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t⁴ Report*

Evidence for the Detection of Non-Endotoxin Pyrogens by the Whole Blood Monocyte Activation Test[1]

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Summary

Threats of pyrogenicity were discovered more than a century ago. Measures to determine the safety of parenterals and, more recently, medical devices and cell therapies for human use have been in place for 70 years. Currently, there are three testing possibilities available: the Rabbit Pyrogen Test, the Limulus Amebocyte Lysate test (Bacterial Endotoxin Test), and test systems using human whole blood or human monocytes, called Monocyte Activation Test (MAT). The MAT is based on the human fever reaction and thus most closely reflects the human situation. Unfortunately, regulations and testing guidelines are not fully harmonized, despite formal international validation. Furthermore, data showing that the MAT is capable of covering the totality of possible pyrogens relevant to humans were not included in the MAT validations of the last decade. For this review we collate evidence from published literature, unpublished data of our own, and results from the international validation study to show that there is overwhelming scientific evidence to conclude that the whole blood MAT reliably detects non-endotoxin pyrogens. Therefore, further validation exercises do not seem warranted.

Keywords: Gram-positive immune stimuli, fungal immune stimuli, non-endotoxin pyrogens, MAT, human whole blood

1 Introduction and background

Fever is one of the cardinal signs of inflammation and very often is related to bacterial or viral invasion of the human body (Dinarello, 1996). As far back as 100 years ago (Hort and Penfold, 1912) it had been realized that substances released from dead bacteria may cause fever. These substances were termed pyrogens, i.e., fever-inducing substances (Kluger, 1991; Moltz, 1993). Whenever they enter the human body or come into contact with the human blood stream, the host's innate defense mechanisms, carried by macrophages, monocytes, dendritic cells, and neutrophils, spring into action (Derijk et al., 1993). This can lead to severe signs of inflammation, shock, multi-organ failure, and sometimes even death (Hartung et al., 1997; Dinarello, 2000; Beutler et al., 2003).

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1 This article is dedicated to the memory of Dr Thomas Montag-Lessing, an inspiring colleague and friend who, sadly, passed away during the preparation of this manuscript.

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To ensure consumer safety, pyrogen testing is necessary for all products intended to overcome the natural barriers of the human body (Ding and Ho, 2001), specifically injectables and medical devices. In 1912 the Rabbit Pyrogen Test (RPT) was introduced into the British Pharmacopoeia following the observation that the application of injectables could cause fever and other serious adverse effects. The RPT was able to reflect the pyrogenicity of bacterial contaminants found in biologic products (Probey and Pittman, 1945) to a certain extent, allowing better safety control. In 1942 it was introduced into the United States Pharmacopeia (USP) as well.

In 1885 it had been discovered that the hemolymph of the horseshoe crab (*Limulus polyphemus*) coagulates in contact with foreign substances (Howell, 1885; Loeb, 1903). This reaction was later traced to bacteria (Bang, 1956) and specifically bacterial lipopolysaccharide (LPS) (Levin and Bang, 1964), a major, conserved, outer surface molecule of Gram-negative bacteria. Currently, LPS is the most potent immune stimulator known, and the detection of LPS contaminations in products entering the human body is of vital importance. Therefore, the Limulus Amebocyte Lysate (LAL) test, also called Bacterial Endotoxin Test (BET), was developed and found its way into the US Pharmacopeia (USP) and others. LPS causes degranulation and destruction of the amebocytes, cells circulating in the hemolymph of the horseshoe crab (Shirodkar et al., 1960).

The LAL test was a significant advancement, replacing the costly and error prone rabbit test. Suddenly, rapid lot and end product testing was possible, and consumer safety was enhanced considerably. However, due to a completely different underlying mechanism, the LAL does not reflect the human fever reaction, the main indicator of human response towards pyrogenic substances (Fig. 1). In fact, as summarized by Brandenburg et al. (2009), the LAL test reacts very differently to LPS in comparison to the human immune response: "… *there was no correlation of the LAL activity with cytokine expression (for example, tumor-necrosis-factor-alpha and interleukins-1 and 6) in mononuclear cells when the 4/2 acyl chain pattern of enterobacterial lipid A was changed, or when the cytokine production induced by LPS from various different species in the whole blood assay was compared with the response from the LAL test*."

By the end of the 1940s, the scientific basis of pyrogenicity and the concept of exogenous/endogenous pyrogens had been explored in detail, as reviewed by Dinarello (2004). It was discovered that the human body releases endogenous fever-causing substances in response to exogenous substances, as some sort of host defense mechanism. More than 30 years later the first endogenous pyrogen, interleukin-1β (IL-1β), was identified (Auron et al., 1984). In the late 80's the first recombinant IL-1β became available, and the development of alternative pyrogen tests employing monocytic cells including whole blood methods started shortly after that (Hartung and Wendel, 1995, 1996). By understanding the underlying mechanisms of the human fever reaction, it was possible to develop test systems reflecting the molecular processes taking place in the human body. This included the identification of non-endotoxin pyrogens (NEP) and various receptors for both LPS and NEP.

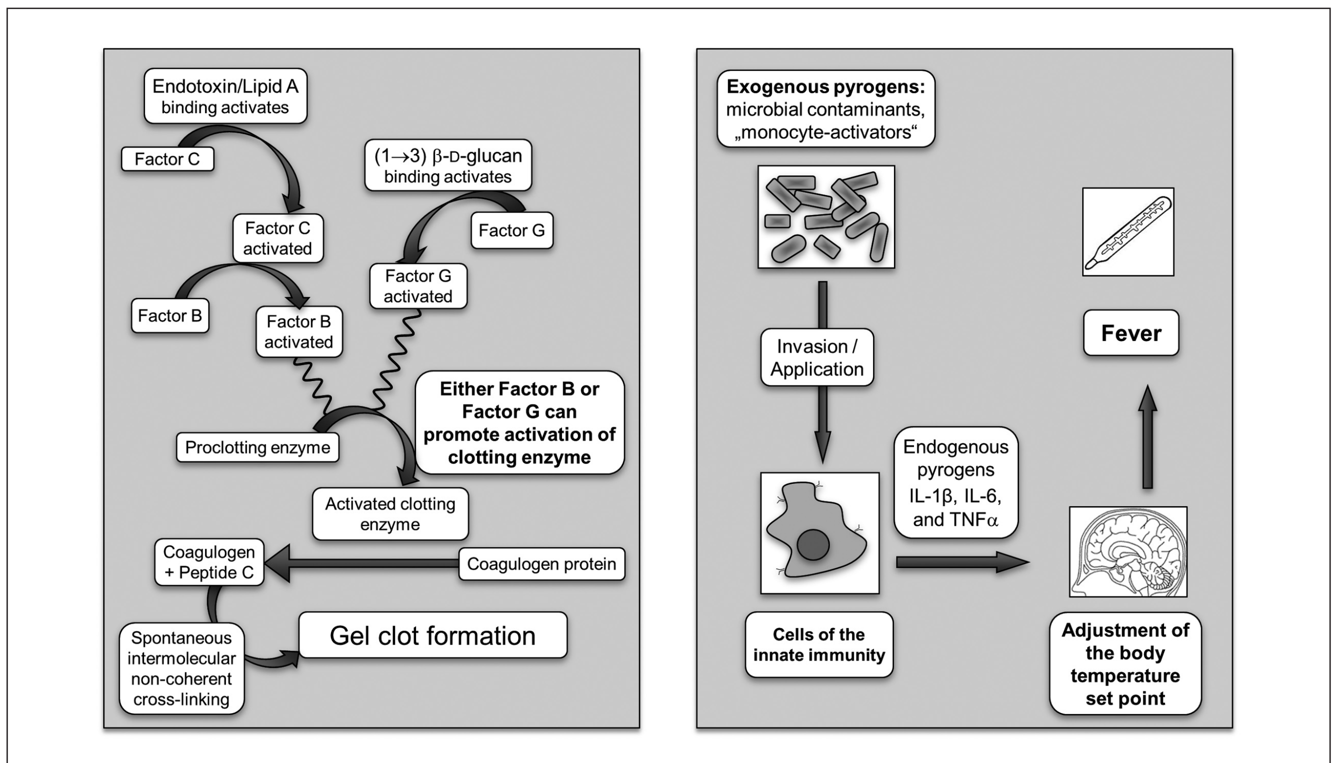

Fig. 1: Cascade of biochemical interactions and reactions leading to gel clot formation in the LAL-assay in comparison to the underlying mechanism of the human fever reaction

1.1 The Rabbit Pyrogen Test (RPT)

Even though fever responses to bacterial pyrogens have been observed in a variety of animals, rabbits were adopted as the standard animal for pyrogen safety testing (Tui and Schrift, 1942). Briefly, the sample is injected and the change in body temperature is measured. For a detailed description of the test see (Weary and Wallin, 1973). The sensitivity of rabbits towards endotoxin preparations depends on the strain used and the experimental conditions, e.g., age, gender, and housing conditions (van Dijck and van de Voorde, 1977; Hull et al., 1993). The most sensitive rabbit strains develop a significant temperature increase upon exposure to 500 pg (i.e., 5 IU) of reference LPS/kg. If the highest permitted volume (10 ml/kg body weight, depending on the drug characteristics) is injected, the resulting detection limit is 500 pg per 10 ml or 50 pg/ml, while the human fever threshold lies around 30 pg/ml (Greisman and Hornick, 1969).

For quality-control purposes a maximum acceptable endotoxin concentration has to be defined. However, the RPT is not suitable for the control of such a limit since it is not a quantitative test, i.e., it gives only a pass/fail result (Bellentani, 1982). Furthermore, the RPT is not suitable for many products, such as radiopharmaceuticals, chemotherapeutics, analgesics, antipyretics, cytokines, dopamine and immunosuppressive agents (Hartung et al., 1998, 2001). Detailed information can be obtained from (Cooper et al., 1972, 1979; Cohen et al., 1986; Booth, 1986). In addition, drugs that influence the central or peripheral mechanisms of body temperature regulation such as antipyretic drugs, steroids, or dopamine (Cranston and Luff, 1972; van Miert and van Duin, 1978; Szreder, 1997; Gagalo et al., 1995, 1996; Gagalo and Matuszek, 1997; Bencsics et al., 1995; Flanigan et al., 1992; Cradock et al., 1986; Kleszynski et al., 1982) cannot be tested in the RPT. The same applies to drugs that can cause immunological reactions (e.g., immunoglobulins; Huszar et al., 2002), oily suspensions, or detergents. Also, the RPT cannot be used for cellular preparations, such as blood components and stem cells (Hartung et al., 2001).

From an animal welfare point of view, the RPT is an animal experiment that subjects rabbits to a certain extent to suffering and pain and, according to animal welfare laws, e.g., in Europe: "*The use of animals for scientific or educational purposes should therefore only be considered where a non-animal alternative is unavailable*." (EU, 2010)

As a true pyrogen test, the RPT is considered to be able to detect endotoxin and non-endotoxin pyrogens (NEPs) alike (Nakagawa et al., 2002); in some cases, however, the RPT remains negative in response to NEP that induce pyrogenic adverse reactions in humans (Martis et al., 2005).

1.2 The Bacterial Endotoxin Test or Limulus Amebocyte Lysate test

The Bacterial Endotoxin Test (BET) refers to a number of tests that detect endotoxins from Gram-negative bacteria based on the clotting reaction of the hemolymph of the horseshoe crab, and therefore it is also called the Limulus Amebocyte Lysate (LAL) test. There are various approaches for measuring the LPS-induced reaction, e.g., by clotting, turbidimetric, or chromogenic measurement (both kinetic or endpoint) (Weary et al., 1982). A recombinant BET (based on a genetically engineered protein from the Carcinoscorpius clotting cascade), which does not consume any animals, has been developed and validated to some extent (Ding and Ho, 2001; Loverock et al., 2010). Special LAL-techniques have been developed for testing very small sample volumes (Jimenez et al., 2010; Gee et al., 2008).

One advantage of the LAL test over the RPT is the possibility of concurrently testing an endotoxin standard that permits the semi-quantitative or quantitative measurement of endotoxins (Weary et al., 1980). The detection limit is usually about 3 pg/ml, i.e., 0.03 EU/ml (1.5 pg/ml final concentration), and the most sensitive test variants detect down to 0.005 EU/ml. By quantifying LPS, the LAL detects the most common and most potent pyrogen known with high sensitivity. However, the assay can only be performed with liquid samples, creating difficulties for the testing of solid materials, such as medical devices (Ross and Bruch, 1982; Roslansky et al., 1991), of which only rinsing solutions can be tested. Similarly, the assay has problems with dialysis fluids (Bohrer et al., 2001), liposomes (Harmon et al., 1997), nanoparticles (Smulders et al., 2012), and cell therapies (Montag-Lessing et al., 2010). Drugs that interfere with the clotting system, i.e., through inhibition (binding of divalent cations such as ethylenediaminetetraacetic acid, citrate, protease inhibitors) or enhancement (high protein content, proteases), cannot be tested with the LAL test (Cooper et al., 1997; Duner, 1995). Furthermore, a number of endotoxin-binding components from plasma are known to mask LPS in the LAL test (Hurley et al., 1991), and due to such interference with the test system, many drugs have to be diluted for testing (al-Khalifa et al., 1989; Elin and Wolff, 1973). The LAL cascade also is triggered by (1,3)-β-d-glucan (Roslansky and Novitsky, 1991; Cooper et al., 1997) and polysaccharides, for example from cellulose filter materials, which can result in false-positive signals (Ikemura et al., 1989; Anderson et al., 2002). Another well-known problem of the LAL test is that substances in the preparation may non-specifically interfere with the assay (Schmidtgen and Brandl, 1995; Moesby et al., 1997).

Immune-stimulating components from Gram-positive bacteria, such as lipoproteins, peptidoglycan, and lipoteichoic acids, or other pyrogens pass the LAL test without resulting in a signal (Morath et al., 2002a). Being specific for endotoxins therefore precludes the BET from being a complete alternative to the RPT (Martis et al., 2005; Huang et al., 2009) because NEP contaminations that are not detectable by this test may cause severe clinical signs.

Further, the LAL does not reflect the inflammatory potency of a specimen in humans (Spreitzer et al., 2002; Williams, 2001; Fennrich et al., 1998). The difference in the endotoxin detection in the LAL-assay and the prediction of pyrogenicity by immune cells of human origin is reflected by different test results on modified LPS-specimens (Brandenburg et al., 2009; Gutsmann et al., 2010; Stoddard et al., 2010). The fact that different LPS species display various LAL activities (not related to their pyrogenicity) further limits the use of the LAL as a pyrogen test (Dehus et al., 2006).

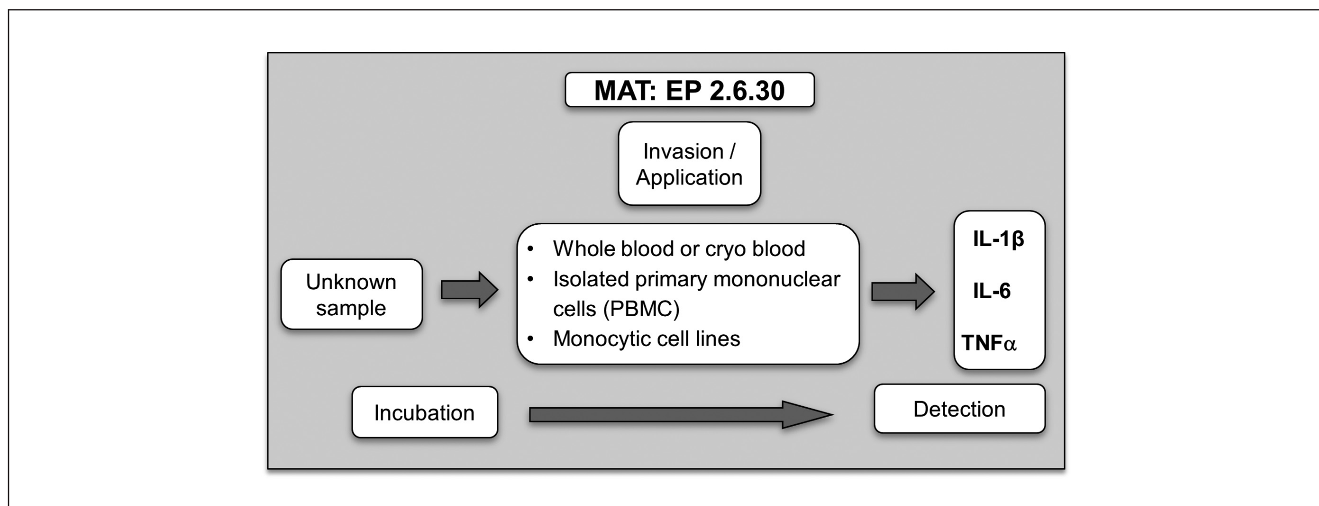

Fig. 2: The biological principle of the "Monocyte Activation Test" as defined by the monograph 2.6.30 in the European Pharmacopoeia (EDQM, 2010)
(IL: interleukin, TNF: tumor necrosis factor).

1.3 Human cell-based assays or the Monocyte Activation Test

The concept of using the human fever reaction for pyrogen testing was pioneered by Dinarello et al. (1984). They used the rabbit test to determine fever-inducing factors secreted from human blood monocytes. The understanding of the human fever reaction and development of ELISA technologies led to the development of test systems based on the *in vitro* activation of human monocytes (Hansen and Christensen, 1990; Werner-Felmayer et al., 1995; Eperon et al., 1997; Moesby et al., 1999; Hartung et al., 2001; Dinarello, 2004; Gaines Das et al., 2004; Schindler et al., 2009) detecting LPS, NEPs, and mixtures (Hermann et al., 2002; Nakagawa et al., 2002; Kikkert et al., 2007). Peripheral blood mononuclear cells (PBMCs) were first used to detect pyrogens by monitoring the release of pyrogenic cytokines (Duff and Atkins, 1982), such as IL-1β, TNF-α, and IL-6. Meanwhile, a number of different test systems, using either human whole blood, cryopreserved blood, peripheral blood mononuclear cells (PBMCs), monocytic cell lines (MONO MAC 6, MM6), or human acute monocytic leukemia cell line (THP-1) as a source for human monocytes, and various read-outs were established (Poole et al., 1988b; Fennrich et al., 1999a; Hartung et al., 1996; Schindler et al., 2004, 2006b; Daneshian et al., 2009), see Figure 2.

