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Advancing Pyrogen Testing: The Role of the Monocyte Activation Test

25. SEP 2024

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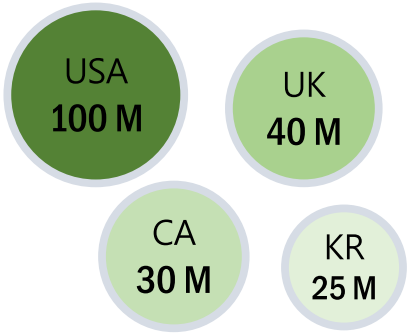
Introduction

Alternative Animal Testing Methods

Testing methods that **do not use animals** can predict and evaluate **efficacy and toxicity**

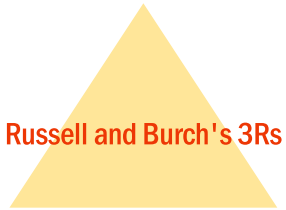
Methods to **reduce the number of animals** emphasizing the **3R principles**

Number of experimental animals



3R principles

Replacement
use of non-animal methods



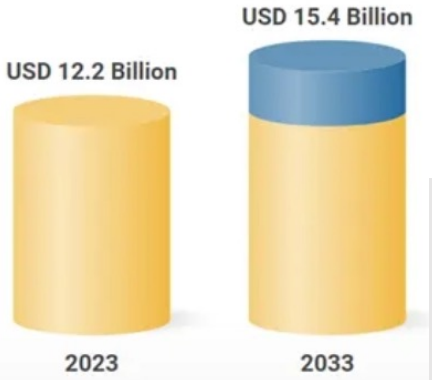
Reduction
minimize the number

Refinement
Enhance techniques

식품의약품안전처
Ministry of Food and Drug Safety

- ARE-Nrf2 In Vitro Skin Sensitisation (OECD TG 442D)
- IL-8 In Vitro Skin Sensitisation (OECD TG 442E)
- In Vitro 3T3 NRU Phototoxicity Test (OECD TG 432)
- In Vitro Skin Irritation (OECD TG 439)

Non-animal Alternative Testing Market Size



Current Status of Alternative Animal Testing Methods and Evaluation

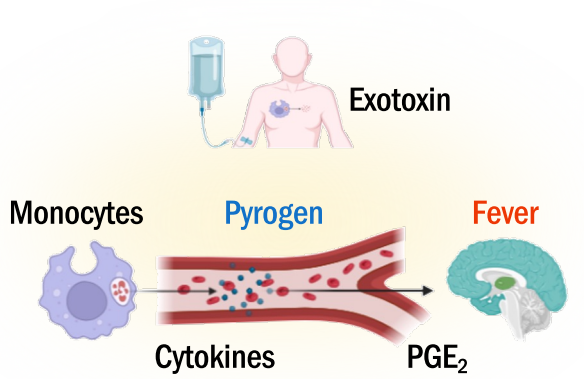
Increase in Demand for Experimental Animals Due to **Enhanced Safety Regulations**

Global emphasis on ethical issues related to the use of experimental animals has led to **ongoing efforts to replace animal testing**

Introduction

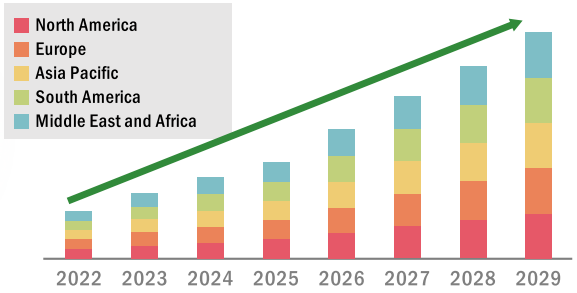
Pyrogen Testing

Pyrogen



Pyrogen Testing Market

Global Pyrogen Testing Market is Expected to Account for USD 2,784.75 Million by 2029



Source: Date Bridge Market Research Market Analysis study 2022

Pyrogen Testing Methods

Pyrogen test assay

Limulus Amoebocyte lysate Test (LAL)

Rabbit Pyrogen Test (RPT)

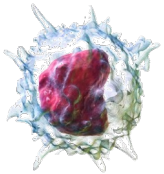
Monocyte Activation Test (MAT)

Monocyte Activation Test (MAT)

