutical quality control: moving away from the rabbit pyrogen test with a ready-to-use monocytic cell line

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Introduction

The batch release of parenteral drugs relies on several quality control tests including the pyrogen test. The standard test is the rabbit pyrogen test (RPT), which has been widely used for decades despite known weaknesses. After the discovery of the reaction of Limulus Amebocyte Lysate (LAL) towards endotoxin the LAL test was progressively adopted by the pharmaceutical industry, as a replacement of the RPT for batch release.
Unfortunately, the LAL test can only detect the presence of one pyrogenic contaminant (lipopolysaccharide, LPS) in a sample, leaving the risk to miss out on other pyrogenic contaminants, often mentioned as non endotoxin pyrogens (NEPs).

In the 90s, the first monocyte activation test (MAT) was patented, opening a new way for *in vitro* pyrogen tests. MAT mimics the human reaction to pyrogens, thanks to the release of cytokines by monocytic cells. Several solutions are commercially available including a test system relying on the use of a ready-to-use monocytic cell line, the Mono-Mac-6 cell line. However, there are still concerns about the ability of monocytic cell lines to detect NEPs.

For this reason, we have demonstrated that the MM6 cells are suitable for the detection of various NEPs targeting different monocytic toll-like receptors, making MM6 cell line-based MAT suitable for RPT replacement.

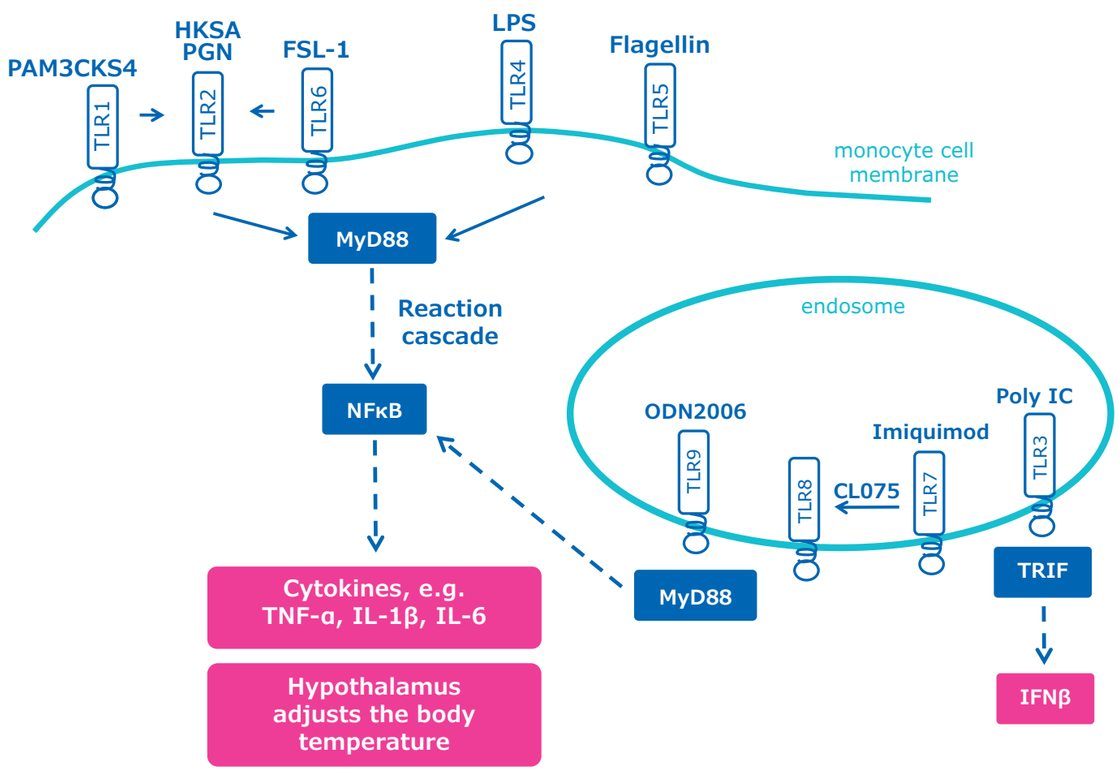
Objectives of this study

- Demonstrate the ability of the MM6 cell line to detect a panel of 10 Toll Like Receptor (TLR) ligands mimicking potential NEP contaminants
- Determine the LOD of each ligand with the test system
- Check the stability of reaction for each ligand showing a dose-dependent response over three batches of MM6 cells

Materials and methods

A ready to use system for MAT using MM6 cells (PyroMAT® system) was used to detect and quantify the following TLR ligands: PAM3CSK4, HKSA, PGN, FSL-1, Poly-IC, Flagellin, Imiquimod, CL075, ODN2006, MDP. [Fig.1] shows the targeted TLR of each ligand.
The range of concentration to be used for the study was determined in a preliminary test using one batch of MM6 cells and was then verified usigng 3 batches of cells.

