ヒトTh2細胞からのIL-4産生を指標に
呼吸器感作性を評価する共培養系の開発

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呼吸器感作性試験法開発の世界的重要性は増している

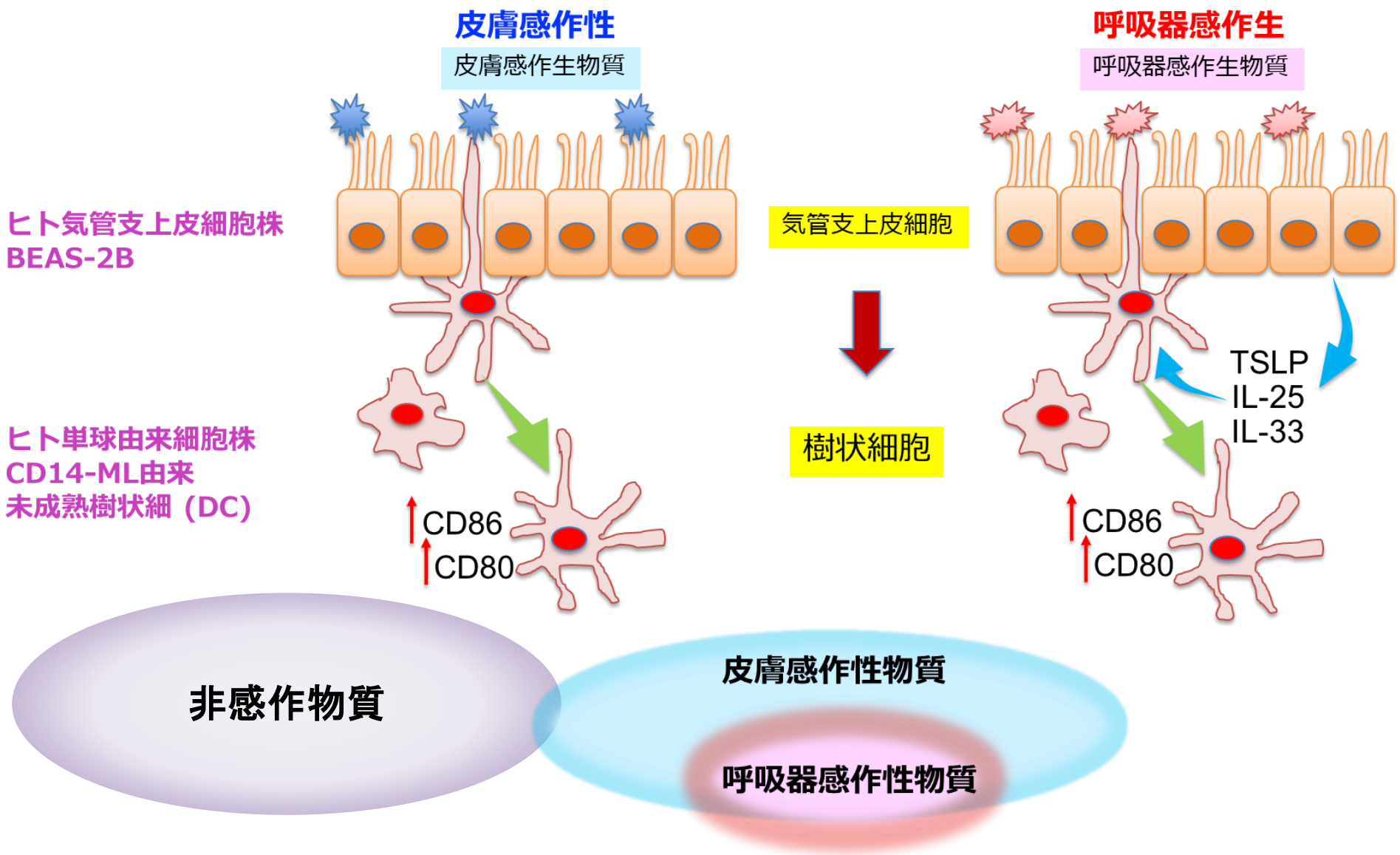
- 化学物質の吸入曝露による健康影響が懸念されている一方、現在のOECDガイドラインであるin vivo吸入曝露試験は多大な費用と時間が課題とされており、毒性発現機構に基づいた効率的で精度の高いin vitro試験法の開発が強く望まれている。

- 特に化学物質の吸入曝露によりアレルギー性喘息を生じる呼吸器感作については、未だ行政が受け入れ可能な試験法が開発されておらず、その重要性から米国よりin vitro呼吸器感作性試験開発を念頭に置いたDetailed Review Paper (DRP)作成の作業計画が2022年秋にOECDに提案され、2023年4月のWNT (World Party of the National Coordinators for the Test Guidelines Program) において承認された。

- 日本においても今年度よりin vitro呼吸器感作性試験のOECDテストガイドライン化を目指した厚生労働科学研究が開始されるなど、試験法開発が行われている。

- 呼吸器感作性試験開発に関する日本の研究動向を整理するとともに、関係者の意見をとりまとめ、DRPを介して的確に世界に発信するために、国立医薬品食品衛生研究所・JaCVAM運営委員会を中心に呼吸器感作性試験資料編纂委員会の設置を提案する。

DCsensRにおける皮膚感作性と呼吸器感作性を区別できる仕組み



皮膚感作性試験の呼吸器感作性試験への応用

Table 1
Behaviour of chemical respiratory sensitisers in *in vitro* skin sensitisation tests.

Chemical respiratory sensitiser	LLNA	DPRA	KeratinoSens/LuSens	h-CLAT
Acid anhydrides				
cis-1,2-Hexahydrophthalic anhydride	+	+	-[a]	+
Maleic anhydride	+	+	−	+
cis-4-Methyl-1,2,3,4-tetrahydrophthalic anhydride	+	ND	ND	ND
Phthalic anhydride	+	+	−	−
Trimellitic anhydride	+	+	−	+
Tetrachlorophthalic anhydride	ND	+[d]	ND	ND
Amines				
Chloramine T	+	+	+	+
Ethylenediamine	+	−	+	+
Piperazine (diethylenediamine)	+	-[d]	ND	ND
Aldehydes				
Glutaraldehyde	+	+	+	+
Diisocyanates				
Diphenylmethane-4-4'diisocyanate	+	+[d]	ND	ND
Hexamethylene diisocyanate	+[a]	+[a]	+[a]	ND
Toluene diisocyanate	+	+[d]	+	ND
Isophorone diisocyanate	+[a]	+[a]	+[a]	ND

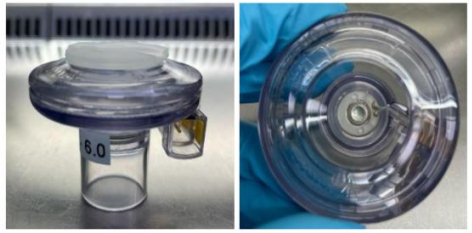
D. Basketter et al. / Regulatory Toxicology and Pharmacology 86 (2017) 101–106

Invitrolize社（ルクセンブルク）で構築された呼吸器感作性試験（ALIsens）

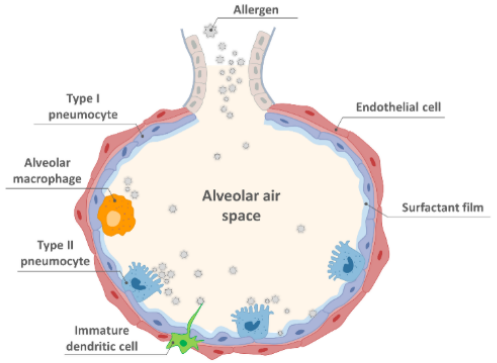
Test system build up.