Pyrogen

1. Endotoxins (LPS from gram negative bacteria)
2. Non-endotoxin pyrogens (gram-positive bacteria, yeaset, mold, virus, etc)

Monocyte



Cytokines

IL-1β,
IL-6,
TNF-α,
IFN-γ

Detection of IL-6 with ELISA

Advantages of MAT

- High Sensitivity and Accuracy (V.S. RPT)
- No Variation Due to Species
- Eliminating ethical concerns
- Detecting all pyrogenic materials
- Reflective of In Vivo Conditions

Recommending the Replacement of RPT with MAT

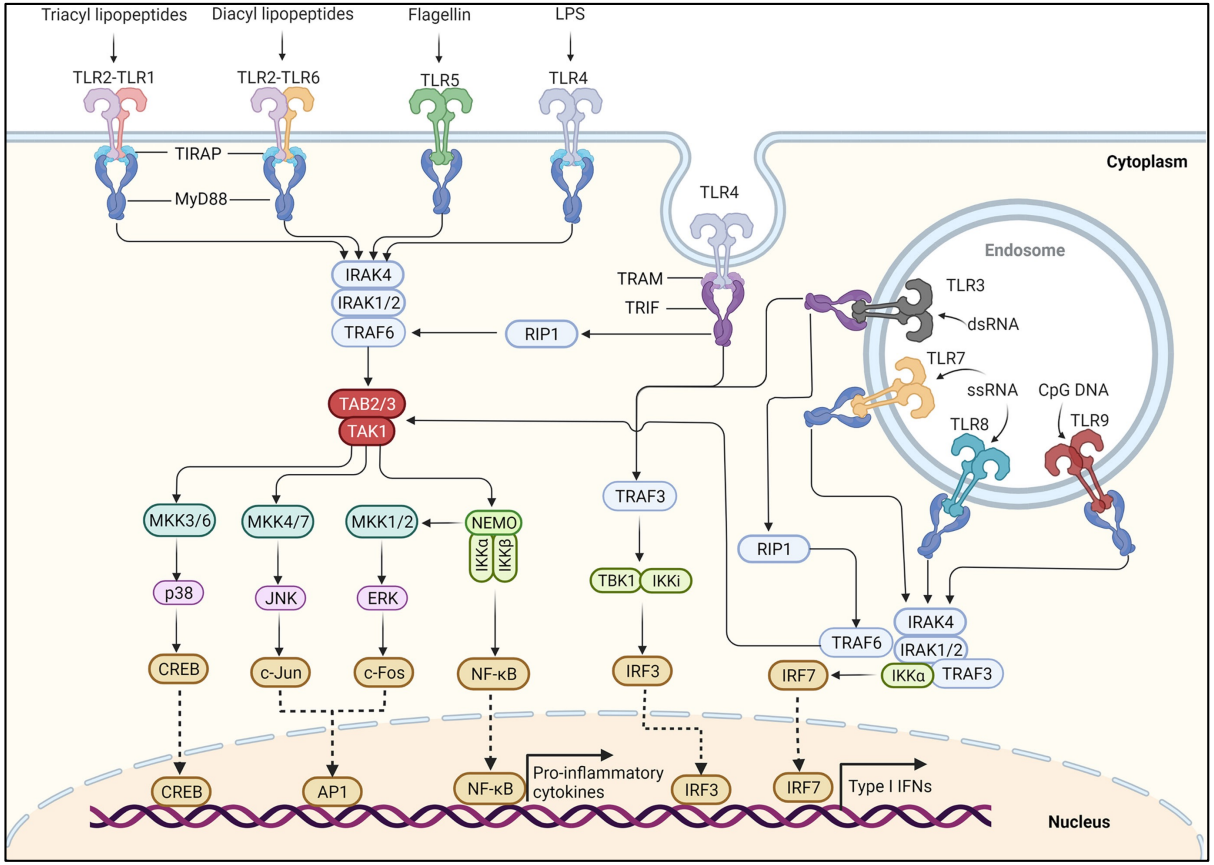
Introduction

Comparison of Pyrogen Testing Methods: RPT vs. LAL vs. Factor C vs. MAT

Assay Type		RPT	LAL	Factor C	MAT
Animal Use		O	X	X	X
Sensitivity (Limit of Detection: LoD)		0.05 EU/ml	0.005 EU/ml	0.005 EU/ml	0.005 EU/ml
Endotoxin	LPS	+	+	+	+
Non – Endotoxin	LTA	+	-	-	+
	Yeast	+	-	-	+
	Virus	+/-	-	-	+
Application	Phamaceuticals	+	+	+	+
	Biologicals	+	+/-	+/-	+
	Medical Devices	+	+/-	+/-	+
	Cell Therapeutics	-	+/-	+/-	+

