Toll-like receptors: tools, assays, and implications for in vitro pyrogen tests
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Chapter 6

Cytokine induction by pyrogens: comparison of whole blood, mononuclear cells, and TLR-transfectants


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Abstract

Given the shortcomings in the measurement of pyrogenic contamination of pharmaceuticals and/or test substances by means of the rabbit pyrogen test and the Limulus amoebocyte lysate (LAL) test, several in vitro pyrogen tests have been developed based on the measurement of cytokine production by monocytes. In this study we measured cytokine production (IL-6, IL-8, IL-1β, and TNF) in diluted whole blood (WB), mononuclear cells (MNC), and HEK cells stably transfected with CD14 and Toll-like Receptor-2 (TLR2) or TLR4, after stimulation with both standard pyrogens and contaminated substances. Our study demonstrated that in MNC, IL-6 production was more sensitive to pyrogen stimulation than IL-1β and TNF production. The sensitivity of WB IL-8 production for pyrogens was comparable with that of MNC IL-6 production, but higher than WB IL-6 production. MNC IL-8 production as readout for pyrogenic stimulation was not useful due to high background IL-8 production. Surprisingly, contaminated culture media potently stimulated WB IL-8 production, but not MNC IL-6 production. Finally, the value of TLR-transfected HEK cells in the detection of pyrogenic contamination as well as the role of IL-10 in interindividual differences in cytokine production, is discussed. To summarize, the results presented herein together with literature data indicate that the measurement of WB IL-8 production may represent an advantageous alternative to the measurement of MNC IL-6 production, for the detection of pyrogenic contamination of pharmaceuticals.
Introduction

Fever is one of the main symptoms of infectious diseases caused by bacteria, viruses, or parasites. However, induction of the fever reaction is not restricted to live microorganisms. As early as 1865 the German surgeon Billroth reported fever reactions following injections with some distilled waters. At present, the discovery of a large family of mammalian receptors termed Toll-like receptors (TLRs), which are expressed on various cells, explains how microbial products of different pathogens can evoke the same biological response, fever. Fever-inducing substances i.e. pyrogens, can be the so-called endotoxins or lipopolysaccharides, cell wall components of Gram-negative bacteria such as *E. coli*, which are recognized by TLR4. Also microbial constituents of Gram-positive bacteria such as peptidoglycan, lipoteichoic acid, as well as products from mycobacteria, yeast or fungi that all stimulate TLR2 can evoke fever. Although much less studied, also polyinosine-polycytidylic acid (poly (I/C)), a synthetic analogue of double-stranded RNA from viruses that stimulates TLR3 is able to induce fever, and it is likely that all ligands that activate TLRs expressed on monocytes, are pyrogens.

To prevent detrimental side-effects of pharmaceutical therapy, the measurement of pyrogens is an important safety precaution for parenterally applied pharmaceuticals. In the early 1940s, as a result of World War II, the use of intravenous therapy, particularly blood plasma or other blood products significantly increased. Occasionally, fever occurred during such therapies and because of this, the role of bacterial contamination as the cause for pyrogenic reactions was studied in more detail. In 1942, a rabbit test for the detection of pyrogens in solutions for parenteral administration was included in the twelfth revision of the U.S. Pharmacopoeia. Soon thereafter, a detailed report of the quantitative and qualitative nature of the thermal response of rabbits to 28 types of bacteria isolated from blood plasma was published, and since then, the rabbit pyrogen test has been commonly used for the detection of pyrogenic contamination of parenteral pharmaceuticals.

The rabbit pyrogen test is able to detect various kinds of pyrogens but has several drawbacks: it requires the use of laboratory animals and is expensive. Also, the rabbit pyrogen test is not a quantitative test, i.e. it only gives a pass/fail result. In addition, both old and recent data suggest that considerable differences exist between humans and rabbits in the response to particular pyrogens. Even more importantly, there are reports that some parenteral products have caused pyrogenic responses in patients even after passing the rabbit pyrogen test.

