Appendix 1. IL-2 Luc assay validation report draft

January, 2019

Report on the international validation study of the IL-2 Luc assay for evaluating the immunotoxic potential of chemicals

Validation Management Team*****

1. Summary

Tohoku University developed a high-throughput screening system to evaluate chemical immunotoxicity. We first established 3 stable lines of reporter cells transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter; THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter and SLR regulated by the G3PDH promoter; and THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter and SLR regulated by the G3PDH promoter. We selected these 4 cytokines because IL-2 and IFN-γ are primarily produced by T cells (a type of adaptive immune cell), whereas IL-8 and IL-1β are primarily produced by monocytes and dendritic cells (types of innate immune cells).

Using these 3 cell lines, we established the Multi-ImmunoTox assay (MITA) in which the effects of chemicals on the IL-2 and IFN-γ luciferase activity of 2H4 cells are evaluated in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io). The effects of chemicals on the IL-1β and IL-8 luciferase activity of THP-G1b and THP-G8 cells, respectively, were examined in the presence of the stimulant lipopolysaccharide (LPS).

To date, we have demonstrated the following:

1) The luciferase activities of the 3 MITA cell lines correspond with mRNA expression in the mother cell lines or in human whole blood cells when stimulated with PMA/Io or LPS in the presence of the 3 representative immunosuppressive drugs dexamethasone (Dex), cyclosporine A (CyA), and tacrolimus (Tac).
2) The MITA indicates that Dex significantly suppresses IL-2, IL-1β, and IL-8 reporter activities, while CyA and Tac suppress IL-2 and IFN-γ reporter activities but have no effect on IL-1β and IL-8 reporter activities. On the other hand, the MITA cannot detect the immunosuppressive effects of an alkylating agent (cyclophosphamide), inhibitors of de novo purine synthesis (azathioprine (AZ), mycophenolic acid (MPA) and mizoribine (MZR)), and an inhibitor of pyrimidine and purine synthesis (methotrexate (MT)).

3) Since sensitization is a major form of toxicity that must be investigated in the immunotoxicity screening of chemicals, we combined the MITA with an *in vitro* sensitization test, the IL-8 Luc assay, recently approved as an OECD test guideline for *in vitro* skin sensitization testing (OECD TG442E) (modified MITA; mMITA). The lead laboratory established a data set of 60 chemicals evaluated by the mMITA. Using this data set, we demonstrated a significant correlation between Lowest Observed Effect Levels (LOELs) or the effect on IL-2 luciferase activity and for that on IFN luciferase activity, and between LOELs for the effect on IL-1β luciferase activity and for that on IL-8 luciferase activity. These results indicated that evaluation of the effects of chemicals on IL-2 luciferase activity and on IL-8 luciferase activity can provide immunotoxicological information almost equivalent to evaluations conducted using IL-2, IFN-γ, IL-1β, and IL-8 luciferase activities. In addition, K-means clustering and hierarchical clustering of the 60 chemicals using the mMITA resulted in the same 6-cluster solution: cluster 1 with preferential suppression of IL-8, cluster 2 with suppression of IL-2 and a positive IL-8 Luc assay result, cluster 3 with suppression of both IL-2 and IL-8, cluster 4 with no effects on IL-2 or IL-8 and a negative IL-8 Luc assay result, cluster 5 with suppression of both IL-2 and IL-8 and a negative IL-8 Luc assay result, and cluster 6 with preferential suppression of IL-8. These data suggest that the mMITA is a promising novel high-throughput approach for detecting unrecognized immunological effects of chemicals and for profiling their immunotoxic effects.

Our final long term goal is to officially validate the MITA for within- and between- laboratory reproducibility and predictivity. Since the IL-8 Luc assay has already been accepted as an OECD test guideline (442E), the purpose of the current effort was to conduct the validation of the IL-2 luciferase reporter assay (IL-2 Luc assay).

The preliminary test trial, Phase 0, was performed by the participating laboratories following explicit explanations of the IL-2 Luc assay procedures and protocol Ver. 008.1E by the lead laboratory, Tohoku University. Three laboratories participated in the Phase 0 study of the IL-2 Luc assay using 5 open labeled chemicals (2-aminoantracene, citral, Chloroquine, dexamethasone and methyl mercuric chloride, 1 set (3 experiments) for each chemical). Most response patterns for the 5 chemicals were similar among the 3 laboratories, except for 2 early experiments conducted by the naïve laboratory. Based on these results, the Validation Management Team (VMT) judged that technical and protocol transfer of the IL-2 Luc assay is acceptable.

In the Phase I study, a total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) were evaluated by 3 experimental sets based on MITA protocol Ver. 008.5E. The average within-laboratory reproducibility was 86.7% (13/15). The between-laboratory reproducibility was 80.0% (4/5). The average predictivity was 93.3% (14/15).

In the Phase II study, between-laboratory reproducibility and predictivity using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting, and 1 undetermined) were evaluated by 1 experiment set based on IL-2 Luc assay protocol Ver. 009.1E. The between-laboratory reproducibility was 80% (16/20) and the average predictivity was 59.6% (34/57)

In the combined results of the Phase I and II studies, the average within-laboratory reproducibility was 86.7% (13/15). The between laboratory reproducibility was 80% (20/25). The average predictivity was 66.7% (48/72).

Although the within- and between-laboratory reproducibilities could satisfy the acceptance criteria for the validation study, the predictivity was not satisfactory. We considered several possible reasons for this unsatisfactory predictivity.

Since the 2H4 cell line used in the IL-2 Luc assay is derived from Jurkat cells, the IL-2 Luc assay cannot evaluate immunotoxic effects of immunosuppressive compounds whose mode of action is the inhibition of DNA synthesis leading to myelotoxicity. Thus, these chemicals should be outside the defined applicability domain for the assay. To overcome this limit, the IL-2 Luc assay requires combination with assays capable of detecting myelotoxicity, such as the conventional 28-day repeat dose toxicity test or *in vitro* myelotoxicity tests. Another possible limitation is for chemicals that need metabolic activation.

Our long term goal is to complete the official validation of the IL-2 Luc assay and the IL-1β Luc assay to provide a novel *in vitro* immunotoxicity screening test by combining the MITA with the OECD test guideline 442E for the IL-8 Luc assay (mMITA).

2. Background

2-1. What is immunotoxicity?

A well-functioning immune system is essential for maintaining the integrity of an organism. Immune dysregulation can have serious adverse health consequences, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Environmental contaminants, food additives, and drugs can target the immune system, resulting in immune dysregulation. Accordingly, the potential for immunotoxicity, which is defined as the toxicological effects of xenobiotics on the function of the immune system, has raised serious concerns from the public as well as regulatory agencies. Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as high cost, ethical concerns, and questionable relevance to risk assessment for humans.

2-2. The current status of *in vitro* approaches to detect immunotoxicants

Now the worldwide vision is promoting alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals used in scientific studies (Adler et al., 2011). The workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (Gennari et al., 2005, Galbiati et al., 2010, Lankveld et al., 2010). In the ECVAM workshop, a tiered approach was proposed. Since useful information can be obtained from regular 28-day general toxicity tests, pre-screening for direct immunotoxicity would begin with the evaluation of myelotoxicity in the proposed tiered approach (Corsini and Roggen, 2017). Compounds that are capable of damaging or destroying bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they are tested for leukotoxicity. Compounds are then tested for immunotoxicity using various approaches such as the human whole-blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction, NK cell assay, T cell–dependent antibody response, dendritic cell maturation assay, and fluorescent cell chip (FCP) assay. Among these assays, the HWBCRA has undergone formal pre-validation, although other techniques are being examined or have been examined in a rigorous pre-validation effort by the ECVAM and other groups. (Fig. 1) However, these assays require fresh rodent or human immune cells, in conflict with animal protection goals. The need for

primary cells may decrease reproducibility and makes the assay unsuitable for high-throughput approaches

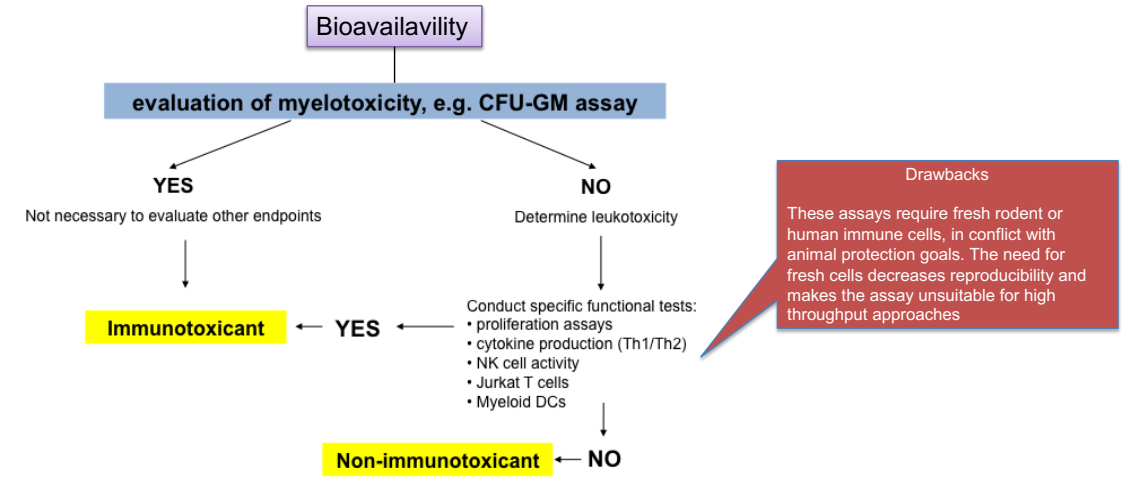


Fig. 1. Decision tree approach for *in vitro* assessment of chemical-induced immunosuppression.