Introduction

TLR signaling pathway and pyrogen



NEP	TLR
Pam3CSK4	1/2
HKSA	2
PGN	2
FSL-1	2/6
Poly-IC	3
Flagellin	5
Imiquimod	7
CL075	7/8
ODN2006	9
MDP	NOD2

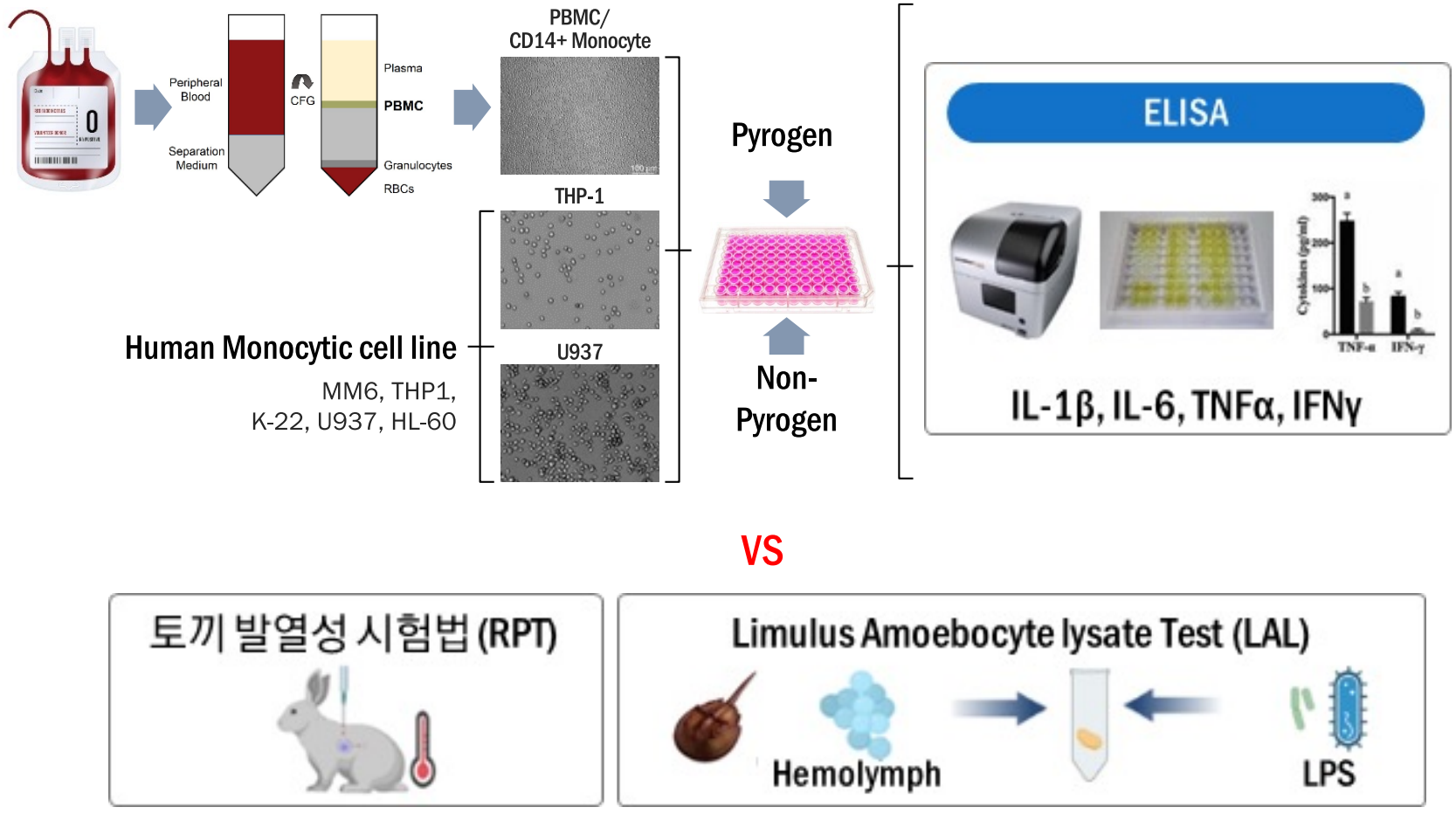
Duan T, *et al. Front Immunol.* 2022.

