



Comparison of a Monocyte Activation Test based on fetal bovine serum and on human AB serum

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Pharmalab, Neuss, 12 November 2019

RESEARCH | DIAGNOSTICS | PHARMACEUTICALS

Topics of the presentation

- Performance of a cryopreserved PBMC-based Monocyte Activation Test (MAT) using fetal bovine serum (FBS) or human AB serum as cell culture supplement
- Case study of analyzing a drug product using FBS or human AB serum as supplement for the MAT assay
- What source of serum to use for the MAT?

Pyrogen testing



- All parenteral administered pharmaceutical products must be free of pyrogenic (fever-inducing) contamination
- Classification of pyrogens:

Non-endotoxin pyrogens	→	<ul style="list-style-type: none">✓ Components from gram-positive bacteria✓ Yeasts & molds✓ Viruses
Endotoxins	→	<ul style="list-style-type: none">✓ Components from gram-negative bacteria: Lipopolysaccharide (LPS)

Pyrogen test vs endotoxin test

Pyrogen tests

Rabbit Pyrogen Test (RPT)



~ 400.000 per year
(worldwide)

Monocyte Activation Test (MAT)



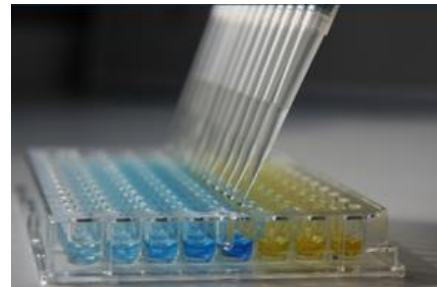
Endotoxin tests

Limulus Amoebocyte Lysate Test (LAL)



- ~ 500.000 per year (USA)
- ~15% mortality rate
- ~ 400.000 per year (Asia)
- Used for consumption after bleeding

Recombinant factor C (rFC)

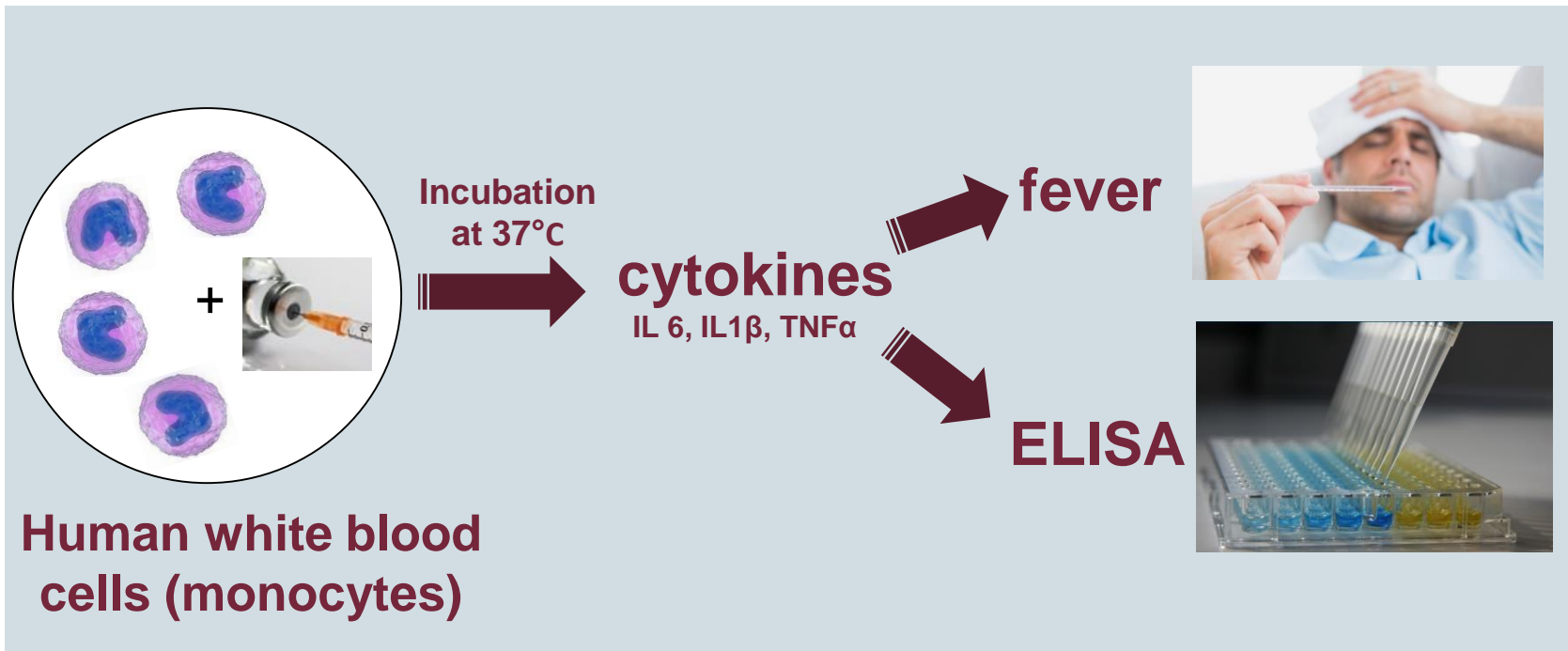


Comparison of pyrogen tests

Pyrogen tests		RPT	BET	MAT
Non-animal, human-based test		-	-	●●●
Detection of endotoxin		●	●●●	●●
Detection of Non-Endotoxin Pyrogens (NEPs)	Human-specific NEP	-	-	●●●
	Bacteria	●●	-	●●●
	Yeasts & molds	●●	-	●●●
	Viruses	●	-	●●●

Monocyte Activation Test:

The human(e) *in vitro* alternative to the RPT



Sanquin

"Together with the donor, we ensure a better life for patients"