2-3. *In vitro* immunotoxicity tests in principle should evaluate effects on both innate and acquired immunity

The immune system comprises innate and adaptive immunity (Fig. 2). Both arms of the immune response function differently and are driven by different populations of cells. In innate immunity, pathogens are recognized through various pattern recognition molecules, such as C-type lectin receptors, toll-like receptors, nod-like receptors, and retinoic acid–inducible gene-I (RIG-I)-like receptors. In addition, a variety of different cells are involved in this type of response, including neutrophils and other types of granulocytes, macrophages, natural killer (NK) cells, innate lymphoid cells, and mast cells. Adaptive immune responses involve specific antigen receptors encoded by rearranged genes, and T cells and B cells play critical roles in these responses.

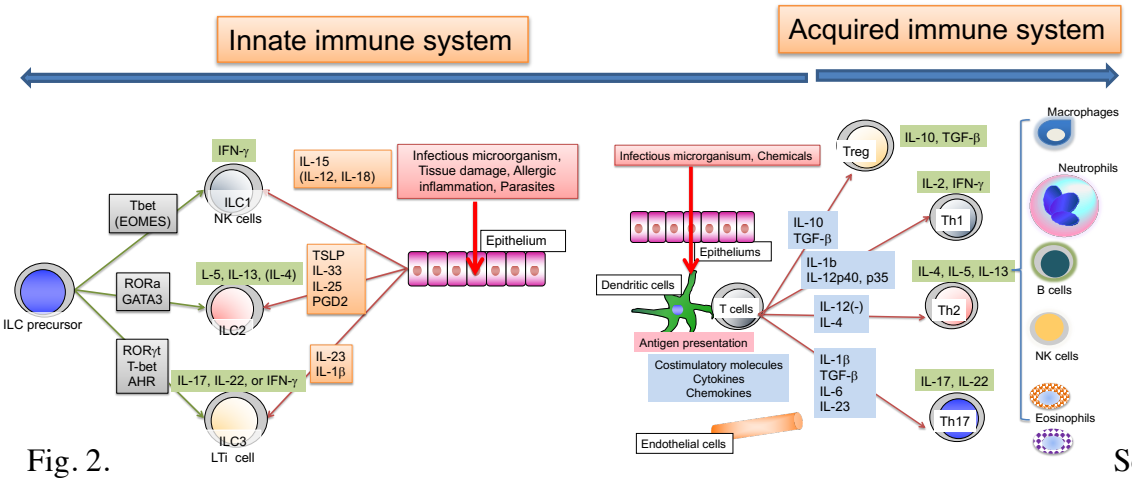


Fig. 2. Schematic representation of the innate immune system and acquired immune system.

Macrophages and dendritic cells (DCs), which act as antigen-presenting cells (APCs), link the innate and adaptive immune responses because they can present antigens to T lymphocytes in the context of major histocompatibility complex (MHC) class I or II molecules and stimulate their proliferation and effector functions after being stimulated via pathogen recognition receptors (Fig. 3). To induce optimal immune responses to various pathogens and minimize autoreactivity, innate and adaptive immune cells produce a vast array of cytokines, chemokines, and chemical mediators and present the molecules required for direct cell-cell interaction on their surface. A variety of

intracellular signaling pathways also play roles in innate and adaptive immune responses.

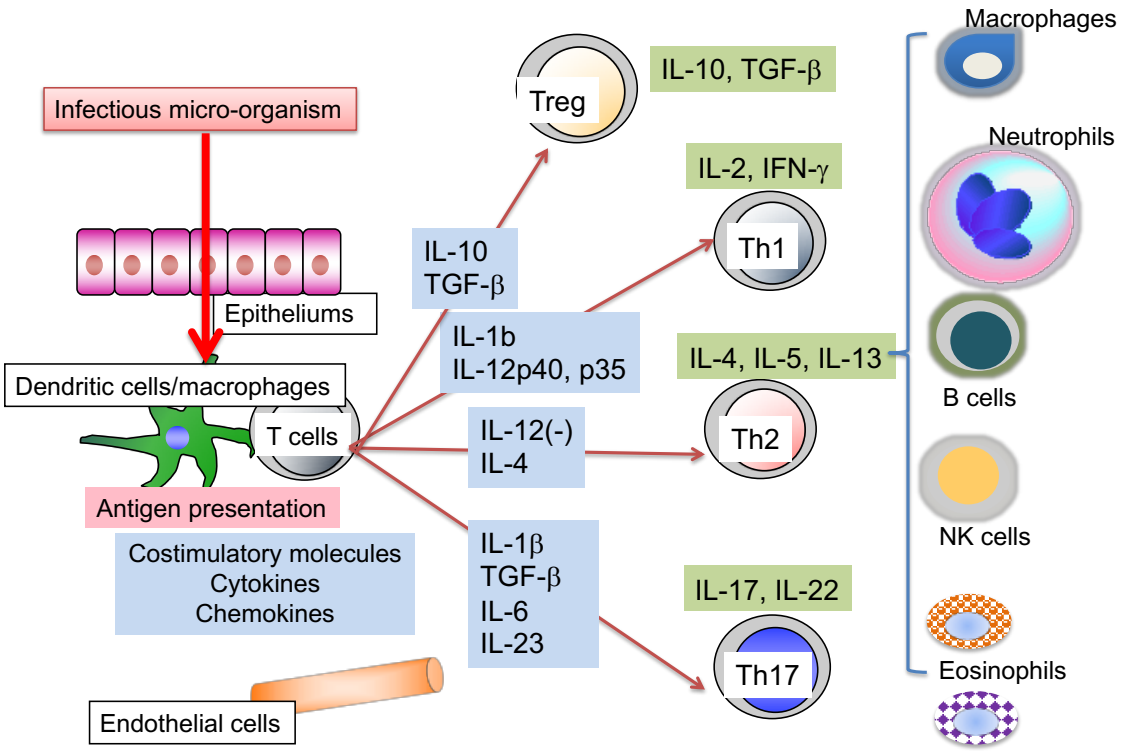
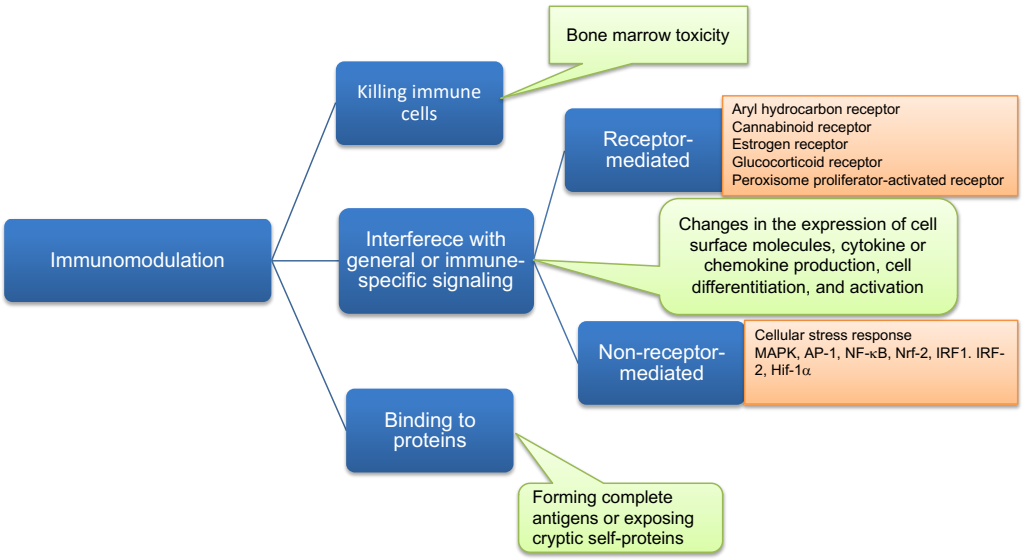


Fig. 3. Dendritic cells link the innate immune response to the acquired immune response.

Theoretically, chemicals can affect the immune system by targeting either the innate immune system or the acquired immune system (Fig. 2 and Fig. 3). Therefore, novel *in vitro* test methods are needed to adequately assess the immunotoxic effects of chemicals on both arms of immune system.

2-4. Mechanism for the induction of immunotoxicity by chemicals

Given the complexity of the immune system, it is unlikely that a single *in vitro* method will be able to detect all immunotoxicants. The mechanisms underlying the immunotoxicity of chemicals can be classified into 3 main categories: 1) killing of immune cells caused by bone marrow toxicity, 2) interference with general or immune-specific signaling leading to changes in the expression of cell surface molecules, cytokines or chemokine production, cell differentiation, and activation, and 3) binding to proteins forming complete antigens or exposing cryptic self-proteins (Fig. 4).



Corsini and Roggen. Overview of in vitro assessment of immunotoxicity DOI: 10.1016/j.cotox.2017.06.016.

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Fig. 4. Main mechanisms of immunotoxicity

Chemicals can interfere with immune-related cell signaling through receptor-mediated pathways using xenobiotic receptors such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), (Hidaka et al., 2017, Elentner et al., 2018) or through non-receptor-mediated ways. In contrast, it is easy to understand the mechanism underlying non-receptor-mediated immunotoxicity by considering the cellular stress response (Kultz, 2005, Fulda et al., 2010). In essence, as long as the stress stimulus does not cross a certain threshold, a cell can cope and survive by mounting an appropriate protective response. Conversely, the failure to activate or maintain a protective response (e.g., when the stressor is too strong) results in activation of stress signaling cascades that eventually activate cell death pathways. Depending on the type of stress and its severity, a cell's response can be manifold. However, most cellular protective responses induced by chemicals can be classified into one of several categories, such as heat shock, unfolded protein, DNA damage, and oxidative stress responses, in addition to the response to danger signals (Gallucci & Matzinger, 2001). These responses are independent of the chemical species (Fig. 5). In addition, these cellular stress responses can affect immune function because they share the same cellular signaling pathways (such as those mediated by MAP kinase, NF-kB, and mTOR) used by the immune response (Milisav, 2011). Indeed, although sensitizers (which are chemicals that induce allergic contact hypersensitivity) include numerous compounds with different molecular structures, it has become clear that their ability to sensitize is based simply

on their reactivity to mainly cysteine residues, which induces a response to oxidative stress (Sasaki & Aiba, 2007). Therefore, although it is assumed that there may be many chemicals with the potential to produce immunotoxicity, only a limited number of assay systems may be required to detect their effects.