Absence of standardized positive control materials for non-endotoxin pyrogens

- **Non-endotoxin pyrogens originate from diverse sources and exhibit varying properties, making standardization challenging**

Research Goal

Establishment of MAT assay

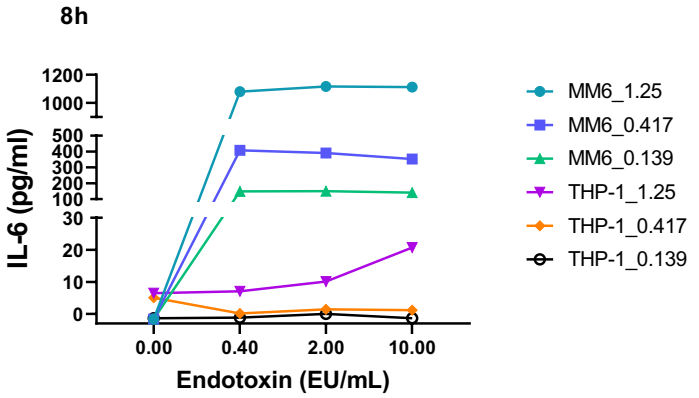


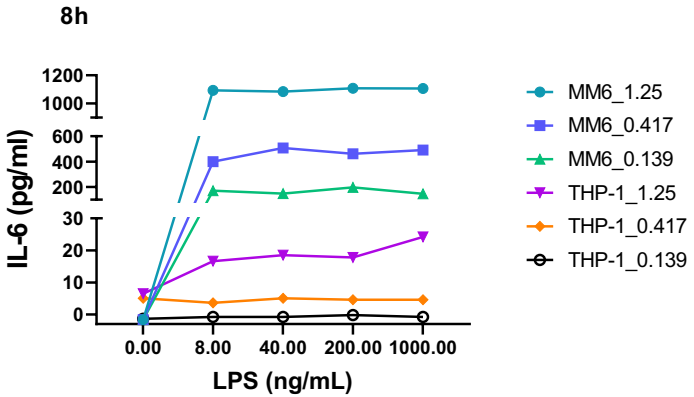
MAT Validation

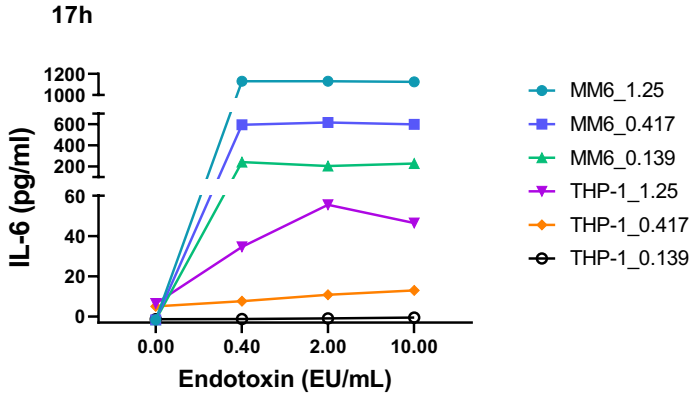
Parameters of MAT assay

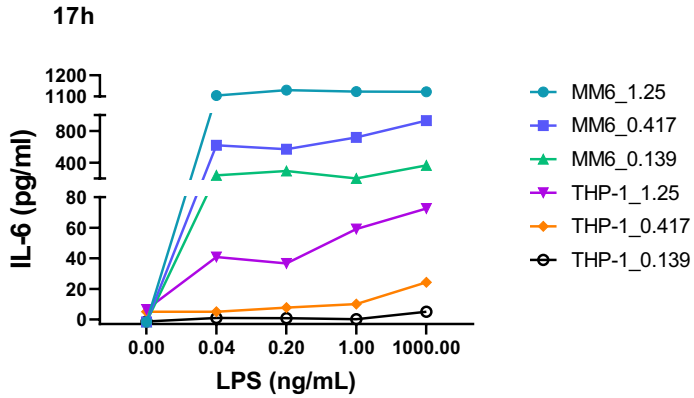
- **Replication: at least 4 concentrations in quadruplicate**
- **Limit of Detection (LOD): 0.005 EU/mL**
- **Accuracy: Recovery rate of 95% or higher (Recovery range: 50-200%)**
- **Precision: Coefficient of Variation (CV) below 10%**
- **Selectivity: No effect of interfering substances on endotoxin detection**
- **Positive control reaction: Strong signal within the expected range**
- **Negative control reaction: Minimal background signal**