Thus, despite these disadvantages of the rabbit pyrogen test only in the 1970s attempts were made to replace the rabbit pyrogen test, when radiopharmaceutical drugs were introduced into clinical practice for which testing in rabbits was deemed immoral.
solution was the bacterial endotoxin test (BET), often referred to as the Limulus amebocyte lysate test (LAL). The principle of the LAL test is that endotoxin causes coagulation of the haemolymph of the American horseshoe crab *Limulus polyphemus*.\(^{15}\) However, the LAL-test for bacterial endotoxin does not detect other bacterial products, whether Gram-positive or Gram-negative, and is susceptible to interference by high protein levels in test substances as well as by β-D-glucans.\(^{16-18}\)

In view of the shortcomings of both the rabbit pyrogen test and the LAL-test, several in vitro pyrogen tests have been developed. The basis of these in vitro pyrogen tests is that pyrogens, via TLRs and other receptors, stimulate cytokine production in monocytoid cells. Bacterial endotoxins stimulate monocytes via interaction with CD14 and TLR4\(^{19}\) to produce IL-1β, IL-6, IL-8, and TNF.\(^{20,21}\) Also microbial constituents of Gram-positive bacteria such as peptidoglycan, lipoteichoic acid, lipoproteins, and lipopeptides, all of which signal through TLR2\(^{4}\) induce cytokine production.\(^{22}\) Finally, although much less studied, also components derived from yeast\(^{23}\) or fungi\(^{24}\) can stimulate cytokine production in human monocytes. A number of different systems using peripheral blood mononuclear cells (MNC), diluted whole blood, as well as monocytoid cell lines Mono Mac 6\(^{25}\) and THP-1\(^{26}\) with various cytokine readouts have been established.\(^{27-29}\)

One of the absolute requirements for in vitro pyrogen test systems is that all reagents used should be free of pyrogenic contaminants. Trace amounts of bacterial constituents, such as endotoxin, occasionally contaminate medium and/or serum that is used for cell culture.\(^{30}\) To illustrate this, commercially available FCS was found to be easily contaminated by endotoxin during the manufacturing process.\(^{31}\) In addition, also plastics for cell culture,\(^{32}\) cell culture plates,\(^{33}\) and blood collection tubes\(^{34}\) were occasionally shown to be contaminated with pyrogens. Self-evidently, pyrogenic contamination of FCS, media, or consumables negatively affects in vitro pyrogen tests and may lead to erroneous results.

At our institute, more than a hundred experiments performed during the last decade strongly suggested that the measurement of IL-8 production in WB could represent a useful alternative to the measurement of MNC IL-6 production for the detection of pyrogenic contamination. Therefore, the aim of the present study was to compare in more detail the cytokine production in the different cell culture systems and to determine which in vitro test is suitable for the detection of pyrogenic contamination. To this end, we compared cytokine production in diluted whole blood, peripheral blood mononuclear cells, and in TLR2/4-transfected HEK cells following stimulation with TLR-ligands.
**Materials & Methods**

**Cells & cell cultures**

Blood was obtained from healthy volunteers after informed consent in line with the Sanquin Ethical Advisory Council. Blood samples were collected from healthy volunteers using endotoxin-free evacuated blood collection tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) containing sodium heparin.

For WB cultures, venous blood was diluted 1/10 with Iscove’s Modified Dulbecco’s Medium (IMDM, Bio Whittaker, Verviers, Belgium) supplemented with 0.1% heat-inactivated endotoxin-free fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands), 15 U/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands), penicillin 100 U/ml, streptomycin 100 µg/ml (Gibco, Merelbeke, Belgium), and 50 µM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). The supplementation of medium with 0.1% FCS was performed to ensure that all stimuli were homogeneously distributed and no stimuli (e.g. LPS) were lost due to adsorption to consumables.

MNC were isolated from freshly drawn blood by separation over a Percoll gradient (d = 1.078, Pharmacia Fine Chemicals, Uppsala, Sweden) and cultured in IMDM supplemented with 5% heat-inactivated FCS, penicillin 100 U/ml, streptomycin 100 µg/ml, 50 µM 2-mercaptoethanol, and 20 µg/ml human transferrin (Sigma-Aldrich). For stimulation experiments MNC were seeded at 4 x 10^4 cells/well in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and stimulated for 16-20 h.