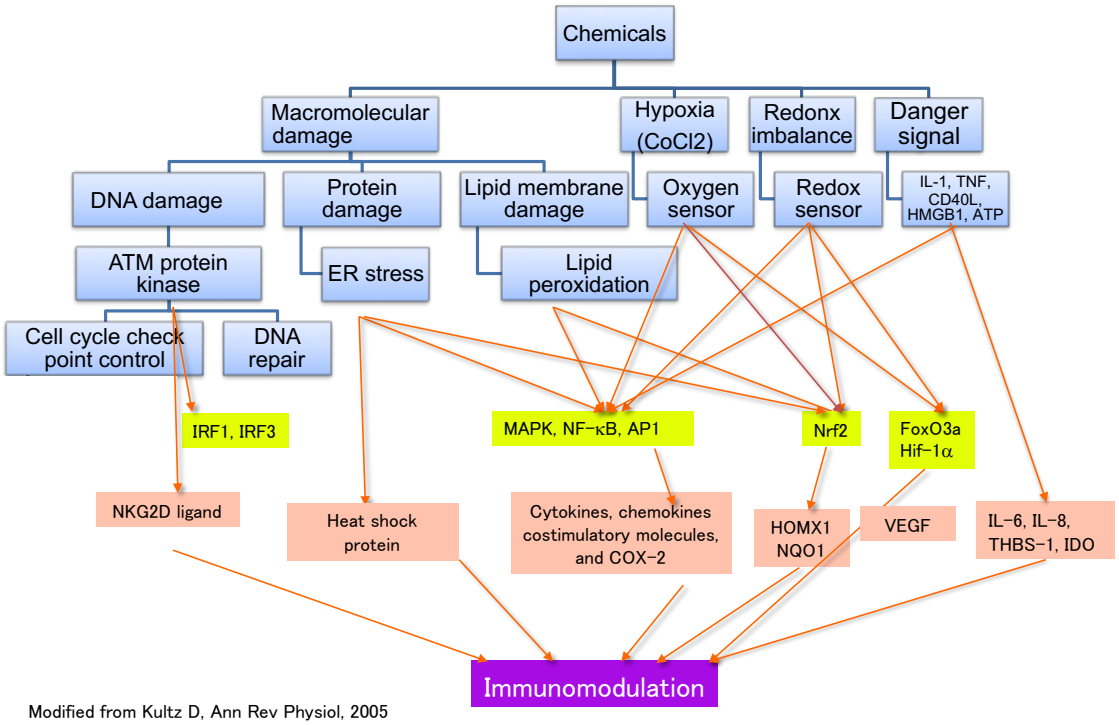


Fig. 5. Cellular stress response and danger signals.

2-5. Multi-ImmunoTox assay (MITA)

Our group developed a high-throughput screening system to evaluate chemical immunotoxicity. We first established 3 stable reporter cell lines transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter (Saito et al., 2011); THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter and SLR regulated by G3PDH promoter (Takahashi et al., 2011); and THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter and SLR by the G3PDH promoter (Kimura et al., 2014). These 4 cytokines were selected because IL-2 and IFN-γ are primarily produced by T cells (adaptive immune cells), whereas IL-8 and IL-1β are primarily

produced by monocytes and dendritic cells (innate immune cells). Using these 3 cell lines, we established the Multi-ImmunoTox assay (MITA). This assay identifies the effects of chemicals on the IL-2 and IFN-γ luciferase activity in 2H4 cells in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), and on the IL-1β and IL-8 luciferase activities in THP-G1b and THP-G8 cells, respectively, in the presence of the stimulant lipopolysaccharide (LPS) (Fig. 6).

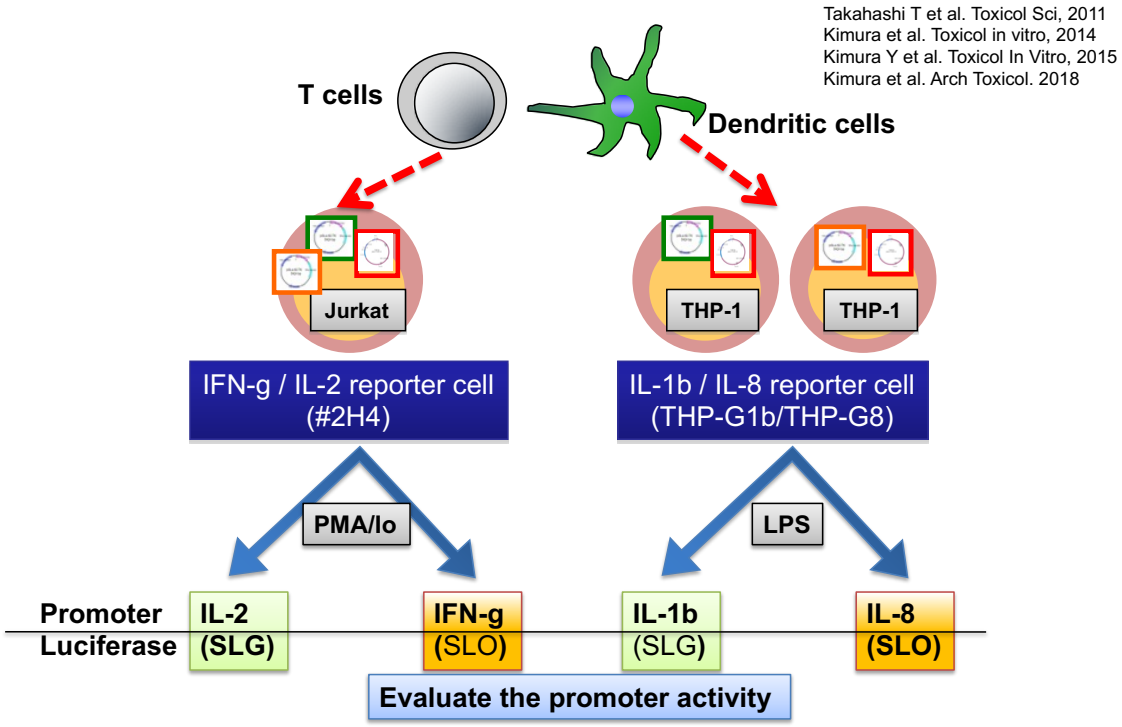


Fig. 6. The Muti-ImmunoTox assay (MITA)

2-6. The luciferase activities of the three MITA cell lines correspond with mRNA expression in the wild type cell lines or in human whole blood cells when stimulated with PMA/Io or LPS in the presence of 3 representative immunosuppressive drugs

After establishing the MITA, we first compared the effects of dexamethasone, cyclosporine, and tacrolimus on the 3 MITA cell lines with those on mRNA expression in the wild type cell lines or in human whole-blood cells stimulated with PMA/Io or LPS. The results confirmed that the MITA correctly reflects changes in mRNA expression in the mother cell lines and whole-blood cells (Kimura et al., 2014).

2-7. The MITA can evaluate the immunotoxicity profiles of well-known immunosuppressive drugs

We next evaluated the performance of the MITA by examining immunosuppressive or immunomodulatory drugs with well-known clinical effects on the human immune system (Kimura et al., 2014). The results obtained with immunosuppressive drugs classified by their principal mechanism of action are shown in Table 1, in which the classification of drugs is based on the review by Allison (Allison, 2000).

The MITA demonstrated that dexamethasone (Dex) significantly suppressed IL-2, IL-1β, and IL-8 reporter activities, while cyclosporine A (CyA) and tacrolimus (Tac) suppressed IL-2 and IFN-γ reporter activities but had no effect on IL-1β and IL-8 reporter activities. However, the MITA could not detect the immunosuppressive effects of the alkylating agent cyclophosphamide, of the inhibitors of de novo purine synthesis azathioprine (AZ), mycophenolic acid (MPA) and mizoribine (MZR), and of the inhibitor of pyrimidine and purine synthesis, methotrexate (MT). These data suggest that the MITA correctly evaluates the effects of chemicals on cytokine expression but cannot detect immunotoxicity associated with the inhibition of DNA synthesis and cell division. This drawback has also been reported for other assays, such as the human whole-blood cytokine release assay (HWBCRA) (Langezaal et al., 2002) and the FCP assay (Wagner et al., 2006). On the other hand, the MITA has the advantage that it can discriminate the effects of chemicals differently on T cells from those on macrophages/dendritic cells.

Table 1. The MITA can detect immunosuppressive effects of representative immunosuppressive drugs

Principal mechanism of action	Drugs	The effects of transcriptional activity			
		IL-2	IFN-γ	IL-1β	IL-8
Immunosuppressing drugs					
Regulation of gene expression	Dexamethasone (Dex)	S	N	S	S
Kinase and phosphatase inhibitors	Cyclosporin A (CyA)	S	S	N	N
	Tacrolimus (Tac)	S	S	N	N
	Rapamycin (RPM)	A	N	N	N
Alkylation	Cyclophosphamide (CP)	N	N	N	N
Inhibition of de novo purine synthesis	Azathioprine (AZ)	N	N	N	N
	Mycophenolic acid (MPA)	A	A	N	N
	Mizoribine (MZR)	N	N	A	A
Inhibition of pyrimidine and purine synthesis	Methotrexate (MTX)	N	A	N	N
Off-label immunosuppressing drugs					
	Sulfasalazine (SASP)	S	S	S	S
	Colchicine	S	N	A	N
	Chloroquine (CQ)	S	N	N	N
	Minocycline (MC)	S	S	N	N
	Nicotinamide (NA)	S	N	S	S
Non-immunomodulatory drugs					
	Acetaminophen (AA)	N	N	N	N
	Digoxin	S	S	N	N
	Warfarin	N	N	S	S

Kimura et al. Toxicol in Vitro 28: 759-769, 2014

*S and A indicates that drugs showed statistically significant suppression in triplicate experiments for each parameter, while N indicates that drugs did not show significant effects.