The response of human cells, horseshoe crab amebocytes (Petri and Fennrich, 2000), and rabbits (Schindler et al., 2003) to Gram-negative endotoxin has been studied extensively, and a comparison reveals a good correlation of the whole blood test with the RPT, with results correlating to the content of pyrogens in the sample (Fennrich et al., 1999a,b; Hartung et al., 2001; Hartung, 2002) as shown in Figure 3. Both RPT and whole blood MAT showed 3-4 log-order differences in potency of different LPS samples. Notably, no such difference in potency was observed for the LAL, which differed by a maximum of one log-order (data not shown).

Five variants of the MAT have been standardized, validated (Hoffmann et al., 2005), and accepted by the European Centre for the Validation of Alternative Methods (ECVAM) and the ECVAM Scientific Advisory Committee (ESAC) as alternatives to the RPT for endotoxin pyrogen detection (ECVAM, 2006), and they proved to have a lower detection limit than the rabbit test. They are more accurate, cost-efficient, and more time-efficient. They are able to detect Gram-positive pyrogens and therefore meet the quality criteria for pyrogen detection, as defined in the recommendations of an ECVAM workshop report (Hartung et al., 2001). It could be concluded that the MAT provides reliable and reproducible results for many final products (Spreitzer et al., 2002; Andrade et al., 2003). As the MAT can detect pyrogens other than endotoxins, results should be presented as endotoxin equivalent units per ml (EEU/ml) as suggested by Montag et al. (2007).

In 2010, the MAT Monograph 2.6.30 was implemented into the European Pharmacopoeia (EDQM, 2010) and since then has been employed as a substitute for detecting Gram-negative endotoxins and NEPs alike in injectables on a case-by-case basis. A similar recommendation was made by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2008, 2009). The same position has been adopted by (FDA, 2009). The most recent guidance[2] states that firms producing products for which pyrogen testing is required may use alternative methods if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, and in other special circumstances. Alternative methods should be subjected to appropriate validation and shown to achieve equivalent or better results compared to the standard method.

[2] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM310098.pdf

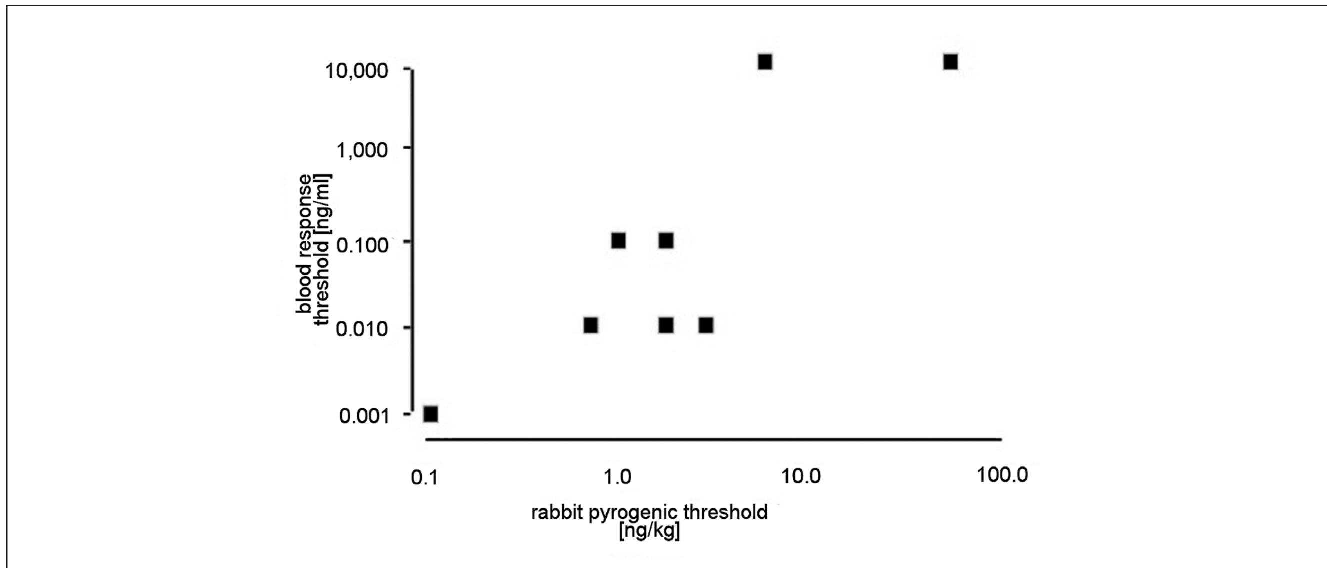

Fig. 3: Pyrogenicity of various endotoxins in rabbits plotted against their whole blood detection limits
Modified from (Fennrich et al., 1998). Endotoxins from *Salmonella typhi*, *Salmonella enteriditis*, *Salmonella abortus equi*, *Pseudomonas aeruginosa*, *E. coli* O55, *E. coli* O127, *Klebsiella pneumonia* and *Shigella flexneri* were used. The lowest concentration inducing significant IL-1β release in whole blood MAT was plotted against literature thresholds doses resulting in positive RPT (Dabbah et al., 1980; Tsuji et al., 1980; Greisman and Hornick, 1969; Keene et al., 1961; Weary et al., 1980; Pearson et al., 1985).

The document also provides guidance for transitioning from one test method to another.

The EMEA encourages the replacement of the RPT by alternative tests such as LAL or MAT in plasma-derived medicinal products (EMEA, 2009). The MAT methods also found acceptance in countries such as Japan, Brazil, and Cuba.

The whole blood MAT (Schindler et al., 2009), also known as the *in vitro* pyrogen test (IPT or IVPT), has the advantage that no cell culture is required and no preparation artifacts occur. The cells are kept and maintained in their natural environment, i.e. plasma. In contrast to the LAL/BET, the whole blood MAT reflects the potency of different LPS species in the rabbit (Fennrich et al., 1999a). Since a cell suspension is used, blood as a reagent can be brought in contact with any material, including medical devices (Hasiwa et al., 2007; Mazzotti et al., 2007) or filters loaded with specimens from air samples (Kindinger et al., 2005). In contrast, cell lines in the validation study have shown drifts and shifts in their responsiveness to pyrogens, letting MonoMac-6 cells fail the prevalidation and the two THP-1 tests fail the validation phase. With the introduction of cryopreserved pooled human whole blood (Schindler et al., 2004), which is pretested by the standards used for blood transfusion, concerns about availability, donor differences, and infectious threats have been overcome. It has also been adapted to rabbit blood, allowing the assessment of species differences in pyrogen detection (Hartung et al., 1998). The test is not disturbed by several components that prohibit LAL or RPT testing, such as aluminum hydroxide in vaccines (Carlin and Viitanen, 2005), lipidic parenterals (Schindler et al., 2006a), toxic or immunomodulatory drugs (Daneshian et al., 2006), water and dialysis solutions (Daneshian et al., 2008), and herbal components with glucan-like structures (Daneshian et al., 2006). It is the only test for which standardized kit versions are internationally available. For these reasons it is more broadly used and more data are available than for other MAT variants. This review thus focuses on the whole blood MAT.

1.4 Non-endotoxin stimuli

As reviewed by (Henderson and Wilson, 1996), the underlying mechanism in the human fever reaction can be provoked not only by LPS but also by many other substances originating from Gram-negative and Gram-positive bacteria. Many other compounds, originating from fungi, yeast, viruses, and parasites also have been shown to induce a human immune reaction and to cause problems when overcoming the natural protective barriers of the human body. Only recently, the relevance of non-endotoxin pyrogens, e.g., lipoteichoic acid (LTA), bacterial DNA (CpG-motive), peptidoglycan, synthetic Toll-like receptor (TLR)-agonists, or endogenous pyrogens has gained more attention, mainly as a cause of human adverse reactions.

A case study

As reported by a major pharmaceutical company, a life-saving drug containing a fermentatively produced drug substance received many reports of adverse drug reactions (ADR), e.g., pain at the injection site, redness, shivering, and fever. The incriminated batches generating these complaints had passed the Bacterial Endotoxin Test (BET) and the Rabbit Pyrogen Test (RPT) without a detectable response. None of the applied physico-chemical methods could distinguish between batches that provoked ADRs and "clean" batches. It became clear that an unknown non-endotoxin pyrogenic contamination was impair-

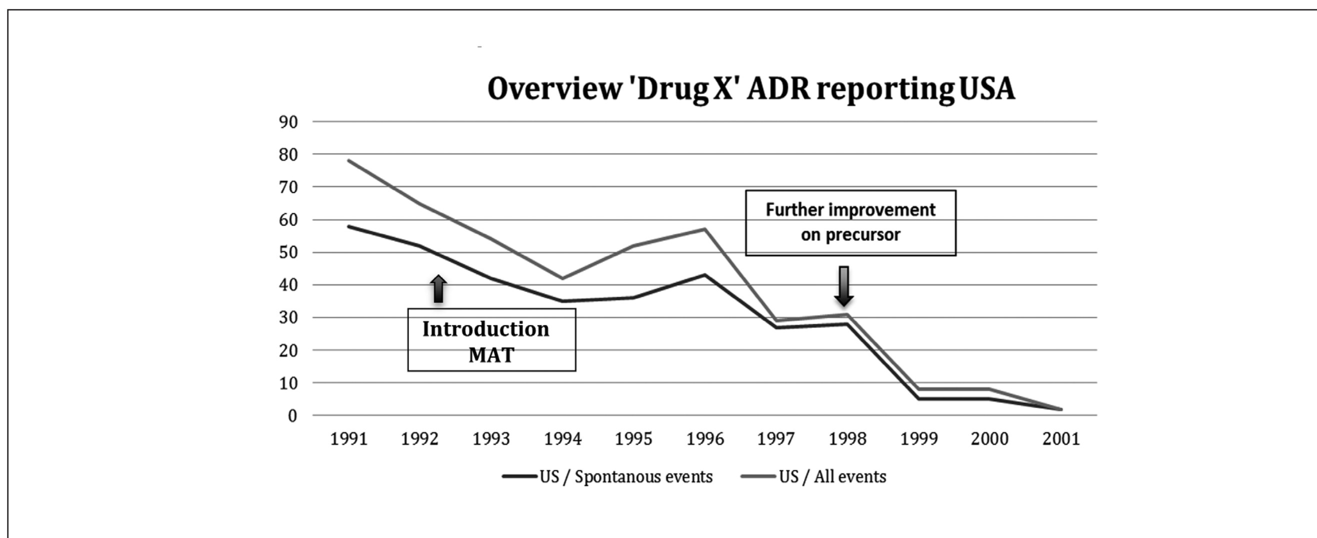

Fig. 4: An example of the clinical value of the MAT: ADR reporting over the years in USA for "drug X"

Tab. 1: Scientific literature showing the relevance of pyrogenic non-endotoxin substances
Adapted from Hartung et al. (2001)

Substances	References
Endotoxin associated proteins	Murphy, 1991; Hitchcock and Morrison, 1984
Peptidoglycans (components of the bacterial cell wall)	Fumarola et al., 1986; Thomsen and Loppnow, 1995
Muramylpeptides (MDP and other subunits of peptidoglycan that synergize with endotoxins)	Dinarello et al., 1978; Johannsen et al., 1991
Porins (proteins from the bacterial cell wall), bacterial outer surface proteins	Benz, 1988; Galdiero et al., 1990; Galdiero et al., 1994
DNA (bacterial)	Elin and Utter, 1980; Won and Lin, 1993; Cowdery et al., 1996; Sparwasser et al., 1997
Lipoteichoic acids and further Gram-positive bacterial cell-wall components	Wexler and Oppenheim, 1979; Vallejo et al., 1996; Toien and Mercer, 1996; see Fig. 12
Superantigens	Huang et al., 1997a; Huang et al., 1997b; Roggiani et al., 1997
Exotoxins	Hackett and Stevens, 1992; Fitzgerald and Pastan, 1993; Bhakdi et al., 1994; Houldsworth et al., 1994; Murai et al., 1996
Lipoarabinomannans (from mycobacteria)	Carson et al., 1988; Rawadi and Roman-Roman, 1996
Fungal components (e.g., mannans, glucans, mannoproteins)	Barwick et al., 1994; Castro et al., 1996
Parasite components (e.g., phosphoinositol)	Bate et al., 1989
Viruses	Barry et al., 1976; Jakeman et al., 1991; Gong et al., 1991; Becker et al., 1991; Alluwaimi et al., 1994; Chang and Shaio, 1994; Kurokawa et al., 1996; Price et al., 1997
Non-microbiological contaminations (e.g., cytokines, media, cells, breakdown products)	Soprana et al., 1994
Solid materials (e.g., medical devices, plastic)	Miller and Anderson, 1988
Drugs (e.g., steroids, bile salts, dapsone, cytokines)	Beloeil et al., 1980; Bodel and Dillard, 1968; Coleman, 1995

ing human safety. After the introduction of the MAT as a testing system in accordance with FDA for batch release, the adoption of several optimization steps and further improvements in the production process, reporting of ADR has decreased to zero over the intervening years (Fig. 4).

An overview of various non-endotoxin substances and the respective literature can be found in Table 1.

Gram-positive bacteria

While endotoxin as the immune-stimulating principle of Gram-negative bacteria is well established and characterized, Gram-positive bacteria are still under investigation. A variety of molecules have been reported to invoke an immune response, e.g., lipoproteins, peptidoglycan, wall teichoic acids, and lipoteichoic acid (LTA) (Bubeck Wardenburg et al., 2006; Morath et al., 2005; Draing et al., 2008; Mirelman et al., 1971; Nakata et al., 2006).

Peptidoglycan (PGN) is the major component of the cell wall of Gram-positive bacteria spiked with lipoteichoic acid (LTA) on its external surface (Baddiley, 1989) and lipoproteins incorporated in the peptidoglycan-layer (Henderson et al., 1996). LTA and lipoproteins are recognized via Toll-like receptors (TLR) 2/6 or TLR2/TLR1 (Jin et al., 2007). Some groups report that peptidoglycan, lipoproteins or LTA play a central role in immune activation (Bubeck Wardenburg et al., 2006; Hoebe et al., 2005; Seo et al., 2008; Boneca, 2005; Dziarski and Gupta, 2005; Lien et al., 1999; Morath et al., 2005, von Aulock et al., 2007), while their ability to induce cytokine release is questioned by others (Hashimoto et al., 2006; Travassos et al., 2004). The core of the discussion is how pure the preparations being tested are and whether minor contaminations with the other components of the cell wall are in fact responsible for immune activation.

To gain more information about the role of the different membrane components of *S. aureus* for immune recognition of whole bacteria, Rockel et al. (2011) compared the immune stimulatory activity of three different *S. aureus* mutant strains lacking either lipoproteins or wall teichoic acids, or possessing a reduced d-alanine content in lipoteichoic acid (LTA) to its corresponding wild type. Inactivated whole bacteria and their purified cell wall components peptidoglycan and LTA, were used to stimulate human whole blood and macrophages from TLR2 wildtype and knock-out mice. They found that whole bacteria from all *S. aureus* strains induced similar amounts of TNF, IL-8, and IL-10 in human whole blood, and none of them was dependent on the presence of TLR2. Highly purified peptidoglycan from all strains, in contrast to LTA, had very low cytokine stimulating activity. Taken together, these results demonstrate that major cell wall alterations do not affect the overall cytokine inducing potential of whole bacteria, and thus cytokine induction appears to be initiated by redundant mechanisms.

A systematic review of membrane components of Gram-positive bacteria responsible as pyrogens for inducing human monocyte/macrophage cytokine release was carried out following the principles of Evidence-based Medicine (Rockel and Hartung, 2012). The authors analyzed the fulfillment of the Koch/Dale criteria (Dale, 1929) for the three membrane components lipoteichoic acid, peptidoglycan, and bacterial lipoproteins. They found enough evidence to conclude that LTA, PGN, and lipoproteins are all potent cytokine inducers that were able to fulfill at least three of Koch and Dale's criteria to prove their role as cytokine inducers in human cells. The presence of LPS was mostly excluded by the LAL assay.

This overview of the state of the art regarding pyrogens from Gram-positive bacteria strongly supports that safety testing for pyrogenic contaminations should be carried out in a way that unknown molecules and mechanisms of recognition of the immune system are taken into account to ensure the safety of human health. Only a test modeling the human immune response to its full extent can ensure an acceptable consumer safety standard.

1.5 Molecular basis of pyrogenic reactions

In mammals, specialized pattern recognition receptors (PRRs) are found mainly on monocytes and macrophages. They recognize pathogen-associated microbial patterns (PAMPs), which include bacterial cell wall components such as LPS of Gram-negative bacteria, and for Gram-positive bacteria, lipoteichoic acid (LTA) and peptidoglycan, as well as lipopeptides, flagellin, viral and fungal components, and bacterial DNA (Underhill and Ozinsky, 2002). Six families of PRRs have been identified that initiate pro-inflammatory signaling pathways, but the most prominent and best studied are the Toll-like receptors (TLRs) (Medzhitov et al., 1997; Akira and Hemmi, 2003; Lien and Ingalls, 2002). This results in a variety of responses triggering the production of pro-inflammatory cytokines (Dinarello, 2000). To underscore their importance, some examples are given (Fig. 5): TLR2 is responsible for the recognition of many PAMPs from Gram-positive bacteria, including bacterial lipoproteins, lipomannans, and lipoteichoic acids. TLR3 is involved in recognizing virus-derived double-stranded RNA. TLR4 is activated mainly by lipopolysaccharide. TLR5 reacts to bacterial flagellin, and TLR9 responds to unmethylated CpG DNA. TLR7 and TLR8 recognize small synthetic antiviral molecules (Jurk et al., 2002) and single-stranded RNA (Heil et al., 2004). They form complex networks, interacting with each other, such as diacylated lipoproteins are recognized via dimers of TLR2 and TLR6, and triacylated lipoproteins require TLR2 and TLR1 (Ozinsky et al., 2000). Furthermore, TLRs are connected to many adapter and accessory molecules. One example is MD-2 and CD14, which form a complex with TLR4 in response to recognition of LPS (Miyake, 2003).