HQ in Amsterdam, The Netherlands

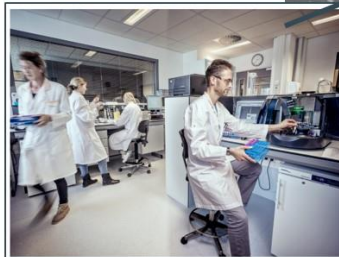
Blood Bank



Reagents



Diagnostic Services



Research

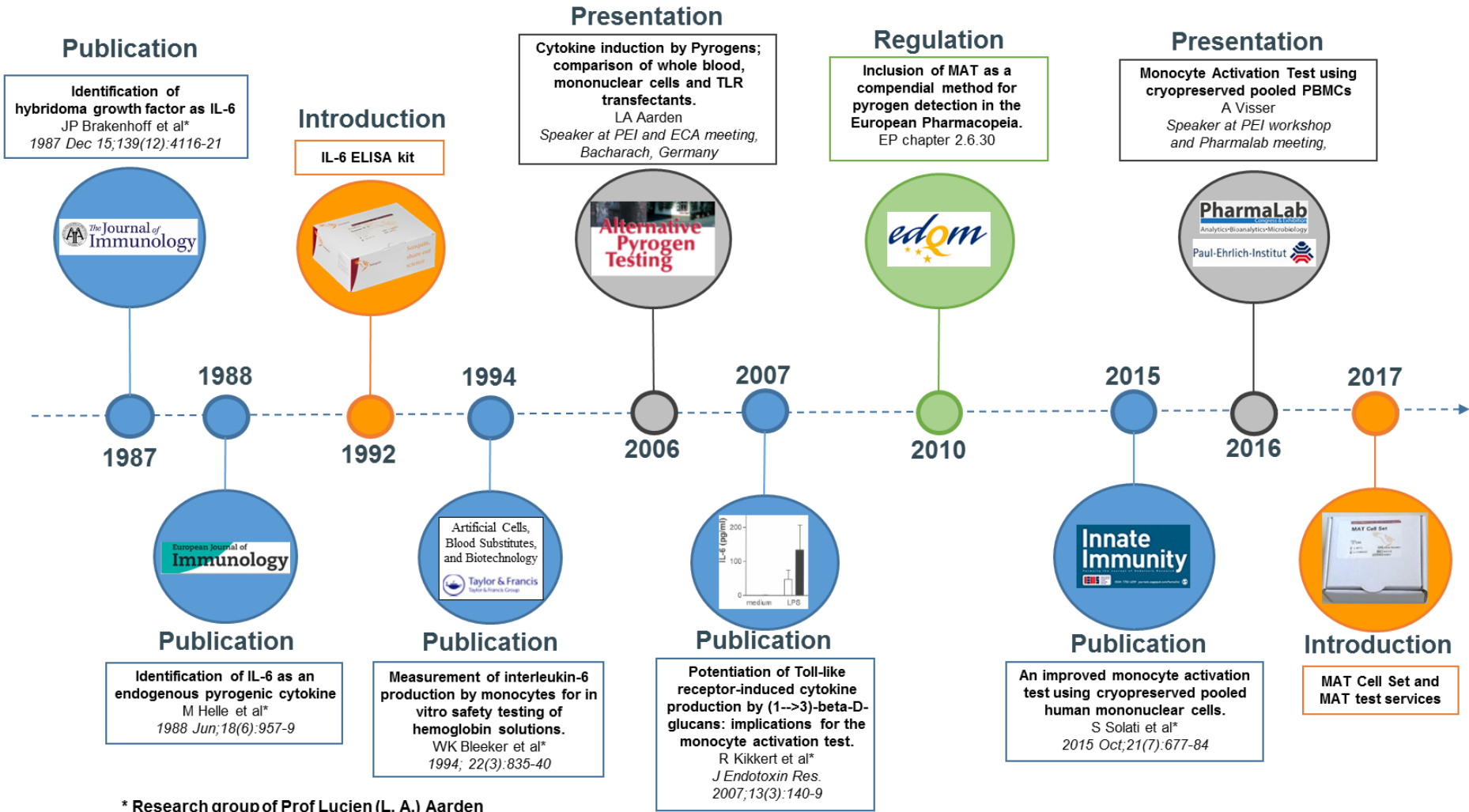


Plasma Products

Facts and figures 2018:

- 2791 employees
- Turnover 448 M euro
- 410,000 whole blood donations
- 310,000 plasma donations

Sanquin and MAT



* Research group of Prof Lucien (L. A.) Aarden

Sanquin MAT kits

Reagents for performing MAT



MAT Cell Set

3 vials MAT qualified cryopreserved pooled PBMCs (for 3 plates)

3 vials dedicated culture medium supplement



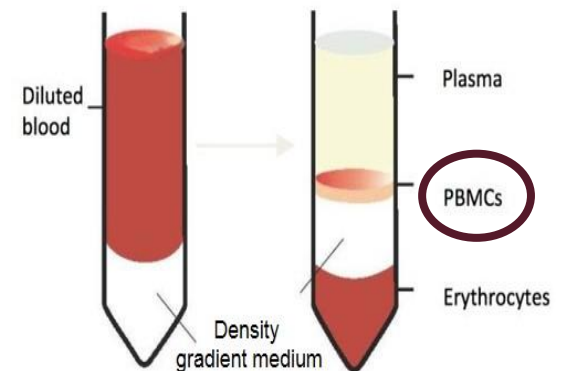
PeliKine IL-6 compact kit

Reagents for IL-6 ELISA (3 plates)

Certification - ISO 13485:2016

Why cryopreserved pooled PBMC* as cell source?

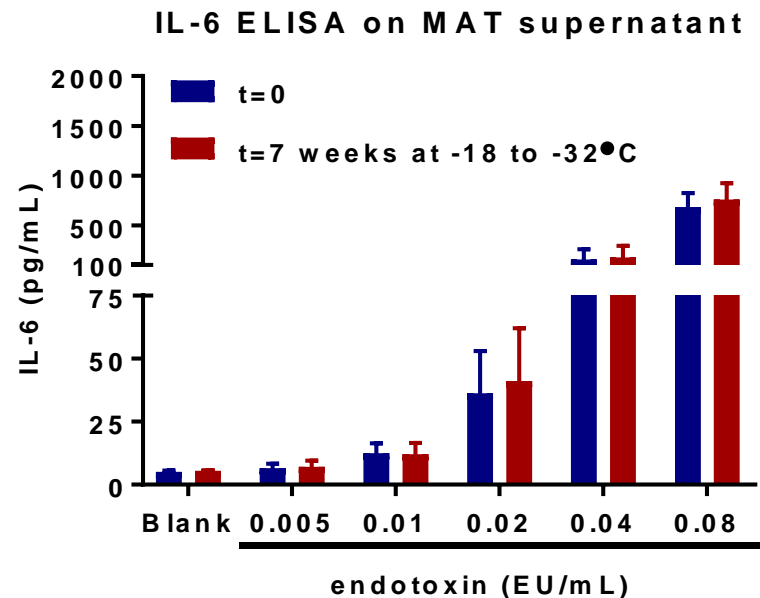
- Comparable reactivity to fresh PBMC
- Available on demand
 - No need take blood and isolate cells prior to each experiment
- Pool of 4 donors takes donor variation into account
- Stable (months at -80°C , years in *liquid N₂*)
- Shipment possible
- Production and extensive qualification of large batches with consistent quality possible



*Peripheral Blood Mononuclear Cells

Why IL-6 as cytokine read-out?

- Demonstrated clinically significant role in fever: Rises in IL-6 levels correlate significantly with rises in body temperature¹⁻⁵
- High sensitivity
- Fully secreted into the medium
- Stable in (frozen) supernatant