2-8. Evaluation of the immunotoxicity of 60 chemicals by the modified MITA (mMITA)

Regulatory authorities worldwide require testing for allergic contact dermatitis (ACD) and appropriate hazard labeling to minimize exposures. Thus, we combined the MITA with an *in vitro* sensitization test, the IL-8 Luc assay, recently approved as an OECD test guideline for *in vitro* skin sensitization testing (OECD TG442E) (OECD, 2017). We designated this combined assay 'modified MITA' (mMITA). We established a data set of 60 chemicals by referring to the publication by Wagner et al. (Wagner et al., 2006) in which they examined 46 chemicals characterized to different degrees for their immunotoxic and immunomodulatory properties using the Fluorescent Cell Chip (FCP) assay. In addition, we also evaluated the chemicals listed in the case studies in the Guidance for Immunotoxicity Risk Assessment for Chemicals published by WHO ((WHO)/ & Meeting, 2012). Since there were several overlaps between the chemicals we examined in our previous publication and those examined by the FCP, our final data set comprised 60 chemicals evaluated by the mMITA (Kimura et al., 2018) (Table 2). Table 2 lists the chemicals that affected the normalized IL-2 luciferase activity in increasing order of their Lowest Observed Effect Level (LOEL), the results of the MITA evaluation (suppression (S), augmentation (A) or no effect (N)), the LOEL for each parameter of each chemical, and the results of the IL-8 Luc assay evaluation (positive (P) and negative (N)).

Table 2. Classification of chemicals by the mMITA in increasing order of the LOEL of the IL-2 Luc assay.

Chemicals	IL-2		IFN-γ		IL-1β		IL-8		IL-8 Luc
	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge
FK 506	S	0.00	S	0.00	A		N		N
Cyclosporine A	S	0.00	S	0.00	N		N		N
Actinomycin D	S	0.00	S	0.01	N		S	0.00	S
Digoxin	S	0.01	S	0.02	N		N		S
Dexamethasone	S	0.01	N		S	0.01	S	0.01	N
Dibenzopyrene	S	0.01	S	0.03	N		N	0.00	N
Pyrimethamine	S	0.04	N		N		N		N
Chloroquine	S	0.05	S	0.02	S	10.00	S	30.00	S
Cisplatin	S	0.24	S	1.22	N		N		S
Hydrocortisone	S	0.34	A	6.27	S	0.34	S	0.34	N
Mitomycin C	S	0.36	N		N		N		S
Citral	S	0.36	S	1.37	N		N		S
Nitrofurazone	S	0.37	A	3.91	A		A	62.50	S
FR167653	S	0.49	S	0.49	S	145.83	S	125.00	N
Amphoterycin B	S	0.78	S	2.08	A	3.13	A	7.82	S
2-Aminoanthracene	S	0.81	S	5.86	S	2.03	N		S
Lithium carbonate	S	0.98	A	116.67	S	0.39	S	0.39	S
Isophorone diisocyanate	S	0.98	N		S	0.98	S	0.98	S
p-Nitroaniline	S	0.98	S	1.95	S	1.47	S	2.45	N
Dibutyl phthalate	S	0.98	S	1.95	S	39.07	S	31.25	N
Formaldehyde	S	1.71	N		S	15.63	S	15.63	S
Benzethonium chloride	S	1.95	S	1.95	S	3.91	N		S
Isoniazid	S	1.97	N		N		S	800.00	N
Chlorpromazine	S	3.91	S	3.91	S	7.81	S	7.81	S
Cobalt chloride	S	3.91	S	9.12	S	3.91	S	125.00	S
Pentamidine isethionate	S	3.91	S	32.55	S	3.91	S	3.91	N
Aluminum chloride	S	3.91	S	62.50	N		N		N
Lead(II) acetate	S	3.91	S	3.91	N		N		N
Hydrogen peroxide	S	7.82	S	31.25	N		N		S
Minocycline	S	8.33	S	5.00	N		N		S
Histamine	S	9.12	A	5.86	N		S	3.91	S
Diethanolamin	S	9.12	N		N		N		S
Nickel sulfate	S	14.32	S	32.55	S	250.00	S	250.00	S
Sulfasalazine	S	36.00	S	1.20	S	7.80	S	1.20	N
Diesel exhaust particles	S	39.07	A	47.53	N		S	62.50	S
Dapsone	S	45.01	S	55.14	S	46.88	S	134.75	N
Sodium bromate	S	125.00	N		N		N		S
Triethanolamine	S	187.50	S	1416.67	N		N		S
Mercuric chloride	N		A	3.91	S	1.95	S	1.95	S
Chloroplatinic acid	N		N		N		S	15.63	S
2-Mercaptobenzothiazole	N		N		N		S	125.00	S
Cyclophosphamide	N		A	168.00	N		N		S
Magnesium sulfate	N		N		S	15.63	N		S
Sodium dodecyl sulfate	N		N		N		N		S
2,4-Diaminotoluene	N		A	62.50	N		S	0.98	N
Ethanol	N		N		N		N		N
Methanol	N		N		N		N		N
Hexachlorobenzene	N		N		N		N		N
Trichloroethylene	N		N		N		N		N
Azathioprine	N		A	40.01	A	9.23	N		N
Mizoribine	N		N		A	5.20	A	7.45	N
Rapamycin	A	0.00	N		A	0.91	N		S
Nicotinamide	A	0.10	A	110.03	S	3.00	S	10.00	N
Colchicine	A	0.29	A	0.06	A	0.02	A	20.00	S
Mycophenolic acid	A	0.38	A	6.24	N		N		S
Methotrexate	A	0.45	A	0.09	N		N		N
Dimethyl sulfoxide	A	3.91	A	625.00	S	66.41	S	3.91	N
Ribavirin	A	15.63	A	187.50	A	5.86	N		N
Warfarin	A	23.33	N		S	30.00	S	0.00	N
Acetaminophen	A	33.33	A	33.33	A	166.67	A	100.00	N

Groups	Suppression of IL-2 promoter activity (LOEL μg/ml)
Group 1	LOEL<0.1
Group 2	0.1≤LOEL<1.0
Group 3	1.0≤LOEL<10
Group 4	10≤LOEL<1000
Group 5	None
Group 6	Augmentation

0.00 of the LOEL menas less than 0.001.

Using this data set, we first demonstrated a significant correlation between LOELs for effects on the IL-2 luciferase assay and those on the IFN luciferase assay, and between LOELs for effects on the IL-1β luciferase assay and those on the IL-8 luciferase assay (Kimura et al., 2018) (Fig. 7). These results indicated that evaluations of the effects of chemicals on the IL-2 and IL-8 luciferase assays can provide immunotoxicological information equivalent to the evaluation of these chemicals using the IL-2, IFN-γ, IL-1β, and IL-8 luciferase assays.

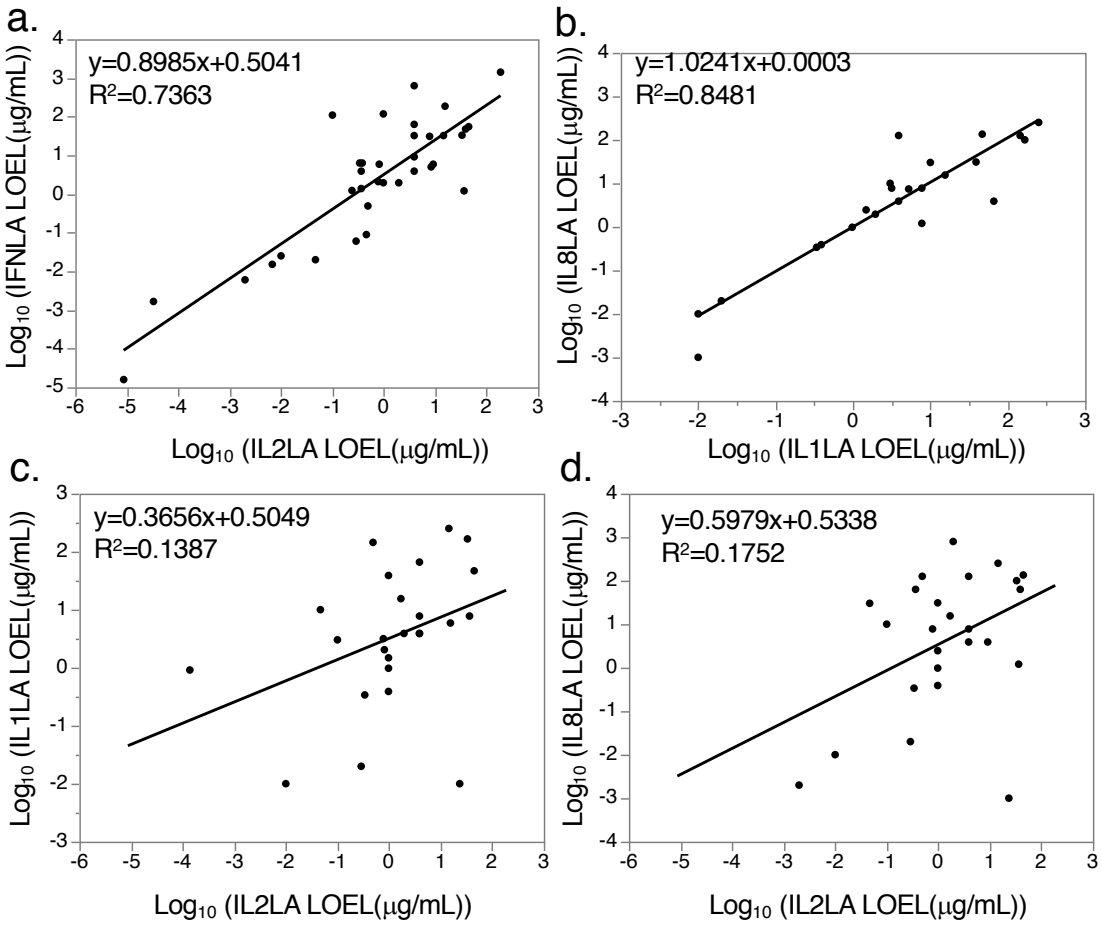


Fig. 7. The correlation between the LOEL for the 4 luciferase assays.