NOD-like receptors (nucleotide oligomerization domain receptors = NLRs) are a family of intracellular pattern recognition receptors that contain more than 20 members in mammals. Only some signaling pathways are understood, as for example NOD1 (nucleotide oligomerization domain), which recognizes d-glutamyl-meso-diaminopimelic acid, a derivative of peptidoglycan, a major component of the bacterial cell wall (Chamaillard et al., 2003; Girardin et al., 2003a). NOD2 is known to interact with muramyl dipeptide (MDP), a structure found in almost all bacteria. The signaling pathway is complex, leading via several subfactors (Kobayashi et al., 2002; Hsu et al., 2007) to IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex) activation and the activation of MAPK (mitogen-activated protein kinase) (Kobayashi et al., 2005) but also to NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and the production of inflammatory cytokines. RIG-I-

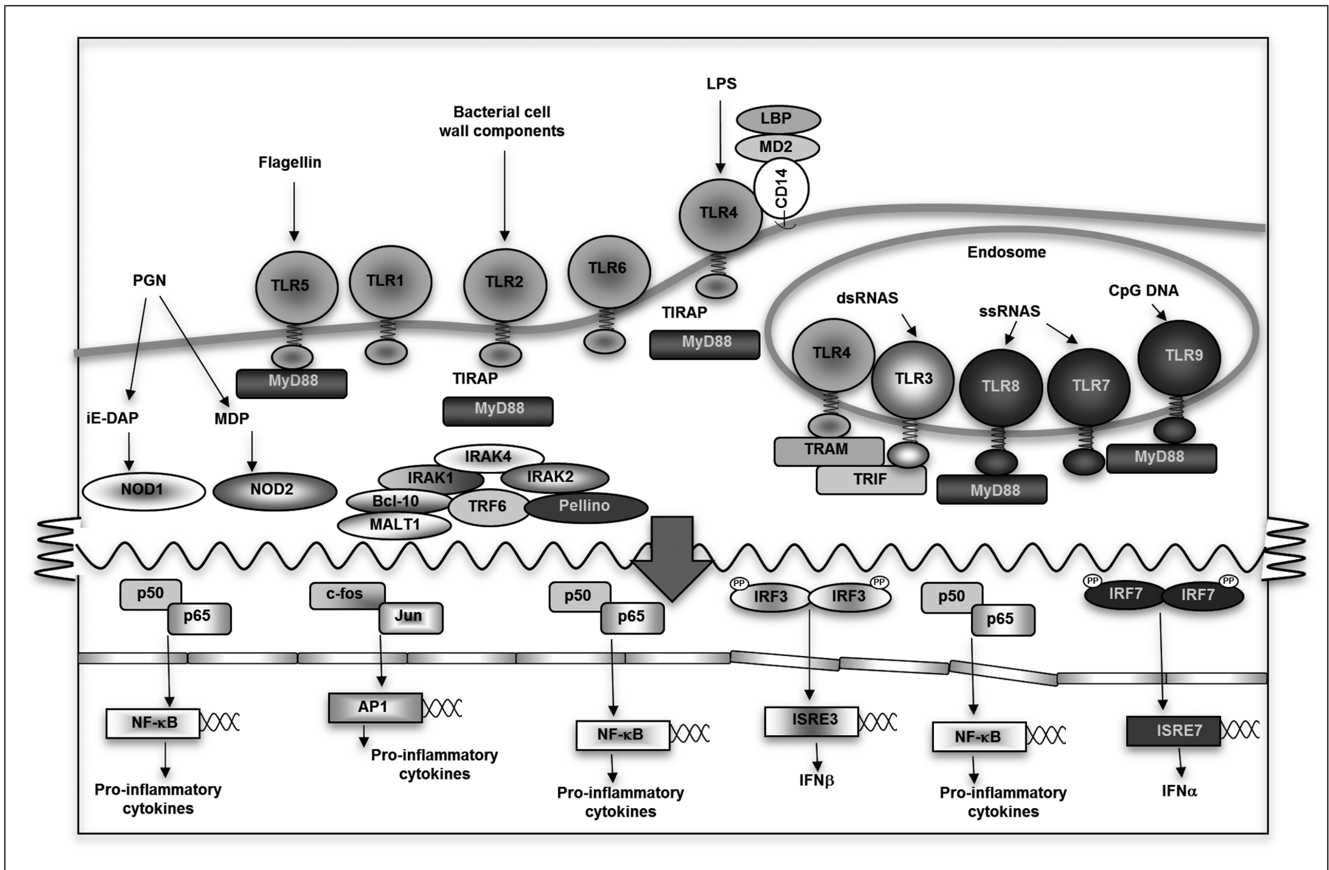

Fig. 5: Toll-like receptors activated by stimuli originating from various sources, leading to the production of pro-inflammatory cytokines
Redrawn with permission from invivogen.com

like-receptors (RLRs) / cytosolic DNA sensors (CDS) are RNA helicases found in the cytosol of the cell, triggering host viral responses. RIG-I and MDA-5 recognize double-stranded RNA (dsRNA), which is an intermediate of virus replication leading to the activation of transcription factors for interferons (Yoneyama and Fujita, 2007).

C-type Lectin receptors (CLRs) are a large family of phagocytic receptors that bind carbohydrate motifs of various pathogens. Some of them are membrane-bound, others freely available. They are present on and in many cell types, macrophages, and dendritic cells. Some of them are well characterized, as for example dectin-1 and dectin-2, which play an important role in the host's antifungal answer. Dectin-1 is the specialized receptor of β-glucans, a major component of the fungal cell wall (Brown et al., 2003), while dectin-2 binds mannose-type carbohydrates and α-mannans (Drummond et al., 2011). Both ways lead to the activation of NF-κB and subsequent secretion of pro-inflammatory cytokines (Gross et al., 2006; Dennehy and Brown, 2007), e.g., IL-1β production (Sancho and Reis e Sousa, 2012).

There are many more receptors, such as CD36 (Hoebe et al., 2005), adapter molecules, signaling pathways, some of them not fully investigated or even yet discovered. Complex recognition mechanisms emerge, where receptor complexes form dynamically in contact with pyrogens (Pfeiffer et al., 2001; Triantafilou et al., 2004a,b, 2006) and internalization plays an additional role (Bunk et al., 2010; Nilsen et al., 2008).

This brief overview illustrates that whole spectrum of host responses is possible when body-foreign substances overcome the body's natural protective barriers. Innate immune mechanisms are intended to protect the human body from invading microorganisms, taking into account that one way might fail by redundancy. Cell lines, being of only one cell type, often having no access to binding molecules from human plasma, and remaining in culture over many passages can only access a limited spectrum of these complex responses, arguing strongly for the use of primary cells *ex vivo* for the detection of pyrogenic contaminations.

2 Evidence for non-endotoxin pyrogen detection in the whole blood MAT

As described above, many components other than LPS are able to evoke an innate immune response, carried mainly by macrophages and monocytes. To prove the suitability of a test, such as the whole blood MAT, to detect a potential threat, such as that of

Tab. 2: Various non-endotoxin stimuli tested with different variants of the Limulus Amebocyte Lysate Assay
The measured endotoxin values exceed the endotoxin content given by the manufacturer by multiple factors. Substances (see also Fig. 9) were prepared as stock solutions according to the manufacturers' instructions. All substances were tested in at least 2 different BET-versions. Gel Clot and Gel Clot II: Gel-Clot: Pyroquant; Method A of European Pharmacopoeia; "reference test". Recombinant LAL: PyroGene, Lonza; Chromogenic kinetic BET, Charles River; Method A of European Pharmacopoeia.

	Gel Clot	rec. LAL	chrom. LAL	PTS	Gel Clot II	Endotoxin content by manufacturer
HKAL	1.2		0.64			<0.125
PAM_3CSK_4	2.4		0.91			<0.125
PGN-SA	1.5		<2.5		1.2	<0.125
Zymosan	120	1.1	63.5			<0.125
Flagellin-BS	1200	481.4				<0.125
Gardiquimod	0.30		0.5			<0.125
MDP			0.075			<0.125
LTA-SA	120	<20	<50	<50	300	<1.25

non-endotoxin pyrogens in parenterals or medical devices, normally a validation study is necessary. One problem of an intense validation study of NEPs is the quality of the available stimuli (Morath et al., 2002a). Even when certified by the producer as endotoxin-free, the subsequent BET (Bacterial Endotoxin Test; LAL) testing often reveals different values (Tab. 2).

One possibility for overcoming this drawback is to block the LPS signal via Polymyxin B (van Miert and van Duin, 1978; Pool et al., 1999) or CD14 antibody. Another possibility is a LAL test of the unknown substance, which normally reveals the LPS content of a sample. Other problems include the disagreement on the structural nature of NEPs, the lack of their broad availability in pure (commercial) form and the lack of reference materials. The following subchapters show examples of clinical materials containing unknown NEP and NEP preparations shown to be covered by the whole blood MAT.

2.1 Case study human serum albumin (HSA)

There are several reports on HSA products that passed RPT and LAL but subsequently elicited pyrogenic episodes in patients (Steere et al., 1978; Poole et al., 1988a,b; Taktak et al., 1991) indicating that these tests do not always provide sufficient safety. As explained in detail by Pool and McLeod (1995), 3 patients receiving HSA from the same lot revealed symptoms such as trembling, shaking, severe rigors, and fever. Previous and subsequent infusion with different lots of HSA did not lead to any of these symptoms. Retesting of the HSA lot by the manufacturer still revealed neither bacterial (LAL) nor pyrogenic (RPT) contamination.

It was shown (van Miert and van Duin, 1978; Pool et al., 1999) that it is possible to block LPS activity by addition of Polymyxin B, whereas Gram-positive pyrogenicity and the assay itself are unaffected. Also, in HSA samples previously positive tested by the RPT, the effect of endotoxin could be blocked by Polymyxin

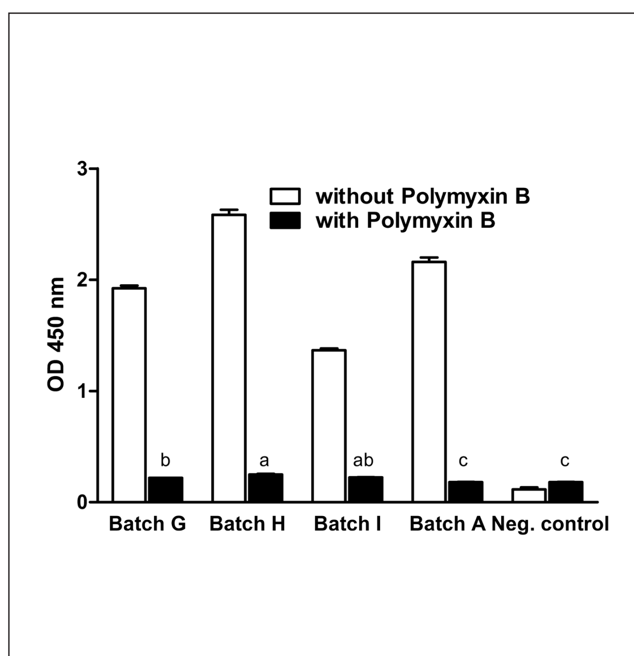

Fig. 6: Effect of Polymyxin B on MAT/IL-1β response in pyrogenic human serum albumin
Each sample was incubated with whole blood with and without Polymyxin B, and the IL-1β response in the supernatant was determined by ELISA. Batch A: Clean HSA sample spiked with 10 EU/ml (assay control); Batches G and I failed the Rabbit Pyrogen Test. Negative control: pyrogen-free physiological saline. Data are represented as mean ±SD of two separate experiments. A low cytokine response significantly different ($p<0.05$) from the assay control remained in the presence of PMB. This appears to be derived from the presence of non-endotoxin pro-inflammatory entities.

B, while the remaining whole blood MAT cytokine signal is due to non-inhibited fractions, not detected by the LAL-assay.

Recently, experimental studies were conducted using various batches of HSA for clinical use comparing the same samples applying the LAL, RPT, and MAT (Perdomo-Morales et al., 2011). The authors concluded that neither the RPT nor the LAL is sufficiently reliable to guarantee consumer safety. The influence of NEPs was investigated, and further LPS-spiking and blocking experiments revealed that all three contaminated batches contained significant concentrations of NEPs identified by the whole blood MAT. Therefore, the MAT is qualified to ensure consumer safety when it comes to pyrogenic contaminations other than LPS (Fig. 6).

Fever reactions caused by a batch of human serum albumin (negative in RPT as well as in the LAL test) also were analyzed, in this case by the Paul-Ehrlich Institute (PEI), the German national control authority. After application of a defined batch of human serum albumin, fever reactions were reported to the PEI. The batch was withdrawn from the market. The manufacturer had tested the product in RPT as a release criterion with negative result. The PEI examined samples of the batch in RPT, whole blood MAT, and in LAL. Negative albumin batches from the same manufacturer served as controls. RPT as well as LAL remained negative. The results of the MAT (5 different donors, at least 15 repetitions per donor) are shown in Table 3. The incriminated batch, negative in LAL as well as in RPT, clearly tested positive in the whole blood MAT.

2.2 Case study infusion solution

An infusion solution containing gelatine (release criterion LAL) induced adverse fever reactions in hospitals. The manufacturer withdrew the incriminated batches from the market and reinvestigated them in LAL and additionally in RPT. The company observed negative LAL but positive RPT results in one batch. However, the most interesting batch, which caused fever in patients, remained negative in LAL as well as in rabbits. The batches were blinded by the manufacturer, sent to PEI, and analyzed in whole blood MAT in parallel with blinded non-incriminated control batches. The results are summarized in Table 4.

The incriminated batches B and C could be identified very clearly in whole blood MAT by donor X by either IL-1β, IL-6, or TNF-α-induction exceeding the cut-off, even the sample remaining negative in RPT. Donor Y detected incriminated batch B via IL-1β, IL-6, or TNF-α-induction, whereas batch C was only detected via IL-1β, the main fever-inducing cytokine. Independent of the chosen cut-off calculation, both donors reacted to the incriminated batches with higher cytokine release as compared to control batch A. The fever causing substances have to be seen as non-endotoxin pyrogens not detectable in LAL. At least one of them is not pyrogenic for rabbits but is for humans.

Tab. 3: Incriminated human serum albumin
Samples were tested using the fresh whole blood assay as described in EDQM (2010). The readout was IL-1β.

Donor	Incriminated batch IL-1 (pg/ml)	Control batch IL-1 (pg/ml)	Quotient incriminated/control
1	79.0	4.0	19.75
2	14.1	3.9	3.61
3	44.3	15.0	2.95
4	20.9	14.9	1.4
5	71.9	3.9	18.44
Mean	**46.04**	**8.34**	**5.52**

Tab. 4: Incriminated infusion solution containing gelatin
The MAT was performed with fresh whole blood; mean of duplicates; 2 donors X and Y; Cut-off calculation: EC = Endotoxin Control; EC1 = 1 pg/ml; EC2 = 300 pg/ml; EC3 = 50 pg/ml; cut-off = mOD450 + ((mOD450K2-mOD450K1)/10).

Batch	LAL test	Rabbit test	Fever in patients	Whole Blood Pyrogen Test		
				IL-1 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
A	negative	negative	no	8.5	28.0	28.2
B	negative	positive	yes	142.6	654.4	67.6
C	negative	negative	yes	421.5	9444.0	116.7
			cut off:	**32.6**	**127.6**	**43.6**

Tab. 5: Testing of complaint batches: Interleukin 6 response of icodextrin-containing dialysate and icodextrin in the PBMC assay from three representative donors
Redrawn from Martis et al. (2005).

	Donor 1 (ng/l)[a]	Donor 2 (ng/l)	Donor 3 (ng/l)
Control medium[b]	45	24	50
Positive controls[c]	12,000	10,000	10,000
Negative control[d]	130	92	150
Icodextrin-containing dialysate (non-complaint batch)	330	350	110
Icodextrin-containing dialysate (complaint batch 1)	5100	7000	970
Icodextrin-containing dialysate (complaint batch 2)	4200	4200	700
Icodextrin raw material (non-complaint batch)	91	100	130
Icodextrin raw material (complaint batch)	9300	780	1600

[a] Interleukin-6 response greater than 500 ng/l judged to be a positive pyrogenic response
[b] Eagle's minimum essential medium with supplemental components
[c] Internal positive control of biosynthetic haemoglobin that passed Limulus Amoebocyte Lysate testing but produced pyrogenic response in RPT and PBMC assay
[d] Glucose-containing standard peritoneal dialysis solution

In conclusion, the whole blood MAT indicates *in vivo* reactions of humans, whereas the rabbit is not able to detect every non-endotoxin pyrogen. Unfortunately, the manufacturer of the infusion solution does not agree to publication of the results, and the identity of the drug cannot be revealed.

2.3 Case study dialysis solution

Dialysis is the only treatment option for patients with chronic, severe kidney disease if no appropriate donor organ is available. Both procedures, renal and peritoneal dialysis, employ the principle of diffusion to remove waste and excess water from the body fluid. In the case of renal dialysis, the blood flows outside the body and is washed by passing a semi-permeable membrane, separating the dialysis solution from the blood. Molecules can pass through the membrane according to the diffusion gradient, returning the "cleaner" blood to the human body. In the case of peritoneal dialysis, the peritoneal membrane is used as a separator inside the body. Nevertheless, tubes and the dialysis solution enter the human body, posing a potential risk to the patient. According to European and US Pharmacopoeia standards, all substances and devices administered as parenterals must be tested for pyrogens. The RPT and the BET still are the standard methods to test tubes, membranes, and the dialysis solution.

Even when applying the most careful standards for dialysis solutions, from time to time adverse drug reaction (ADR) complaints arise (Karanicolas et al., 1977; Mangram et al., 1998; Benevent et al., 1984; Tuncer et al., 2000; Gokal et al., 1981), for example in 2001-2003, when a global recall was issued for a certain icodextrin-containing dialysate. Patients using these batches complained about abdominal pain, nausea, vomiting, diarrhea, fever, and emitted a cloudy dialysate.

Martis et al. (2005) investigated the patients' reports and the incriminated batches. The complaint batches did not produce a signal in the LAL assay, nor did they show a body temperature rise in rabbits. To establish a relationship between the clinical symptoms and the dialysis solution, they then used an *ex vivo* pyrogen test measuring IL-6 response in freshly isolated human peripheral blood mononuclear cells (PBMCs) from healthy donors after exposure to the test substance as described by Poole et al. (1988b) and later developed as one of the MAT versions. They found a correlation between the incriminated batches and an increased cytokine release, suggesting the presence of NEP contaminations (Tab. 5).