Human Epithelial Kidney 293 (HEK) cells stably transfected with CD14, CD14-TLR2 or CD14-TLR4 were a kind gift from Drs. D. Golenbock and E. Latz, Worcester, MA, USA and have been described elsewhere. Transfected HEK cells were cultured in IMDM supplemented with 5% heat-inactivated FCS (Bodinco), penicillin 100 U/ml / streptomycin 100 µg/ml (Gibco), 50 µM 2-mercaptoethanol (Sigma-Aldrich), and 5 µg/ml puromycin (Sigma-Aldrich). For stimulation experiments cells were seeded at 5 x 10^4 cells/well in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and stimulated the next day. HEK-CD14-TLR4 cells were stimulated in the presence of 5% human serum as described elsewhere. After 16-20 h of stimulation supernatants were harvested for determination of IL-8 production.

All cells were cultured in 200 µl wells in flat-bottom microtitre plates (Nunc, Roskilde, Denmark) at 37 °C in the presence of 5% CO₂, in a humidified incubator.
Materials

Four lots of fetal calf sera (1: lot S03125, 2: lot S04253, 3: lot 151923, 4: lot 151966) were obtained from Bodinco, Alkmaar, The Netherlands, and one lot FCS (5: lot 10270-106 40F9545K) was purchased from Invitrogen, Leek, The Netherlands. As a reference, a previously characterized FCS, also from Bodinco, was used (6: lot S01933).

*Staphylococcus aureus* Cowan I (SAC; Pansorbin) was from Calbiochem, La Jolla, CA, USA and *E. coli* LPS serotype O55:B5 was from Sigma. A neutralizing monoclonal antibody against IL-10 (αIL-10, BT-10) was a kind gift from Dr. J. Wijdenes (Diaclone, Besancon, France).

Blood collection heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) are routinely tested in this institute’s laboratory for the presence of endotoxins or other stimulatory contaminants. A particular batch that failed this test was used to investigate its stimulatory properties. To this end, 0.5 ml IMDM was incubated in 9 ml vacutainers, for 2 h at room temperature.

Cytokine measurements

After stimulation (16-20 h) of cells, supernatants were harvested and stored at -20 °C until assayed. IL-1β, IL-6, IL-8, and TNF were determined by ELISA kits (Peli-Kine-compact, Sanquin Reagents, Amsterdam, the Netherlands), according to the manufacturer’s instructions. The plates were read in an ELISA-reader (Labsystems Multiskan Multisoft, Helsinki, Finland) at 450 nm, with 540 nm as a reference.

Results

**IL-1β, IL-6, IL-8, and TNF production after stimulation of WB and MNC with pyrogens**

To determine which *in vitro* assay for the detection of pyrogenic contaminants in drugs or medium ingredients was most suitable, WB from four different donors and MNC isolated from the same donors were stimulated with *Staphylococcus aureus* cells (SAC) and *E. coli* lipopolysaccharide (LPS), which are ligands for TLR2 and TLR4, respectively. After stimulation for 16-20 h supernatants were harvested and cytokine production (IL-1β, IL-6, IL-8, and TNF) was measured. In MNC, but not in WB, IL-8 production was characterized by high background production, up to 5 ng/ml. (fig. 1A and B). MNC exhibited also slightly increased background IL-6 production (approximately 40 pg/ml, fig. 1C and D). In contrast, background production of neither IL-6 nor IL-8 was observed in whole blood. Also, no background production of IL-1β, and TNF was observed either in MNC or in WB (fig.1 E-H). Although MNC exhibited dose-dependent IL-8 production in
response to low concentrations of SAC and LPS, the dynamic range of WB IL-8 production was much larger at higher concentrations of these stimuli (fig. 1A and B). IL-6 production in MNC was more sensitive to low SAC and LPS stimulation as compared to IL-6 production in WB (fig. 1C and D). SAC was the optimal stimulus for IL-1β production, both in MNC and WB (fig. 1G). SAC, at low concentrations, dose-dependently stimulated TNF production in MNC cells, whereas for substantial TNF production in WB much higher concentrations of SAC were required (fig. 1E). No differences between MNC and WB were found in TNF and IL-1β production after LPS stimulation. Both cytokines were only moderately produced after stimulation with rather high LPS concentrations (fig. 1F and H). Because the measurement of IL-6 and IL-8 as readout for pyrogenic contamination was superior to IL-1β and TNF production, we only measured IL-6 and IL-8 in subsequent experiments.