Next, we demonstrated that K-means clustering and hierarchical clustering of the 60 chemicals based on the LOEL for their effects on IL-2 and IL-8 promoter activities, and the judgment by the IL-8 Luc assay, resulted in the same 6-cluster solution: cluster 1 with preferential suppression of IL-8, cluster 2 with suppression of IL-2 and a positive IL-8 Luc assay result, cluster 3 with suppression of both IL-2 and IL-8, cluster 4 with no effects on IL-2 or IL-8 and a negative IL-8 Luc assay result, cluster 5 with suppression of both IL-2 and IL-8 and a negative IL-8 Luc assay result, and cluster 6 with preferential suppression of IL-8 (Kimura et al., 2018) (Fig. 8 and Fig. 9). These data suggest that the mMITA is a promising novel high-throughput approach for detecting unrecognized immunological effects of chemicals and for profiling their immunotoxic effects.

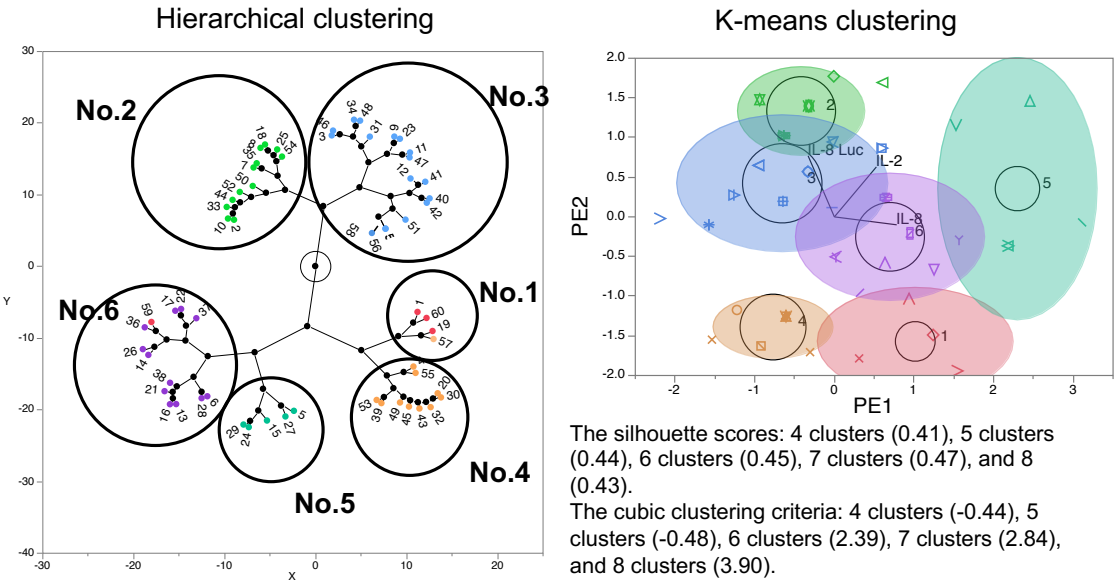


Fig. 8. Clustering of 60 chemicals by the mMITA

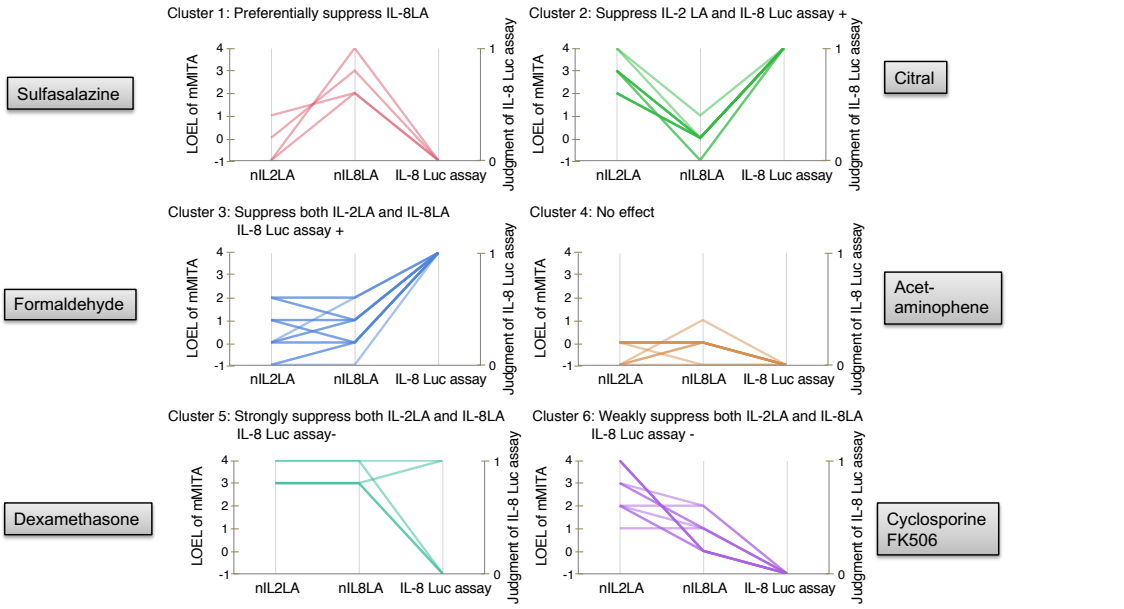


Fig. 9. Characteristics of each cluster and their representative chemicals

2-9. The process of validation of the mMITA

Our final goal is to officially validate the mMITA for within- and between- laboratory reproducibility and predictivity. Since the IL-8 Luc assay had already been accepted as OECD test

guideline 442E, in the current study we conducted the validation study for the IL-2 luciferase assay and the IL-1β luciferase assay using a tiered approach. The first step was the conduct of a validation study of the IL-2 luciferase reporter assay (IL-2 Luc assay).

2-10. The Adverse Outcome Pathway (AOP) of chemicals that affect IL-2 transcription

Immune dysregulation may have serious impacts on human health, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Pivotal immune elements of these diseases are the development of antigen-specific effector T-helper type (Th2) cells, Th1 cells, Th17 cells, and regulatory T cells (Treg cells) that are associated with clinical features and disease progression. Consequently, identifying the immunotoxicity of chemicals requires clarifying their effects on the development of these T cells (reviewed by Kaiko et al., 2008).

IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. IL-2 promotes Th1 differentiation by inducing IL-12Rb2 (and IL-12Rb1), promotes Th2 differentiation by inducing IL-4Ra, inhibits Th17 differentiation by inhibiting gp130 (and IL-6Ra), and drives Treg differentiation by inducing IL-2Ra. IL-2 also potently represses IL-7Ra, which decreases survival signals that normally promote cell survival and memory cell development (reviewed by Liao et al., 2011). It is therefore conceivable that chemicals that affect IL-2 release by T cells could significantly impact immune function; consequently, we focused on the regulation of IL-2 transcription and attempted to construct an AOP with transcriptional dysregulation of IL-2 as a central key event.

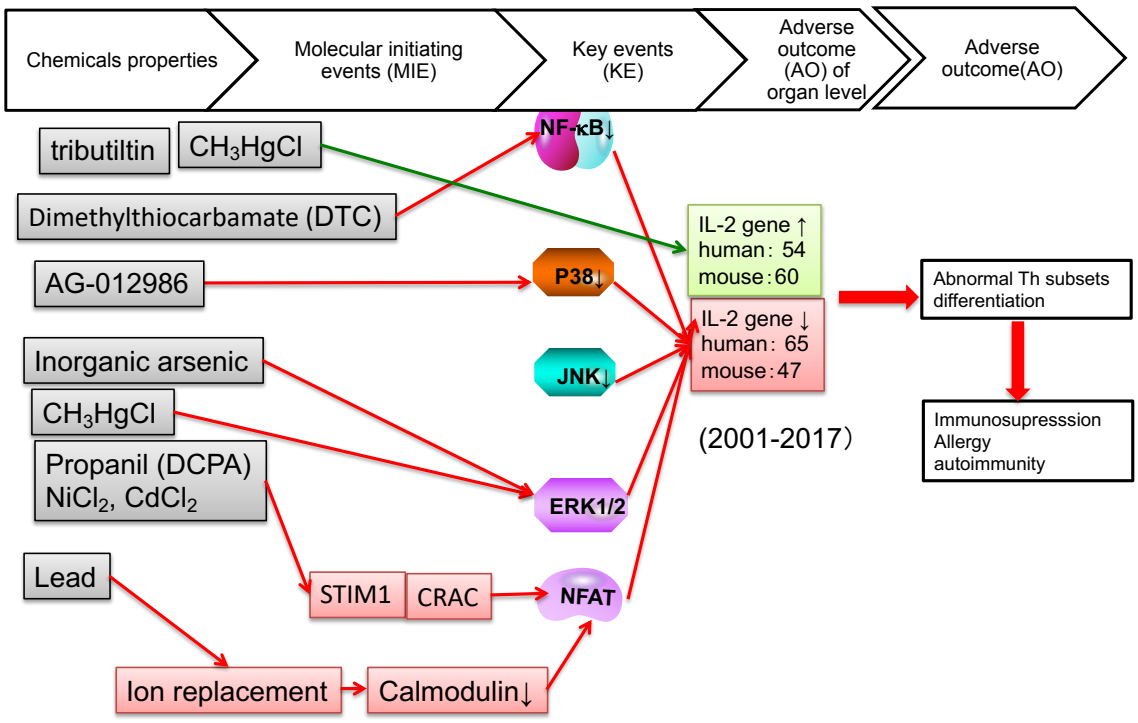
IL-2 mRNA is transcribed after T cell receptor stimulation. Therefore, chemicals that affect any pathway leading to IL-2 transcription after T cell activation can induce dysregulation of IL-2 mRNA and protein expression by T cells. In antigen presentation, T cells are stimulated by T cell receptor (TCR) with co-receptor CD4 or CD8 and CD28. The TCR with CD4 or CD8 recognizes the major histocompatibility complex (MHC)–peptide complex, which results in activation of the SRC kinase Lck and subsequent phosphorylation of immunoglobulin family tyrosine (Y)-based activation motifs (ITAMs) in the CD3 complex (Y-p). This leads to recruitment and phosphorylation of ζ-chain-associated protein (ZAP70), which phosphorylates adaptor proteins, resulting in activation of phospholipase Cγ1 (PLCγ1) and the guanine triphosphatase RAC. PLCγ, in turn, promotes Ca^{2+} mobilization and RAS activation. The combination of these upstream events leads, by complex signaling cascades, to activation of the mitogen-activated protein (MAP)