¹Helle M, Eur J Immunol. 1988

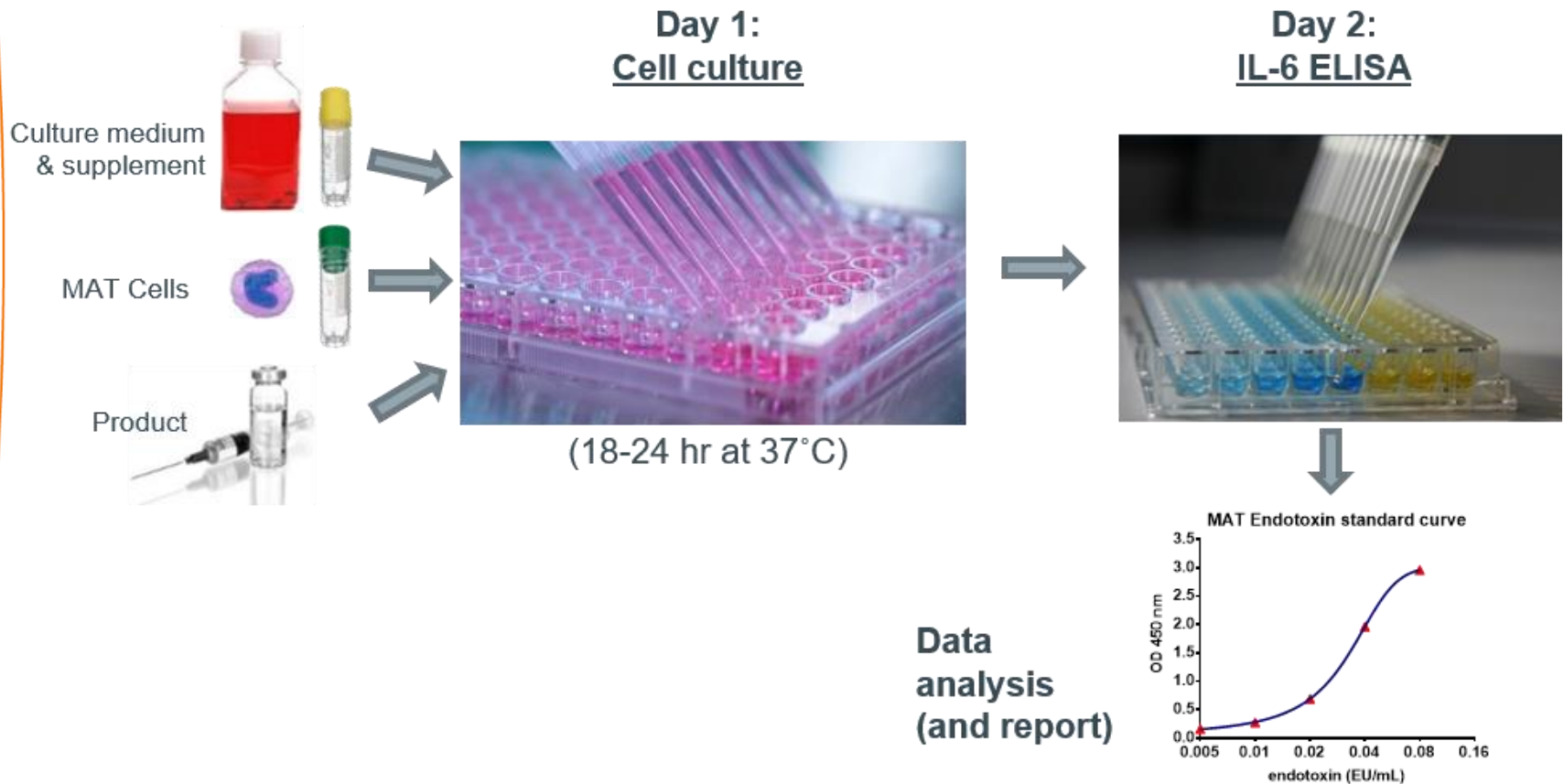
²Engel A, Infection 1994

³Cartmell T, J Physiol. 2000

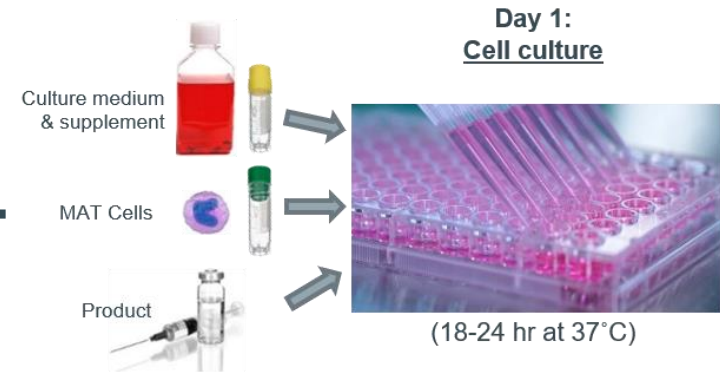
⁴Haarbrink M, The Journal of Infectious Diseases, 2000

⁵Spittler A, Clinical Infectious Diseases, 2000

Overview assay procedure



Cell culture step of the MAT



- Monocytes are usually cultured in the presence of serum as a source for growth factors and other proteins
- Current MAT assay at Sanquin was historically developed and validated using FBS as serum source
- European Pharmacopoeia chapter 2.6.30 states the following:

“PBMC or monocytic cell lines, in culture medium and with either the donor’s own plasma or AB serum, are typically used at a final cell density of $0.1-1.0 \times 10^6$ cells per well, tube or other receptacle. For monocytic cell lines, heat-inactivated foetal bovine serum may be substituted for AB serum.”

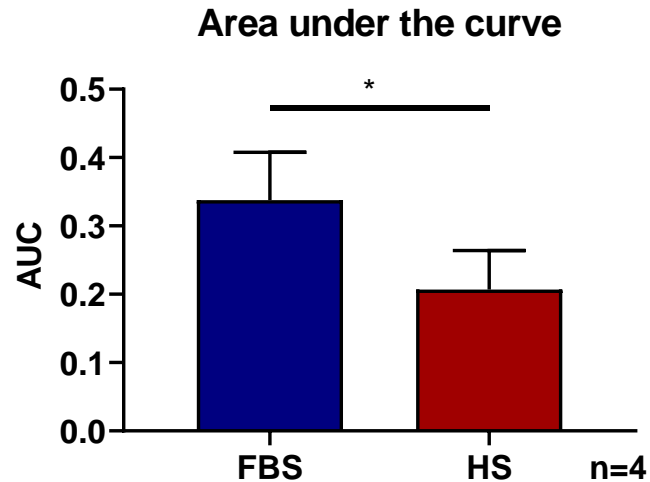
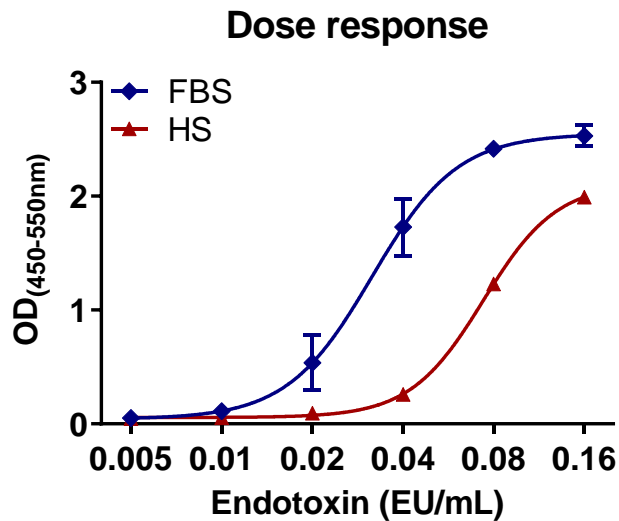
Aim of this study

To compare the performance of a cryopreserved PBMC-based Monocyte Activation Test (MAT) using fetal bovine serum (FBS) or human AB serum (HS) as cell culture supplement

- Reactivity towards endotoxin and non-endotoxin pyrogens
- Consequences of serum heat-inactivation
- Case study: Testing of a pharmaceutical product

