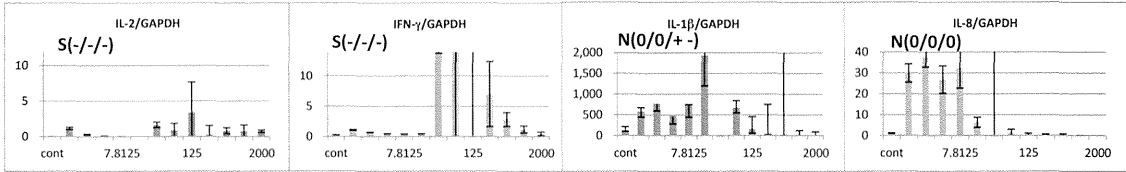
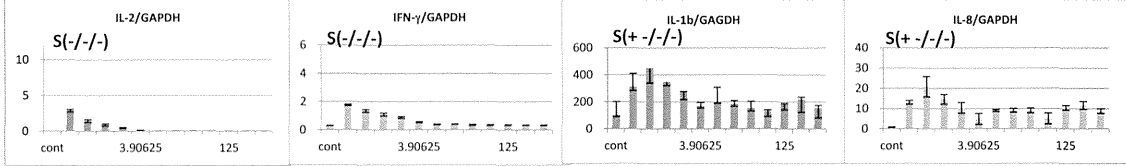