- Day 7: exposure at the ALI using Vitrocell Cloud systems
- 500 µL liquid mix in the Aerogen nebulizer
- Chemical in vehicle : PBS = 1:1 (v/v)

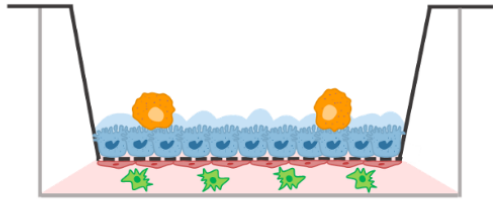
Vitrocell Cloud 6



Aerogen nebulizer



Alveolus representation



ALIsens representation

- Macrophage (THP-1 differentiated with PMA)
- Alveolar type II pneumocyte – epithelial cell (A549)
- Endothelial cell (EA.hy926)
- Dendritic-like cell (THP-1)
- Surfactant
- Cell culture medium

Award Winning Technology

LUSH PRIZE SUPPORTING ANIMAL-FREE TESTING

*Scientists involved in the ALIsens tech were LIST employees at that time.

PETA SCIENCE CONSORTIUM INTERNATIONAL e.V.
Advancing 21st Century Toxicology

FIT4 START
Accelerator programme in Luxembourg with a 150k€ award.

Lush Prize

Dr. Aline Chary

Dr. Arno Gutleb

Characterisation:

- Alveolar barrier
- Carefully characterised
- ALI exposure
- Lung inflammation and irritation
- Respiratory sensitisation
- Activation of dendritic cells

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powered by cells

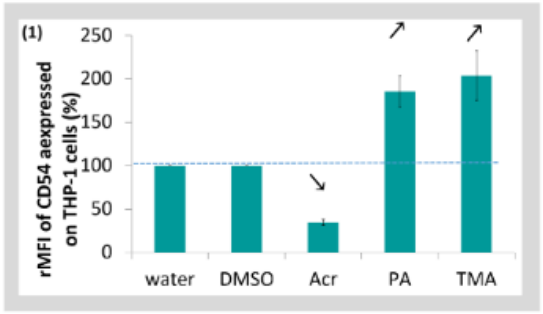
Our Office

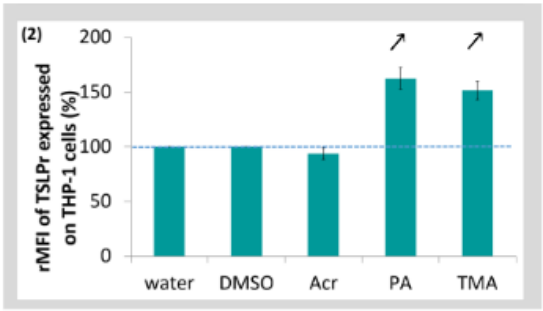
41 rue du Brill
L-4422 Belvaux
Luxembourg

Chary et al. ALTEX 36(3) 2019 及び　Invitrolize社https://invitrolize.com/の資料より引用

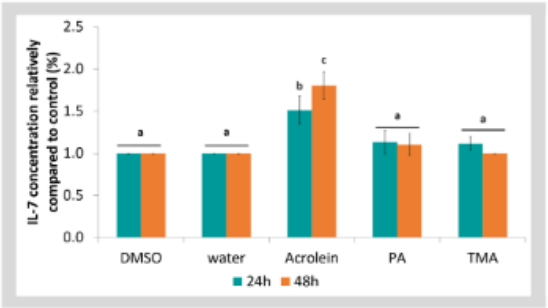
ALIsensでの呼吸器感作性試験評価

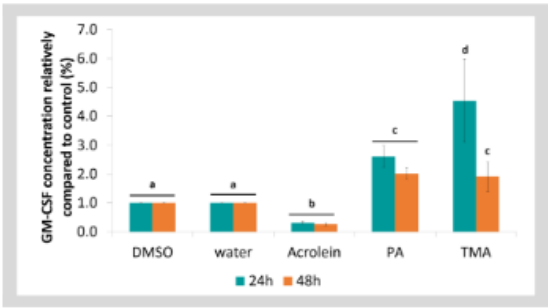
FACS measurements





Cytokines pattern





- Acr = Acrolein > respiratory irritant
- PA = Phthalic anhydride > respiratory sensitizer
- TMA = Trimellitic anhydride > respiratory sensitizer

Comparative pattern of effects

	Acrolein		PA		TMA	
	24h	48h	24h	48h	24h	48h
MCP-1	↘	=	=	↗	=	=
MIP-3a	↘	↘	↗	↗	↗	↗
IL-6	↘	↘	↗	↗	↗	↗
IL-7	↗	↗	=	=	=	=
RANTES	=	↗	↗	↗	↗	↗
GM-CSF	↘	↘	↗	↗	↗	↗
IL-10	↘	↘	↗	↗	↗	=

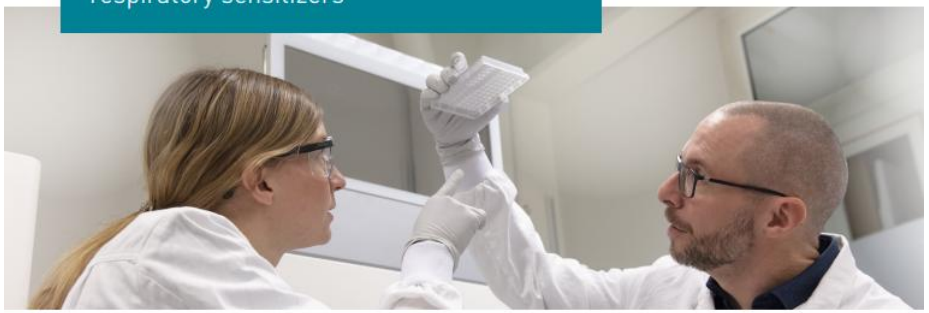
- Definition of respiratory irritation/sensitization markers
- Panel of cytokines able to identify respiratory sensitizers
- Possibility to discriminate between irritants and sensitizers

Invitrolize社https://invitrolize.com/の資料より引用

SENZAGEN社（スウェーデン）で構築された呼吸器感作性試験 (GARDair)

SENZAGEN

GARD®air
A predictive test for chemical respiratory sensitizers



GARDair is an *in vitro* test capable of identifying chemical respiratory sensitizers.