FBS vs HS as serum source in the MAT: reactivity towards endotoxin

Endotoxin



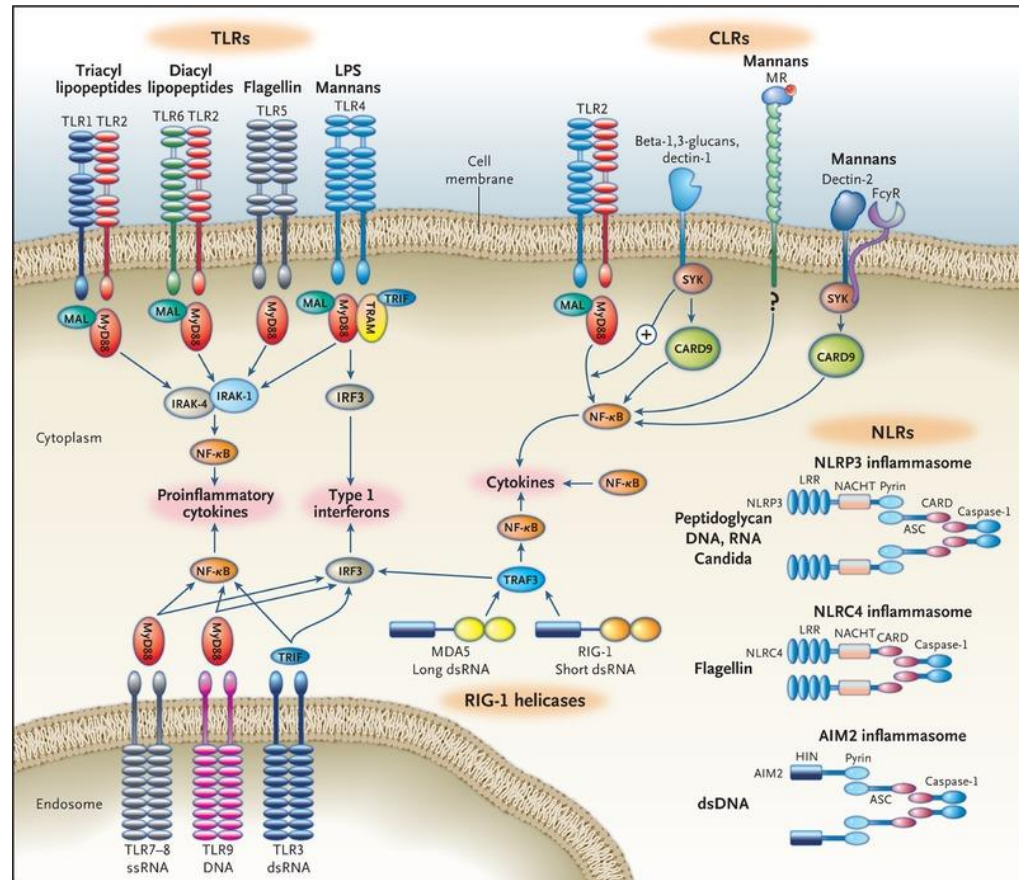
Serum source	LOD (EU/mL)
FBS	≤ 0.01
HS	≤ 0.03

→ Lower reactivity with HS

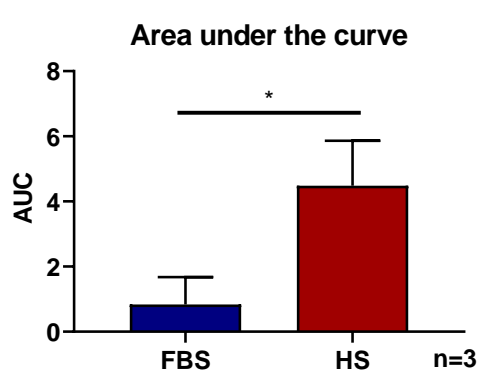
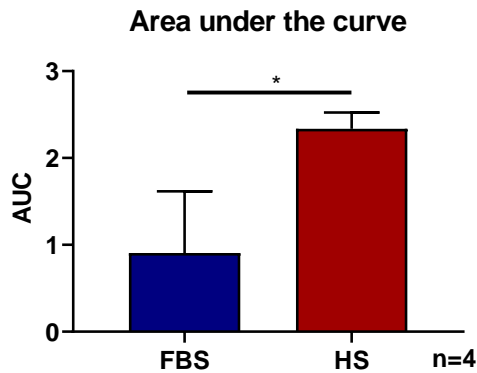
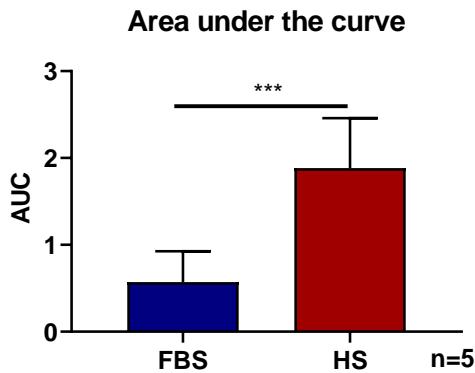
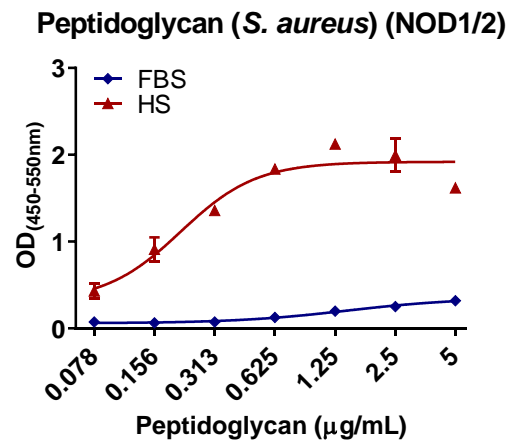
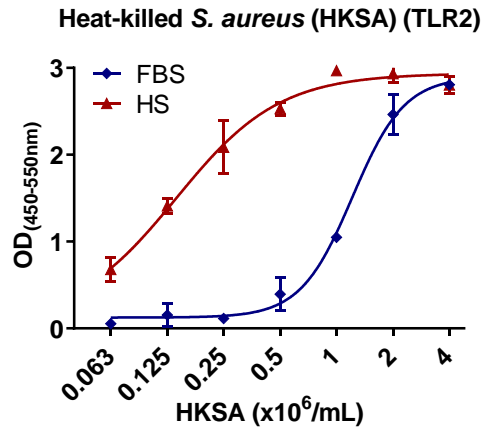
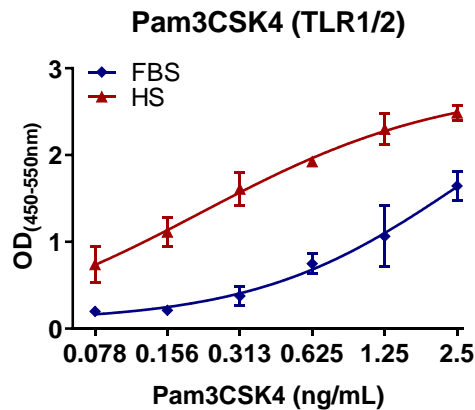
Pattern recognition receptors (PRRs) alert the immune system to the presence of microbial infections

- Pyrogens are detected by different PRRs:

- Toll-like receptors, e.g.:
 - Endotoxin
 - Flagellin
 - Triacylated lipopeptides (Pam3CSK4)
 - R848 (Resiquimod)
- NOD like receptors, e.g.:
 - Peptidoglycan
- C-Type lectin receptors, e.g.:
 - Beta-glucan
- RIG like receptors
- Cytosolic DNA Sensors

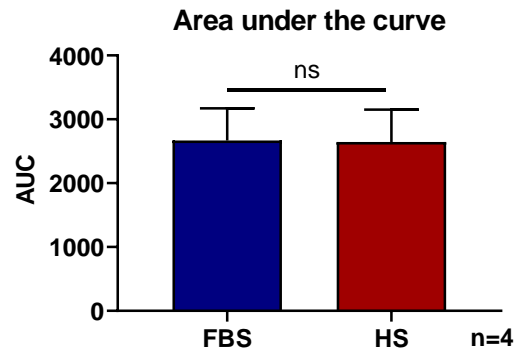
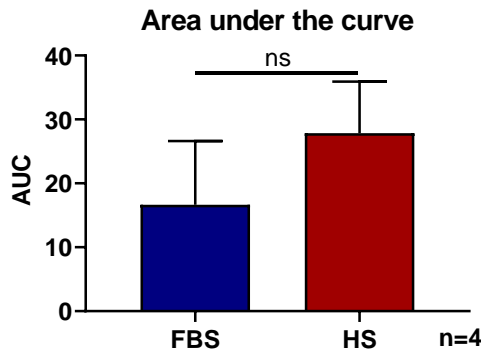
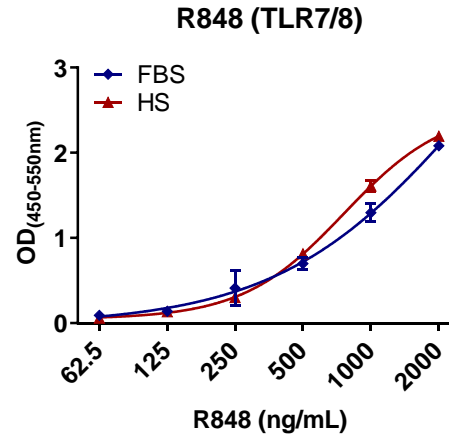
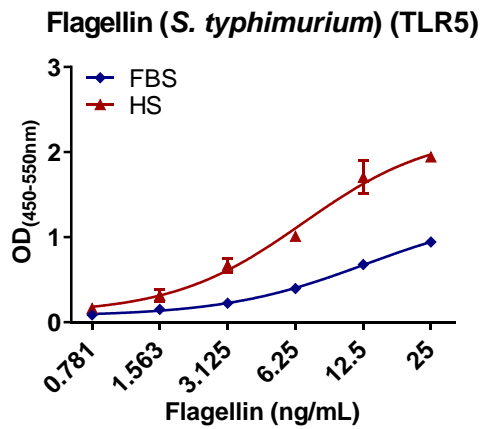