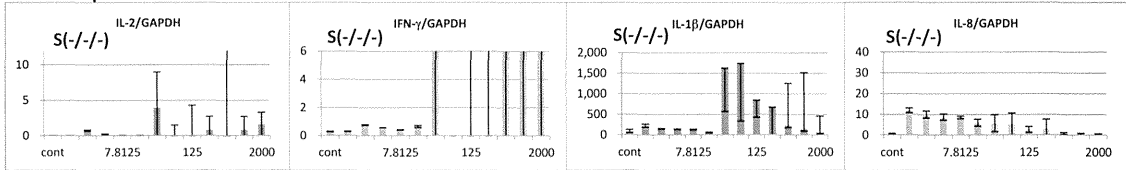
Benzethonium chloride



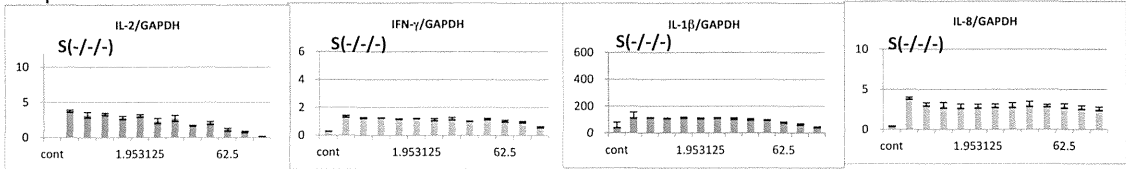
Dibutyl phtahlate



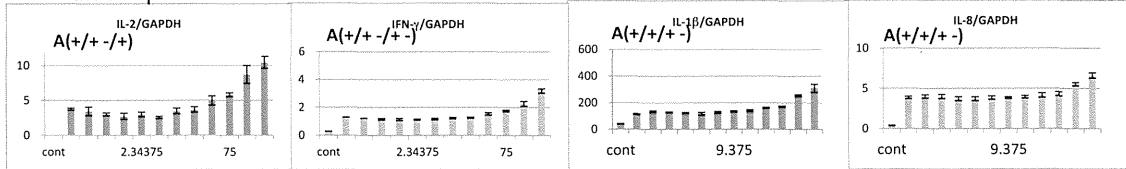
Chlorpromazine



Dapsone



Acetaminophen



Ethanol

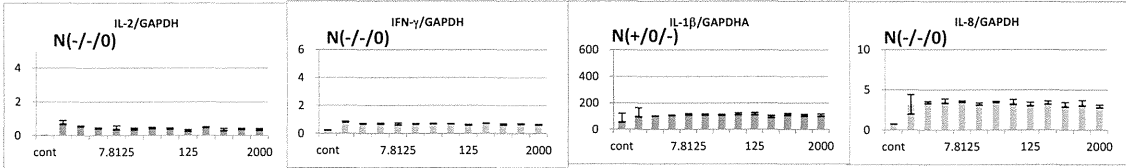
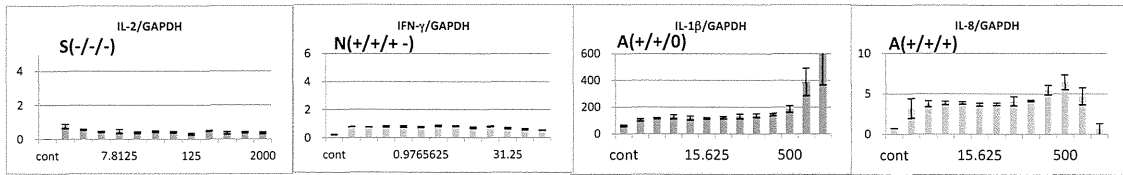
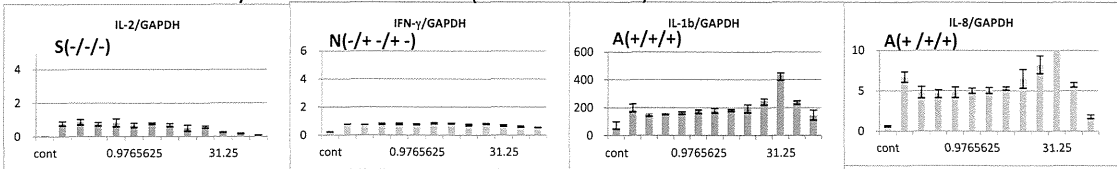


図5 #2H4 細胞 (IL-2 および IFN- $\gamma$ )、THP-G1  $\beta$  細胞 (IL-1 $\beta$ ) および THP-G8 細胞 (IL-8) を用いた 10 物質の施設内再現性

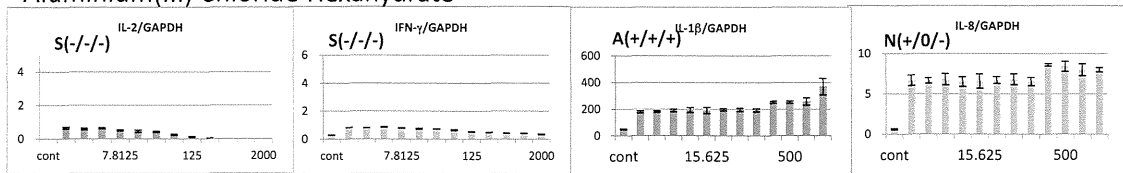
Sodium bromate



5-Nitro-2-furaldehyde semicarbazone (Nitrofurazone)



Aluminium(III) Chloride Hexahydrate



4-Nitroaniline

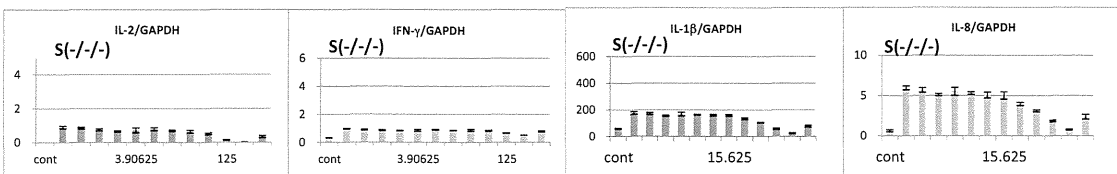


図5 #2H4 細胞 (IL-2 および INF-γ)、THP-G1β 細胞 (IL-1β) および THP-G8 細胞 (IL-8) を用いた 10 物質の施設内再現性 (続き)