Features and Benefits

Test system
- Human dendritic-like cell line: SenzaCell®.

Solvent
- DMSO and H_2O within standard protocols.

What it measures
- Gene expression profile of 28 genomic biomarkers.

Readout
- Binary prediction: Respiratory sensitizer or non-sensitizer.

Recommended use
- With 95% specificity, GARDair is recommended for use as an R&D tool to identify and rule out respiratory sensitizers in candidate ingredients.*

Short turnaround time
- 4-8 weeks for standard studies.

Low sample requirement
- 0.5g (solid) or 1ml (liquid).

Compliance
- GLP or non-GLP.

Inter-laboratory ring trial

Method

A inter-laboratory ring trial was performed in three independent laboratory with blind assessment of 29 compounds, including expected respiratory sensitizers, skin sensitizers and non-sensitizers.

Table 1. The GARDair inter-laboratory ring trial results demonstrate high reproducibility as well as outstanding predictive performance.

Accuracy	74%
Sensitivity	53%
Specificity	95%
Within Laboratory Reproducibility	52-72%
Between Laboratory Reproducibility	79%

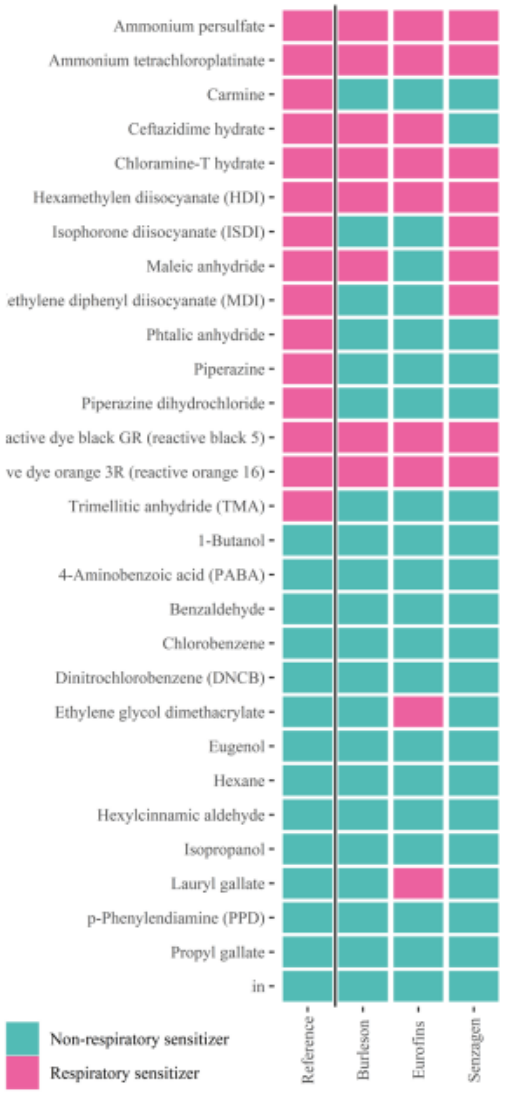


Figure 1. GARDair inter-laboratory ring trial results from three independent laboratories.

SENZAGEN (https://senzagen.com/)より引用

GARDairでの呼吸器感作性試験評価

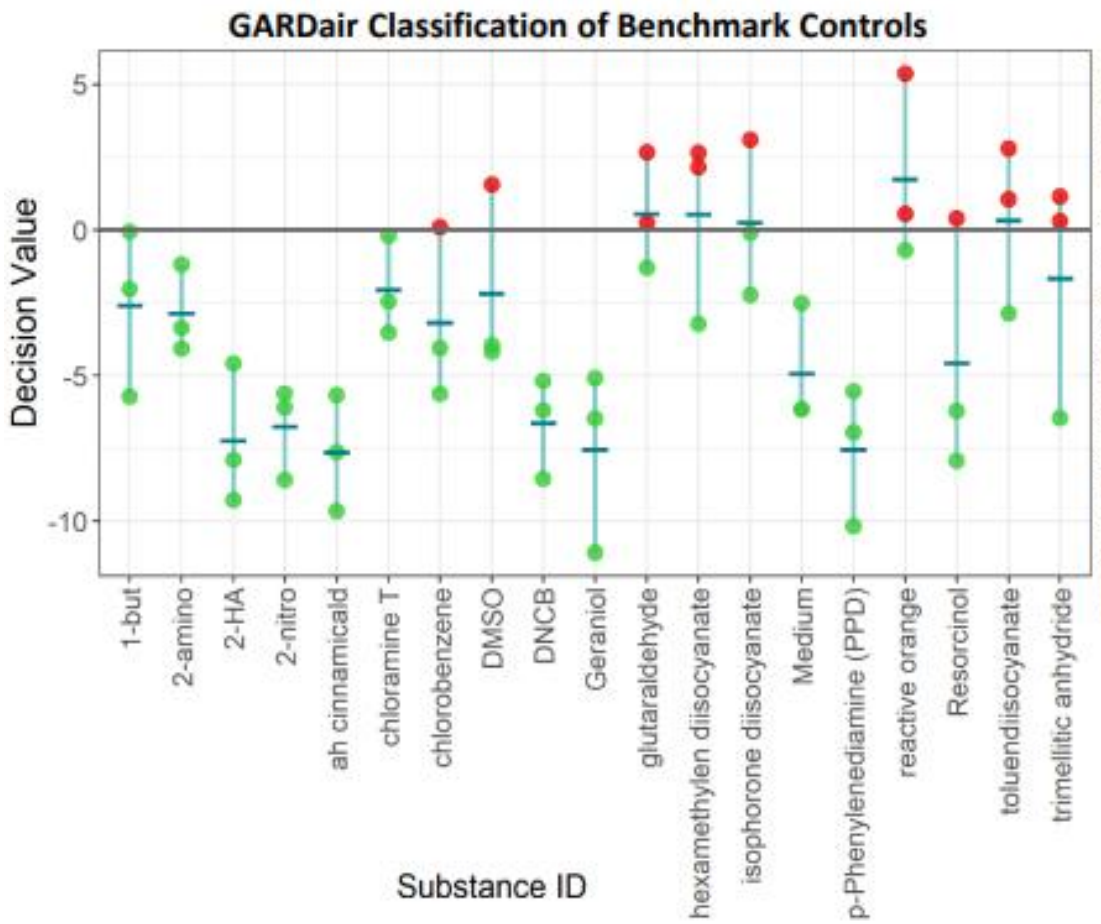


Figure 1: GARDair Decision Values of benchmark controls: Any test substance with a mean DV > 0 (n=3) is classified as a respiratory sensitizer. Out of the 7 assayed respiratory sensitizers, 5 are accurately classified as such by GARDair. No false positives were generated. Thus, the sensitivity and specificity are estimated to 71% (5/7) and 100% (12/12), respectively, with an overall predictive accuracy of 89% (17/19).