Stimulation of TLR-transfectants with SAC and LPS

Next, we sought to investigate whether human embryonic kidney cells (HEK) stably-transfected with CD14/TLR2 or CD14/TLR4 were appropriate for the screening of pyrogenic contaminants in test substances. To this end, HEK-CD14-TLR2 (HEK-TLR2) and HEK-CD14-TLR4 (HEK-TLR4) cells were incubated with different concentrations of SAC and LPS, and after 16-20 h of stimulation supernatants were harvested for measurement of IL-8 production as readout of NFκB activation. Specificity of TLR2 and TLR4 for SAC and LPS, respectively, was checked: SAC only stimulated IL-8 production in HEK-CD14-TLR2 cells, and not in HEK-CD14-TLR4 cells, and conversely, LPS only stimulated IL-8 production in HEK-CD14-TLR4 cells, and not in HEK-CD14-TLR2 cells. SAC, a ligand for TLR2, already at minute concentrations, dose-dependently increased IL-8 production in HEK-TLR2 cells (fig. 2A). In contrast, much higher concentrations of LPS were required to observe substantial IL-8 production in HEK-TLR4 cells (fig. 2B). Finally, HEK-TLR4 cells showed somewhat higher background IL-8 production compared to HEK-TLR2 cells.

Stimulation of WB, MNC, and HEK-TLR transfectants by pyrogenic contaminants in FCS

Studies in our laboratory on the role of TLRs in the production of pro-inflammatory cytokines in whole blood and MNC cultures were occasionally hampered by stimulatory material present in routinely used reagents, which gave rise to high background cytokine production. In those circumstances, all (new) components of the experimental conditions were carefully analysed and compared with previous successful experiments that were not impeded by high background cytokine production. Prior to the acquisition of a new FCS batch, aliquots of six different FCS batches were tested for the presence of contaminating...
Fig. 1. Production of IL-1β, IL-6, IL-8, and TNF after stimulation of WB and MNC with pyrogens. IL-8 (A and B), IL-6 (C and D), TNF (E and F), and IL-1β (G and H) were measured after stimulation of WB (open circles) and MNC (closed circles) with SAC (A, C, E and G) and LPS (B, D, F, and H; see Materials & Methods). Data represent mean cytokine production ± SEM of four different donors. A representative of two identical experiments is shown.

substances that could affect background cytokine production in cell culture. Given our experience with the relative high frequency of false-positive and false-negative results in LAL-assays, we tested different batches of fetal calf sera in a WB assay. This demonstrated the presence of stimulatory material in FCS 4, as it markedly induced IL-8 production up to 30,000 pg/ml (fig. 3A).

Surprisingly, a strong dose-dependent effect of FCS 4 on WB IL-8 production was observed, whereas IL-8 production in MNC was only moderately increased at higher concentrations of FCS 4 (fig. 3B). IL-6 production in WB after FCS 4 stimulation was only present at the highest concentration FCS 4 tested (10%, fig. 3C), whereas IL-6 MNC production was only moderately increased at higher FCS 4 concentrations as compared to WB and MNC IL-8 production (fig. 3B and C).

Finally, also IL-8 production in HEK-TLR2 cells was dose-dependently increased by FCS-4, whereas no such effect was observed in HEK-TLR4 cells (fig. 3D and E), which suggested that the pyrogenic contaminant in FCS 4 was not endotoxin. In addition, experiments with polymyxin-B, a polycationic compound that specifically inhibits LPS, also demonstrated that the pyrogenic contaminant in FCS 4 was not endotoxin (data not shown).
Fig. 2. Stimulation of TLR-transfectants with pyrogens. HEK-CD14-TLR2 (A) and HEK-CD14-TLR4 cells (B) were stimulated with SAC and LPS, respectively. Data represent mean IL-8 production ± SEM of duplicate samples. One representative of at least three experiments is shown.

Stimulation of WB, MNC, and HEK-TLR transfectants by pyrogenic contaminants in vacutainers

Also medium resuspended in vacutainers that were previously demonstrated to contain stimulatory substances, was tested both in WB, MNC, and HEK-TLR cells. Already at the lowest concentration of contaminated medium tested (2.5%), IL-6 and IL-8 production, both in WB and MNC, were markedly increased compared to background production (fig. 4A and B). HEK-TLR2 cells, and not HEK-TLR4 cells, dose-dependently produced IL-8 following stimulation with culture medium resuspended in contaminated vacutainers, suggesting that also this stimulus does not exert its effects via TLR4 (fig. 4C and D).