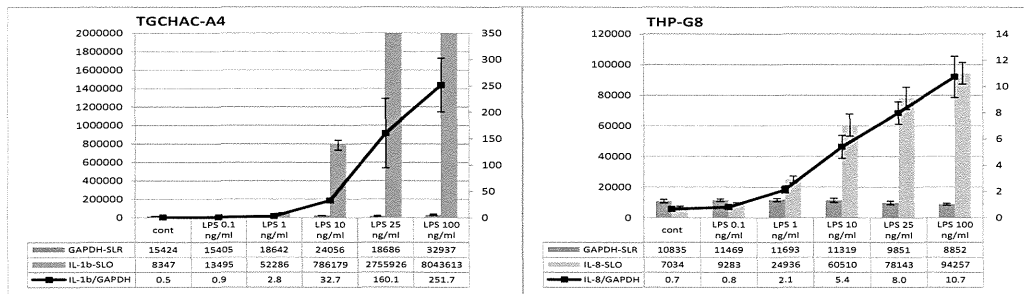


図6 TGCHAC-A4 細胞 (左) および THP-G8 細胞 (右) の LPS に対する反応性

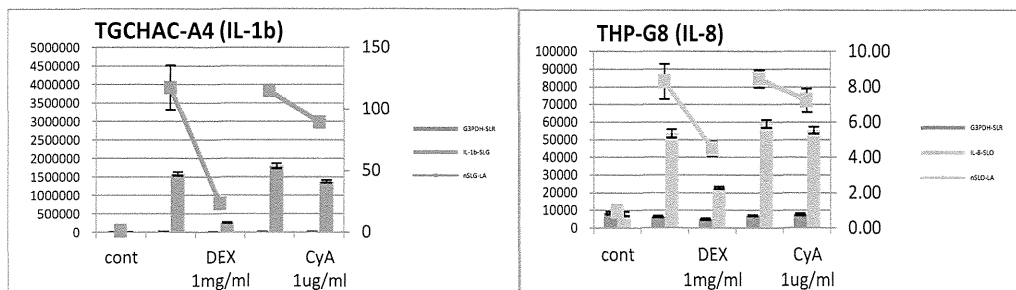
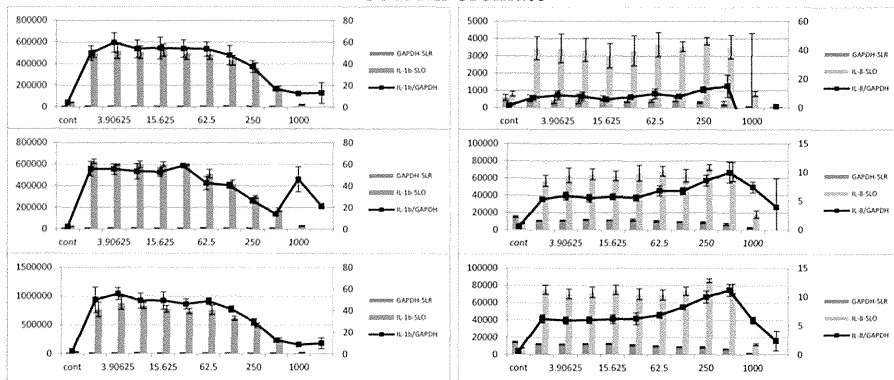
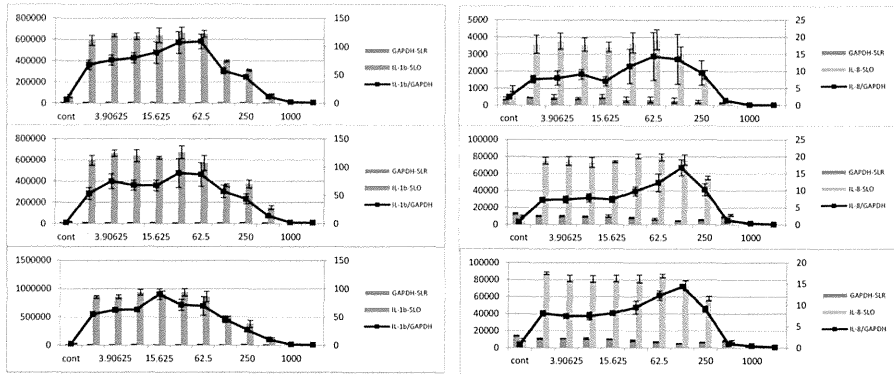