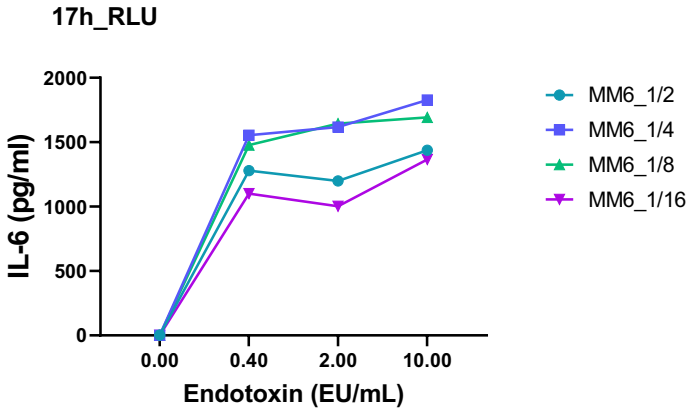
MAT Validation

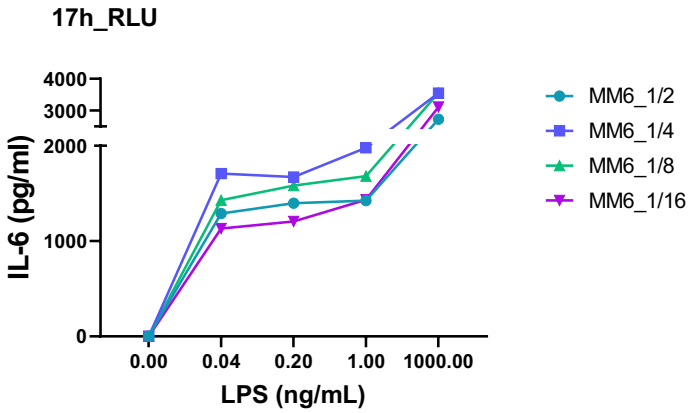








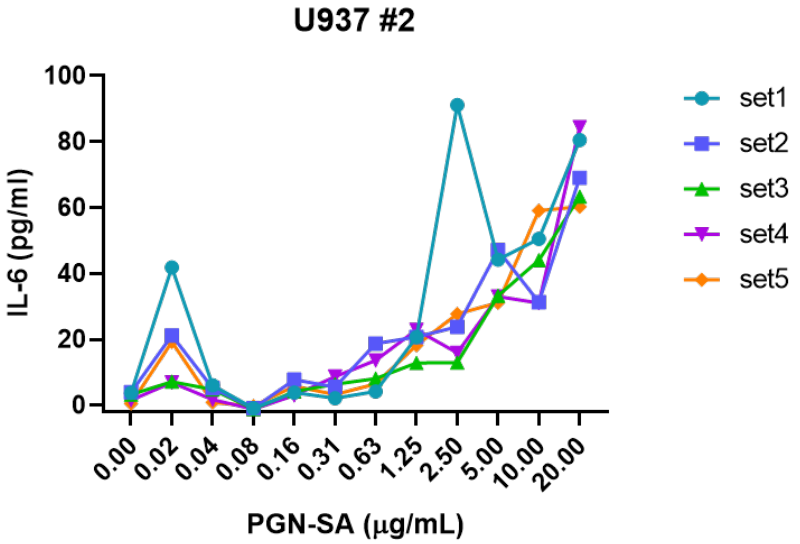




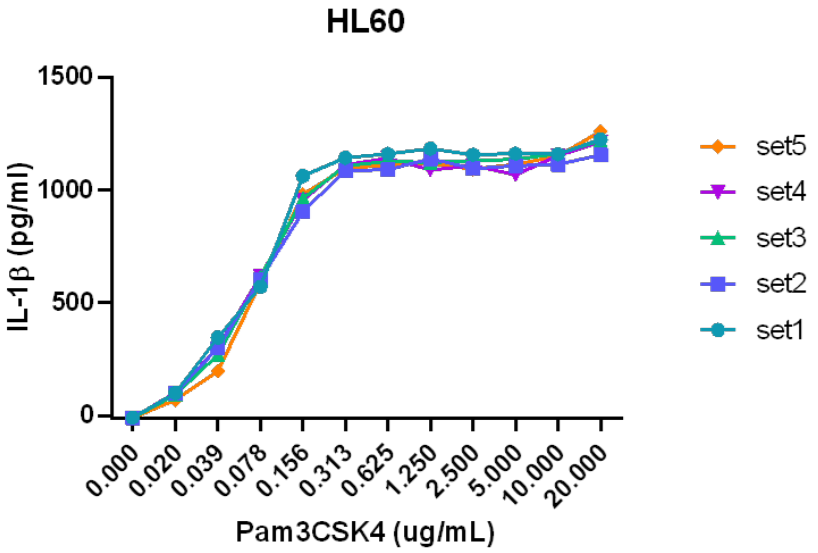
1EU/mL = 0.1ng/mL LPS(E.coli)
10EU/mL=1ng/mL LPS
100EU/mL=10ng/mL LPS
1,000EU/mL=100ng/mL LPS
10,000EU/mL=1ug/mL LPS

MAT for NEP detection

	Conc. μg/mL	IL6(pg/mL)						
		abs1	abs2	abs3	abs4	abs5	Mean	SD
PGN-SA	0.00	3.707317	3.878049	3.195122	1.5	0.487805	2.6	1.5
	0.02	41.85366	21.12195	7.097561	6.853659	19.17073	19.2	14.3
	0.04	6	5.146341	4.780488	1.731707	0.878049	3.7	2.3
	0.08	-0.95122	-0.95122	-1.19512	-1.19512	-0.21951	-0.9	0.4
	0.16	3.804878	7.707317	4.170732	2.95122	5.756098	4.9	1.9
	0.31	2.097561	5.512195	6.365854	8.682927	3.195122	5.2	2.6
	0.63	4.170732	18.68293	8.073171	13.56098	6.487805	10.2	5.9
	1.25	20.63415	20.7561	12.82927	22.82927	18.19512	19.0	3.8
	2.50	91.12195	23.80488	12.95122	15.87805	27.70732	34.3	32.3
	5.00	44.17073	47.09756	33.19512	32.95122	31.12195	37.7	7.4
	10.00	50.5122	31.2439	44.04878	31	59.04878	43.2	12.2
	20.00	80.5122	69.04878	63.31707	84.41463	60.26829	71.5	10.6