kinases: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, as well as phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt). Together, these signals promote different events, including the activation of transcription factors, which result in gene expression and, presumably, T-cell function. On the other hand, CD28 might associate, in its unphosphorylated state, with the serine/threonine phosphatase protein phosphatase 2A (PP2A). Upon T-cell stimulation, CD28 undergoes phosphorylation on its intracellular tyrosine residues (Y), presumably resulting in dissociation from PP2A and recruitment of phosphatidylinositol 3-kinase (PI3K) and growth-factor-receptor-bound protein 2 (GRB2). Activation of PI3K, which induces phosphorylation of phosphatidylinositol (PI) into phosphatidylinositol 3-phosphate (PIP3), might promote activation of protein kinase B (PKB/Akt), followed by activation of nuclear factor-κB (NF-κB), resulting in BCL-XL upregulation that favors T-cell survival. Akt activation might also promote interleukin-2 (IL-2) production. PI3K is negatively regulated by phosphatase and tensin homologue (PTEN). The carboxy-terminal proline (P)-rich region might promote IL-2 production and proliferation, perhaps by recruiting and activating Lck (reviewed by Alegre et al., 2001).

Many chemicals have been reported to affect IL-2 transcription or production. Any component of these signaling cascades can be a potential target of these chemicals, but the mechanism by which they affect IL-2 transcription or production remains largely unknown.

Based on recent advances in immunology, we tentatively propose the following AOP for immunosuppression focusing on IL-2 transcription. Figure 10 shows the AOP with representative chemicals that affect IL-2 transcription. From 2001 to 2007, 54 chemicals were reported to augment IL-2 gene or protein expression in human and 60 chemicals had this effect in mice, while 65 chemicals in human and 47 chemicals in mice were reported to decrease IL-2 gene or protein expression, as determined by a PubMed search.

Fig. 10. AOP for dysregulation of Th subset differentiation triggered by disrupted IL-2 transcription.



3. Objective of the study

The objective of the present validation study was to determine the usefulness and limitations of the IL-2 Luc assay in MITA as a non-animal screening method to detect and assess the immunotoxicity of chemicals.

The specific objectives of the study were to establish:

1) “Transferability”, i.e., the extent to which a laboratory can adapt and easily implement the IL-2 reporter assay;

2) “Between or inter-laboratory reproducibility”, i.e., the extent to which results agree among different laboratories;

3) "Within or intra-laboratory reproducibility", i.e., the extent to which results agree in the same laboratory; and

3) “Predictivity”, i.e., the extent to which the *in vitro* results agree with the known immunological profiles of the chemicals.

4. Test Method and modification

4-1. IL-2 reporter cell, 2H4

The Jurkat human acute T lymphoblastic leukemia cell line (ATCC, Manassas, VA, USA), was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing Antibiotic-Antimycotic (Invitrogen) and 10% Hyclone^{TM} fetal calf serum (Thermo Fisher Scientific, Waltham, MA) (Jurkat growth medium) at 37°C with 5% CO_2. The luciferase reporter assay system was constructed using 3 luciferases that emit green light (Stable luciferase green; SLG), orange light (Stable luciferase orange; SLO), and red light (Stable luciferase red; SLR) using a single substrate. Namely, we constructed three luciferase vectors, pSLG-test/Hyg^r, pSLO-test/Neo^r, and pSLR-test/Pur^r, by ligating the *Bam*HI/*Sac*I site of resistant gene vectors containing one of three resistant genes, hygromycin (SLG), neomycin (SLO) or puromycin (SLR), SV40 promoter, and HSVtk polyA into luciferase gene vectors, pSLG-test, pSLO-test and pSLR-test (Toyobo, Osaka, Japan), respectively. The activities of the luciferases can be measured simultaneously and quantitatively with optical filters. This system can rapidly and easily monitor the expression of multiple genes (Nakajima et al., 2005, Noguchi et al., 2008).

4-2. Chemical treatment of 2H4 cells and measurement of luciferase activity

Based on previous reports (Saito et al., 2011, Takahashi et al., 2011), 2H4 cells (2×10^5 cells/50 μl/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were pretreated with different concentrations of individual chemicals for 1 h. The 2H4 cells were then stimulated with 25 nM PMA and 1 μM ionomycin (PMA/Io) for 6 h. Three luciferase activities (SLG luciferase activity (SLG-LA), SLO luciferase activity (SLO-LA), and SLR luciferase activity (SLR-LA)) were simultaneously determined using a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) and Tripluc luciferase assay reagent (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturers' instructions. Use of the 2H4 cell line enabled measurement of SLO-LA driven by the IL-2 promoter (IL2LA), SLG-LA driven by the INF-γ promoter (IFNLA), and SLR-LA driven by G3PDH (GAPLA) in 2H4 cells. We accounted for the variation in cell number and cell viability after chemical treatment by normalizing the data for IL2LA (nIL2LA) or IFNLA (nIFNLA) by dividing IL2LA or IFNLA,

respectively, with GAPLA in the 2H4 cells. In addition, we calculated % suppression, % augmentation, and Inh-GAPLA as follows:

% suppression = (nIL2LA of 2H4 cells treated with chemicals/nIL2LA of non-treated 2H4 cells) x 100;

% augmentation = (1-(nIL2LA of 2H4 cells treated with chemicals/nIL2LA of non-treated 2H4 cells)) x 100;

Inh-GAPLA = GAPLA of 2H4 cells treated with chemicals/GAPLA of untreated cells. Definitions of these terms are provided in Table 3.

Table 3. Definition of the parameters in the IL-2 Luc assay.

Abbreviation	Definition
SLG-LA (SLG-luciferase activity)	Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
SLO-LA (SLO-luciferase activity)	Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
SLR-LA(SLR-luciferase activity)	Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
Normalized SLG-LA (nSLG-LA)	SLG-LA/SLR-LA
Normalized SLO-LA (nSLO-LA)	SLO-LA/SLR-LA
Inhibition index of SLR-LA (I.I.-SLR-LA) (Cytotoxic effect of chemicals)	SLR-LA of #2H4 treated with chemicals/SLR-LA of untreated #2H4
% suppression (Effect of chemicals on IL-2 promoter)	(1-(nSLG-LA of #2H4 treated with chemicals) /(nSLG-LA of non-treated #2H4)) x 100

4-3. Criteria to determine the effects of chemicals on T cells

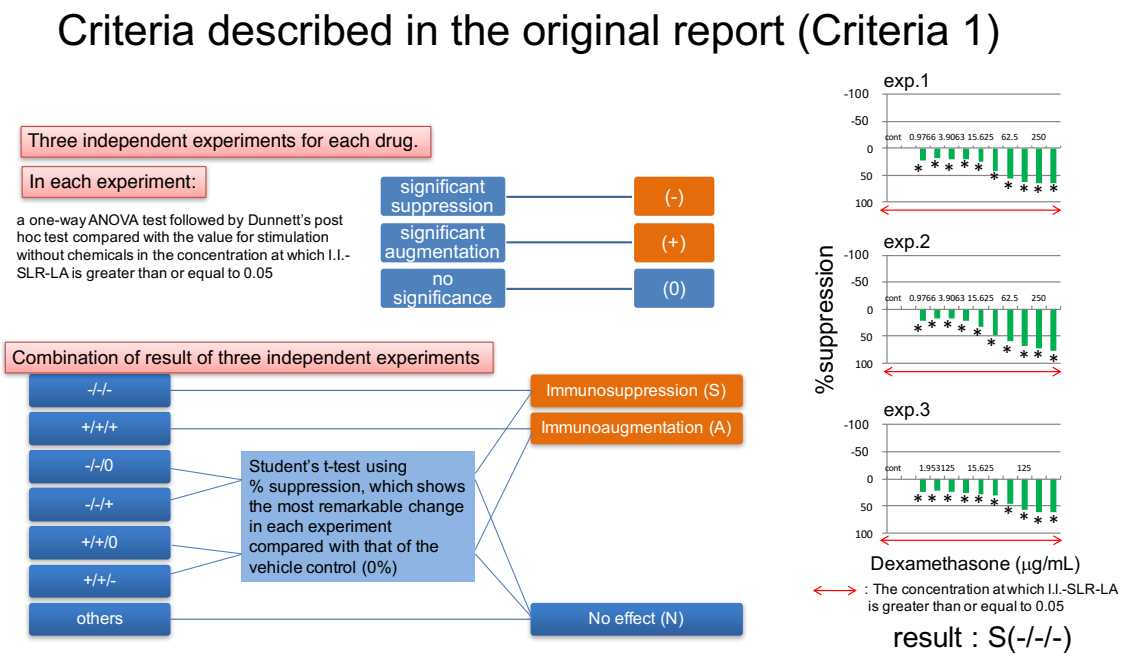
During the validation study, we modified the criteria to determine the effects of chemicals on T cells to determine the criteria for the MITA.