The role of IL-10 in TLR ligand-induced cytokine production

We hypothesized that the large interindividual differences in the production of IL-10, an anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines such as TNF, IL-1β, IL-6, and IL-8, may bring about a large variation between donors in monocyte/neutrophil cytokine production. Therefore, we hypothesized that stimulation of monocytes with TLR-ligands in the presence of a neutralizing antibody against IL-10, would result not only in enhanced cytokine production, thereby increasing the sensitivity for contaminating substances, but also in a reduced variation in cytokine production. Incubation of LPS-stimulated WB with a neutralizing mAb to IL-10 indeed strongly
Fig. 3. Stimulation of WB, MNC, and HEK-TLR transfectants by pyrogenic contaminants in FCS.

A: IL-8 production in WB after stimulation with 5% (white bars) and 10% (black bars) of six different batches of FCS. Data represent mean IL-8 production ± SEM of duplicate samples of one donor. One of two donors is shown. B and C: IL-8 production (B) and IL-6 production (C) in WB (open circles) and MNC (closed circles) after stimulation with FCS 4. Data represent mean cytokine production ± SEM of four different donors. One of two similar experiments is shown. D and E: IL-8 production in HEK-CD14-TLR2 (D) and HEK-CD14-TLR4 cells (E) after stimulation with FCS 4. Data indicate mean ± SEM of duplicate samples. A representative of two experiments is shown.
potentiated IL-8, IL-6, and TNF production (Fig. 5A-C), but not IL-1\(\beta\) production (Fig. 5D). However, \(\alpha\)-IL-10 did not inhibit the interindividual differences in cytokine production compared to stimulation in the absence of \(\alpha\)-IL-10 (Fig. 5A-D). Similar results were obtained when other TLR-ligands, such as SAC, monocyte-activating lipopeptide 2, Pam\(_3\)Cys-SK4KK, lipoarabinomannan, peptidoglycan, zymosan (all TLR1/2 or TLR2/6) or flagellin (TLR5), were used for stimulation (data not shown). Incubation of MNC with TLR-ligands in the presence of the \(\alpha\)-IL-10 mAb gave similar results (data not shown). Also incubation of either WB or MNC at higher cell density (1/2-diluted instead of 1/10-diluted for WB, or 200,000 cells instead of 40,000 cells for MNC), did not affect the large interindividual variations in cytokine production (data not shown).

**Discussion**

Ample experience from many previous experiments and from the current study, as well as literature data\(^{30-34}\) emphasize the necessity of frequent testing of all components of cellular pyrogen assays, in particular when new batches of materials are purchased. In our laboratory, when testing media or drugs for the presence of contaminating substances prior to use in cellular assays, we arbitrarily measured IL-6 or IL-8 production in either WB or MNC, depending on the type of other ongoing experiments. Recently, we reported that under defined experimental conditions IL-8 production was observed in WB without concomitant IL-6 production,\(^{40}\) suggesting that differences exist between the regulation of IL-8 and IL-6 production, which may be of relevance when testing pyrogens. Since the measurement of IL-6 production by MNC as a readout for pyrogenic contamination of substances has been internationally validated,\(^{28,29}\) and no published data are available about the use of IL-8 as a readout for pyrogenic contamination, we compared IL-6 and IL-8 production both in WB and MNC following stimulation with pyrogens. Because IL-1\(\beta\) production as a readout for contamination has been strongly advocated,\(^{41,42}\) we also included the measurement of this cytokine in our study.

Following activation of TLRs on monocytes by pyrogens, monocytes respond with the production of the pyrogenic cytokines IL-1\(\beta\), IL-6, and TNF as well as IL-8. In agreement with other research groups\(^5,28,29\) we confirm that IL-6 is to be preferred over IL-1\(\beta\) and TNF as readout for MNC cytokine production. The concentrations of IL-6 induced by SAC or LPS were higher than the induced concentrations of IL-1\(\beta\) and TNF. Moreover, IL-6 was induced at lower concentrations of pyrogen than IL-1\(\beta\) and TNF (Fig. 1). The comparison between IL-6 and IL-8 is less clear cut. Our data as well as literature data\(^{43,44}\) indicate that MNC IL-8 production is not useful as a readout for pyrogen contamination because of high
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Fig. 4. Stimulation of WB, MNC, and HEK-TLR transfectants by pyrogenic contaminants in vacutainers.