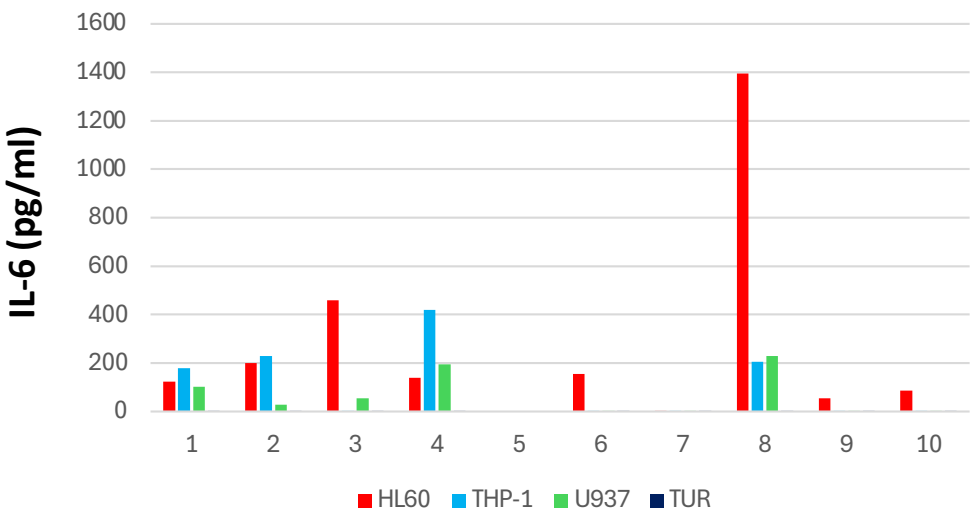


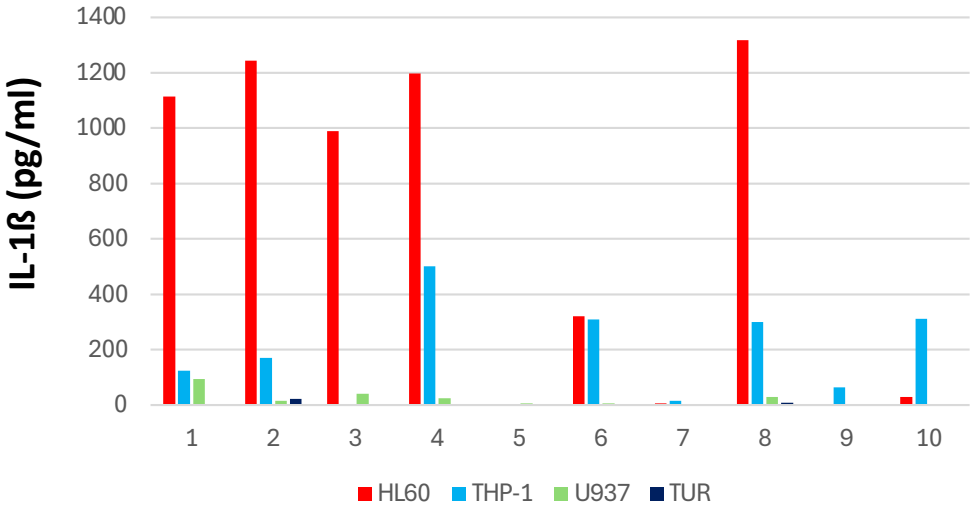
	Conc. ug/mL	IL1B(pg/mL)						
		abs1	abs2	abs3	abs4	abs5	Mean	SD
Pam3CSK4	0	-30.0588	-33.5882	-31.2353	-32.4118	-31.8235	-31.8	1.3
	0.0195	100.5294	99.94118	92.29412	101.7059	69.94118	92.9	13.3
	0.0391	347	299.9412	269.9412	302.2941	196.4118	283.1	55.7
	0.0781	570.5294	604.6471	614.0588	617.5882	589.3529	599.2	19.4
	0.1563	1062.882	904.6471	962.8824	954.6471	981.1176	973.2	57.6
	0.3125	1142.882	1088.176	1107.588	1111.706	1097	1109.5	20.8
	0.625	1160.529	1090.529	1127	1141.706	1111.706	1126.3	26.9
	1.25	1184.059	1139.941	1122.882	1089.353	1118.176	1130.9	34.9
	2.5	1157.588	1095.235	1129.941	1108.765	1093.471	1117.0	27.0
	5	1162.294	1108.176	1136.412	1068.176	1114.647	1117.9	35.0
	10	1159.353	1114.647	1160.529	1152.882	1151.118	1147.7	18.9
	20	1224.647	1156.412	1221.706	1213.471	1260.529	1215.4	37.6



Non-Endotoxin Pyrogen (NEP)