Stevenson et al. British Toxicology Society Annual Congress 2018

Canonical Pathway	*-log(p-value)*	*Regulated molecules*[1]
Oxidative phosphorylation	*17.62*	ATP5E, ATP5I, ATPK, COX Vb, COX VIIa-2, NDUFA1, NDUFA13, NDUFA2, NDUFA3, NDUFA6, NDUFA7, NDUFA9, NDUFAB1, NDUFB10,NDUFB4, NDUFB6, NDUFB8, NDUFB9, NDUFC1, NDUFS4, NDUFS5, NDUFS6, NDUFS8, NDUFV2, UQCR10, UQCRQPC
Ubiquinone metabolism	13.29	NDUFA1, NDUFA13, NDUFA2, NDUFA3, NDUFA6, NDUFA7, NDUFA9, NDUFAB1, NDUFB10, NDUFB4, NDUFB6, NDUFB8, NDUFB9, NDUFC1, NDUFS4, NDUFS5, NDUFS6, NDUFS8, NDUFV2
Granzyme B signaling	4.72	Bid, Caspase-2, Lamin A/C,LAMP2,**Smac/Diablo**, tBid, Tubulin alpha
FAS signaling cascades	3.79	Bid, c-FLIP (S), Caspase-2, DAXX, Lamin A/C, **Smac/Diablo**, tBid
Cytoplasmic/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim	3.57	Bid, DAXX, DLC1 (Dynein LC8a), DLC2 (Dynein LC8b), **Smac/Diablo**, tBid
Inhibitory PD-1 signaling in T cells	3.27	**BCL2L1**, **CD8**, **CD8 alpha**, CD80, CD86, MHC class II, PTEN
HSP60 and HSP70/ TLR signaling pathway	3.22	CD80, CD86, MD-2, MEK1/2, MHC class II, MyD88, Ubiquitin
Astrocyte differentiation from adult stem cells	3.17	**HES1**, ID1, ID2, ID3, MEK1/2, **SOX1**
Apoptotic TNF-family pathways	3.06	Apo-2L(TNFSF10), **BCL2L1**, Bid, Caspase-2, **Smac/Diablo**, tBid
TNFR1 signaling pathway	3.00	Bid, c-FLIP (S), Caspase-2, jBid, **Smac/Diablo**, tBid
Role of Nek in cell cycle regulation	2.80	**Histone H1**, Ran, Tubulin (in microtubules), Tubulin alpha, Tubulin beta
ATP/ITP metabolism	2.70	5'-NTC, ADSL, APRT, POLR2G, POLR2J, PPAP, RPB10, RPB6, RPB8, RRP41
Generation of memory CD4+ T cells	2.51	**BCL2L1**, CD80, CD86, **IL7RA,** MHC class II
Dynein-dynactin motor complex in axonal transport in neurons	2.48	DYNLL, DYNLT, Tctex-1, **TMEM108**, Tubulin (in microtubules), Ubiquitin
Antigen presentation by MHC class II	2.44	HLA-DM, HLA-DRA1, MHC class II
IL-33 signaling pathway	2.36	Histone H2A, Histone H2B, MEK1/2, MyD88, **ST2L**, Ubiquitin
Insulin regulation of translation	2.27	eEF2, eIF4A, **eIF4B**, eIF4G1/3, MEK1(MAP2K1)
TNF-alpha-induced Caspase-8 signaling	2.23	Bid, c-FLIP (S), Caspase-2, PP2A regulatory, tBid
Antigen presentation by MHC class I	2.18	**CD8,CD8 alpha**, PSMB5, **PSME3**
Main pathways of Schwann cells transformation in neurofibromatosis type 1	2.18	**BCL2L1, Calmodulin,** MEK1(MAP2K1), MEK1/2, Neuregulin 1,PTEN
Granzyme A signaling	2.08	**Histone H1**, Histone H2B, Lamin A/C, LAMP2
G-CSF-induced myeloid differentiation	2.08	G-CSF receptor, MEK1/2, Myeloblastin, PERM
Substance P mediated membrane blebbing	2.07	MRLC, Tubulin (in microtubules), Tubulin alpha
Role of IAP-proteins in apoptosis	2.03	Bid, **Smac/Diablo**, tBid, Ubiquitin

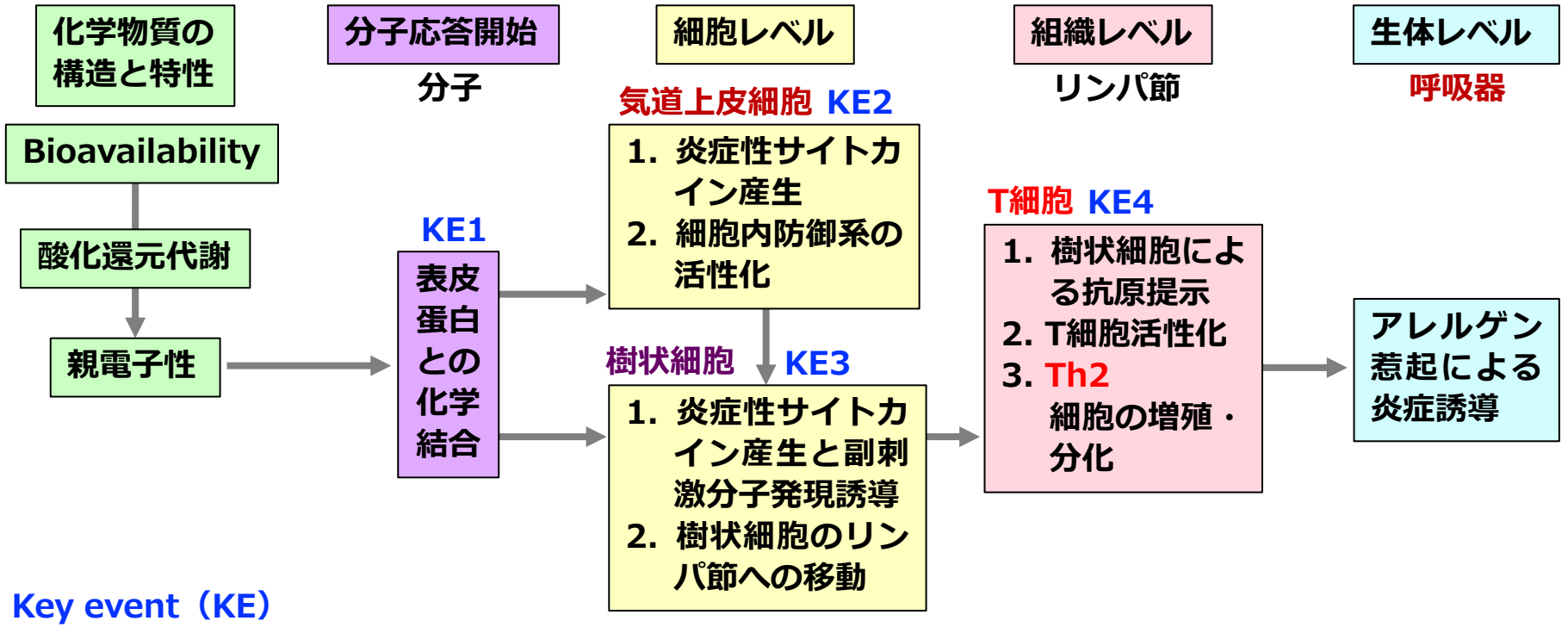
[1]Molecules indicated in bold are present in GRPS.