After IMDM was incubated in vacutainers known to contain pyrogenic contaminants (see M & M), WB, MNC, and TLR-transfectants were stimulated with this contaminated IMDM. **A and B:** IL-8 (A) and IL-6 (B) production in WB (open circles) and MNC (filled circles) after stimulation with contaminated vacutainer medium. Data represent mean cytokine production ± SEM of four different donors. A representative of at least three experiments is shown. **C and D:** IL-8 production in HEK-CD14-TLR2 (C) and HEK-CD14-TLR4 cells (D) after stimulation with contaminated vacutainer medium. Data represent mean ± SEM of duplicate samples. One of two experiments is shown.

background IL-8 production. Our studies after background cytokine production also indicated that MNC could effectively be cultured at 40,000 cells per well. Using this number of MNC economizes on blood/cells needed for in vitro pyrogen testing. In addition, at 40,000 cells per well, background cytokine production (IL-6/IL-8) was lowest, and specific cytokine production (induced by TLR-ligands) was highest when culture medium
containing endotoxin-free FCS was used (see materials and methods), as compared to medium containing AB-serum or autologous serum (data not shown). Therefore, all MNCs were cultured at 40,000 cells/well in medium containing FCS.

WB IL-8 production closely resembled MNC IL-6 production, but was more sensitive to pyrogenic stimulation than WB IL-6 production (fig. 1). Surprisingly, particular substances such as contaminated FCS, dose-dependently stimulated WB IL-8 production, whereas the MNC IL-6 production was less sensitive (fig. 3 C and D). IL-6 has also been the preferred readout because it is secreted entirely into the medium, whereas IL-1β and TNF remain largely intracellular, raising the possibility that substances affecting cell-permeability may influence IL-1β and TNF levels, rather than IL-6. Indeed, following synthesis of IL-1β, a secondary stimulus is required for its release. Also, certain bacterial components stimulate the production of IL-6, but not IL-1β and TNF.

In man and experimental animals, IL-6 has been demonstrated to be the principal circulating pyrogen, and studies with IL-6 knockout mice demonstrated that the pyrogenic activity of IL-1β and TNF is entirely dependent on IL-6. Finally, it has been claimed that IL-6 is the analyte in blood that correlates best with pyrogenicity. Thus, there are ample arguments for the use of IL-6, rather than IL-1β and TNF, as readout in cellular assays for the detection of pyrogenic contamination. However, in these studies no comparison was made between IL-6 and IL-8 production levels, nor was the role of IL-8 in the induction of fever investigated.

In this study we demonstrate that in addition to IL-6, also IL-8 can be reliably used as readout for pyrogenic contamination. Moreover, WB IL-8 production was induced by lower concentrations of particular stimuli than were required to induce IL-6 production in MNC. Despite the fact that IL-8, like IL-6, is produced in large quantities following stimulation with pyrogenic substances, its role in the induction of fever has been much less studied. Nonetheless, data are available which demonstrated that at least in rats, IL-8 is able to induce fever. Unlike IL-6, which is entirely dependent on the generation of prostaglandin E2 for the induction of fever, IL-8 induces fever in a prostaglandin-independent manner, mediated by corticotrophin-releasing factor. Also in favour of the use of IL-8 as a readout are a number of studies which demonstrated that plasma IL-8 levels, next to IL-6, were predictive of fever and strongly correlated with pyrogenicity in febrile patients. In one of these studies, IL-8 levels correlated even better with pyrogenicity than IL-6 levels. Thus, IL-8 can be readily detected in WB following incubation with pyrogenic contaminants, it can be regarded as a genuine pyrogenic cytokine and it correlates strongly with pyrogenicity in man.
Fig. 5. The role of IL-10 in TLR ligand-induced cytokine production.

WB was stimulated with LPS in the absence and presence of a neutralizing antibody to IL-10 (10 µg/ml) for 16-20 h after which IL-8 (A), IL-6 (B), TNF (C), and IL-1β (D) production in the supernatant was measured. Data represent mean cytokine production ± SEM of four different donors. A representative experiment is shown.