FBS vs HS as serum source in the MAT: reactivity towards non-endotoxin pyrogens (NEP) (1)



→ Higher reactivity with HS

FBS vs HS as serum source in the MAT: reactivity towards non-endotoxin pyrogens (NEP) (2)



→ Comparable reactivity

Conclusions (1)

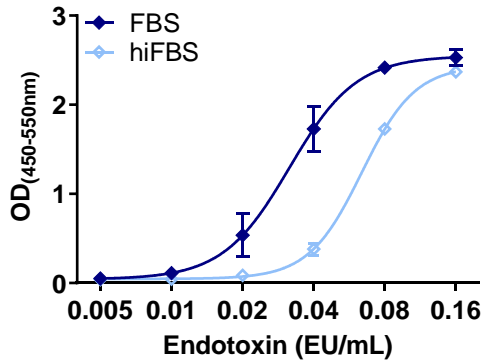
- Use of HS results in lower reactivity towards endotoxin compared to FBS
 - Higher Limit of Detection
 - Lower area under the curve
- Use of HS results higher reactivity towards most tested NEPs
 - Pam3CSK4 → Higher
 - HKSA → Higher
 - Peptidoglycan → Higher
 - Flagellin → No significant difference
 - R848 → No significant difference

Heat-inactivation of serum

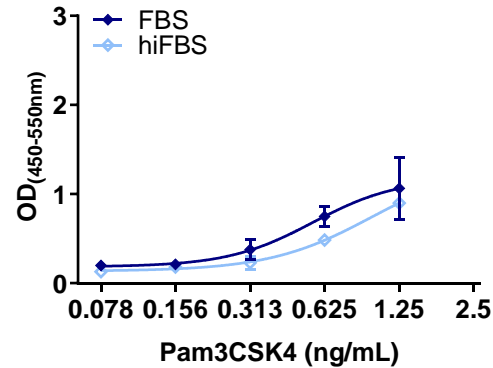
- Heat-inactivation (heating to 56°C for 30 minutes) of serum is usually done to:
 - inactivate complement, a group of proteins present in sera that are part of the immune response.
 - Destroy mycoplasma in serum. However, because most serum suppliers filter through 0.1 µm filters to remove mycoplasma before distribution, this is not usually necessary.
- Serum is often heat-inactivated without any evidence of beneficial effect, simply because an earlier protocol calls for such action
- Heat inactivation also reduces or destroys serum growth factors and should only be performed when there is a compelling reason

Heat-inactivation of FBS: Reactivity towards pyrogens (1)

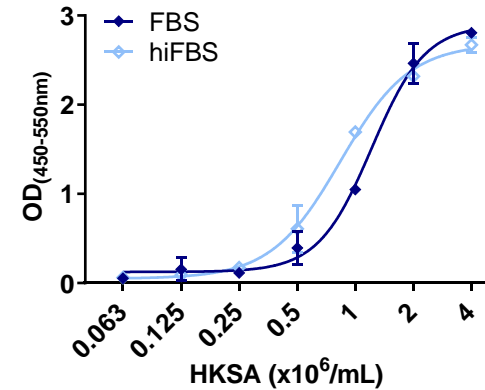
Endotoxin (TLR4)



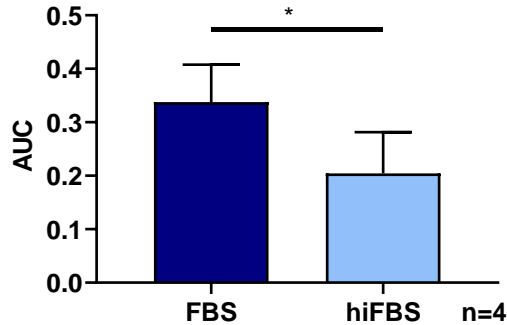
Pam3CSK4 (TLR1/2)



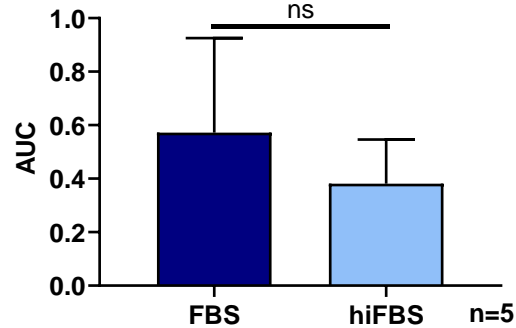
Heat-killed *S. aureus* (HKSA) (TLR2)



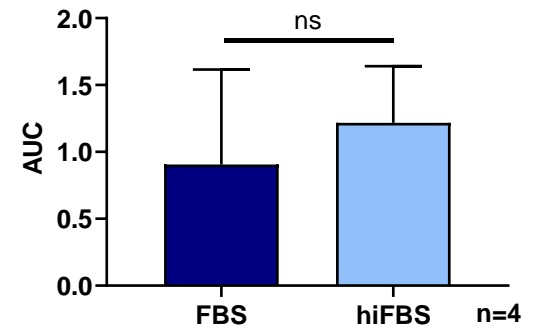
Area under the curve



Area under the curve

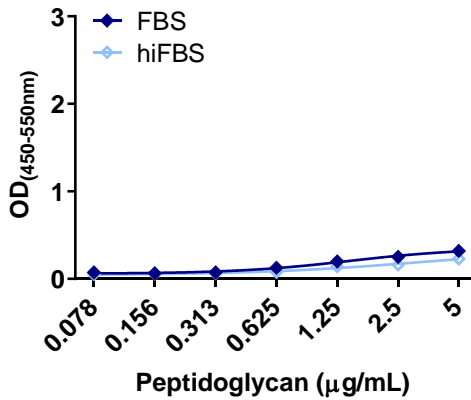


Area under the curve

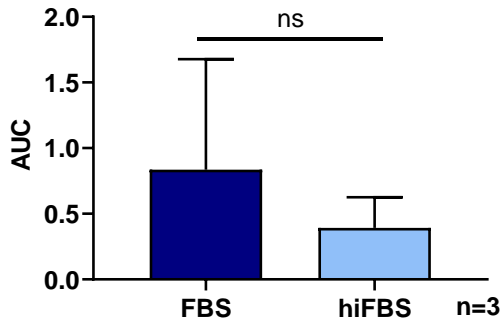


Heat-inactivation of FBS: Reactivity towards pyrogens (2)