doi:10.1371/journal.pone.0118808.t004

Forreryd et al. PLOS ONE 2015

SENZAGEN社https://senzagen.com/より引用

感作性化学物質による呼吸器アレルギー発症までの有害性発現経路AOPと各試験法

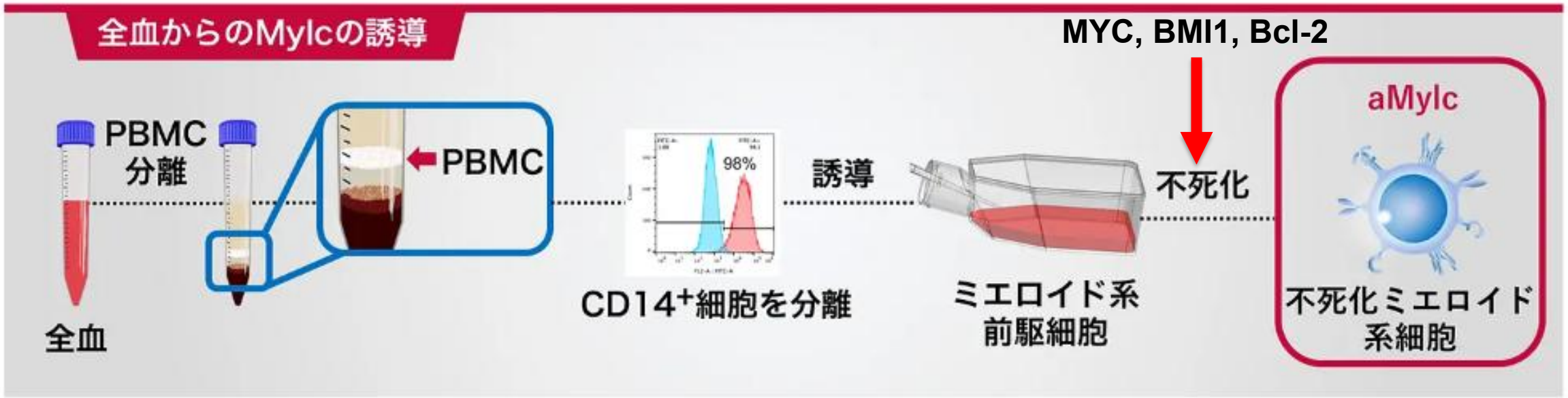


Key event（KE）

	in chemico	*in vitro*	*ex vivo*	*in vivo*
皮膚感作性試験：	DPRA ADRA	角化細胞：KeratinoSens, LuSens 樹状細胞：h-CLAT, U-SENS, IL-8 Luc Assay, GARD™Skin	LLNA	GPMT
呼吸器感作性試験：		DC共培養系	2ステップDC/T共培養系	

Sullivan et al. Appl In Vitro Toxicol 2017

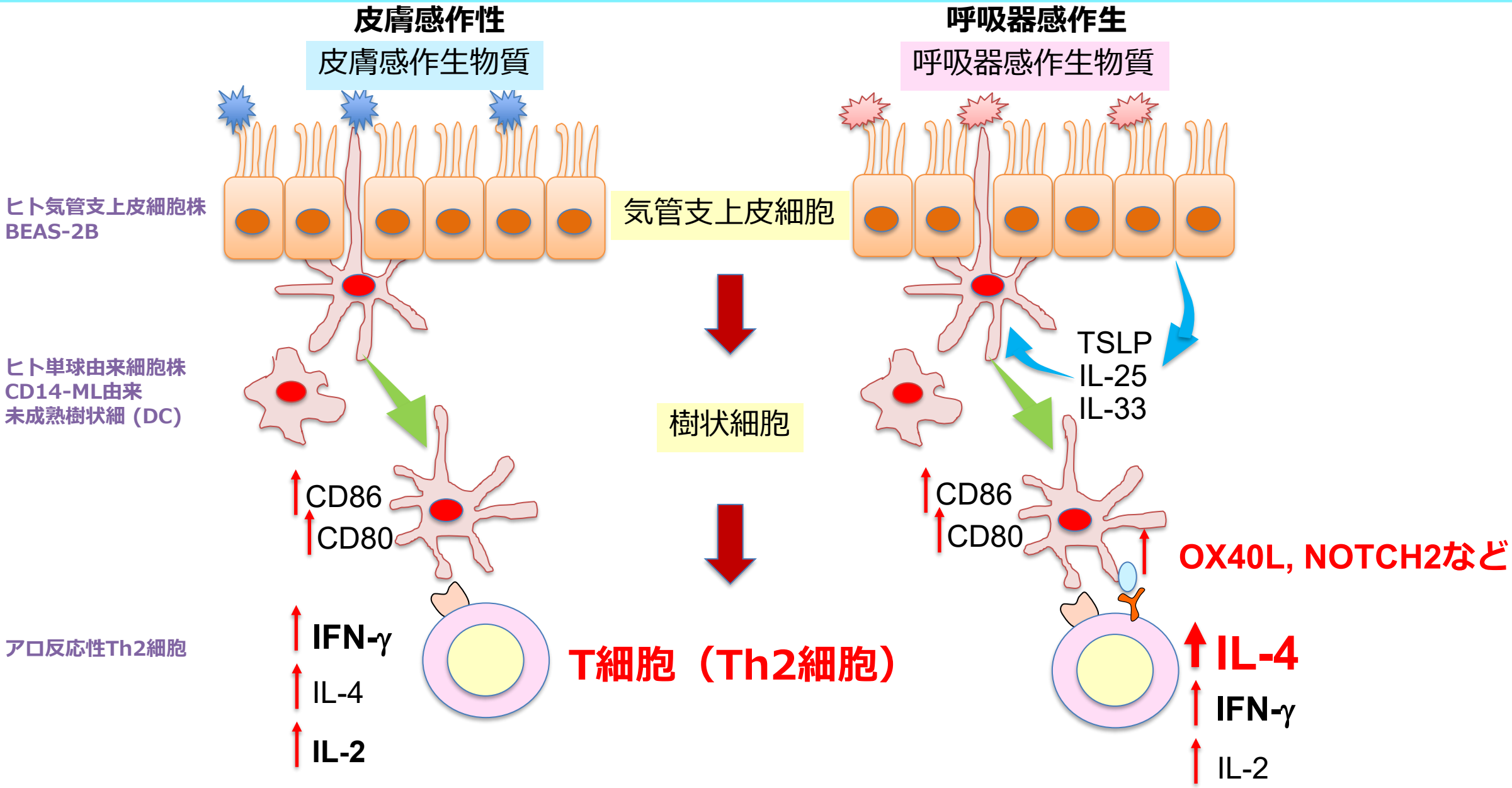
ヒトPBMC由来単球細胞株CD14-ML (aMylc)