Analysis of the samples in the silkworm larvae plasma test confirmed that peptidoglycan was part of the non-endotoxin contamination, and investigation of the production process revealed the Gram-positive bacteria *Alicyclobacillus acidocaldarius* as the originating source of the contamination (Martis et al., 2005). Noteworthy, it was shown that the whole blood MAT can test dialysis solutions with adequate sensitivity (Daneshian et al., 2008).

2.4 Biofilms

Another example is the detection of biofilm contamination in the dialysis tubing. Biofilms are aggregates of microorganisms adhering to each other and to surfaces, producing an extracellular polymeric matrix. They can be found in many different settings causing various problems such as fever and infections (Hall-Stoodley et al., 2004; Lear and Lewis, 2012; Karatan and Watnick, 2009). Especially in dialysis tubes, which are meant to overcome the human natural barrier, the presence of these biofilms may be detrimental. The fluids passing through the

tubing may bring biofilm components into contact with the human immune system, evoking an unwanted response. Many of the biofilm-creating bacteria are of Gram-positive origin and do not reveal any signal response in the LAL. A recent publication (Marion-Ferey et al., 2005) showed that the amounts of endotoxin equivalents measured by the MAT were significantly higher than the endotoxin measured in the Limulus Amebocyte Lysate (LAL) test, again indicating a contribution of NEPs to detrimental effects. The authors concluded that the LAL assay is insufficient to guarantee a representative quantification of pyrogenic contaminations that could be hazardous to the patients' health.

2.5 Pyrogen detection in immunomodulatory, toxic and high volume parenterals by whole blood MAT

A solution for evaluation and quantification of pyrogenic burden for toxic and immune-modulatory drugs was developed by Daneshian et al. (2006). In this study the whole blood MAT was modified on the basis of human albumin-linked beads that are able to adsorb pyrogenic molecules from solutions. The modified test was termed AWIPT (adsorb, wash, *in vitro* pyrogen test), as it included adsorption steps of the pyrogens to albumin-linked beads and washing steps before the whole blood MAT (*in vitro* pyrogen test) measurement, as shown for the example of the chemotherapeutic drug daunorubicin in Figure 7.

This approach was subjected to further development to quantify pyrogenic contaminations at very low concentrations in water and dialysis solutions, arguing that even very low concentrations of contaminations add up to a significant threat in the case of dialysis where the patient is exposed to large volumes of fluid at each session. It could be shown that pyrogens at very low concentrations can be accumulated from a large volume of fluids on human albumin-linked beads and detected in the whole blood MAT (the procedure was names AcWIPT) (Daneshian et al., 2008). Much lower concentrations of pyrogens, i.e. LPS and LTA, (e.g. 0.1 pg/ml LPS i.e. 0.001 EU/ml) can be measured, as shown in Figure 8. These data show hat modifications of the whole blood MAT can achieve considerably lower detection limits to meet demands for high-volume parenterals, dialysis solutions and intrathecal applications. Furthermore, absorption of pyrogens, washing off inhibitory components and subsequent measurement with the whole blood MAT allows measurements of previously not testable materials. Beside the daunorubicin example, paclitaxel, cisplatin, gentamicin, liposomal amphotericin B, and prednisolone were tested successfully (Daneshian et al., 2006).

2.6 General ability of the whole blood MAT to respond to selected NEPs

To ensure consumer safety for products intended to enter the human body, test systems must be in place that are able to model the complexity and the efficiency of the human system. The MAT, using human immune response related cells, is able to do that, and scientifically there are no doubts that monocytes are able to detect and react to non-endotoxin pyrogens via their specialized receptors, as demonstrated in Figure 9.

The variety of NEPs and endotoxin used in Figure 9 are described in the following:

– HKAL: heat killed *Acholeplasma laidlawii*: *A. laidlawii*, a member of the mycoplasma family, is a bacterium without a cell wall. Despite the absence of classical modulins found in microbial cell walls, such as LPS and LTA, mycoplasma are potent activators of macrophages (Takeuchi et al., 2000). Heat-killed mycoplasma such as HKAL induce higher stimulation of macrophages than lipoproteins from other bacteria, even at low

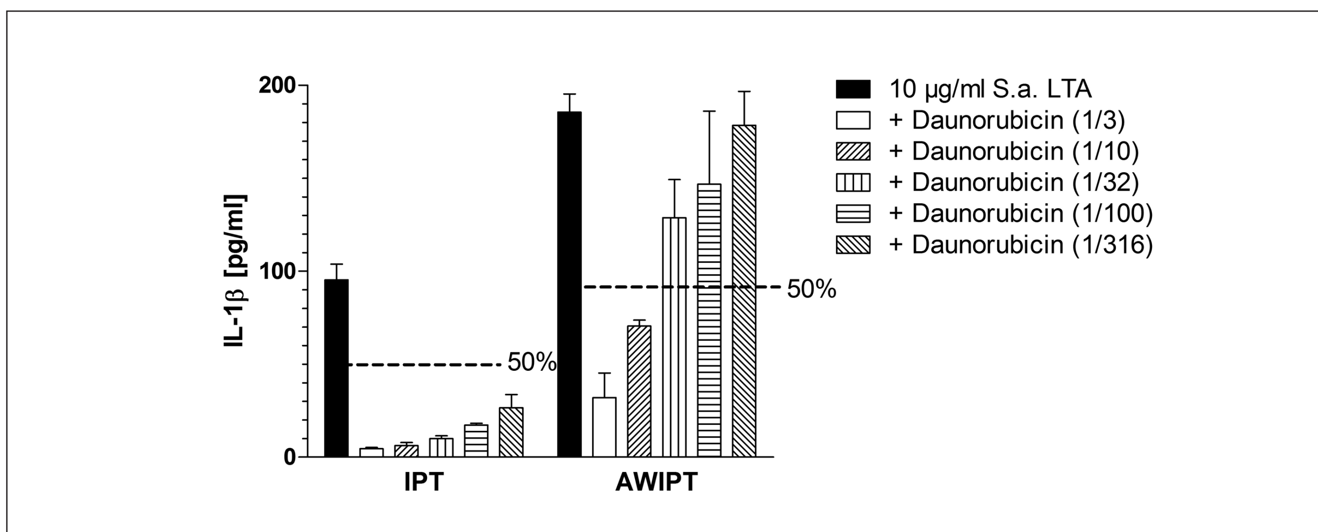

Fig. 7: Detection of endotoxin spikes in dilutions of liposomal Daunorubicin
Liposomal Daunorubicin (2 mg/ml) was diluted in series and spiked with 10 µg/ml S. aureus LTA. Samples of these dilutions were employed in the IPT and the AWIPT in parallel. IL-1β was measured by ELISA. Data are given as means ±SD of triplicates. 50% of cytokine secretion in response to the LTA spike are marked by a dashed horizontal line. Figure and legend adapted from (Daneshian et al., 2006).

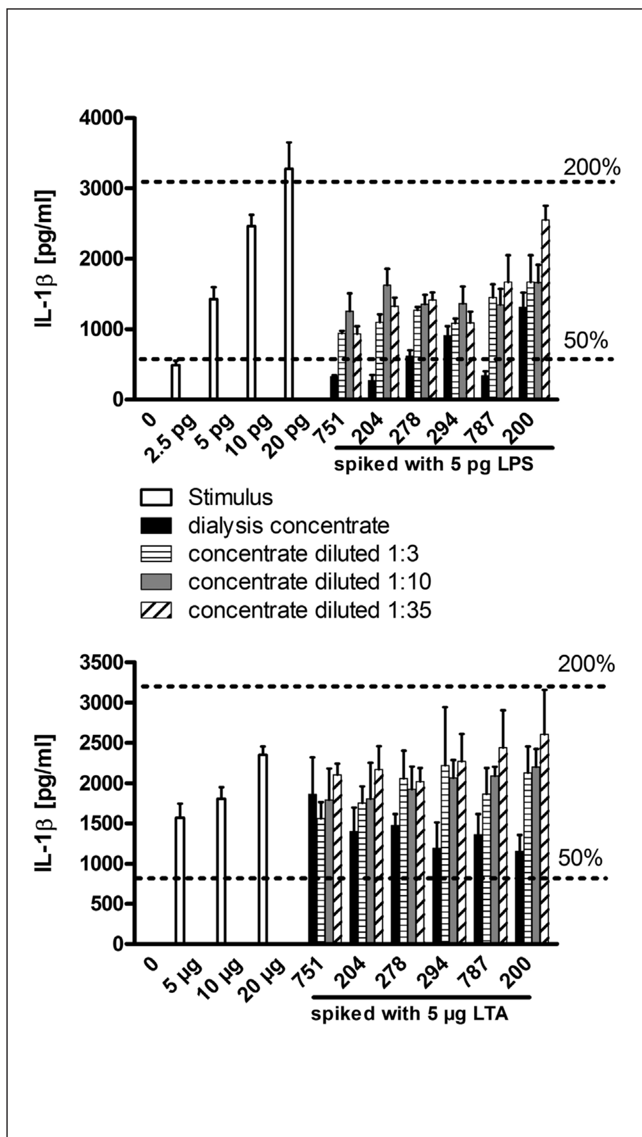

Fig. 8: Sensitive spike retrieval in some dialysis concentrates and all tested dialysis working solutions by AcWIPT
50 ml dialysis concentrates and working solutions (1/35) spiked with 5 pg LPS or 5 μg LTA were employed in the AcWIPT. In parallel, an LPS or LTA concentration/ response curve was performed in the AcWIPT. The dotted line shows 50% and 200% spike retrieval. Figure and legend adapted from (Daneshian et al., 2008).

concentrations (Muhlradt et al., 1997). The response is mediated by TLR2 and MyD88. Stimulation with HKAL induces rapid activation of NF-κB and the production of pro-inflammatory cytokines.

– Pam_3CSK_4: synthetic bacterial lipoprotein: Bacterial lipoproteins are a family of pro-inflammatory cell wall components found in both Gram-positive and Gram-negative bacteria. The stimulatory activity of bacterial lipoproteins resides in their acylated amino terminus. Pam_3CSK_4 is a synthetic tri-palmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins. Pam_3CSK_4 is a potent activator of the pro-inflammatory transcription factor NF-κB (Aliprantis et al., 1999). Its recognition is mediated by TLR2, which cooperates with TLR1 through their cytoplasmic domain to induce the signaling cascade (Ozinsky et al., 2000).

– PGN: Peptidoglycan is a major surface component of Gram-positive bacteria. PGN was described to be a potent activator of NF-κB and TNF-α, and its immune-stimulatory response was determined to be mediated by TLR2 (Takeuchi et al., 1999). However, recent studies with purified PGNs from different bacteria have revealed that these PGNs are not sensed via TLR2, TLR2/1 or TLR2/6 (Travassos et al., 2004). The PGN signal is lost after removal of lipoproteins or LTAs from bacterial cell walls. These data suggest the immune-stimulatory activity of PGN is triggered by other pattern recognition proteins (PRPs), such as NOD1 and NOD2 (Girardin et al., 2003b), intracellular PRPs that detect PGN degradation products, and PGRPs, for which the role in PGN response is still unknown (Dziarski, 2004).

– Zymosan: Zymosan, an insoluble preparation of the cell wall from *Saccharomyces cerevisiae*, activates macrophages via TLR2, which cooperates with TLR6 and CD14 (Ozinsky et al., 2000). Zymosan is also recognized by Dectin-1, a phagocytic receptor expressed on macrophages and dendritic cells, which collaborates with TLR2 and TLR6, enhancing the immune responses triggered by the recognition of zymosan by each receptor (Gantner et al., 2003).

– Flagellin (*Bacillus subtilis*): Flagellin from both Gram-positive and Gram-negative bacteria is recognized by Toll-like receptor 5 (TLR5). The flagellin used here was from the Gram-positive bacterium *Bacillus subtilis*. Activation of the receptor stimulates the production of pro-inflammatory cytokines, such as TNF-α, through signaling via the adaptor protein MyD88 (Gewirtz et al., 2001; Hayashi et al., 2001). TLR5 can generate a pro-inflammatory signal as a homodimer, suggesting that it might be the only TLR participating in flagellin recognition. However, TLR5 may require the presence of a co-receptor or adaptor molecule for efficient ligand recognition and/or signaling (Tallant et al., 2004).

– LTA: Lipoteichoic acid is a major immune-stimulatory component of Gram-positive bacteria. LTA shares many of the biochemical and physiological properties of LPS and has long been suspected of causing Gram-positive sepsis (Bone, 1993, 1994; Holm, 1982; Sriskandan and Cohen, 1999; Tanowitz and Chan, 2000). LTA is an amphiphile, formed by a hydrophilic polyphosphate polymer linked to a neutral glycolipid and stimulates immune cells through TLR2 to produce TNF-α and other inflammatory cytokines (Schwandner et al., 1999).

– WHO-LPS: Lipopolysaccharide (reference material): WHO International Standard Endotoxin (WHO-LPS 94/580, Escherichia coli O113:H10:K(-)) is a lipopolysaccharide originating from Gram-negative bacteria representing the best-characterized and strongest inducer of immune responses known so far (Poole et al., 1997).

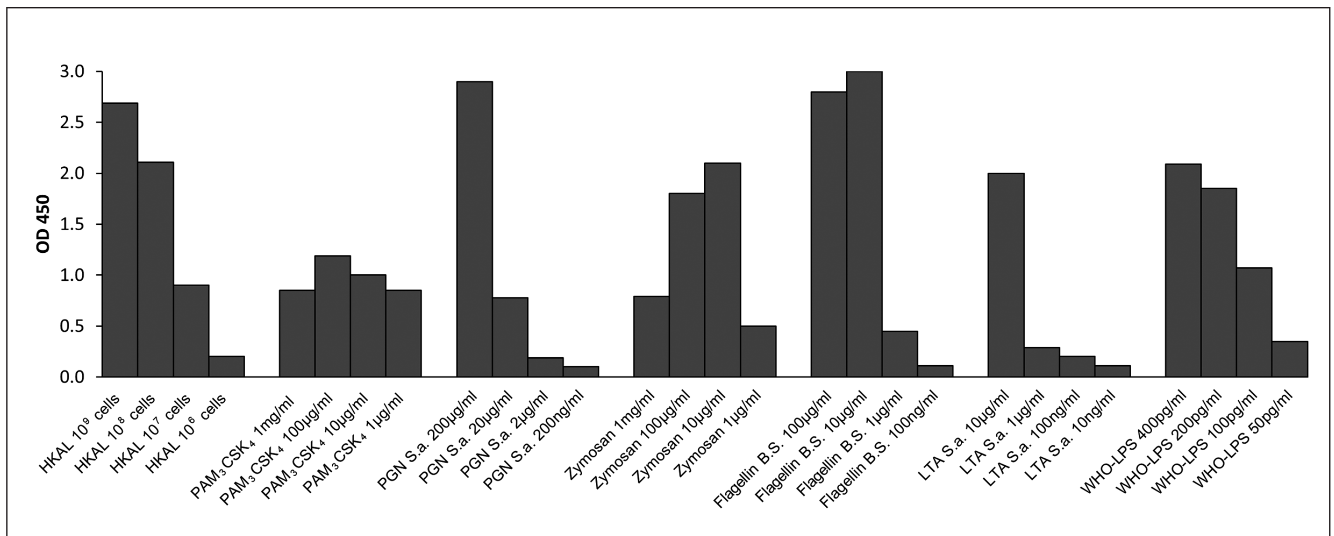

Fig. 9: Various stimuli tested in the MAT

Immune response stimulating substances originating from various sources in comparison to LPS (cryopreserved blood, pool of 5 donors). After determination of the individual endotoxin content (see Tab. 2) the stock-solutions employed in the BET were diluted in 10 fold-steps and compared with WHO-LPS in a MAT. Blood of 5 donors was pooled and stored at -80°C; storage time did not exceed 41 days; and a Dizytokine-ELISA was performed (detects both IL-1β and IL-6 as a sum signal without discriminating between them). The endotoxin content of the first three stimuli is in the same low range (see Tab. 2), but the MAT results indicate clear dose-response differences.

2.7 Evidence for lipoteichoic acid (LTA) detection in the whole blood MAT

The literature on LTA as an immune stimulus is flooded by work using endotoxin contaminated and degraded commercial materials (Morath et al., 2002a). A breakthrough attributing LTA a role as a major pyrogen of Gram-positive bacteria was the development of an isolation procedure that results in active but endotoxin-free material (Morath et al., 2001). Note that this isolation was guided by using the whole blood MAT to detect the biological activity and the LAL to exclude endotoxin contamination.

LTA gives consistent responses in both fresh and cryopreserved blood from single or pooled donors (Spreitzer et al., 2008). Another study confirmed "*no donor-dependecy was found to highly purified lipoteichoic acid from the Gram-positive bacteria Bacillus subtilis and Staphylococcus aureus, known to be mediated via TLR-2 and TLR-6*" (Carlin and Viitanen, 2005). The largest comparison included 154 donors showing no non-responders to LTA in the whole blood MAT (Aulock et al., 2006). A systematic review by Rockel and Hartung (Rockel and Hartung, 2012) addressed LTA as one candidate NEP and included only publications following a detailed search strategy. Sixteen publications with data for endotoxin exclusion revealed a release of pro-inflammatory cytokines such as IL-1β, IL-6, IL- 8, and TNF, or the anti-inflammatory cytokines IL-10 and G-CSF, in human whole blood, PBMCs, THP-1 cells, human primary monocytes, or neutrophils after LTA stimulation (Rockel and Hartung, 2012), see Table 6.

LTA has been shown to be an immune activator in humans *in vivo* (Knapp et al., 2008). A number of receptors appear to be involved in LTA recognition including CD14 (Fan et al., 1999), TLR-2 (Lehner et al., 2001; von Aulock et al., 2004; Lotz et al., 2004; Michelsen et al., 2001; Opitz et al., 2001; Liljeroos et al., 2007), TLR-6, complement factor L-ficollin (Lynch et al., 2004; Mayilyan et al., 2007), CD36 (Hoffman, 2000, Hoebe et al., 2005), and LBP (Fan et al., 1999; Mueller et al., 2006). This stresses again the need for primary cells from different donors to show a physiological reaction, arguing against cell lines and single receptor transfected approaches.