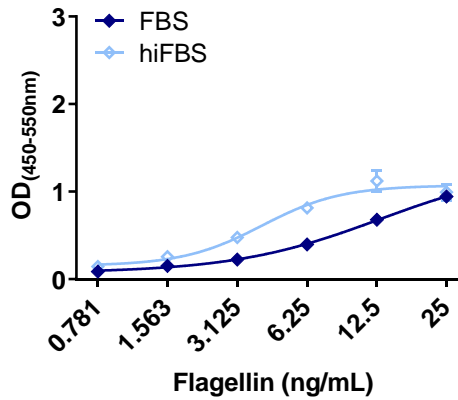
Peptidoglycan (*S. aureus*) (NOD1/2)



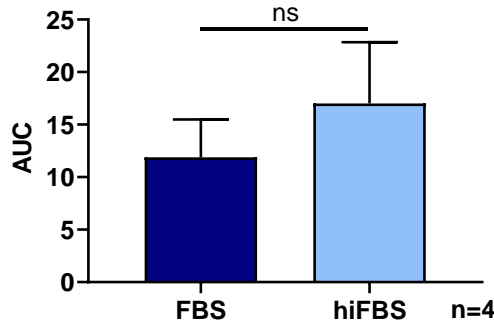
Area under the curve



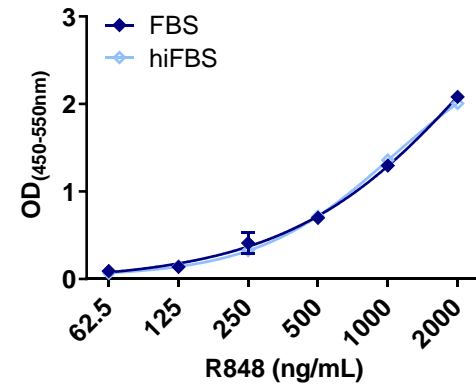
Flagellin (*S. typhimurium*) (TLR5)



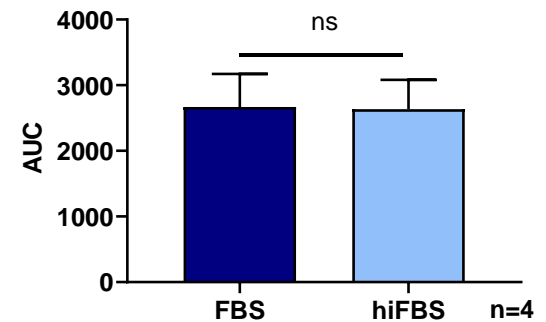
Area under the curve



R848 (TLR7/8)

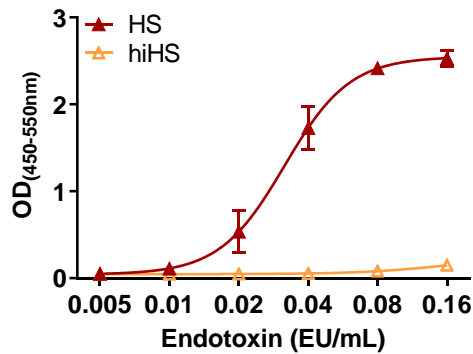


Area under the curve

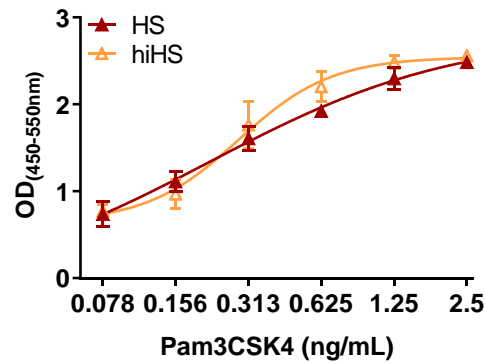


Heat-inactivation of HS: Reactivity towards pyrogens (1)

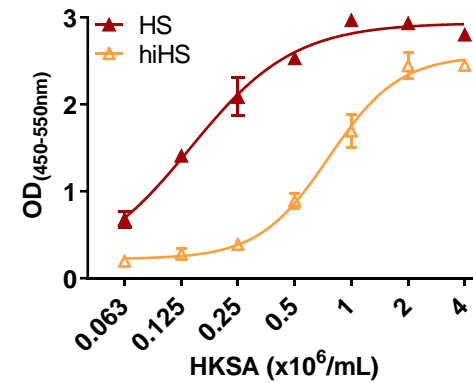
Endotoxin (TLR4)



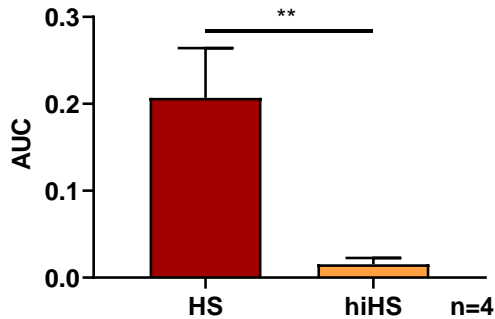
Pam3CSK4 (TLR1/2)



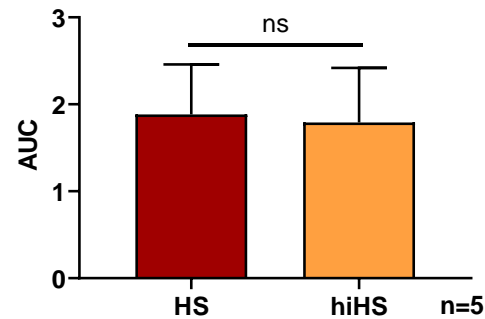
Heat-killed *S. aureus* (HKSA) (TLR2)



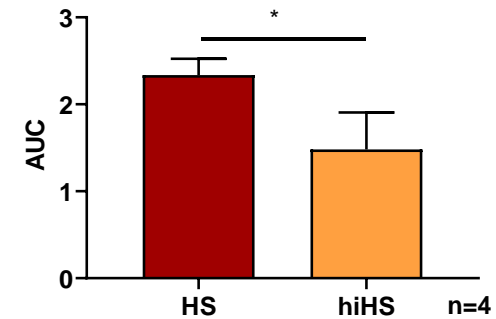
Area under the curve



Area under the curve

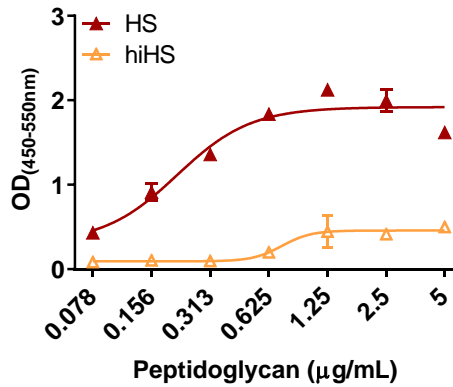


Area under the curve

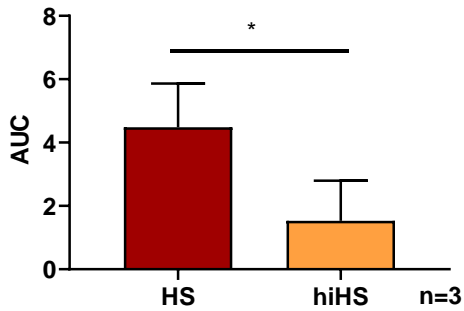


Heat-inactivation of HS: Reactivity towards pyrogens (2)

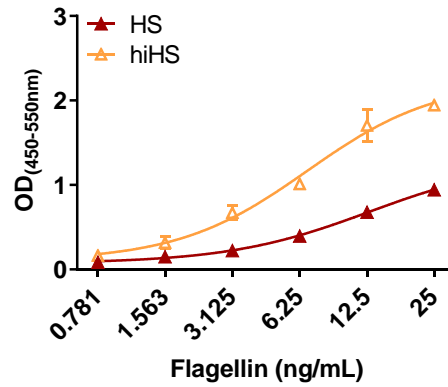
Peptidoglycan (*S. aureus*) (NOD1/2)