4-3-1. Criteria used in our first publication describing the MITA.

We used the following Criteria 1 in our first publication describing the MITA.

Three independent experiments were conducted for each chemical. For each experiment, a one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. If chemicals showed statistically significant immunosuppression or immunostimulation in 3 experiments, they were judged as immunosuppressive or immunostimulatory drugs, respectively. If chemicals showed statistically significant immunosuppression or immunostimulation in only 2 independent experiments, they were judged as potential immunosuppressive or immunostimulatory drugs, respectively. If not, they were judged as ineffective. Then, for potential immunosuppressive

or immunostimulatory drugs, we selected their percent suppression or percent augmentation (negative percent suppression) in 3 experiments that showed the most significant change, calculated their percent suppression or percent augmentation, and statistically compared suppression or augmentation by the chemicals with that of the vehicle control in 3 different experiments by the Student's t-test. Only when chemicals demonstrated statistical significance were they judged as immunosuppressive or immunostimulatory, respectively(Kimura et al., 2014).



After the pre-validation study, in addition to the original criteria (Criteria 1), two new criteria were proposed by the statistician (Criteria 2, Criteria 3). These 3 criteria were used temporarily and one of these criteria would be adopted after the Phase I validation study.

4-4. Bioluminescence system

In a typical dual-reporter assay, firefly luciferase from *Photinus pyralis* (FLuc) is used as the experimental reporter and Renilla luciferase is used as the internal control reporter. This internal control reporter connects to a constitutively expressed promoter, such as the herpes simplex virus thymidine kinase promoter, cytomegalovirus (CMV) immediate-early promoter, or simian virus 40 (SV40) promoter. This assay system is commercialized as a Dual-Luciferase Reporter Assay System by Promega Corporation. In this system, both luciferase activities are measured sequentially from single extracts on the basis of their bioluminescent substrate specificity. Firefly luciferase activity is measured first by adding firefly D-luciferin, and then Renilla luciferase activity is measured by adding coelenterazine (another name for Renilla luciferin), with

concomitant quenching of firefly luciferase luminescence. Finally, firefly luciferase activity is normalized by Renilla luciferase activity as the promoter activity (Michelini et al., 2014; Nakajima and Ohmiya, 2010; Roda et al., 2004).

An alternative chemical test using a cell-based assay requires the analysis of a large number of samples. It is therefore preferable to use an improved assay system whereby gene expression can be monitored simultaneously in a one-step reaction in single extracts. Beetle luciferases emit red luminescence during reaction, compared to the green emitted by firefly D-luciferin. The two colors can be divided using an optical filter. The dual color-reporter assay is based on the color difference between beetle and firefly luciferases and is sold commercially as the Tripluc Reporter Assay System by TOYOBO (Nakajima et al., 2004; Nakajima et al., 2005).

In the IL-2 Luc assay, the triple-color assay system consisted of a green-emitting luciferase (SLG; λmax = 550 nm) (? et al., ?) for the gene expression of the IL-2 promoter, an orange-emitting luciferase (SLO; λmax = 580 nm) (Viviani et al., 2001) for the gene expression of the IFN-γ promoter, and a red-emitting luciferase (SLR; λmax = 630 nm) (Viviani et al., 1999) for the gene expression of the internal control promoter, GAPDH. The three luciferases emit different colors upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (Nakajima et al., 2005). First, the total relative light units (F0) are measured in the absence of the filters. Then, the F1 and F2 values that passed through the R56 filter (>560-nm long-pass filters) or the R60 filter (>600-nm long-pass filters), respectively, is measured. The three luciferase activities are calculated using the simultaneous equation shown below by substituting the F0, F1 and F2 values. In this equation, G, O and R are the activities of the green-, orange- and red-emitting luciferases, respectively, κG_{R56}, κO_{R56} and κR_{R56} are the transmission coefficients of the green-, orange- and red-emitting luciferases of the R56 filter, respectively, κG_{R60}, κO_{R60} and κR_{R60} are the transmission coefficients of the green-, orange- and red-emitting luciferases of the R60 filter, respectively.

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Luminescence activity is measured using a filtered 96-well microplate luminometer (for example, Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). It is necessary to calibrate the luminometer in each

experiment to ensure reproducibility (Niwa et al., 2010). Recombinant green-, orange- and red-emitting luciferases are available for this calibration.

5. Validation Management Structure

5-1. Validation Management Team (VMT)

Trial Coordinator:	Hajime Kojima (Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Kawasaki, Japan), VMT trial coordinator, Chemical supplier and Management of quality control
Lead laboratory:	Setsuya Aiba (Tohoku University, Miyagi, Japan), Developer of this assay, Test method, expertise underlying science Yutaka Kimura (Tohoku University, Miyagi, Japan)
International expert members	
EU liaison:	Emanuela Corsini (Milan Univ., Italy), Test system expertise, validation expertise, immunotoxicity expertise Erwin L. Roggen (3Rs Management and Consulting ApS, Denmark), Test system expertise, validation expertise, immunotoxicity expertise
ICCVAM liaison:	Dori Germolec (NTP/NIEHS, USA), Immunotoxicity expertise
JSIT liaison:	Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.), Immunotoxicity expertise
Data management team:	Takashi Omori (Kobe University, Kobe, Japan), Data analysis, biostatistics dossier
Chemical Selection Committee	Setsuya Aiba (Tohoku University) Yutaka Kimura (Tohoku University Hajime Kojima (JaCVAM) Emanuela Corsini (Milan Univ) Erwin L. Roggen (3Rs Management and Consulting ApS) Dori Germolec (NTP/NIEHS) Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.)

Participating Test Facilities	Test Facility 1: Hatano Res. Inst., FDSC, Study Director (SD): Kohji Yamakage Test Facility 2: AIST, Tsukuba, SD: Rie Yasuno Test Facility 3: AIST, Takamatsu, SD: Yoshihiro Nakajima

5-2. Management office

Hajime Kojima (JaCVAM)
3-25-26 Yodomimati Kawasaki, Kawasaki, 210-9501
TEL: +81-44-270-6600
h-kojima@nihs.go.jp

5-3. Meetings held

27-28/1/2016 (Mitoya, Sendai, Japan)
1st International VMT Meeting
Subjects: Kick-off meeting for the MITA assay
VMT members: Corsini, E., Roggen, E., Germolec, D.(telephone), Inoue, T., Kageyama, S., Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M., Yasuno, R., Ohmiya, Y., Omori, T., Kojima, H., Tanabe, S., Venti, S.
Participating laboratories: AIST(Tsukuba), HRI

13/9/2016 (Skype-meeting)
Meeting by Skype
Subjects: Result of the phase 0 study and proposal of the revised protocol
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

4-5/2/2017 (Nayamachi community hall, Kyoto, Japan)
2nd International VMT Meeting
Subjects: Validation results, discussion and suggestion
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M.,

Yasuno, R., Nakajima, Y., Omori, T., Mori, A., Kobayashi, M., Kojima, H., Venti, S.

Participating laboratories: AIST(Tsukuba), HRI, AIST(Takamatsu)

18-19/11/2017 (Umeda Center Building, Osaka, Japan)

3rd International VMT Meeting

Subjects: Validation results, discussion and suggestion

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M., Yasuno, R., Nakajima, Y., Omori, T., Mori, A., Kobayashi, M., Kojima, H., Venti, S.

Participating laboratories: AIST(Tsukuba), HRI, AIST(Takamatsu)

29/3/2018 (Skype-meeting)

Meeting by Skype

Subjects: Proposal of the revised protocol

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

10/4/2018 (telephone-meeting)

Meeting by telephone

Subjects: Understanding the unexpected results in the IL-2 Luc assay

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

4-6/10/2018 (Kobe Univ., Kobe, Japan)

4th meeting for the MITA Validation study

Subjects: Validation report for the IL-2 assay

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T. Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Yasuno, R., Nakajima, Y., Omori, T., Takagi, Y., Mashimo, N., Kado, Y., Kojima, H., Venti, S.

Participating laboratories: AIST(Tsukuba), HRI, AIST(Takamatsu)

6. Study Design

The aim of this phase is to (pre)validate the IL-2 Luc assay method to assess transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals.

The validation study (Phase I and Phase II trials) was conducted by 3 laboratories, based on the study design and schedule shown in Tables 8 and 9 and using the test chemicals shown in Tables 10 and 11. The methods were described above in section 4: 'Test Method 4.1 IL-2 Luc assay', and the precise protocol is described below in section 8: 'Protocol 8.2 Protocol for the IL-2 Luc assay'.

Table 4. The number of chemicals analyzed in the validation study

Studies	Within-Laboratory	Between-laboratories	Predictivity
I	5	5	5
II		20	20
Total	5	25	25

7. Test Chemicals

The selection process for the test chemicals for the IL-2 Luc assay validation study is described below.

In addition, the chemical categories or physical state and chemical properties (e.g., solid, liquid, etc.) are included in the tables of these test chemicals in order to investigate the applicable domain.

Table 5. Breakdown of the IL-2 Luc assay validation study

Phase	The number of the test substances	The number of the repetitions	Examination	Date of experiment start
Pre	5	1	Between- laboratory transferability (Non-coded)	July, 2016
I	5	3	Within- and between- laboratory reproducibility (Coded)	September, 2016
II	20	1	Between- laboratory reproducibility and predictivity (Coded)	May, 2017

7-1. Basic rule for chemical selection

The selection of test chemicals by the Chemical Selection Committee (CSC) in the VMT was based on published papers on *in vivo* immunotoxicity tests and validation studies for *in vitro* alternative assays on immunotoxicity test methods.