Note that the description of a first inhibitor of LTA but not LPS action (Draing et al., 2008) provides a tool to distinguish this NEP from LPS in complex pyrogen mixtures. Furthermore, a clear structure/function relationship for LTA inducible cytokine release in whole blood strongly supports its crucial role in immune recognition (Morath et al., 2001, 2005; Grangette et al., 2005). The strongest evidence for LTA representing a Gram-positive pyrogen comes from synthetic LTA (Stadelmaier et al., 2003; Morath et al., 2002b), which was active in the whole blood MAT. Subsequent work included the definition of a minimal structure by synthetic variants (Deininger et al., 2003; Figueroa-Perez et al., 2005, 2006; Stadelmaier et al., 2006). However, it appears that, as opposed to Gram-negative bacteria where LPS is the predominant pyrogen with some synergy of peptidoglycan/muropeptides (Traub et al., 2004), Gram-positives have redundant PAMPs as elimination of a single one does not blunt cytokine release (Rockel et al., 2011).

A key problem in understanding the nature of the Gram-positive pyrogen is the obvious difference in potency, typically a factor of 10^3. However, first it must be recalled that different LPS vary in potency by a factor of 10^5 (Dehus et al., 2006), and we are somehow blinded by the fact that almost all LPS research is done with the highly potent LPS from Enterobacter

or *Salmonella*. Second, the relative potency depends on the cytokine measured, with some cytokines such as IL-8 and G-CSF even more strongly induced by LTA than by LPS (von Aulock et al., 2003). Furthermore, it appears that a key difference between LTA and LPS is that the former requires presentation on a surface (Deininger et al., 2008) resulting in a 10^3 increase in potency. Note that LTA is the only NEP for which such an increase in potency has been shown, making it a prime candidate as the leading structure for recognition of Gram-positive bacteria by human monocytes in the MAT. This makes LTA both a prime candidate for MAT NEP reference materials and key for detectability to establish the reliability of MAT for NEP determination.

2.8 Evidence for Peptidoglycan and muropeptide detection in the whole blood MAT

The same exercise (Rockel and Hartung, 2012) was done for peptidoglycan (9 publications, two using the whole blood MAT) and synthetic, recombinant, or isolated lipoproteins (8 publications, none using the whole blood MAT, i.e., (Wang et al., 2002; Schromm et al., 2007; McCurdy et al., 2003; Schroder et al., 2004; Sieling et al., 2003; Wu et al., 2008; DiRita et al., 2000; Giambartolomei et al., 2002). Note that PGN and muropeptides synergize with LPS and LTA in the whole blood MAT (Traub et al., 2006; Holtkamp et al., 2010), which is not seen in the BET. Work using synthetic muropeptides supports this biological activity (Traub et al., 2004), see Table 7.

Tab. 6: Summary of the literature fulfilling the criteria using the whole blood MAT stimulated with LTA
Modified from Rockel and Hartung (2012). Noteworthy, other variants of the MAT also fulfilled the criteria of this study: Kim et al. (2007a), Uehara et al. (2002), Bucki et al. (2008), Kim et al. (2008), Mueller et al. (2006), Kim et al. (2007b), Henneke et al. (2005), Grangette et al. (2005), Schroder et al. (2003), Jacinto et al. (2002), Plitnick et al. (2001), Into et al. (2007).

References	Substances	Sources of substances	Cytokines	Cells	Endotoxin exclusion
Deininger et al., 2003	LTA	Synthetic	IL-1β, TNF, IL-6, IL-8, IL-10	Whole blood	LAL
Deininger et al., 2007	LTA	Synthetic	TNF, IL-8	Whole blood	LAL
Deininger et al., 2008	LTA	Isolated	IL-8, IL-1β, IL-6, TNF, G-CSF, IL-10	Whole blood	LAL
Draing et al., 2006	LTA	Isolated	TNF, IL-8, IL-10, G-CSF, IL-1β	PBMC, whole blood	LAL
Draing et al., 2008	LTA	Isolated	TNF	Whole blood, PBMC	LAL
Hasiwa et al., 2007	PGN, LTA	Commercial, isolated	IL-1β	Whole blood	LAL
Levels et al., 2003	LTA	Commercial	TNF	Whole blood	LAL (<19ng/mg LTA)
Meron-Sudai et al., 2008	LTA, Pam_3CSK_4	Isolated	IL-1β	Whole blood, monocytes	LAL
Morath et al., 2001	LTA	Isolated	TNF	Whole blood	LAL
Morath et al., 2002a	LTA	Isolated, synthetic	TNF, IL-1β, IL-6, IL-10	Whole blood, PBMC	LAL
Morath et al., 2002b	LTA	Synthetic	TNF	Whole blood	–
Stadelmaier et al., 2006	LTA	Synthetic	TNF, IL-8	Whole blood	–
Wang et al., 2000	LTA, PGN	Isolated	TNF, IL-1β, IL-6	Whole blood	Polymyxin B

Tab. 7: Summary of the literature fulfilling the criteria using PGN as stimulus
Modified from Rockel and Hartung (2012). Noteworthy, other variants of the MAT also fulfilled the criteria of this study:
Wolfert et al. (2002), McCurdy et al. (2003), Natsuka et al.(2008), Uehara et al. (2005), Into et al. (2007), Eriksson et al. (2006), Uehara et al. (2006).

References	Substances	Sources of substances	Cytokines	Cells	Endotoxin exclusion
Wang et al., 2000	LTA, PGN	Isolated	TNF, IL-1β, IL-6	Whole blood	Polymyxin B
Langer et al., 2008	PGN	Isolated	TNF	Whole blood	Polymyxin B

Comparing the three major cell wall components of Gram-positive bacteria, the systematic review of Rockel and Hartung (2012) shows that they play an important role in stimulation of the human innate immune system. However, the authors conclude that LTA is the most probable main non-endotoxin stimulus of cytokine production in humans, as they also addressed the criteria of Bradford Hill (Hill, 1965) for assessing evidence of causation. These criteria (strength, consistency, specificity, temporality, biological gradient, plausibility, coherence, and experiment) are only fulfilled completely by LTA out of the three components.

2.9 Evidence for the detection of fungal pyrogens by whole blood MAT

Next to bacteria and viruses, yeasts as well as filamentous fungi may pose a microbial challenge to the human immune system (Johnson, 2000). Few yeasts and filamentous fungi are generally considered to be human pathogens, but medical case reports about a variety of fungal species previously considered to be non-pathogenic are accumulating (Stark et al., 2003; Lyratzopoulos et al., 2002; Patterson, 2005). The fever inducing potential of fungi has long been known (Braude et al., 1960). Two classes of conserved surface structures of fungi have been proposed to be immune-stimulating non-endotoxins: glycans and structures containing fatty acids. The surface glycans, e.g., β(1-3) and β(1-6) glucans such as zymosan, laminarin, lichenan, and curdlan interfere with the LAL-test (Vassallo and Limper, 1999). The rabbit test does not mirror the human situation regarding pro-inflammatory cytokine release upon stimulation with glucans, as the concentration of glucan needed to induce fever in rabbits (about 100 μg/ml - 300 μg/ml) is far higher than for humans (0.01 μg/ml - 0.1 μg/ml) (Nakagawa et al., 2002; Ellis et al., 2008; Obayashi et al., 1995). This high amount of glucan also causes the development of severe adverse effects, e.g., splenomegaly, in rabbits (Williams et al., 1988).

Evaluation of the pyrogenic capacity of the surface lipophilic compounds of fungi in LAL or the rabbit test is also difficult, as these surface compounds are modified with glycans, e.g., with GPI-anchors (Costachel et al., 2005). Moreover, there are hints from glucan research pointing to impurities and lipophilic contamination of these preparations, which may play a causative role in the inability of even modified LAL tests – Glucatell – to adequately mirror the pyrogenic capacity of fungal stimuli by producing "*falsely greater positive results*" (Ikeda et al., 2005; Cherid et al., 2011).

The pyrogenic activity of fungal spores was evaluated in the human whole blood MAT, using spores from more than 44 different pathogenic and non-pathogenic filamentous fungi and yeasts (Daneshian, 2006). Fungi in general proved to be highly active in the whole blood test, though differences between different strains exist (Fig. 10A). The use of the LPS inhibitor Polymyxin B demonstrated that the pyrogenic potency is not due to LPS contaminatinos (Fig. 10B).

In this approach the MAT assay proved to be an adequate system for evaluating the pyrogenic potential of fungal stimuli, directly reflecting the human reactivity, as comparative studies with LPS, LTA, and glucans regarding the kinetics of cytokine production, donor variance, and the pyrogenic activity of fungal glucans other than zymosan reveal (see below).

Cytokine patterns induced by fungal spores

The comparison of spore-induced cytokine patterns with those of LPS, LTA, and zymosan showed that the fungal immune-stimulatory pattern is more similar to LTA of Gram-positive bacteria than to LPS or zymosan, because of the high levels of IL-8 release and lack of IFNγ induction (Daneshian, 2006)

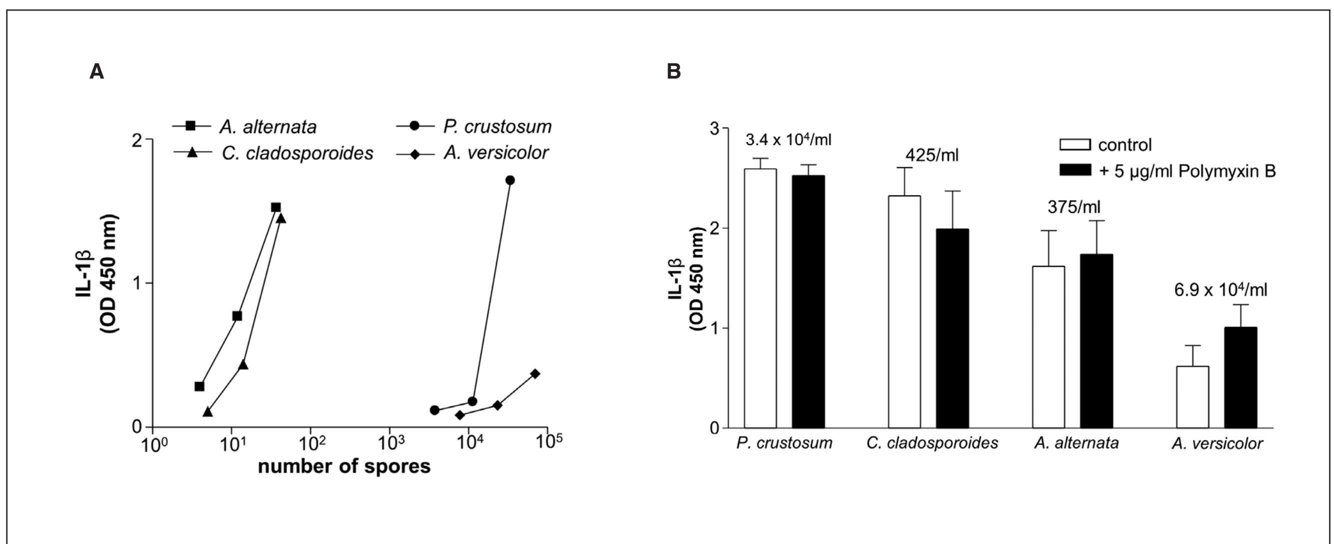

Fig. 10: Cytokine release of human whole blood upon stimulation with various fungi is not inhibited by Polymyxin B
A, Fungal spores (*Alternaria alternata*, *Cladosporium cladosporoides*, *Penicilium crustosum*, *Aspergillus versicolor*) induce the release of IL-1β by human whole blood. B, Co-incubation of Polymyxin B does not block cytokine release, indicating that LPS is not responsible for the cytokine induction. For experimental details see supplementary file A at www.altex-edition.org

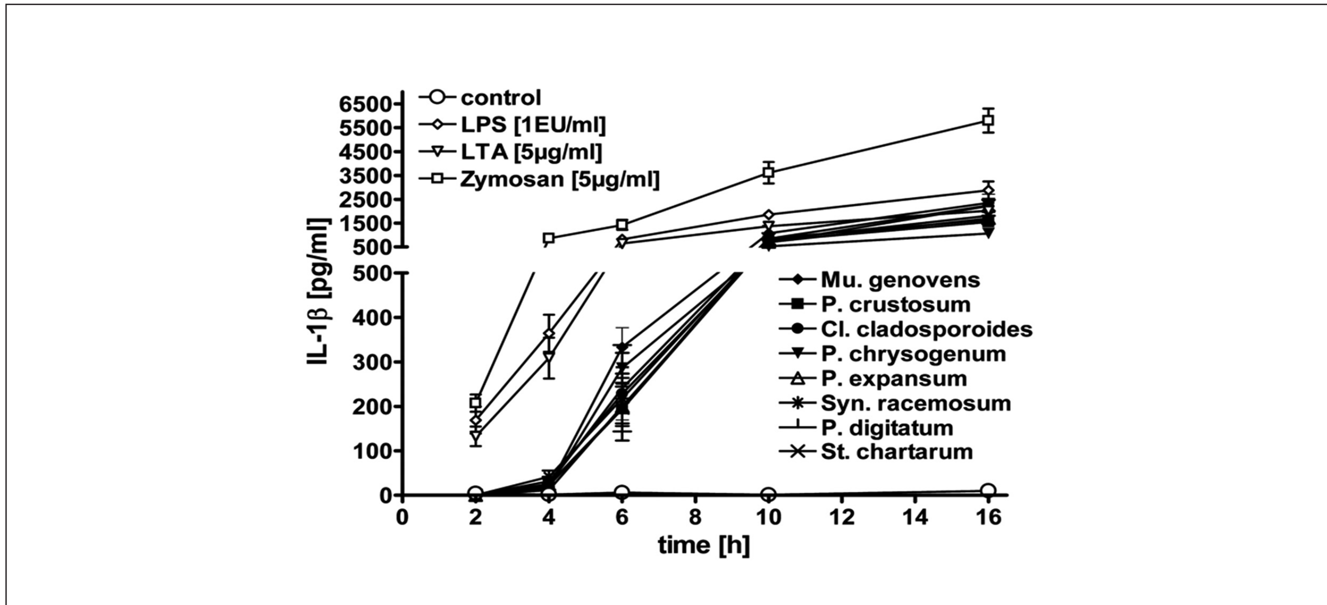

Fig. 11: Delayed kinetics of IL-1β production in response to fungal spores in comparison to LPS, LTA, and zymosan in human whole blood

Human whole blood was stimulated with the LPS, LTA, and zymosan or fungal spores, and IL-1β release was determined by ELISA at the given time points. 8 of 44 tested fungal species are shown exemplarily; for the stimulation with fungal spores, spore counts representing 10 mm^2 spore surface area were employed (Daneshian, 2006). For experimental details see supplementary file A at www.altex-edition.org

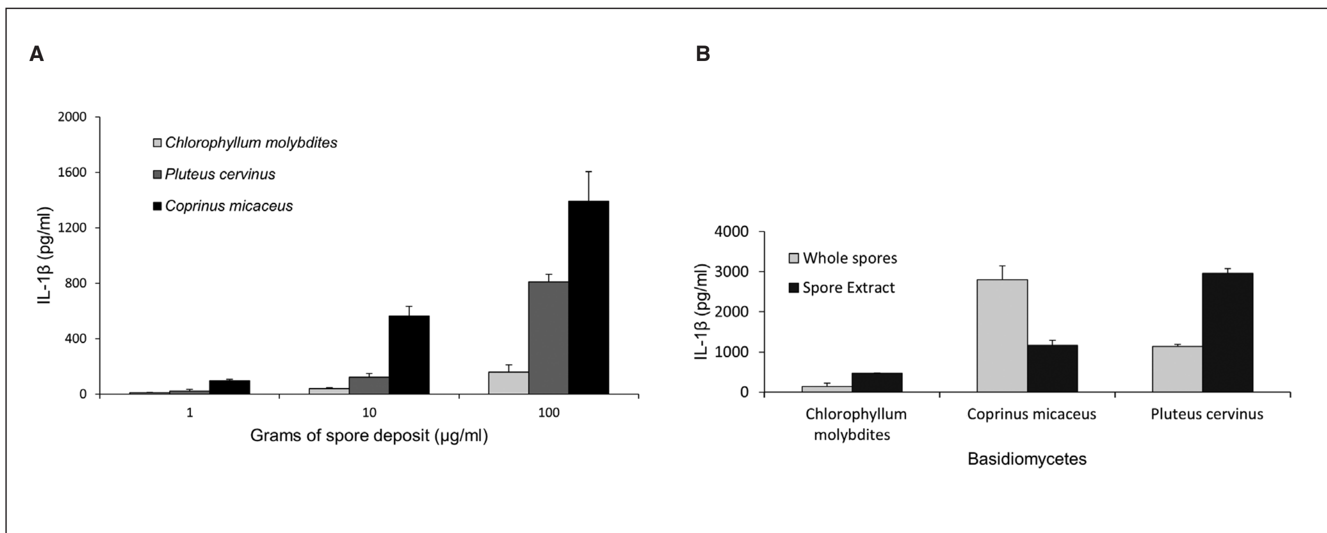

Fig. 12: IL-1β production in response to fungal spores and spore extracts of basidiomycete fungi in cryopreserved human whole blood

A, Spore deposits from basidiomycetes *Chlorophyllum molybdites*, *Pluteus cervinus*, and *Coprinus micaceus* were weighed into centrifuge tubes. Spore suspensions were prepared (1 mg spore deposit/ml) in pyrogen-free water (PFW) and diluted to 100µg/ml, 10µg/ml and 1µg/ml in PFW. Thawed cryopreserved human whole blood was stimulated for 18 hours with different suspensions of spore deposits from three different basidiomycete fungi, and IL-1β release was determined with ELISA. B, Spore suspensions (100 µg spore deposit/ml) were prepared in pyrogen-free Phosphate Buffered Saline (pfPBS) in microcentrifuge vials. The vials were sonicated for 30 min, centrifuged at 500 g for 5 min, and the supernatant was diluted 1:2 in pfPBS. Whole spore suspensions of basidiomycetes (100 µg spore deposit/ml) and 100 µl of extract were incubated in WBA to compare the proinflammatory potency between whole spores and spore extracts. Thawed cryopreserved human whole blood was stimulated with 100 µl of spores suspension (100 µl/ml) or 100 µl of spore extract for 18 hours, and IL-1β release was determined with ELISA. Spore extracts were prepared by disrupting 100 µl/ml of spore in pyrogen-free water.

also described previously for LTA (Hermann et al., 2002; von Aulock et al., 2003). The impurities, possibly endotoxin contaminations, in commercially available glucans, e.g., zymosan, may be the reason for this stimulus eliciting a different pyrogenic reaction than the other fungal stimuli tested (Ikeda et al., 2005).