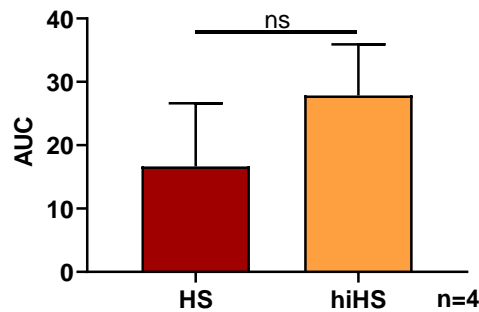
Area under the curve



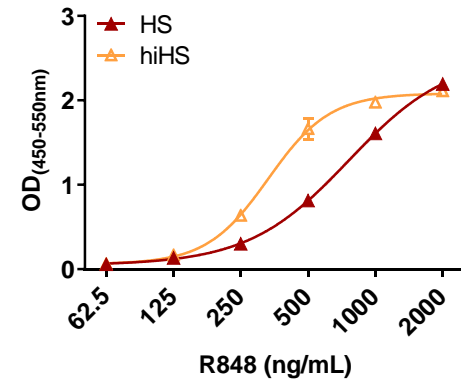
Flagellin (*S. typhimurium*) (TLR5)



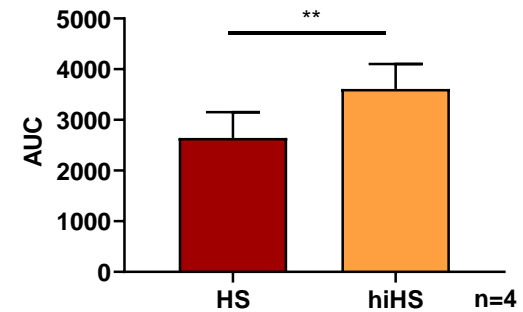
Area under the curve



R848 (TLR7/8)



Area under the curve



Conclusions (2)

- Heat-inactivation of FBS:
 - Results in reduced reactivity towards endotoxin
 - No significant effects on reactivity towards NEPs
- Heat-inactivation of HS:
 - Results in almost complete loss of reactivity towards endotoxin
 - Varying effects on reactivity towards NEPs:
 - Pam3CSK4 → no effect
 - HKSA → Lower
 - Peptidoglycan → Lower
 - Flagellin → no effect
 - R848 → higher

FBS vs HS as serum source: Product testing

- MAT using HS as cell culture supplement results in a higher limit Of Detection (LOD), thereby reducing the Maximum Valid Dilution (MVD) of a product:

$$\text{MVD} = \frac{\text{CLC} \times \text{C}}{\text{LOD}}$$

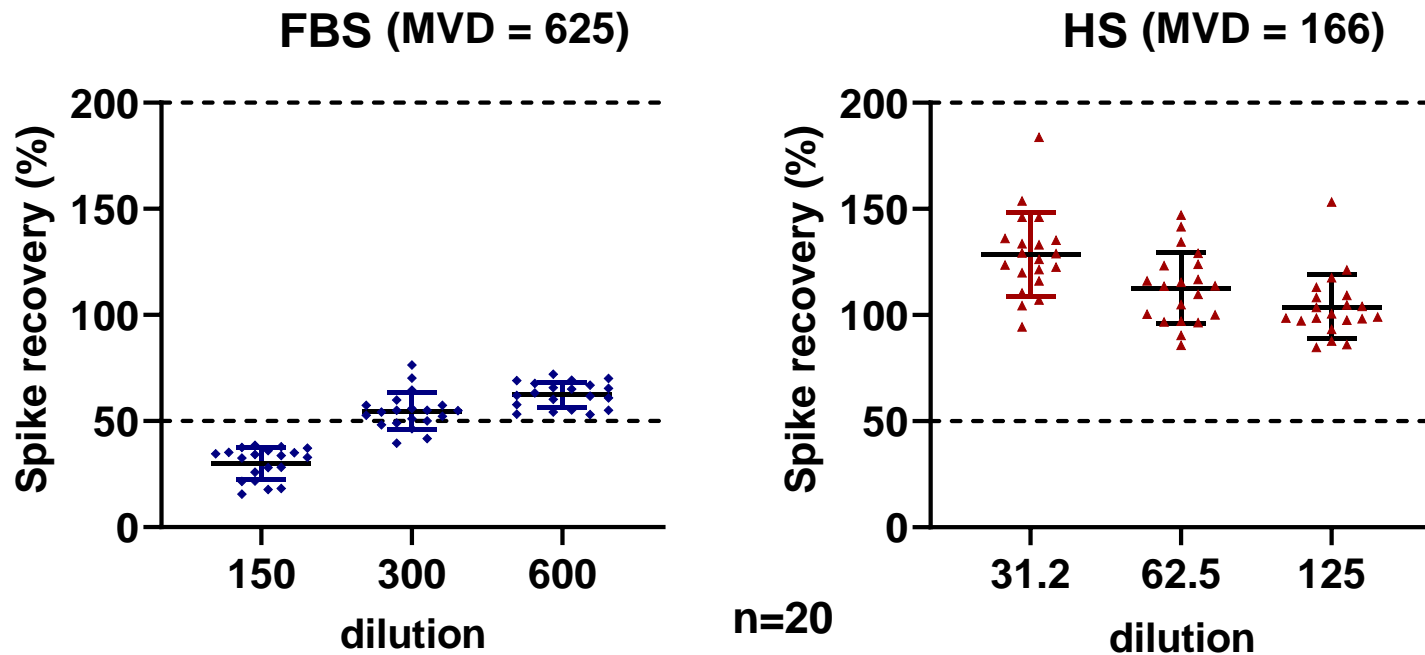
- Maximum Valid Dilution (MVD): The maximum allowable dilution of a sample at which the contamination limit can be determined.
- CLC = contaminant limit concentration
- C = concentration of test sample

Case study: Analyzing a blood-derived product in the MAT using FBS or HS as serum source

- Product is known to cause interference
- CLC of the product = 5
- LOD of FBS-based MAT = 0.008
 - MVD therefore is 625
- LOD of HS-based MAT = 0.03
 - MVD therefore is 166

$$\text{MVD} = \frac{\text{CLC} \times \text{C}}{\text{LOD}}$$

FBS vs HS as serum source in the MAT: Results of endotoxin spike recovery of the product



- Valid spike recovery (50-200%)
 - Product tested in MAT based on FBS requires $\geq 300x$ dilution
 - Product tested in MAT based on HS requires $\geq 31.2x$ dilution

Conclusions (3)