7-1-1. The applied selection criteria

- information on mode/site of action
- coverage of a range of relevant chemical classes and product classes
- quality and quantity of reference data (*in vivo* and *in vitro*)
- high-quality data derived from animal and (if available) human studies
- information on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of a range of toxic effects/potencies
- chemicals that do not require metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as implicated by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- cost

In the first phase of the selection procedure, the CSC identified and collected several existing lists of potential chemical immunotoxicants, such as NTP IMMUNOTOX, EPA candidate list. An extensive literature search was performed by the CSC in order to ensure that all the pre-selected chemicals fulfilled the selection criteria described above. In addition, it was decided that at least 20% of the total chemicals to be tested should provide negative results (i.e., not immunotoxic) in order to increase the statistical power of the data analysis.

7-1-2. Chemical Acquisition, Coding and Distribution

Laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were assessed using coded chemicals. Coding was supervised by JaCVAM, in collaboration with CSC. CSC was responsible for coding and distributing the test chemicals, references, and controls for the validation study.

7-1-3. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing hazards identification and exposure controls/personal protection for each chemical. The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

7-2. Pre-validation study

Transferability of this assay was checked using five non-coded chemicals (2-aminoanthracene, citral, chloroquine diphosphate salt, dexamethasone and methylmercury(II) chloride) (Appendix 1) in 4 test facilities, including the lead laboratory. These chemicals were selected by the CSC.

7-3. Validation study -Phase I trial

Within- and between-laboratory reproducibility of this assay was checked using 5 coded chemicals in 3 test facilities, as shown in Table 6 (Appendix 2). These chemicals were selected by CSC based on the in-house data set of the lead laboratory. The chemicals were coded by JaCVAM as shown in Table 10 and distributed to the test facilities.

Table 6. Chemical code list on the phase I validation trial for IL-2 Luc assay

No.	Chemical	CASRN	MW	Supplier	Catalog No.	Content	Physical characteristics	Lot	Storage	Purity	LabA	LabB	LabC	LabD
											TOHOKU unv.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU
1	Dibutyl phthalate	84-74-2	278.34	Wako	021-06936	500mL	Liquid	TLN0112	RT	98.0+% (Capillary GC)	MIA003A	MIB014A	MIC027A	MID036A
											MIA004B	MIB017B	MIC026B	MID033B
											MIA007C	MIB016C	MIC023C	MID034C
2	Hydrocortisone (for Cell Culture)	50-23-7	362.46	Wako	080-10194	50g	Solid	SAH3714	RT	97% (HPLC)	MIA005A	MIB017A	MIC029A	MID038A
											MIA007B	MIB019B	MIC028B	MID035B
											MIA009C	MIB018C	MIC025C	MID037C
3	Lead(II) acetate trihydrate (Deleterious substances)	6080-56-4	379.33	Sigma-Aldrich	316512-100G	100g	Solid	09901TS	RT	99.999% trace metals basis	MIA007A	MIB018A	MIC021A	MID310A
											MIA008B	MIB011B	MIC210B	MID037B
											MIA001C	MIB110C	MIC027C	MID038C
4	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	305.82	Kanto Chemical	48028-31	25g x2	Solid	403N2204	RT	>95.0% (T)	MIA009A	MIB110A	MIC023A	MID037A
											MIA010B	MIB013B	MIC027B	MID039B
											MIA003C	MIB017C	MIC029C	MID310C
5	Nickel (II) sulfate hexahydrate	10101-97-0	262.85	Wako	146-01171	100g	Solid	LKQ2263	RT	99.0-102.0% (as NiSO4・6H2O) (Titration)	MIA001A	MIB012A	MIC025A	MID034A
											MIA002B	MIB015B	MIC024B	MID031B
											MIA005C	MIB014C	MIC021C	MID032C

7-4. Validation study -Phase II trial

Twenty test chemicals were selected by CSC for between-laboratory reproducibility as shown in Table 7 (Appendix 3). The chemicals were coded by JaCVAM as shown in Table 7 and distributed to the test facilities.

Table 7. Chemical code list on the phase II validation trial for IL-2 Luc assay

	Chemical	Cas.no.	LabA	LabB	LabC	LabD	Note	State	Storage	Supplier	Lot
			TOHOKU univ.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU					
1	2,4-Diaminotoluene	95-80-7	MIA401	MIB515	MIC618	MID702	Deleterious	S	RT	Wako	CDF0347
2	Benzo(a)pyrene	50-32-8	MIA413	MIB516	MIC601	MID703		S	RT	TCI	M8DFD
3	Cadmium chloride	10108-64-2	MIA403	MIB502	MIC602	MID714	Deleterious	S	RT	Wako	DEE3332
4	Dibromoacetic acid	631-64-1	MIA406	MIB518	MIC610	MID720		S	RT	ALDRICH	BCBR5175V
5	Diethylstilbestol	56-53-1	MIA420	MIB509	MIC611	MID711		S	RT	SIGMA	BCBR9766V
6	Diphenylhydantoin	630-93-3	MIA412	MIB510	MIC615	MID704		S	RT	SIGMA	SLBB3874
7	Ethylene dibromide	106-93-4	MIA407	MIB507	MIC605	MID705	Deleterious	L	RT	Wako	KWG5479
8	Glycidol	556-52-5	MIA408	MIB505	MIC607	MID712		L	2-8℃	ALDRICH	MKBX5752V
9	Indomethacin	53-86-1	MIA409	MIB508	MIC609	MID715		S	RT	SIGMA	122K0718
10	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	MIA411	MIB517	MIC612	MID707		S	RT	Fluka	SLBF8371V
11	Nitrobenzene	98-95-3	MIA402	MIB519	MIC603	MID701	Deleterious	L	RT	Sigma-Aldrich	SHBG5577V
12	Urethane, Ethyl carbamate	51-79-6	MIA415	MIB520	MIC604	MID719		S	RT	Sigma-Aldrich	WXBC3505V
13	Tributyltin chloride	1461-22-9	MIA404	MIB506	MIC613	MID713	Deleterious	L	RT	TCI	2442A-IQ
14	Perfluorooctanoic acid	335-67-1	MIA414	MIB514	MIC614	MID718		S	RT	TCI	O3U70
15	Dichloracetic acid	79-43-6	MIA416	MIB511	MIC606	MID716	Deleterious	L	RT	Sigma-Aldrich	SHBH3492V
16	Toluene	108-88-3	MIA417	MIB512	MIC616	MID706	Deleterious	L	RT	Sigma-Aldrich	J5136
17	Acetonitril	75-05-8	MIA405	MIB501	MIC617	MID708	Deleterious	L	RT	Wako	KWH4805
18	Mannitol	69-65-8	MIA418	MIB503	MIC619	MID717		S	RT	Wako	LKP4362
19	Vanadium pentoxide	1314-62-1	MIA419	MIB504	MIC608	MID709	Deleterious	S	RT	Wako	SAE6958
20	o-Benzyl-p-chorolophenol	120-32-1	MIA410	MIB513	MIC620	MID710		S	RT	Wako	KPQ0988

7-5. Acceptance criteria

The within-laboratory reproducibility for the all test facilities was done by an independent biostatistical analysis using coded five chemicals, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I study.

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibility in the phase I and II study. At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the trial coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle). The proportion of concordance between-laboratory reproducibility should be equal or more than 80% as acceptance criteria.

8. Protocols

8-1. Overview of the IL-2 Luc assay

An overview of the IL-2 Luc assay is shown in Fig. 9. In addition, the final protocol of the present test (version 023E) is provided as attached Appendix 4 and the procedures are described in detail below.

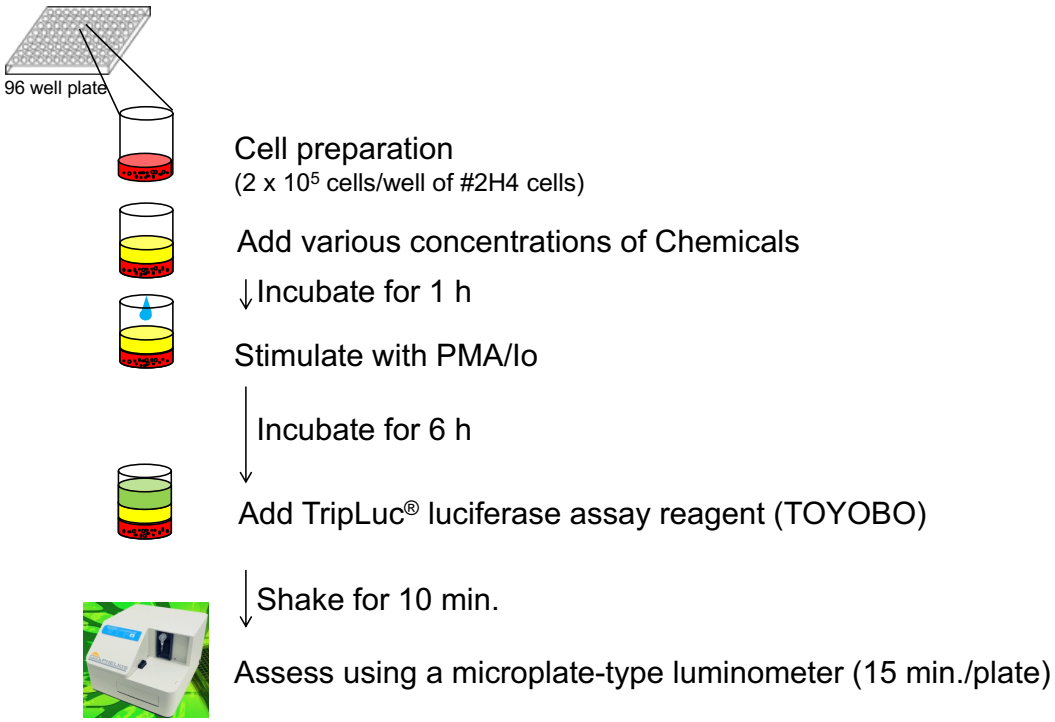


Fig. 11. Overview of the IL-2 Luc assay