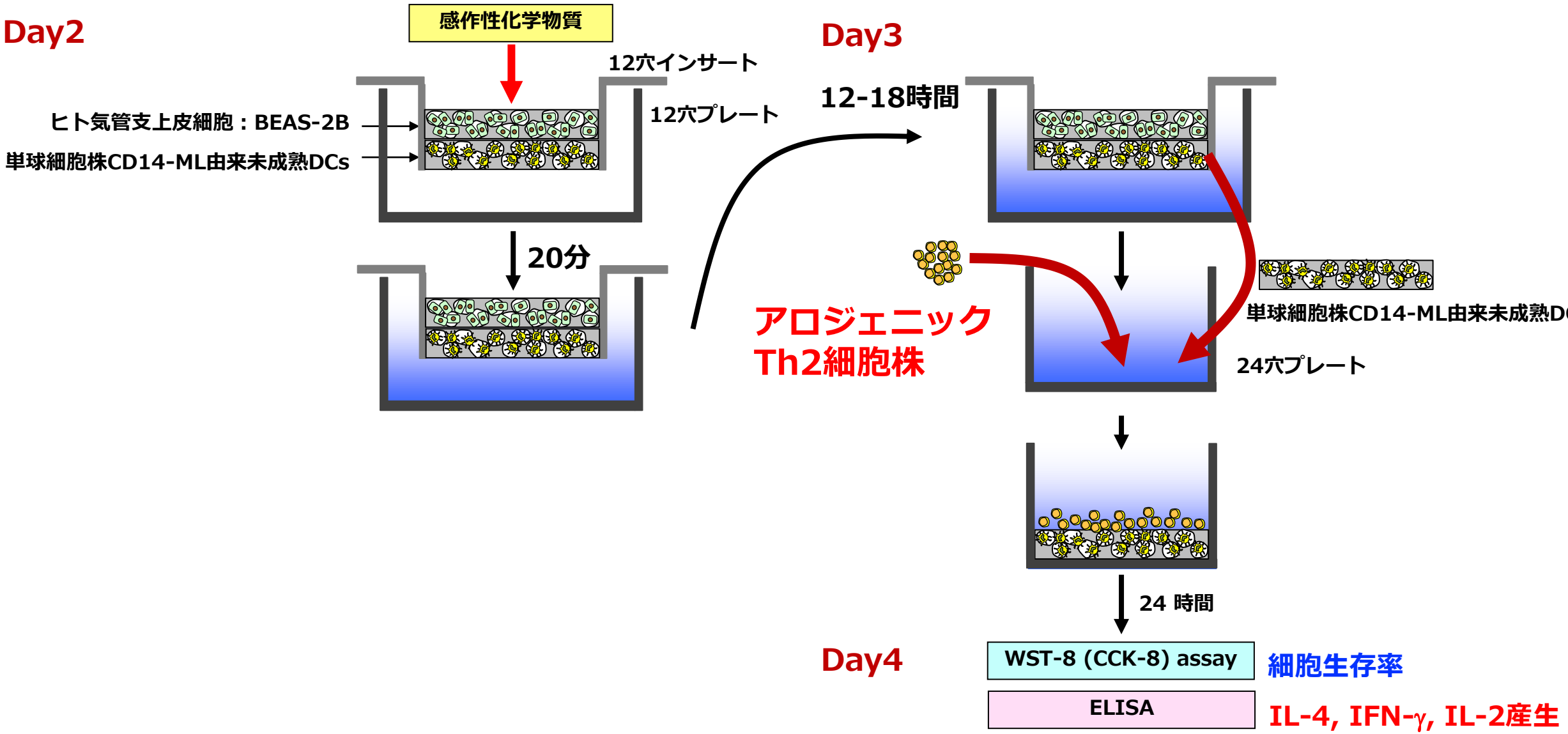
血液中の末梢血単核細胞(PBMC)から分化誘導したMylc細胞はaMylcと言い、上記の様に誘導します。