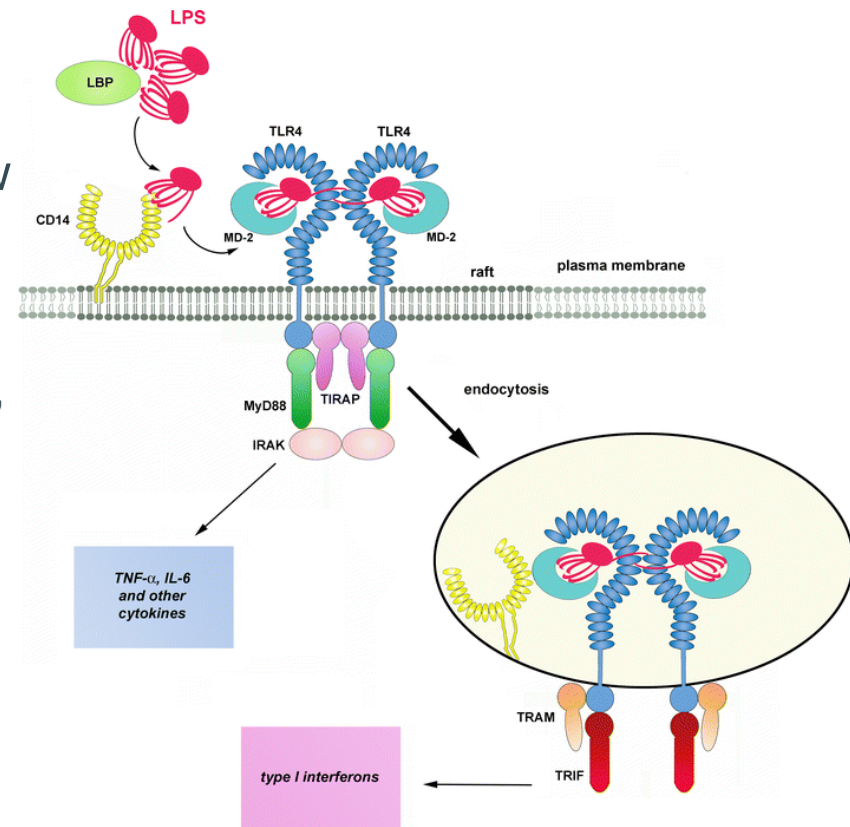
- Product can be tested at lower dilutions in the HS-based MAT assay compared to the FBS-based one to have valid spike recoveries

Summary

- MAT based on HS shows in most cases higher reactivity towards NEPs but lower reactivity towards endotoxin compared to the FBS-based MAT
- Consequences of heat-inactivation
 - FBS
 - Results in reduced reactivity towards endotoxin
 - No significant effects on reactivity towards NEPs
 - HS:
 - Results in almost complete loss of reactivity towards endotoxin
 - Varying effects on reactivity towards NEPs:
- Product testing using the FBS- or the HS-based MAT assay
 - Although the HS-based system has a higher LOD for endotoxin, the product required lower dilution compared to the FBS-based MAT to have valid spike recoveries

Discussion

- Why would the MAT based on HS show lower reactivity towards endotoxin?
 - Differences in LPS-binding protein (LBP) content
 - Differences in serum lipoprotein (e.g. LDL, VLDL) content (have been shown to inactivate LPS^{1,2})
 - Presence of anti-LPS antibodies
- Why would heat-inactivation reduce reactivity towards endotoxin?
 - Heat sensitivity of LBP³
- Valid spike recoveries at lower dilutions for a blood-derived product with the HS-based MAT
 - Inhibiting factor already present in HS?

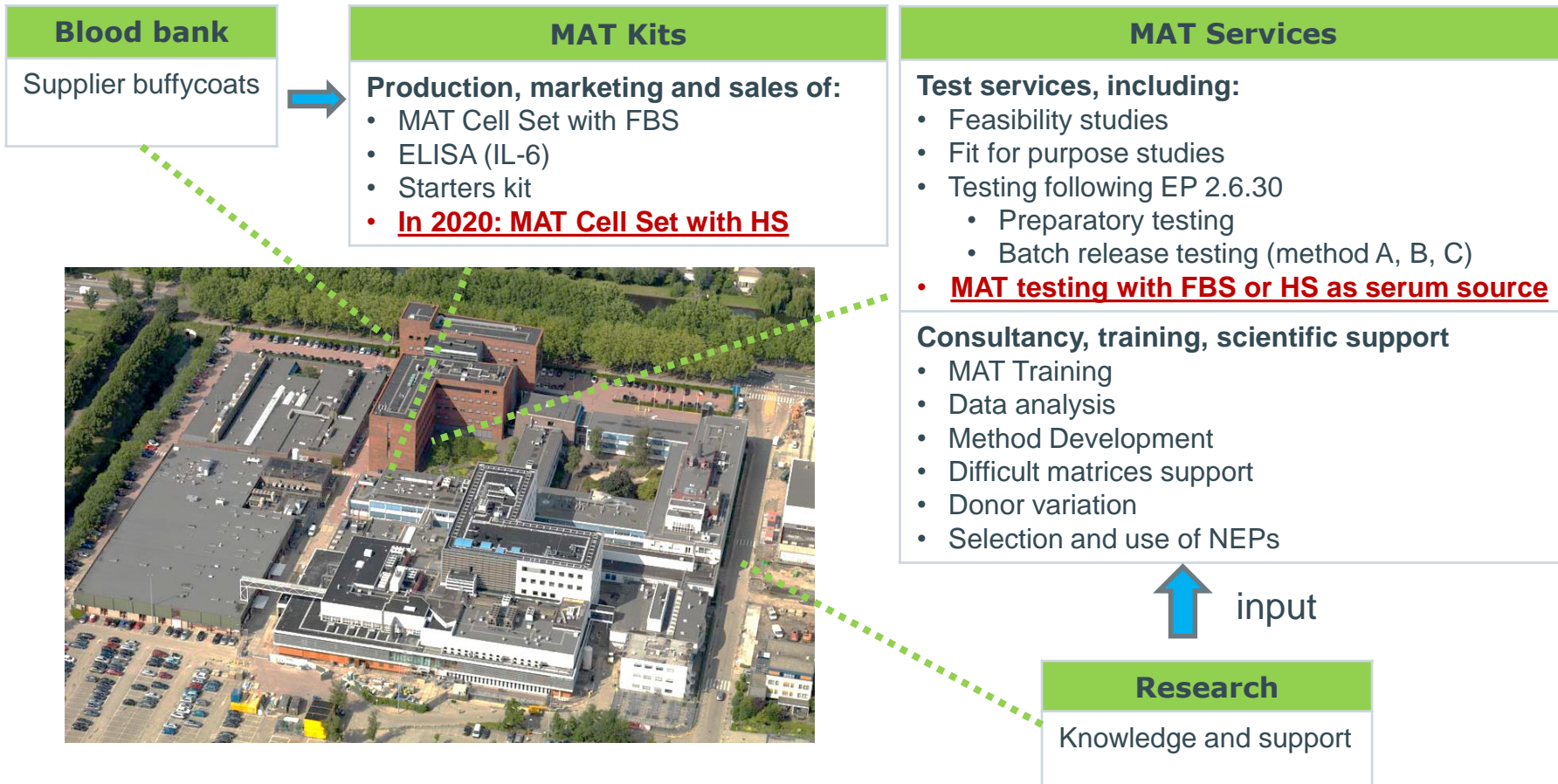


1) Berbee JF, J Endotoxin Res 2005
 2) Wendel M, Intensive Care Med 2007
 3) Meszaros K, Infection and Immunity 1995

What source of serum to use for the MAT?

- Depends on the type of product
 - HS-based MAT would be preferred for blood/plasma-derived products (Valid spike recoveries at lower product dilutions)
 - FBS-based MAT may be more suitable for testing vaccines, especially vaccines against diseases for which the donor of the HS may already have antibodies
- If the highest sensitivity towards endotoxin is required, FBS may be the best choice
- For the highest sensitivity towards NEPs, HS may be the best choice
- Avoid heat-inactivated serum, especially when using HS

Sanquin's MAT Center of Expertise



Everything under the same roof

Acknowledgements

MAT test services

MAT Kits

Astrid Visser
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Elisa Teunissen
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Maarten Koot
Paulien Doodeman
Rita Ramdas
Fereshte Rezai

Research

Anja ten Brinke
Miranda Dieker-Meijer
Tineke Jorritsma
Lucien Aarden

Blood collection

Blood bank

Susan Cuvalay
Marcia van den Eijnden

Research

The Monocyte Activation Test



The human(e) alternative
to the Rabbit Pyrogen Test

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