Figure 1: Target TLRs of NEP ligands



Results

The different samples were used in a dose screening to determine the limit of detection of the monocyte activation test. For this, at least four dilutions of every NEP were run individually in the assay. The non-endotoxin pyrogens tested were all detected, with values over the cut-off of the respective assay.
The limit of detection of each NEP was determined to be the lowest concentration giving a signal ≥ 0.05 EU/mL and are detailed in [Table 1]. The contaminations with MDP and ODN2006 could be detected but not quantified, as the calculated values were below the validated limit of quantification, 0.05 EU/mL.

The stability of the reaction over several batches of cells was evaluated. For this evaluation, only those pyrogens showing a dose-dependent response were used, as only for those would any differences in cell reactivity be expected to have a large influence on quantification of the contamination. The results are shown in [Graph1].

Graph 1: Stability of NEP detection over three batches of cells

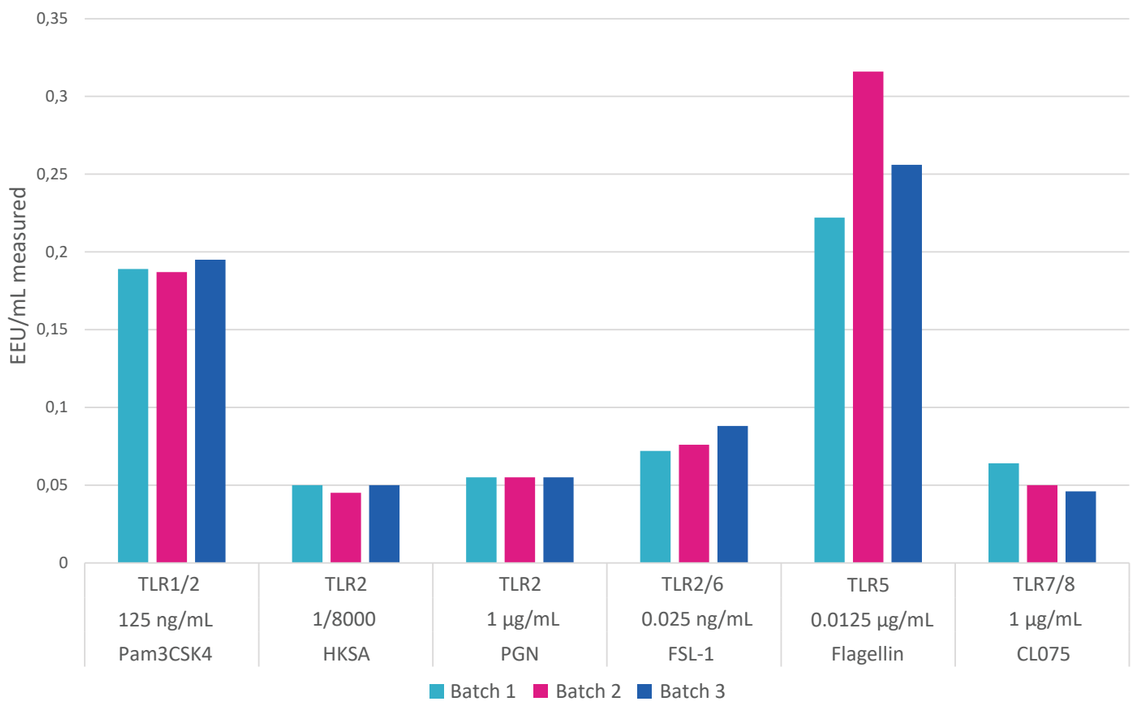


Table 1: LOD of tested ligands and corresponding quantification in EEU/mL obtained with the test system

NEP	LOD	EEU/mL measured
PAM3CSK4	0,125 µg/mL	0,055
HKSA	1/8000	0,054
PGN	1,25 µg/mL	0,063
FSL1	0,025 ng/mL	0,072
Poly IC	250 µg/mL	0,04*
Flagellin	0,0125 µg/mL	0,222
Imiquimod	100 µg/mL	0,088
CL075	1,25 µg/mL	0,084
ODN2006	100 µg/mL	0,027*
MDP	100 µg/mL	0,042*

*estimated quantification given for information. Signal obtained below the cut-off value of the test.

Discussion

In general, non-endotoxin pyrogens recognized by a cell surface receptor did show a dose-dependent increase of pyrogenicity in the monocyte activation test, but dose-dependency was less pronounced for pyrogens recognized by intracellular receptors. This indicates that the internalization process is an important factor in the reaction and that the quantification of non-endotoxin pyrogens with intracellular receptors might be hampered by the need for the cells to internalize the pyrogens.

With this study, we have demonstrated that the Mono-Mac-6 cells can detect a wide range of ligands targeting various TLRs, including intracellular ones.
The MAT-based PyroMAT® system also shows a reproducible reaction to reference standard endotoxin and non-endotoxin pyrogens.
The determined LOD of the system for each ligand could be used in further assays to compare different method sensitivities, for example between RPT and MAT.

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