Mican Technologies社ホームページより引用

気管支上皮細胞、DCおよびT細胞から構成されるDC/TsensRを用いた皮膚感作性と呼吸器感作性を区別できる3次元共培養系モデル



単球細胞株CD14-ML由来DCとアロジェニックTh2細胞株を用いた DC/TsensRの構築方法

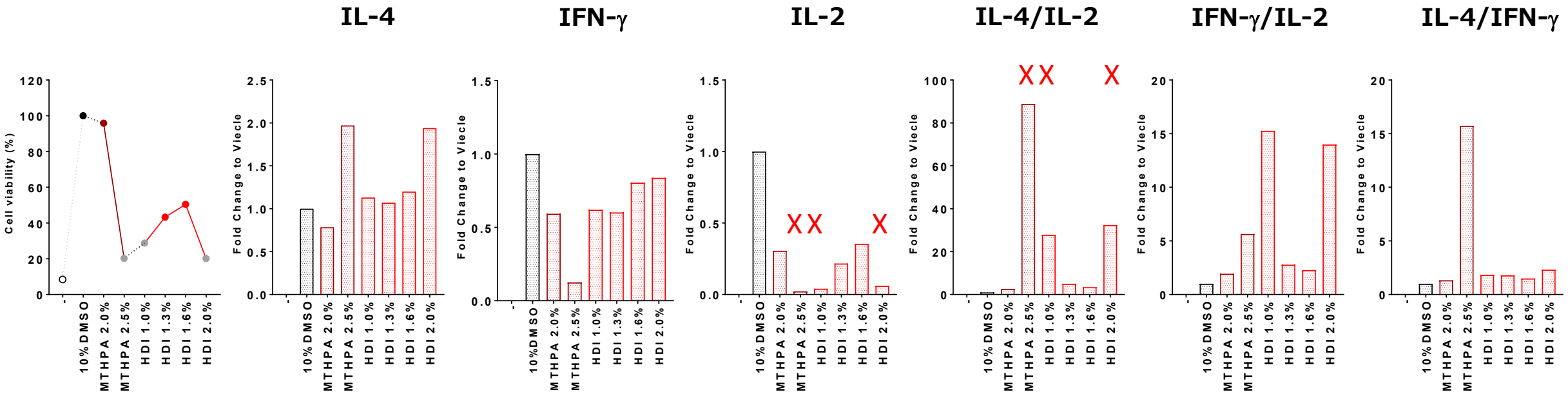


今回の試験 (DC/TsensR) に用いた非感作(3)、皮膚(11)、呼吸器(9)感作性化学物質

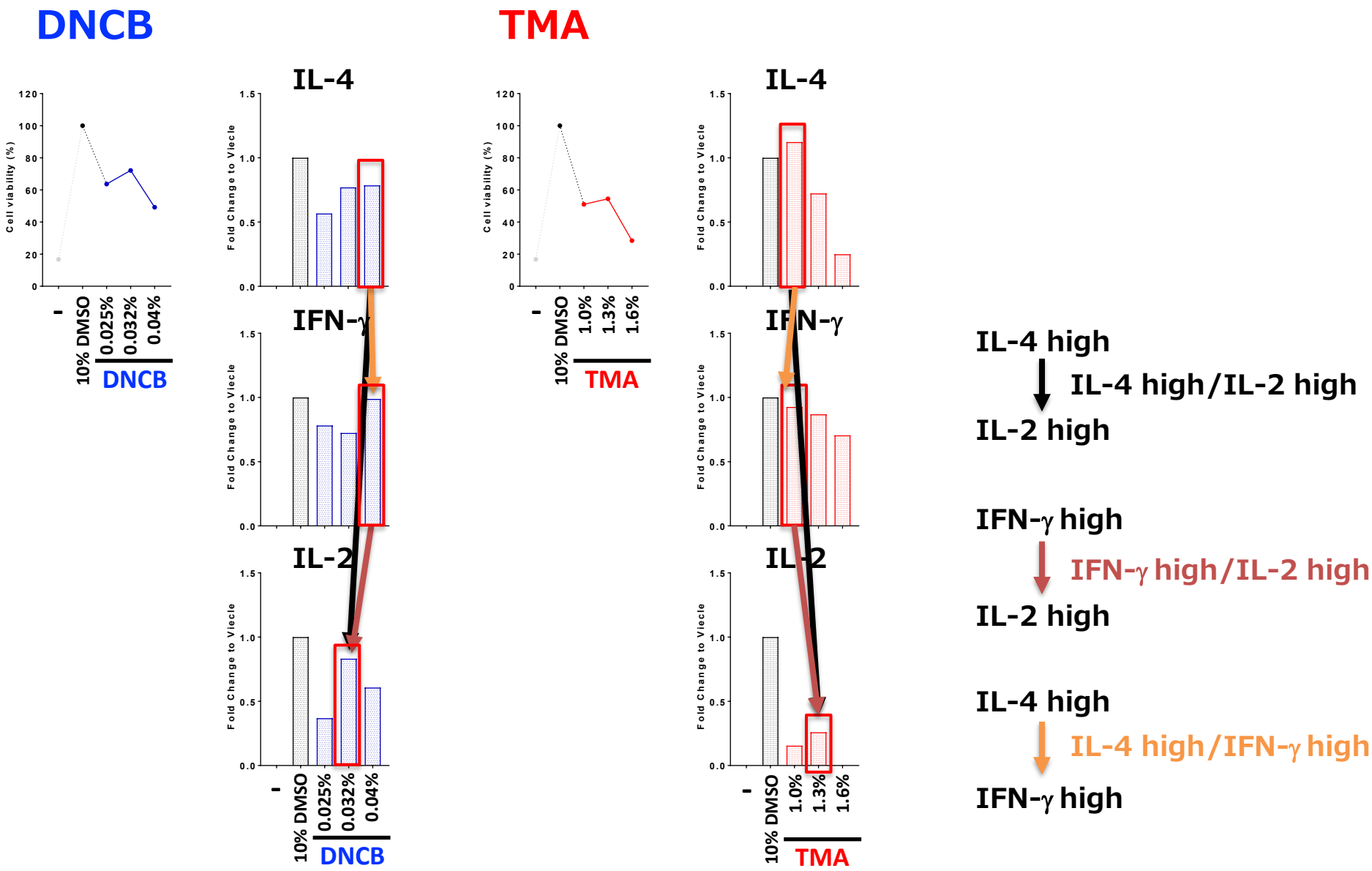
	非感作性化学物質	皮膚感作性化学物質	呼吸器感作性化学物質
1	4-Aminobenzoic acid	1,4-Benzoquinone (BQ)	Chloramine T (Ch-T)
2	α-Isomethylionone	4-Nitrobenzylbromide (NBB)	Trimellitic anhydride (TMA)
3	Benzyl benzoate	2,4-Dinitrochlorobenzene (DNCB)	Hexahydro phthalic anhydride (HHPA)
4	n-Hexane	Oxazolone (OXA)	Hexamethylene diisocyanate (HDI)
5	Isopropanol	Formaldehyde (FA)	Ortho-phthaldialdehyde (OPA)
6		2-Mercaptobenzothiazole (MBT)	Piperazine (PZA)
7		Isoeugeno (IEU)	Toluene diisocyanate (TDI)
8		Eugenol (EU)	Glutaraldehyde (GA)
9		Phenyl benzoate (PB)	Methyltetrahydrophthalic anhydride (MTHPA)
10		Butyl glycidyl ether (BGE)	
11		Ethylene glycol dimethacrylate (EGDM)	

1. Ponder J, et al. (2022). "In Litero" Screening: Retrospective Evaluation of Clinical Evidence to Establish a Reference List of Human Chemical Respiratory Sensitizers. *Front Toxicol 4*, 916370.
2. Sadekar N, et al. (2021). Identifying a reference list of respiratory sensitizers for the evaluation of novel approaches to study respiratory sensitization. *Crit Rev Toxicol 51*, 792-804.