Apart from its relevance in fever, the measurement of WB IL-8 offers additional advantages. A particular practical advantage is that after collection of donor blood, WB can immediately be put in culture, whereas the isolation of MNC requires additional purification procedures and time. In addition, the dynamic range in IL-8 production is much larger than in IL-6 production, i.e. the induced production of IL-8 relative to IL-8 background production is much larger than this ratio for IL-6 (fig. 6). FDA release criteria
for several pharmaceutical blood products state that the endotoxin content should be less than 0.5-1.33 EU/ml (~50-133 pg/ml of a control standard endotoxin, such as the *E. coli* strain used in our experiments). This indicates that the critical endotoxin concentration for a reliable pyrogen test is approximately 50 pg/ml. At this LPS concentration, the ratio of IL-8 production induced in WB relative to background IL-8 production is much larger than this ratio for MNC IL-6 (fig. 6B). Finally, since IL-8 is also produced by activated neutrophils, the pyrogen test using WB/IL-8 also detects possible stimulation of neutrophils, which may be of relevance when testing parenteral pharmaceuticals. Therefore, in addition to the well-validated use of IL-6 as readout in MNC cultures, we advocate the measurement of WB IL-8 production as a sensitive readout for pyrogenic contamination of pharmaceuticals.

HEK-cells stably transfected with CD14/TLR2 or CD14/TLR4 are powerful tools in determining whether a contaminating substance is an agonist for TLR2 and/or TLR4. Knowledge of TLR-stimulatory properties of test substances may give important clues as to the source of contaminants present in the test substance. Therefore, also HEK-TLR2 and HEK-TLR4 cells were stimulated with SAC, LPS, FCS-4, and medium from contaminated vacutainers. The sensitivity of HEK-CD14-TLR2 cells was comparable with that of WB and MNC when stimulated with SAC, a specific TLR2 agonist. However, the sensitivity for LPS of HEK-TLR4 cells compared with that of WB or MNC was much lower, indicating that WB and MNC, rather than HEK-CD14-TLR4 cells, are to be preferred in cellular assays to detect endotoxin contamination. The difference in sensitivity between HEK-TLR2 and HEK-TLR4 cells for their respective ligands may be caused by the high background IL-8 production in HEK-TLR4 cells. Despite the fact that substantial IL-8 production in HEK-TLR4 cells is only observed at an LPS concentration of approximately 100 pg/ml, both FCS-4 and contaminated vacutainer medium only stimulated HEK-CD14-TLR2, and not HEK-CD14-TLR4, which strongly suggested that the contaminants present in FCS4 and contaminated vacutainer medium is different from endotoxin.

Because interindividual differences between donors in WB and MNC cytokine production after pyrogen stimulation may necessitate test substances to be tested in eight instead of four blood donors, we sought to reduce these interindividual differences. To this end, we stimulated WB with pyrogens in the presence of an IL-10 neutralizing antibody. However, unexpectedly, incubation with anti-IL-10 merely increased, rather than inhibited the interindividual differences. Since it was recently demonstrated that interindividual differences in the immune response to LPS were dependent on the level of TLR4 expression on immune cells, it is likely that this differential expression of TLRs, rather than differences in IL-10 production is responsible for the large interindividual differences observed.
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Fig 6: Pyrogen-induced IL-6 and IL-8 production in WB and MNC relative to background cytokine production.
Cytokine production after stimulation of WB and MNC with SAC (A), LPS (B), FCS 4 (C) and contaminated vacutainers medium (D) was plotted relative to background cytokine production (see figures 1 and 3). The different combinations of assay (WB or MNC) and readout (IL-8 or IL-6) are indicated (WB/IL-8 filled circles, MNC/IL-8 filled squares, WB/IL-6 open circles, MNC/IL-6 open squares). Data represent mean cytokine production relative to background cytokine production from one representative experiment using 4 different donors.

To summarize, in this study we demonstrated that for the detection of pyrogenic contaminants in test substances or parenteral pharmaceuticals, IL-8 production in WB is a reliable readout that represents a valuable alternative to the well-established measurement of IL-6 production in MNC. We would like to emphasize that we do not claim that the measurement of WB IL-8 is a better readout for pyrogenic contamination than the measurement of MNC IL-6. However, we are confident that our results will give an incentive to the area of pyrogen testing and will contribute to the ongoing discussion on alternative pyrogen testing using the monocyte activation test.
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