Monocytic cell line and NEP

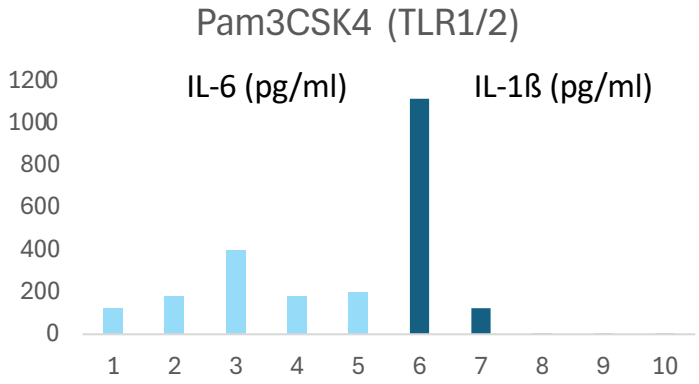


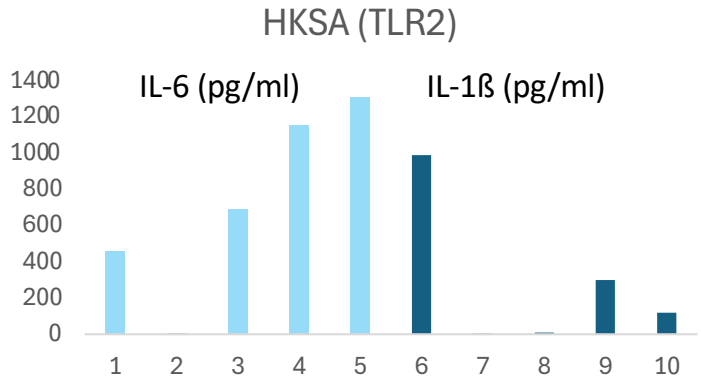


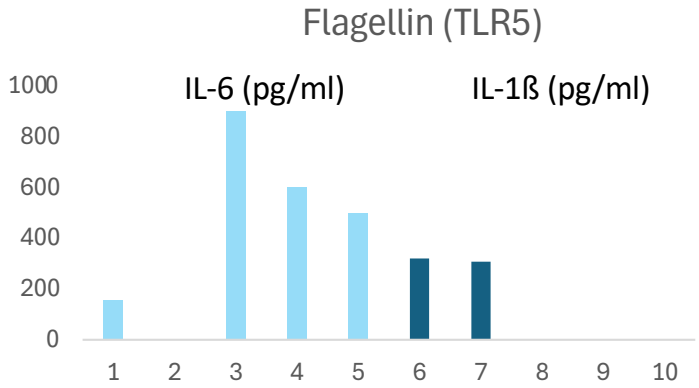
	NEP	TLR
1	Pam3CSK4 (20μg/mL)	TLR1/2
2	PGN-SA (20μg/mL)	TLR2
3	HKSA (10^6/ml)	TLR2
4	FSL-1 (20μg/mL)	TLR2/6
5	Poly I:C (20 μg/mL)	TLR3
6	Flagellin (2 μg/mL)	TLR5
7	Imiquimod (20μg/mL)	TLR7
8	CL075 (20 μg/mL)	TLR7/8
9	ODN2006 (20μg/mL)	TLR9
10	MDP-1 (20μg/mL)	NDO2

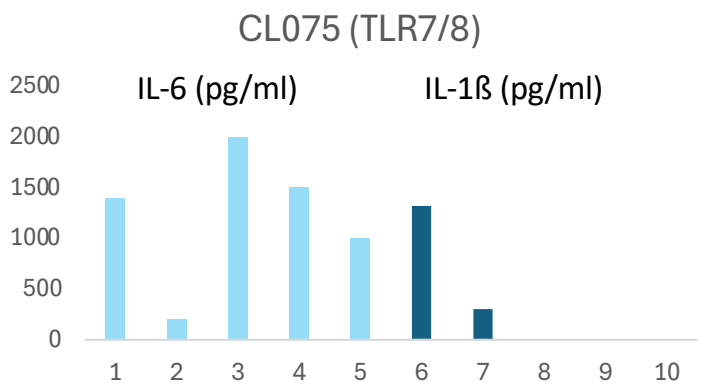
Non-Endotoxin Pyrogen (NEP)

PMBC and NEP









	Cell	Cytokine
1	HL60	IL-6
2	THP-1	
3	PBMC#1	
4	PBMC#2	
5	PBMC#3	
6	HL60	IL-1ß
7	THP-1	
8	PBMC#1	
9	PBMC#2	
10	PBMC#3	

Future Direction

Establishment of MAT assay

- Collection and exchange of information from domestic and international related organizations
- Establishment of monocyte activation test method using human monocytic cell lines and human peripheral blood monocytes, analysis of monocyte phenotypic changes
- Optimization and validation of test methods, investigation of the mechanism of action of pyrogens
- Development and establishment of test methods through the performance of in-house assay validation tests in comparison with previous research results
- Verification of licensed plasma fractionation products and research on monocyte activation mechanisms and functions
- Verification and comparison with the rabbit pyrogen test method

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