Kinetics of IL-1β production in response to fungal stimulation
The kinetics of cytokine induction in blood in response to the purified stimuli LPS, LTA, and zymosan were compared with those of 44 fungal spore species. Eight species are shown representatively in Figure 11. LPS, LTA, and zymosan led to significant IL-1β and also TNF production after 2 hours, whereas cytokine release in response to all of the 44 tested mold and yeast species started only after at least 6 hours incubation, indicating the facilitated recognition of purified reference stimuli compared with whole spores. These data also show clearly that the fever-inducing capacity of fungal spores can be evaluated with the MAT method and that there is a very similar reaction to exposure to a certain total fungal surface area (here 10 mm^2), pointing to common fungal immune-stimulatory structures.

Comparison of the IL-1β production in response to whole spores and spore extracts from basidiomycete fungi
The induction of IL-1β in blood in response to whole spores and spore extracts from three basidiomycetes (which together with Ascomycotafor form the higher fungi) species (*Chlorophyllum molybdites*, *Pluteus cervinus*, and *Coprinus micaceus*) were compared (Rivera-Mariani et al., 2012). Dose-dependent production of IL-1β in response to a suspension of spore deposit is observed in Figure 12A, with spores of *C. micaceus* showing higher IL-1β inducing potency. In Figure 12B, the IL-1β inducing capacity of whole spores versus spore extract was evaluated. Whole spores and spore extracts differentially induced IL-1β in blood, indicating that whole spore and extract have different IL-1β inducing capacities. These data also show that whole blood MAT can evaluate the fever-inducing capacity of spores and spore extracts from higher fungi as well as mitosporic fungi.

Variance of donor responsiveness to fungal stimuli
The use of human peripheral blood cells for evaluation of fever-inducing capacities of endotoxins and non-endotoxins raises the question of donor variance. To examine the phenomenon of

Fig. 13: Comparison of various blood donors and their response to fungal spores
A: IL-1β induction in blood from 16 donors in response to fungal spores and to LPS (1 EU/ml), LTA (5 μg/ml) and zymosan (5 μg/ml).
B: IL-1β induction per 10^6 monocytes in blood from 16 donors; The IL-1β response to spores from seven fungal species is illustrated exemplarily from 33 species tested in total. Data are means ±SD of triplicates (Daneshian, 2006). For experimental details see supplementary file A at www.altex-edition.org

donor-related differences in their responsiveness upon stimulation with endotoxins and non-endotoxins, 16 blood donors were recruited, all of whom stated that they felt healthy, had normal white blood cell counts, and had no known allergies. The ability of their blood to react to fungal spores of 32 species and to LPS, LTA, and to zymosan by measuring IL-1β release was compared (Daneshian, 2006).

Figure 13A shows for six arbitrarily selected spores that, although the absolute response of the donors differs, the ranking of the different stimuli in the response of the individual blood donors is the same. For example, *Candida albicans* elicits the strongest IL-1β response from every donor, and *Aspergillus fumigatus* induces hardly any cytokine response in any of the donors.

As, in blood, IL-1β is predominantly produced by the monocytes, the question was addressed whether the differences in the absolute response of the different donors is determined by their individual monocyte counts. For that purpose, IL-1β release was normalized to the respective monocyte counts determined prior to stimulation. Figure 13B shows that this normalization to monocyte counts leveled the IL-1β response of the donors. Thus, the inter-individual variations clearly are determined by the differences in the number of monocytes. The similarity of the responses to stimulation with spores of fungal species also strongly suggests the existence of common immune-stimulatory structures. Apparently, the MAT method is suitable not only as a robust tool in safety sciences but also for addressing questions in basic research.

Evaluation of pyrogenic activities of glucan with MAT

Some α- and β-glycosidically linked sugars, i.e., laminarin, lichenan, curdlan, and mannan, have been suggested to inhibit fungal activation of dectin-1, a receptor involved in recognition of fungal pyrogenic structures. These sugars were subjected to the MAT system in a comparative study to evaluate their fever-inducing potential (Daneshian, 2006).

The compounds induced significant IL-1β release in human blood with the maximal stimulating concentration of 10 μg/ml of either compound inducing as much IL-1β as 5 μg/ml zymosan (Fig. 14A). The IL-1β inducing capacity dropped significantly between 10 μg/ml and 100 μg/ml stimulus, and 500 μg/ml of the sugars no longer induced IL-1β release. The corresponding Alamar blue viability test (Fig. 14B) demonstrated that the vi-

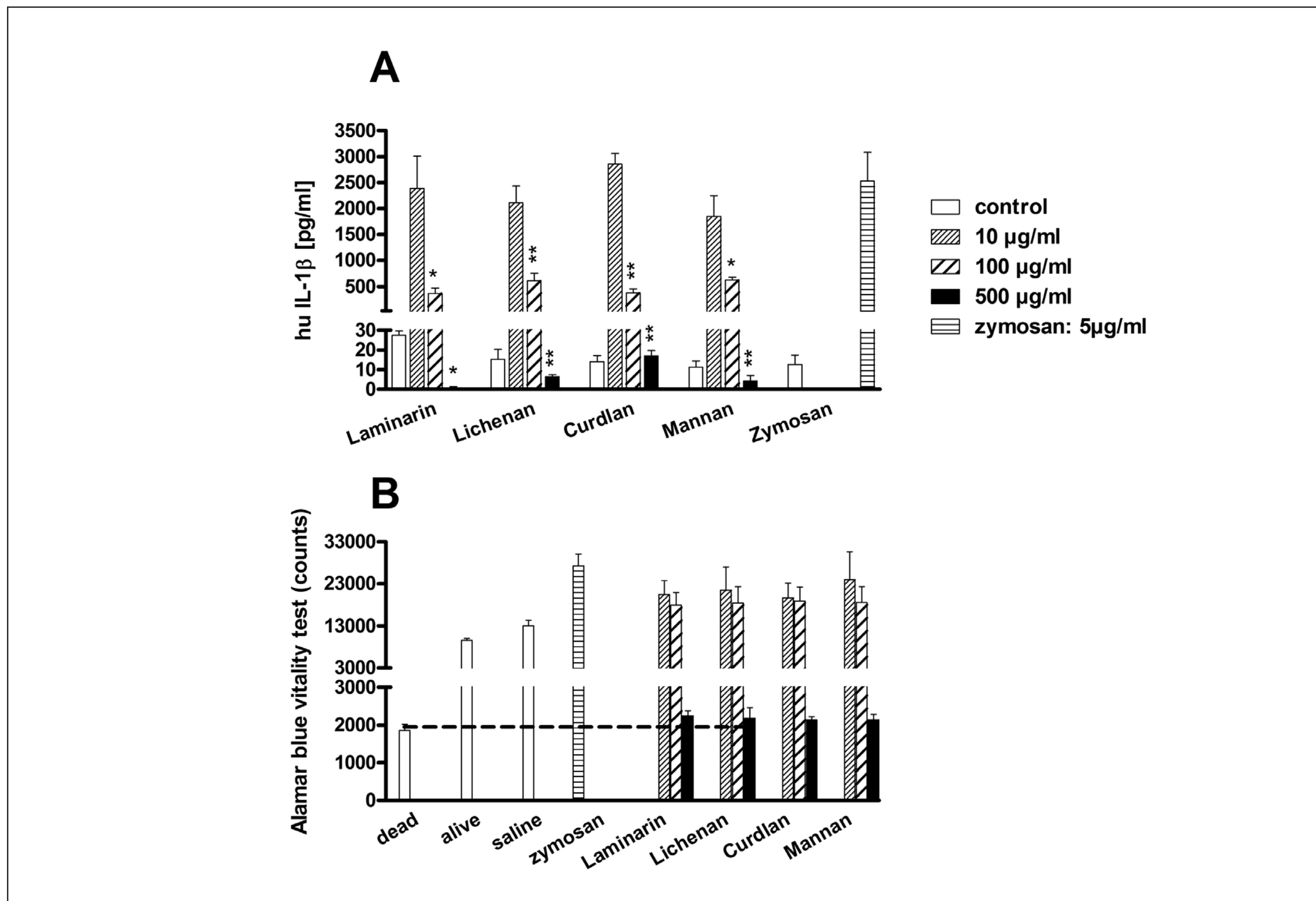

Fig. 14: Putative dectin-1 antagonists are pyrogenic and toxic
Whole blood was incubated with dectin-1 antagonists (laminarin, lichenan, curdlan, mannan) and (A) IL-1β release (ELISA) and (B) Alamar blue metabolism were measured in the same samples; *, p≤0.05; ***, p≤0.001 of 100 or 500 μg/ml antagonists versus 10 μg/ml of the same stimulus (Daneshian, 2006). For experimental details see supplementary file A at www.altex-edition.org

ability of the cells decreased with the increasing sugar concentration and that 500 μg/ml of the sugars reduced the viability to background levels. Co-stimulation of blood cells with zymosan and subtoxic concentrations of dectin-1 antagonists resulted in additive effects on IL-1β production (data not shown).

These findings show clearly that the whole blood MAT methodology has the capacity to evaluate the pyrogenic potential of purified fungal glucans. Notably, due to findings of this study the postulated ability of these glucans to inhibit the dectin-1 pathway (Brown and Gordon, 2001) could be refuted, as the chosen concentrations of these glucans in the dectin-1 study are evidently toxic to the cells.

Responsiveness of human blood compared to murine alveolar macrophages

A critical question is, especially for inhaled pyrogens where the primary contact with immune cells is in the lung not the blood, whether blood monocytes reflect the response of tissue macrophages in their response to NEP. Blood monocytes possess the full range of pattern recognition receptors (PRRs), and they are also the precursors of tissue macrophages, but the question remains if the MAT could model the immune reaction in the different tissues. In this regard, the most sophisticated tissue to model seems to be the lung, as there are no human alveolar macrophage cell lines available at the moment. A study was conducted that aimed to analyze and to compare cytokine patterns of a large number of different filamentous fungal species and yeasts that are common in Central Europe and North America and include both pathogenic and non-pathogenic fungi (Daneshian, 2006).

As the main exposure to fungal spores occurs in the lung, it was investigated whether the response of human blood reflects the response of alveolar macrophages. Therefore, the TNF induction by spores of 21 fungal species in the murine alveolar macrophage cell line MH-S was compared with that in human blood.

Figure 15A shows that there is a strong positive correlation (r>0.99) between the human and the murine TNF response upon fungal stimulation; note that every point in the plot corresponds to spores of a different fungal species. Figure 15B shows the

Fig. 15: Fungal spores induce comparable TNF release in human whole blood and alveolar macrophages
10, 20, 30 and 100 mm^2 of fungal spore surface area of 21 fungal species were incubated with human blood or with the murine alveolar macrophage cell line MH-S (5x10^5 cells). A: comparison of the AUCs for murine TNF (mu-TNF) and the corresponding human TNF (hu-TNF). B: quotients of AUC's of human and murine TNF induced by the respective fungal spores. C: shows comparison of human and murine TNF amounts released upon stimulation with 30 mm^2 of spore surface area of some of the respective fungi (Data are means ±SD of quadruplicates of two different experiments) (Daneshian, 2006). For experimental details see supplementary file A at www.altex-edition.org

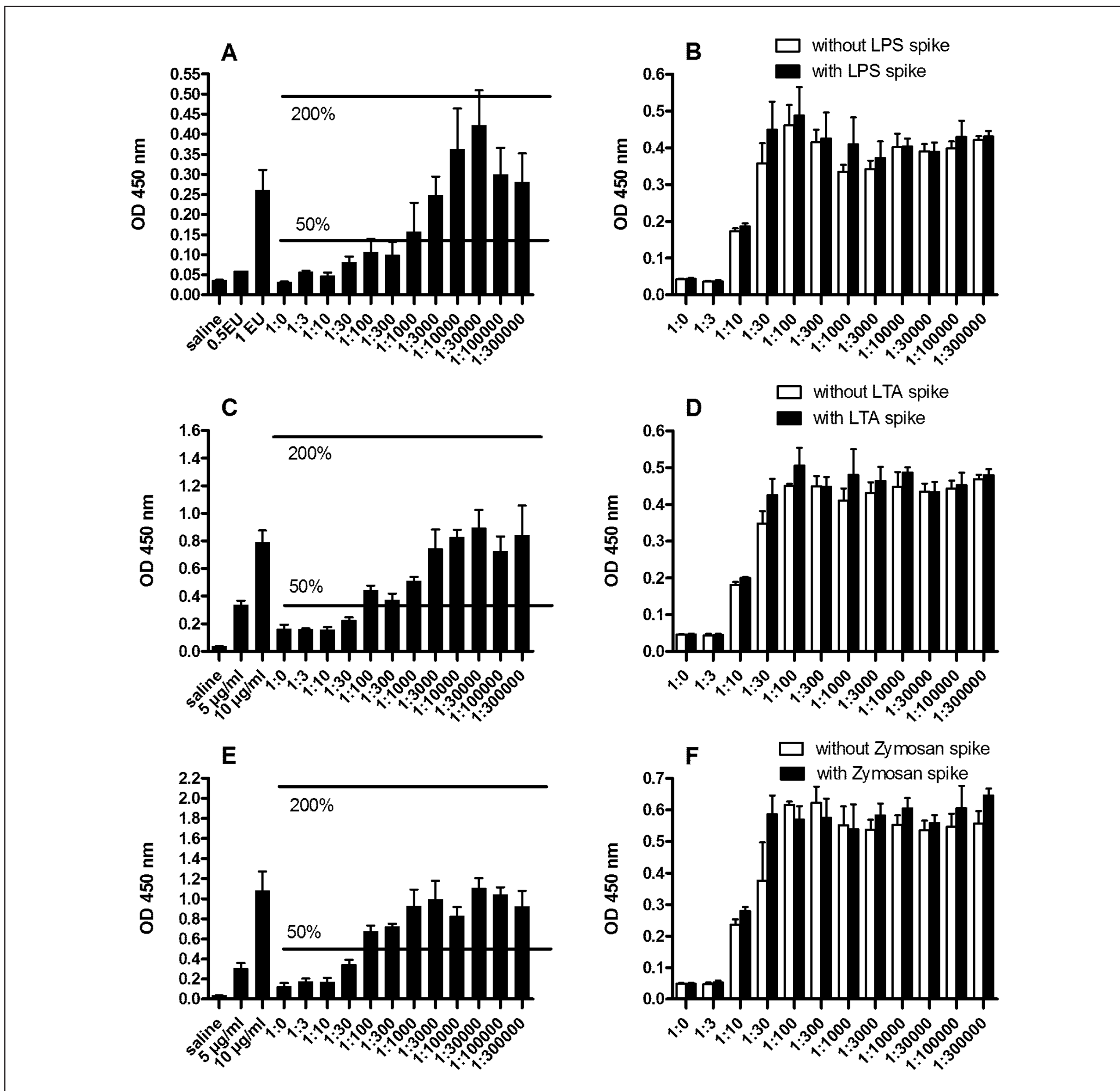

Fig. 16: Pyrogenicity of fluid coolant with MAT

A: Fluid coolants were diluted in 3-fold steps up to 1:300,000; the probes were spiked with 100 pg/ml LPS from *E. coli* O-113 [1EU] and the samples were treated according to the standard protocol of the *in vitro* pyrogen test (MAT). The margin of spike recovery was set corresponding to the LAL protocol, i.e., between 50% and 200% of the cytokine response compared to the spiked saline control. B: the cells were after the incubation subjected to Alamar blue viability test; the filled columns show the viability of cells exposed to different dilutions of the spiked coolant samples, and the white columns show the viability of cells exposed to non-spiked coolant samples. C: fluid coolants were diluted in 3-fold steps up to 1:300,000; the probes were spiked with 10 µg/ml LTA from *Staphylococcus aureus*, and the samples were treated due to the standard protocol of the *in vitro* pyrogen test (MAT). D: after the incubation the cells were subjected to Alamar blue viability test; the filled columns show the viability of cells exposed to different dilutions of the spiked coolant samples, and the white columns show the viability of cells exposed to non-spiked coolant samples. E: fluid coolants were spiked with 10 µg/ml Zymosan from *Saccharomyces cerevisiae*, and the samples were treated according to standard protocol of the *in vitro* pyrogen test (MAT). D: after the incubation the cells were subjected to Alamar blue viability test; the filled columns show the viability of cells exposed to different dilutions of the spiked coolant samples, and the white columns show the viability of cells exposed to non-spiked coolant samples. Data are means ±SD of triplicates. For experimental details see supplementary file A at www.altex-edition.org

Tab.8: Scientific literature showing the detection of non-endotoxin pyrogens by human whole blood cytokine release

Non-endotoxin stimuli	References
Mycobacterial stimuli including: Mycobacterium tuberculosis antigen *M. leprae* proteins *M. leprae* purified antigens	Bertholet et al., 2011 Geluk et al., 2010 Kamble et al., 2010
Bacterial components released from planktonic and biofilm cells	Oscarsson et al., 2008
Whole Gram-negative bacteria	Frieling et al., 1997 Rarick et al., 2006 Jemmett et al., 2008 Voropaev et al., 2002 Bodet and Grenier, 2010 Goris et al., 2011 Schroder et al., 2005 Pool et al., 1998
Unusual LPS	Mathiak et al., 2003 Bodet and Grenier, 2010 Nakamura et al., 2004
Whole Gram-positive bacteria	Frieling et al., 1997 Strunk et al., 2010 Segura et al., 2006 Fredheim et al., 2011 Mereghetti et al., 2009 Jagger et al., 2002 Tietze et al., 2006 Nakagawa et al., 2002 Pool et al., 1998
Bacterial culture supernatants	Schindler et al., 2001 Hanage and Cohen, 2002
Peptidoglycan	Schrijver et al., 1999 Hadley et al., 2005 Nakagawa et al., 2002
Superantigens	Hermann et al., 2003 Langezaal et al., 2002 Driessen et al., 1995
Toxoids from *Corynebacterium diphtheriae*	Carlin and Viitanen, 2005
Porins	Cusumano et al., 1997
Flagellin	Bachmann et al., 2006
RNA (poly-IC, i.e. synthetic analogue of viral double-stranded RNA)	Nakagawa et al., 2002
Viruses	Lagrelius et al., 2006 Fischer et al., 2001
Fungal stimuli including: – *C. albicans* – *A. fumigatus* – *Agaricus blazei murill* – Molds – Glucan	Murciano et al., 2007 Lagrelius et al., 2006 Jouneau et al., 2011 Popa et al., 2005 Popa et al., 2009 Johnson et al., 2009 Forland et al., 2011 Kruger et al., 2004 Engstad et al., 2002 Wouters et al., 2002
Lectins	Van Wauwe et al., 1995 Banerjee and Mohanan, 2011
Bioaerosols and dust	Liebers et al., 2009 Zucker et al., 2006 Sigsgaard et al., 2000
Trinitrophenol	Banerjee and Mohanan, 2011

quotient of the areas under the curve for human and murine TNF. The minimum ratio was found to be 0.8 and the maximum 1.1, whereas the MH-S cells produced more TNF than human blood cells in 19 of 21 cases. Figure 15C shows the amounts of murine and human TNF induced upon stimulation with 30 mm^2 spore surface of some of the tested species.