DC/TsensRを用いた皮膚と呼吸器感作性化学物質識別評価法においては30%以下細胞生存率が結果に影響を与える



DC/TsensRを用いた 皮膚と呼吸器感作性化学物質識別評価法 1

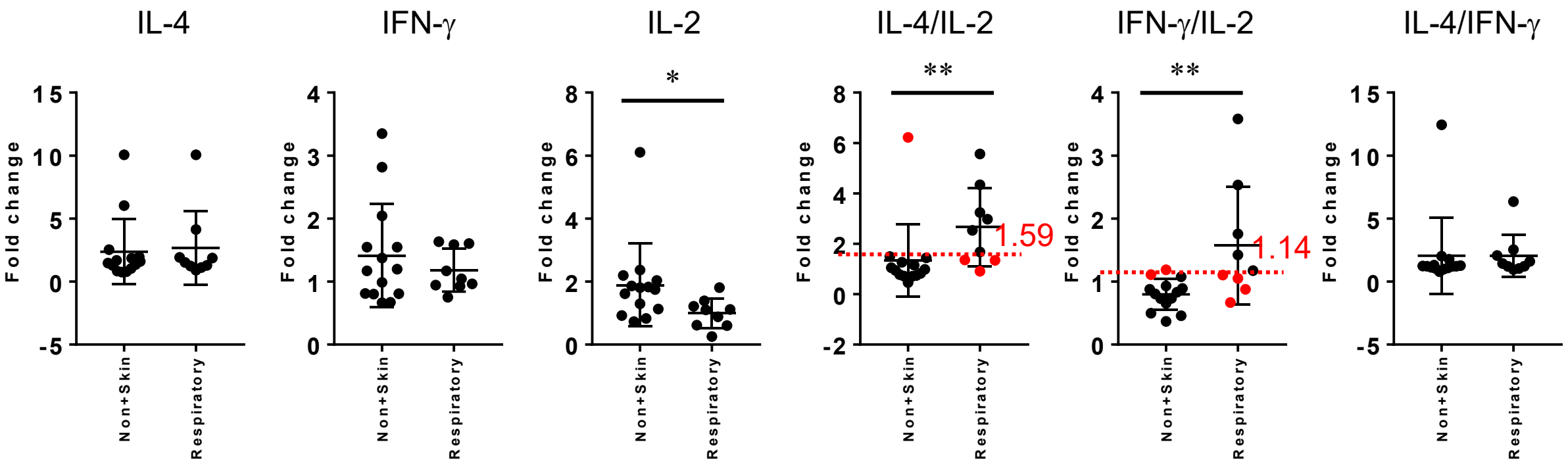


IL-4 high
↓
IL-4 high/IL-2 high
IL-2 high

IFN-γ high
↓
IFN-γ high/IL-2 high
IL-2 high

IL-4 high
↓
IL-4 high/IFN-γ high
IFN-γ high

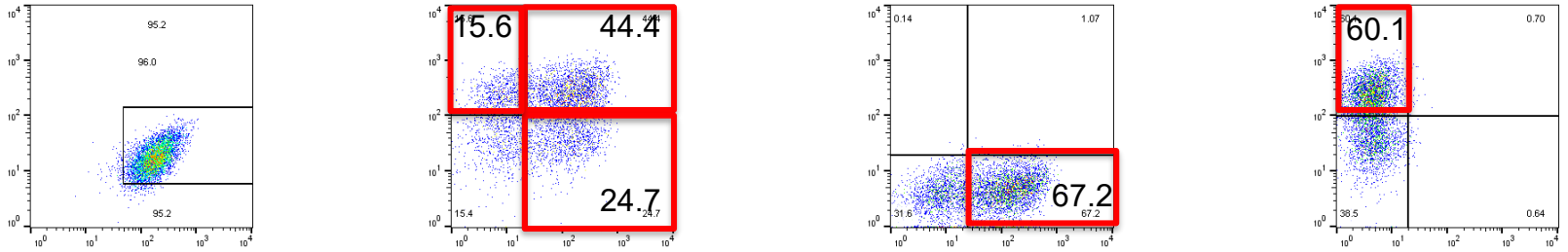
DC/TsensRを用いた解析によりIL-2で補正したIL-4およびIFN-γ産生増強比率での皮膚と呼吸器感作性の識別が可能 (評価法1)



ヒト末梢血由来ナイーブCD4+T細胞をTh2分化条件のもとアロジェニックDCによる頻回刺激で作製したアロ抗原特異的Th2T細胞株の限界希釈により得られたTh2クローンのサイトカイン産生能

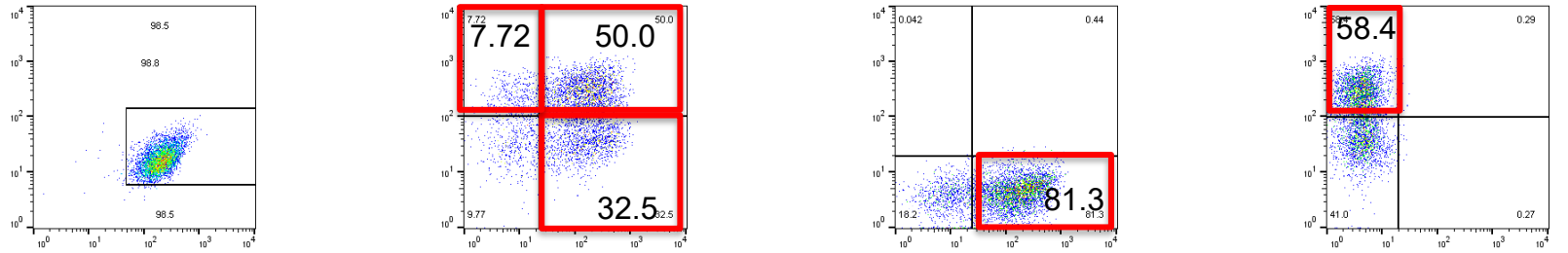
限界希釈1回目

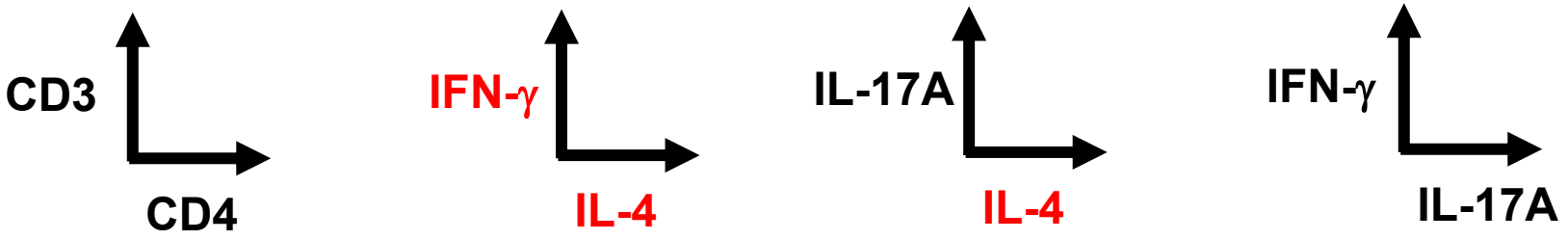
Th2 clone #2



限界希釈2回目

Th2 clone #4-3





結果のまとめ

- 試験を行った皮膚感作性物質11種類、呼吸器感作性物質9種類、非感作物質3種類において評価法１では、ELISAでのIL-4/IL-2産生を指標に感度66.7%、特異度92.9%、正確度76.5%で呼吸気感作性物質を区別することができたが、感度、正確度が低下してしまった。

- DC/TsensRで得られた結果とDCsensRで得られた結果が一致する傾向を示したことは系の信頼性を担保する結果となり得る。

ヒトTh2細胞からのIL-4産生を指標に呼吸器感作性を評価する共培養系 (DC/TsensR) の開発：実験予定

3年目	• 生物学的独立試行回数（N=3）を実施し、施設内再現性実験を完遂し論文化する（分担：片平、溝口）。 • 最適な Th2 ク ローン細胞を作製し、クローン化していないTh2細胞株との反応性の違いを検討する（分 担：溝口）。