This study reveals the potential of the MAT test to reflect the reactivity and responsiveness of differentiated tissue macrophages as well. This finding predestined the MAT methods for prediction of the modulatory status of the tissue-specific immune response upon exposure to stimulating compounds. It seems reasonable to assume that the combination of MAT-based assays with valid viability assays also poses a potent tool for evaluation of immune-toxic effects. These findings suggest that whole blood MAT measurements are relevant also for tissue responses to NEP.

The MAT method for evaluation of toxic complex mixtures

A major problem with toxic complex mixtures is the evaluation of their pyrogenic activity, as these compounds cannot be tested in animals due to their toxic and severely impairing effects, nor can they be subjected to LAL because of inhibitory components (Yum et al., 2010; Ishiguro et al., 2007; Brent, 2001). Temperature transfer fluids, fluid coolants, are of broad use in industrial facilities but also are used as household chemicals, e.g., as antifreeze agents in cars. The composition of compounds in coolants varies depending on their area of use. They can contain ethylene glycol, diethylene glycol, propylene glycol, polyalkalyne glycol, liquefied propane, haloalkanes, nanoparticles, aluminium, copper, brass, solder, cast iron, lead, and mercury *inter alia*. As microorganisms are known to be a source of contamination for fluid coolants, which are recirculated warm over months (Herwaldt et al., 1984) and aerosols formed during work processes can be inhaled, it is of major interest to have a fast and reliable system to evaluate the pyrogenic activity in these mixtures as a hint for the putative microbial threat.

The association of the German automotive industry and the Austrian Social Insurance for Occupational Risks (AUVA) both cooperated with the University of Konstanz to establish a methodology for the evaluation of the pyrogenic and toxic capacity of fluid coolants. For these studies, the provided coolants were diluted in series up to a dilution of 1:300,000 in 3-fold steps, and each dilution spiked with LPS (Fig. 16A,B) and non-endotoxins, i.e., LTA (Fig. 16C,D), and zymosan (Fig. 16E,F). The cytokine response compared with the viability of the cells could indicate possible interference of the mixture with the spike recovery.

As Figure 16 shows, for every mixture the relevant dilution factor has to be defined, which would lead to the spike recovery compared to spiked buffer or saline (panels A, C, E). The additional viability test with the cells in the same setup would show whether the cells are able to react in different coolant dilutions and if there are indications for interference between the mixture and the immune response (panels B, D, F).

The results of these studies revealed that some coolants had to be diluted more to enable the spike recovery but needed fewer dilution steps to retain cell viability. Still, it could be shown that it is possible to determine the pyrogen content as opposed to RPT and LAL. These data suggest that various pyrogens can be determined in toxic complex mixtures such as coolant fluids. These findings are seminal for further design of studies aiming to evaluate the pyrogenic contamination in toxic complex mixtures employing the MAT methodology and possible links to workplace related illnesses.

2.10 Evidence for other immune stimuli detection in the whole blood MAT

The whole blood cytokine release test has been used broadly to assess diverse immune stimuli. Examples from the literature are listed here (Tab. 8):

Taken together, these studies suggest that essentially all presumed pyrogens and pyrogenic microbiological preparations have at some point been shown to induce cytokine release in the whole blood model.

3 Validation excercises and comparison to RPT and LAL/BET

Beside the formal validation study, which led to validity statements in Europe, the US, and Japan and subsequent regulatory acceptance at least as an endotoxin test (notably the status of the LAL/BET, which was acceptable to replace about 90% of rabbit testing), a number of formal evaluations / validations have been reported with regard to NEP detection by the whole blood MAT. They are sumarized here.

Tab. 9: List of the six alternative methods included in the project

Method's basic principle	Expert laboratory	Read out	Abbreviation
Whole blood	NIBSC, London (UK)	IL-6 (Interleukin-6)	blood-IL6
Whole blood	STZ InPut, Konstanz (D)	IL-1β (Interleukin-1β)	blood-IL1
PBMC (peripheral blood mononuclear cells)	Novartis, Basle (CH)	IL-6 (Interleukin-6)	PBMC-IL6
THP-1 (monocytoid cell line)	University of Berne (CH)	TNF-α (Tumor Necrosis Factor-α)	THP-1-TNF
THP-1 (monocytoid cell line)	University of Innsbruck (A)	Neopterin	THP-1-Neo
MM6 (monocytoid cell line)	RIVM, Bilthoven (NL)	IL-6 (Interleukin-6)	MM6-IL6

3.1 Formal ECVAM validation study

The following chapter is adapted from "*Human(e) Pyrogen Test: Final Report, QLRT-1999-00811, Quality of Life and Management of Living Resources, Comparison and Validation of Novel Pyrogen Tests Based on the Human Fever Reaction, Human(e) Pyrogen Test*" (Hoffmann et al., 2005).

As agreed at an ECVAM workshop (Hartung et al., 2001) the overall aim of the Human(e) Pyrogen Test project, starting in February 2000, was to develop, evaluate, and validate methods based upon the human fever reaction to replace the RPT. All of the methods are built upon responses of human leukocytes, particularly monocytes, which release inflammatory mediators (endogenous pyrogens) in response to pyrogenic contaminants (exogenous pyrogens). However, the cell-based *in vitro* assay systems differ with regard to the cells employed (whole blood, isolated peripheral blood mononuclear cells, or established monocytic cell lines), the mediator determined (Interleukin-1β, Interleukin-6, Tumor Necrosis Factor-α, and Neopterin) and the precise set-up of the test. In Table 9 a summary of the six alternatives is given.

After intensive interaction and co-operation to evaluate and compare the methods that formed the basis for the study, a pre-validation and finally a validation study were carried out addressing several questions, but primarily, the issues of relevance, transferability, precision, and predictive capabilities were addressed. Overall, a successful study was conducted such that several methods were recommended for consideration to replace the rabbit pyrogen test. Based on this study, the introduction of the MAT into the European Pharmacopoeia was possible (EDQM, 2010).

The true status of artificially contaminated test substances was defined by the amount of the endotoxin spike. Here, a threshold value was agreed to, on which the choice of spike was based. This led to an approach incorporating positive and negative controls by which comparability, general applicability, as well as robustness of all methods was increased. By means of a two-classification, i.e., pyrogenic vs. non-pyrogenic, a balanced approach was chosen, providing equivalent information on both parameters of interest, namely sensitivity and specificity. In parallel, LTA (Morath et al., 2001, 2002a,b) was provided from the University of Konstanz for unblinded testing to gather information about each method's performance with regard to non-LPS bacterial cell wall material. All MAT variants were able to detect endotoxin-free LTA.

Non-endotoxin substances

Because the validation study has already been published in detail (Hoffmann et al., 2005), we will focus here on the unpublished data concerning the performance of the tests towards non-endotoxin substances, which are listed in Table 10.

The testing was performed in series of dilutions of the substances according to a provided protocol. The samples comprised several substances, such as lectins, Gram-positive endotoxins, and substances giving false positive results in the Bacterial Endotoxins Test (BET).

Tab. 10: List of non-endotoxin, pyrogenic substances

Substance	derived from	Diluent	Concentration	Dilutions tested
Curdlan	*Alcaligenes faecalis*	0.2 N NaOH	2.5 mg/ml	10 µg/ml, 1 µg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml
β-Glucan	Barley	0.2 N NaOH	2.5 mg/ml	as above
β-Glucan	*Saccharomyces cervisiae*	0.2 N NaOH	0.5 mg/ml	as above
Zymosan	*Saccharomyces cervisiae*	saline		as above
PHA-E (Erythroagglutinin)	*Phaseolus vulgaris* (kidney bean)	PBS	500 µg/ml	as above
PHA-L (Leucoagglutinin)	Kidney bean	PBS	500 µg/ml	as above
Monophosphoryl Lipid A	Rd Mutant of *E. coli* F 583		100 µg/ml	as above
Glucan standard				undiluted, 1:5, 1:10, 1:20, 1:50
Endotoxin Code C	*Pseudomonas aeruginosa* (virulent strain)	saline	4 µg/ml	4 µg/ml, 400 ng/ml, 40 ng/ml, 4 ng/ml, 400 pg/ml, 40 pg/ml
Endotoxin Code G	*Pseudomonas aeruginosa* (non-virulent strain)	saline	4 µg/ml	4 µg/ml, 400 ng/ml, 40 ng/ml, 4 ng/ml, 400 pg/ml, 40 pg/ml
LTA	*Bacillus subtilis*	saline	50 µg/ml	50 µg/ml, 5 µg/ml, 1 µg/ml

Tab. 11: Results of the validation study part B, non-endotoxin substances

	blood-IL1[a]	blood-IL6
Curdlan	1000	1000
Glucan-Barley	negative	negative
Glucan-Yeast	not done	negative
Zymosan	negative	10,000
PHA-L	100	10,000
PHA-E	negative	negative
Lipid A	10,000	1000
Glucan STD	negative	negative
Endotoxin-C	4	40
Endotoxin-G	0.4	40
LTA	5000	500

[a] Smallest concentration [ng/ml] or dilution of Validation B substances active in the respective method. The results are expressed in mass units/ml.

The glucans curdlan and the monophosphoryl-Lipid A are not immuno-active (pyrogenic) in humans, in reasonable concentrations but give positive responses in the BET. PHA-L is known to be immuno-active (pyrogenic), in contrast to PHA-E, regarded as a negative control for PHA-L; corresponding BET results are not known. LTA is a BET-negative formulation but is expected to be immuno-active (pyrogenic), i.e., inducing cytokine-release in humans. The two *Pseudomonas aeruginosa* endotoxins were included since they are neither very potent in the BET, nor in the rabbit, but are considered likely contaminants of parenteral drugs. Generally, the endotoxin coded G was expected to be more potent than that coded C. Similarly, the response of the new methods to zymosan was of interest.

For each substance the smallest concentration (in ng/ml) inducing a response in the respective method was reported. In Table 11 a summary of the results for the two whole blood MAT tests is presented.

The whole blood variant using cryopreserved blood was validated only in a catch-up validation study (Schindler et al., 2006b), which was not included in this phase. However, these NEPs and LPS from other bacterial strains were tested in a PhD Thesis by S. Schindler (Schindler, 2006) using the whole blood MAT.

The three glucans (curdlan, barley glucan, and zymosan), which give false positive results in the BET, were not active at the highest concentration of 10,000 ng/ml assessed in the MAT, in which they were tested. Curdlan, which reacted in the BET, induced a cytokine response in the MAT generally only at a concentration of some 1000 ng/ml. Also, zymosan and monophosphoryl-Lipid A were inactive or active only in very large doses in the new methods. The MAT identified correctly the immunostimulatory PHA-L, while the variant leading only to lectin-mediated hemagglutination (PHA-E) was negative. The

Tab. 12: Detection of different LPS and NEP in cryopreserved whole blood MAT
Detection limits were calculated using one-way ANOVA (Graph Pad Prism Software, San Diego, USA).

Bacterial strain LPS	Donors	Detection limits
S. typhosa	Donor 1	**30 pg/ml**
	Donor 2	**50 pg/ml**
	Donor 3	**50 pg/ml**
S. typhimurium	Donor 1	**250 pg/ml**
	Donor 2	**250 pg/ml**
	Donor 3	**250 pg/ml**
Serratia marcescens	Donor 1	**75 pg/ml**
	Donor 2	**75 pg/ml**
	Donor 3	**30 pg/ml**
	Donor 4	**75 pg/ml**
	Donor 5	**30 pg/ml**
	Donor 6	**50 pg/ml**
***E. coli* O128**	Donor 1	**100 pg/ml**
	Donor 2	**200 pg/ml**
	Donor 3	**400 pg/ml**
***E. coli* O111:B4**	Donor 1	**0.5 EU/ml**
	Donor 2	**0.5 EU/ml**
	Donor 3	**0.25 EU/ml**
	Donor 4	**0.5 EU/ml**
	Donor 5	**0.25 EU/ml**

Tab. 13: Testing of non-endotoxin pyrogens in cryopreserved whole blood MAT
Detection limits were calculated using one-way ANOVA (Graph Pad Prism Software, San Diego, USA).

Substance	Positive/negative
Curdlan	negative
Glucan, barley	negative
Zymosan	positive at 10 µg/ml
PHA-L	positive at 100 ng/ml
PHA-E	negative
Monophosphoryl-Lipid A	positive at 10 µg/ml
Glucan standard	negative
Endotoxin Code C	positive at 1:1000
Endotoxin Code G	positive at 1:1000
LTA	positive

results for the PHA-L differed slightly between the methods. It was most reactive in the whole blood MAT using IL-1β as readout. However, the PHA-L was more reactive than the PHA-E, which was largely inactive. Endotoxin Code C and Code G represent LPS from *Pseudomonas* provided by NIBSC. They are weak endotoxins in mammals, and are overestimated in the LAL. They were correctly classified as pyrogenic. The potencies of the *Pseudomonas* endotoxins C and G differed somewhat in the different methods. Monophosphoryl-Lipid A has no pyrogenic or immuno-stimulatory effect in mammals. It is false-positive in the LAL. It was correctly classified negative. Similarly, LTA, which represents the putative counterpart to LPS in Gram-positive bacteria, was positive both in the MAT and the cryopreserved whole blood MAT. Taken together, the assays did not react to the LAL false-positive glucans and curdlan but reacted to the LAL-false negative LTA.

Drugs

A preparation of human serum albumin (HSA) and the antibiotic Gentamicin were chosen as a proof of principle to demonstrate that the MAT works as well for real-life samples. These two drugs had previously been associated with adverse drug reactions, including fever. The goal was to see whether the new methods were able to detect the contamination. Different concentrations of endotoxin spike were added to the samples for interference testing and positive product control.

Two samples of a human serum albumin (HSA) were tested. One of these was essentially pyrogen-free and had been released on the basis of negative RPT and BET results; the other one was a contaminated lot that was associated with adverse reactions in recipients. The clean lot was used to determine an interference free dilution of the HSA. The response of this dilution was assessed by means of an LPS standard curve. Dilution response curves also were produced for the contaminated lot.

The same approach was taken with two lots of Gentamicin. Again, one of these was clean and one was associated with adverse reactions in recipients. The problematic preparation of Gentamicin was therapeutic grade material that had satisfied US FDA batch release requirements. The preparation was negative in the BET and in independent BETs carried out in a number of laboratories.

For the HSA, the THP-1-Neo, MM6-IL6, whole blood-IL6 and PBMC-IL6 were able to distinguish the contaminated lot from the pyrogen-free lot. For Gentamicin all but the THP-1-Neo could distinguish the lots because of severe interference in the cell line test. Although the interference free dilutions differed, the other four methods could discriminate the pyrogen-free and the contaminated lot.

Ten drugs were spiked with different amounts of LPS (Hoffmann et al., 2005) tested blindly for all assays in three laboratories each forming the core of the validation study. Interestingly, also the NIBSC, as well as the Norwegian Institute of Public Health (NIPH), i.e., two National Control Authorities, conducted LAL-testing of the drugs of the validation study with two sets of spikes each. Table 14 provides the results of these tests.

The outcome of the LAL testing, which was conducted by experienced laboratories, is surprising. Both labs missed some positive samples. The NIBSC also had a lot of false positive results. Therefore, a critical review of the LAL seems to be appropriate; however, this was not the scope of the validation study.

Tab. 14: Result of LAL-testing of the 10 spiked drugs used in the MAT validation study

NIPH		"Truth" –	"Truth" +	Σ
PM	+	**12**	**4**	16
	–	**0**	**8**	8
Σ		12	12	24
NIBSC		**"Truth" –**	**"Truth" +**	**Σ**
PM	+	4	2	6
	–	**8**	**10**	18
Σ		12	12	34

Conclusions of the validation study

The six different methods included in the validation study share a common methodical core consisting of an incubation of monocytoid cells with the sample and an ELISA read-out. This approach is well established and was shown once more to be suitable for measuring fever-related contaminations. The study proved that all four endpoints, IL-1β, IL-6, TNF-α, and neopterin, correlate with LPS in a way that is suitable for detecting pyrogens. Furthermore, both cell lines and primary cells were shown to be appropriate for assessing pyrogens. Further differences between the methods, e.g., with regard to materials employed or duration of incubation, are present but were optimized as much as possible for every single method during the project. It was realized that all six methods have their inherent value.

During the study it was repeatedly shown that every method is appropriate for pyrogenicity testing, although individual advantages and disadvantages were present. On the one hand, the methods based on fresh human blood revealed high robustness, transferability, and practicability and thus are promising for routine testing. On the other hand, cell line approaches offer the advantage of the availability of cells and of genetic manipulation for optimization with regard to specific purposes.

With regard to endotoxin, they perform similarly well compared to the established methods, primarily the RPT and also the BET. It was concluded that, in general, all methods work well for non-endotoxins. Substances known or considered to be either problematic in the BET (Glucans, LTA) or in the RPT give similar results in all six methods, matching with the expectation of the pyrogenic potential of these formulations. The fresh blood approaches may be closest to humans, whose fever reaction is to be predicted.

All novel tests have the potential to be quantitative tests. Compared to LAL, they even offer the advantage of reflecting

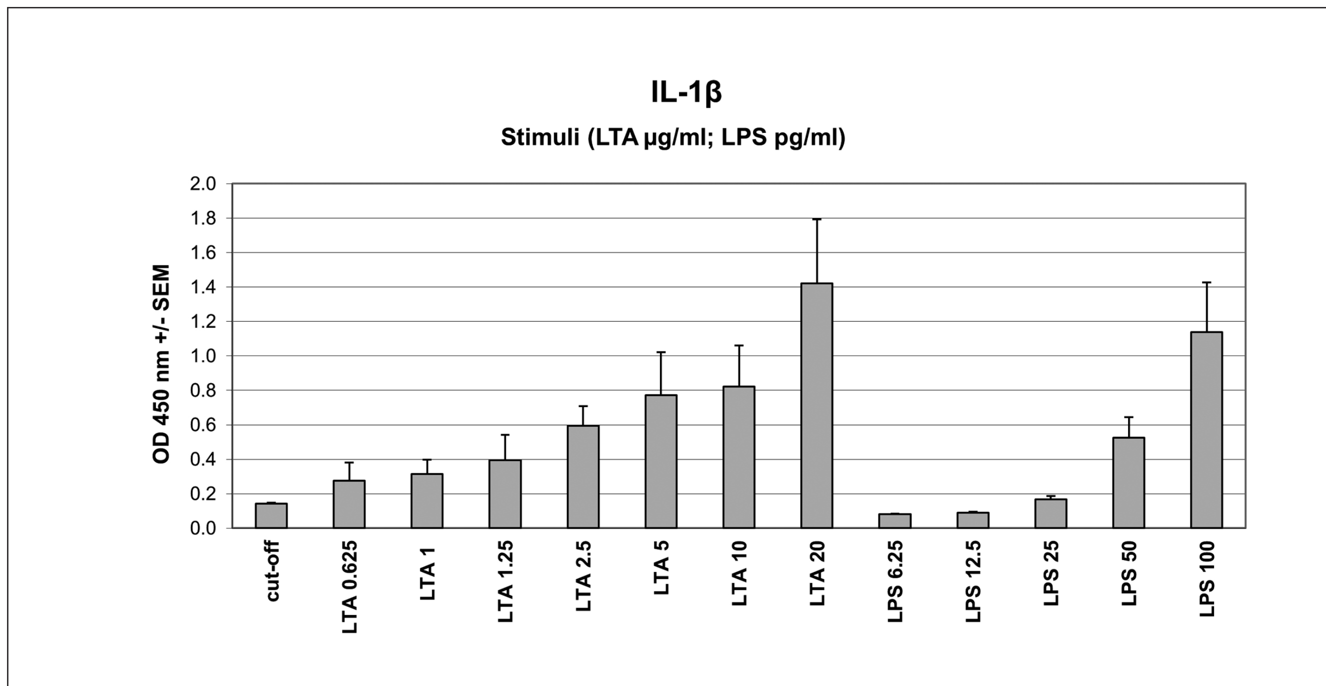

Fig. 17: Purified LPS and LTA are both detectable in the MAT/IPT.
The MAT was performed with fresh blood of three donors, every sample in duplicates; mean of three donors +/-SEM. Comparable results were obtained with IL-6 and TNF-α.

the potency of different LPS from different species (which, in fact, makes the LAL a "pseudo-quantitative" test). The time and financial frame of the study did not allow the development and specific validation of quantitative tests. The validation study also produced initial evidence that all the MATs detect NEPs, both in drugs that caused fever reactions in patients, and employing isolated NEPs. However, no ring trials as to spike recovery of NEPs were included.

3.2 Validation of the whole blood MAT for Gram-positive pyrogens by the French Health Products Safety Agency (Afssaps French OMCL)

A validation of the commercial kit using fresh human whole blood for *in vitro* pyrogenicity testing of Gram-positive bacteria was reported in 2006 (Francois et al., 2006): "*The two conventional tests to detect pyrogen contaminants in injectable pharmaceutical drugs are the Rabbit Model and the Limulus amebocyte lysate (LAL) test. To replace these models, a new system on human whole blood is developed, using the release of Interleukin 1 beta (IL1beta) after cell stimulation with Gram-positive and Gram-negative pyrogens. The purpose of this study was to validate the kit using the quantitative ELISA enzyme immunoassay. The assay is divided into two parts: blood cell stimulation with Lipopolysaccharides (LPS) and Lipoteichoic acid (LTA) and quantitation of IL1beta using the ELISA method. In each assay, blood from a particular donor was stimulated with the Endotoxin Standard and with a sample of a commercial antibiotic preparation (Clavulanic acid/Ticarcillin) spiked with the Endotoxin Standard. LTA from Bacillus subtilis and a sample of diphtheria toxoid also were used. At least six assays were tested. A polynomial regression of the Endotoxin Standard series showed a correlation coefficient greater than 0.99. The spiked antibiotic sample recoveries were 50-121%. The LTA quantitation limit was 0.1 microg/ml, and the range of detection of pyrogens from Gram-positive diphtheria toxoid was 0.77 to 2.5 EEU/ml. The IL1beta production varied markedly between donors. However, the coefficient of variation was less than 20% intra-assay. In conclusion, the kit can be used for the quantitative and qualitative detection of pyrogens from Gram-negative and Gram-positive bacteria.*"

3.3 Paul-Ehrlich-Institute evaluation of the whole blood MAT and LTA

A study carried out by the Paul-Ehrlich-Institute, the German National authority for sera and vaccines, has validated the whole blood MAT in comparison to the RPT using LPS spiked clinical samples (Spreitzer et al., 2002). This work was expanded to a number of other biologicals, such as immunoglobulins and cell therapies (Montag et al., 2007). Furthermore, they analyzed the response of the different pyrogen test systems to LTA in detail. Purified LTA is defined by its extraction method and contains 10 times less endotoxin according to the gel clot LAL assay than the standard preparation. At concentrations ranging from 1 ng to 10 µg/ml, it highly activates TLR2.

The same purified LTA stock solution (20 µg/ml) was tested in the BET, the RPT, and the whole blood MAT. The LAL showed less than 0.03 IE/ml (<3 pg/ml) of endotoxin for the LTA stock solution, while 100 pg/ml of LPS S.a.e. (*Salmonella*

abortus equi) revealed an endotoxin amount of 1.2 IE/ml. The same substances were subjected to the RPT and LTA showed no pyrogenic (or unusual/adverse) reaction within the triple-groups receiving 10, 100, or 200 μg LTA/kg (0.5, 5, or 10 ml of LAL-tested stock solution with 20 μg LTA/ml) during 180 minutes (temperature) and one week subsequent observation (behavior, weight), while the LPS solution did not pass the rabbit test. Only the MAT was able to react to both substances in a concentration-dependent manner (Fig. 17).

These data are in line with findings in a variant of the whole blood MAT using rabbit blood, which showed a strongly attenuated reaction of the rabbit blood cells to LTA, while there was no difference to human blood for LPS (Schindler et al., 2003). The authors concluded: "*This finding suggests that the In vitro Pyrogen Test (IPT) which uses human blood to detect contaminations, e.g. of injectable drugs, might predict the human reaction to the contamination better than the 'gold standard' rabbit pyrogen test*."

3.4 Evaluation by the Japanese National Institute for Environmental Health Sciences

Nakagawa et al. (2002) compared the whole blood MAT and a MonoMac-6 cell MAT with the rabbit assay, concluding: "*The reliability of an in vitro pyrogen test system based on pro-inflammatory cytokine release from human monocytic cells was assessed by comparison with a test system based on a human whole blood culture as well as with the conventional rabbit pyrogen test. … The cytokine-producing responses of MM6-CA8 cells correlated significantly with the responses of cultured human whole blood, which represents an ex vivo culture test system reproducing pyrogen-induced cytokine production in the human body. In terms of cytokine inducibility, the pyrogens were ranked in the order endotoxin > PG > poly (I . C) > SAC [peptidoglycan, S. aureus] in both culture systems, a ranking which almost agreed with the ranking of their pyrogenicity as assessed by the rabbit pyrogen test*."

3.5 Other validations of the whole blood MAT for NEP

A number of other evaluations of the whole blood MAT took place after the validation study, but most were restricted to LPS. A validation study for a trivalent vaccine from both Gram-negative and Gram-positive bacteria was conducted successfully (Carlin and Viitanen, 2005). Pool et al. assessed the whole blood MAT for batches of human serum albumin, which passed the LAL but were pyrogenic in rabbits, showing that the assay detects these samples (Pool et al., 1999). They conclude: "*This study shows that pyrogenic substances other than endotoxin can contaminate batches of pharmaceutical products and that results obtained using the Limulus Amoebocyte Lysate (LAL) assay does not necessarily indicate the pyrogenic status of pharmaceutical products. The WBC [whole blood cell] assay for pyrogens, having a broader sensitivity range than the LAL assay, is a better indicator of the pyrogenic status of pharmaceutical products*."

Taken together, these studies show that evaluations for NEP came to similar results as those for endotoxin, i.e., the whole blood MAT does cover various NEP and allows their reliable detection.

4 Discussion

This review aimed to compile evidence that the whole blood MAT is suitable to detect NEPs, extending previous formal validation studies and peer reviews for endotoxins. Thus, the question is whether the assay is reproducible and reliable for this purpose. The answer is easy for the first part. There is no indication that test reproducibility varies between endotoxin and NEP. Even for complex bioaerosols (26 dust samples), it was found (Liebers et al., 2009): "*Using cryo-preserved blood the coefficient of variation (CV) regarding the interassay variability was below 21% for all cytokines measured*."

Any evaluation for NEPs is hampered by the fact that no agreement as to the nature of Gram-positive bacterial (with the possible exception of LTA), fungal, and viral pyrogens exists. Nor are there reference materials. For none of these NEPs has fever induction in rabbits or humans been clearly shown under exclusion of endotoxin contaminations. However, there is no doubt of the fever inducing capacity of all of these pathogens. Evidence compiled here clearly shows that all of these classes induce cytokines in human blood. Similarly, there are a number of examples that materials that produced fever in patients were positive in the test. Larger systematic evaluations are not possible, as incriminated samples are part of criminal investigations; their testing usually is not in the interest of the manufacturer, and the stability of the NEP typically is not known, making it difficult to interpret negative test data in such cases.

If LTA is taken as the best-established Gram-positive pyrogen and NEP, there is clear evidence for its presence in essentially all Gram-positive bacteria studied so far: Rockel and Hartung identified fifty-eight publications showing an isolation of LTA using different purification variants for 81 different bacterial strains (Rockel and Hartung, 2012). LTA has consistently been detected with whole blood MAT in many laboratories for many bacterial species. A formal one-center validation was carried out by the French and German (as well as the Japanese one but for NEPs other than LTA) national authorities, and it was also part of the European validation study where it was shown that all variants of the MAT were capable of detecting LTA.

Safety testing of products is of utmost importance and is the biggest concern of regulatory agencies and manufacturers. Therefore, many laws, regulations, and guidance documents, national and international, are in place to ensure that only products that were subjected to adequate safety testing reach the market and the consumer. Special concern has to be given to products intended to overcome the body's natural protective barriers, such as injectables and medical devices. Beside their general biocompatibility, they may be contaminated with microorganisms and their components, such as pyrogens during production. If so, they can evoke, once in the human body and in contact with the human immune system, severe clinical signs of inflammation, e.g., fever, sepsis, and sometimes even multiple organ failure and subsequent death.

Several tests are prescribed in the European and US Pharmacopeia that are able to detect pyrogens to a greater or lesser extent. The first test in place was the rabbit pyrogen test (RPT). Its potential to detect pyrogens of all origins, with some exceptions, has been known for years. But its drawbacks, including the lack of pyrogen quantification, its inability to test certain substances, like radiopharmaceuticals, the time it takes as well as the costs, led to its replacement, to a large extent, by the LAL. The LAL is a potent test for LPS, the main immune-stimulating component of Gram-negative bacteria, but misses all other possible stimuli. The test is quantitative (though not reflecting potency in humans) and therefore is an improvement compared to the rabbit test in this regard. Nevertheless, the LAL cannot be regarded as a true pyrogen test due to its inability to model the processes taking place in the human body in response to various pyrogenic compounds.

There are several reports of clinical adverse fever reactions, where substances, e.g., HSA and dialysis products, were defined "pyrogen-free" by the LAL and/or the RPT were administered to patients. Further investigation revealed that non-endotoxin substances were responsible for these adverse events. More and more companies, for this reason, use their own version of the MAT to reduce ADR problems.

When taking a closer look into the molecular processes of the human response towards bacterial, viral, fungal, and other immune-stimulating components, one has to recognize, that a pyrogen test must be able to detect the full spectrum of relevant pyrogens. Taking into account that most of the details were only discovered during the last decade, and many of them are still not fully understood, a true pyrogen test must still be based on the same principles. This means that, at this stage, the MAT tests based on primary monocytes have to be favored. The whole blood variant has some practical advantages, but there is no indication that isolated PBMC or monocytes cannot similarly detect NEP. This review has taken into consideration a number of different protocols of the whole blood MAT; the experience of the authors is that the test is remarkably robust to such alterations. However, it remains for each and every product to be shown that a given variant is suitable to detect endotoxin and NEP. An in-house validation has to be carried out for every product to be tested. The suitability of the MAT for detection of Gram-positive stimuli, which are missed by the LAL, can be considered a scientific fact supported by overwhelming evidence. There is no indication that any relevant performance criteria have been ignored in the analyses performed. In view of the accumulated knowledge and aspects of time and costs, the need to undertake yet another full validation study to prove the general applicability of the MAT is highly questionable.

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Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test

Supplementary File A

Cultivation of fungi
Filamentous fungi collected from environmental sources and expertly identified were grown at 23°C on malt extract agar (MEA) plates; yeasts were cultivated on saubauroud agar plates (Merck, Darmstadt, Germany). Fungi were plated on the surface of polycarbonate membrane filters with a pore diameter of 0.8 µm (Millipore, Billerica, MA, USA). After 3-6 days the filters with the fungal spores were removed and transferred to 50 ml falcons with 10 ml 0.9% saline (Berlin Chemie AG, Berlin, Germany). Vigorous mixing led to detachment of the spores from the membrane filters. Spores were separated from fruit bodies and remaining hyphae by polyamide filters (Franz Eckert GmbH, Waldkirch, Germany); filters with pore diameters of 20 or 40 µm were used, depending on the fungal spore size. Fungal spore purity was verified by microscope. Fungal spore counts were determined in a Neubauer cytometer.

Stimuli and inhibitor
Lipopolysaccharide (LPS) from *Escherichia coli* O-113 (National Institute for Biological Standards and Controls, Hertfordshire, UK), 100 pg/ml of *E. coli* 0-113 LPS is defined as one endotoxin unit (EU). Lipoteichoic acid (LTA) from *Staphylococcus aureus* was isolated by n-butanol extraction according to the protocol from Morath et al. (2001). Polymyxin B, Zymosan A, laminarin, lichenan, curdlan and mannan were purchased from Sigma, Deisenhofen, Germany.

Whole blood incubation
Differential blood cell counts were performed with a Pentra 60 (ABX Diagnostics, Montpellier, France) to exclude donors with acute infections. Heparinized whole blood was diluted tenfold in 0.9% saline (Berlin Chemie AG, Berlin, Germany) in polypropylene vials (Eppendorf, Hamburg, Germany) and stimulated with fungal spores, LPS, LTA or zymosan. After incubation for 20 h at 37°C in a humidified atmosphere with 5% CO_2, the vials were shaken and spun down. Supernatants were stored at -80°C until cytokine measurement.

Alveolar macrophages
The murine alveolar macrophage cell line MH-S (ATCC, CRL-2019), derived by SV40 transformation of an adherent cell-enriched population of mouse alveolar macrophages, was cultured in a humid atmosphere at 37°C and 5% CO_2 in RPMI 1640 with 2 mM L-glutamine (Cambrex, Vervies, Belgium) and 5 mM ultraglutamine 1 (Cambrex), adjusted to contain 1.5 g/l sodium bicarbonate (PAA, Cölbe, Germany), 4.5 g/l glucose (Sigma), 10 mM HEPES (PAA), 1 mM sodium pyruvate (PAA), 0.05 mM 2-mercaptoethanol (Sigma), 10% heat inactivated FCS and 1% penicillin/streptomycin. Cells were plated at 2.5 x 10^5 cells/well in 96-well culture plates (Greiner bio-one, Frickenhausen, Germany) for at least two hours to allow adherence before stimulation.

Vitality test
The Alamar blue reduction assay was employed to measure the activity of the cellular mitochondria as turnover of Alamar blue (Biosource, Camarillo, USA) to the fluorescent product resorufin, which can be detected at 544 nm excitation and 590 nm emission wavelengths. Blood cells from incubation vials were spun down and resuspended in 200 µl prewarmed 10% Alamar blue in RPMI 1640 (Cambrex, Verviers, Belgium). After 2 h incubation at 37°C in a humid atmosphere with 5% CO_2 the vitality was quantified in a fluorometer. 100 µl fresh blood cells were employed as positive control, and 100 µl blood cells were killed by incubation with 10% DMSO for 15 minutes to provide a negative control.

Coolant
Samples of different fluid coolants were kindly provided by the Austrian Social Insurance for Occupational Risks (AUVA). The fluid coolants were subjected to gradual dilution (150 mM saline), in 3-fold steps up to 1:300,000. A series of diluted coolant samples were spiked with 100 pg/ml LPS from E. coli O-113 [1EU], in parallel with 10 µg/ml LTA from *Staphylococcus aureus* and also with with 10 µg/ml Zymosan from *Saccharomyces cerevisiae*. After 2 h spiking time the samples were treated according to the standard protocol of the in vitro pyrogen test (MAT). The margin of spike recovery was set corresponding to the LAL protocol, i.e., between 50% and 200% of the cytokine response compared to the spiked saline. In parallel to the MAT testing the cells were subjected to a Alamar blue viability test after the incubation.

Statistics
Statistical analyses were performed using GraphPad Prism program 4.01 (GraphPad Software, San Diego, USA). Statistics on 3 or more groups were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. For two groups we used the unpaired t-test. Data are means ± SEM. A p-value ≤ 0.05 was considered significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. All statistical analyses are based on raw data. For calculation of the minimal cytokine inducing spore count or spore surface area, linear regression was performed on increasing mean data points from a dose response curve in Excel (Microsoft, Redmond, CA, USA).