図7 TGCHAC-A4 細胞 (左) および THP-G8 細胞 (右) の DEX および CyA に対する反応性

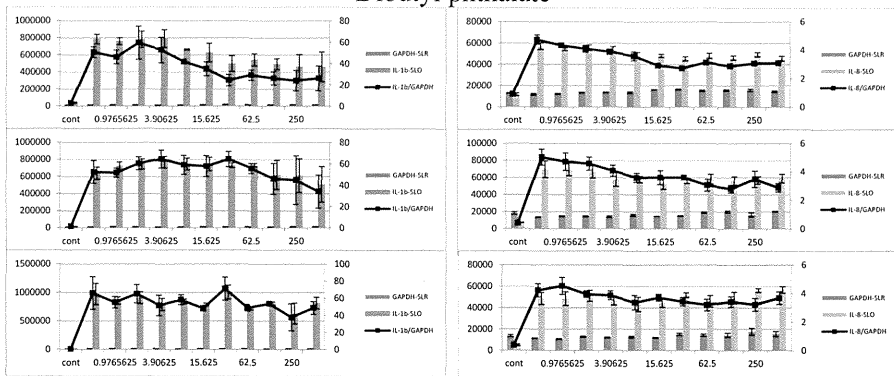
### Sodium bromate



### Nickel sulfate



### Dibutyl phthalate



### 2-MBT

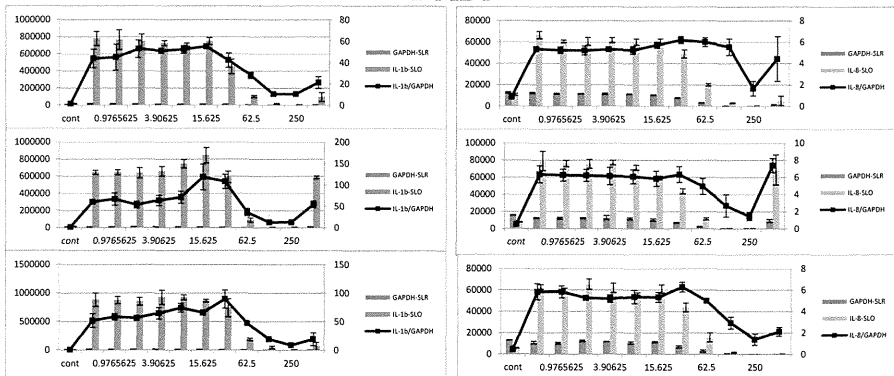


図9 TGCHAT-A4細胞(左)およびTHP-G8細胞(右)における4物質の施設内再現性

#### D. 考察

新たな*in vitro*毒性評価試験法開発に際しては、技術移転性、施設内再現性および施設間再現性の高い試験法開発は重要な課題である。*In vitro*免疫毒性評価を目的に開発されたMITAの技術移転性、施設内および施設間再現性を確認するため、14物質の反復実験（3回）の結果、用量反応性に依存しない特異な反応性を示す傾向が認められた。原因を検討した結果、細胞不活化剤添加に使用している連続分の添加操作に原因があることが明らかとなった。今後、プロトコルを完成させる上で貴重な情報となり得ると考えられる。

この添加操作を改善して再試験を実施し計10物質について繰り返し実験を行ったが、実験毎の発光量の違いが大きい印象があるものの、標準化することにより多少のバラツキは認められるもの実験毎の反応性はほぼ同様の結果を示し、施設内再現性は良好であると考えられた。

また、THP-G1β細胞の問題点を克服するために新たに開発されたTGCHAC-A4細胞は、今回試験したいずれの化合物に対しても安定した反応を示し、用量反応性を見る限り、非常に良好な施設内再現性が得られる試験系であると考えられた。

#### E. 結論

MITAのバリデーション試験に先立ち、技術移転性、施設内および施設間再現性を検討し、良好な結果が得られたと考えられる。一方、細胞賦活化剤添加操作の注意点や新たに開発されたTGCHAC-A4細胞の利用をプロトコルに反映させることにより、MITAは*in vitro*毒性評価試験法としてより安定した結果が期待される。

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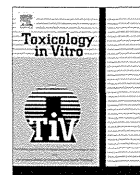
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研究成果の刊行物・別刷



## Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs



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### ABSTRACT

We established a luciferase reporter assay system, the Multi-ImmunoTox Assay (MITA), to evaluate the effects on key predictive *in vitro* components of the human immune system. The system is composed of 3 stable reporter cell lines transfected with 3 luciferase genes, SLG, SLO, and SLR, under the control of 4 cytokine promoters, IL-2, IFN- $\gamma$ , IL-1 $\beta$ , and IL-8, and the G3PDH promoter. We first compared the effects of dexamethasone, cyclosporine, and tacrolimus on these cell lines stimulated with phorbol 12-myristate 13-acetate and ionomycin, or lipopolysaccharides, with those on mRNA expression by the mother cell lines and human whole blood cells after stimulation. The results demonstrated that MITA correctly reflected the change of mRNA of the mother cell lines and whole blood cells. Next, we evaluated other immunosuppressive drugs, off-label immunosuppressive drugs, and non-immunomodulatory drugs. Although MITA did not detect immunosuppressive effects of either alkylating agents or antimetabolites, it could demonstrate those of the off-label immunosuppressive drugs, sulfasalazine, chloroquine, minocycline, and nicotinamide. Compared with the published immunological effects of the drugs, these data suggest that MITA can present a novel high-throughput approach to detect immunological effects of chemicals other than those that induce immunosuppressive effects through their inhibitory action on cell division.

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### 1. Introduction

Environmental contaminants, food additives, and drugs can target the immune system, resulting in adverse health effects, such as the development of allergies, autoimmune disorders, cancers, and other diseases. Accordingly, immunotoxicity, which is defined as the toxicological effects of xenobiotics on the functioning of the immune system, has raised serious concerns from the public as well as regulatory agencies. Currently, the assessment of chemical immunotoxicity relies on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as expense, ethical concerns, and eventual relevance to risk assessment for humans. Therefore, European policy is promoting alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals employed for scientific studies (Balls et al., 1995).

A 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 (Galbiati et al., 2010; Gennari et al., 2005; Lankveld et al., 2010). In that workshop, a tiered approach was proposed, since useful information can be obtained from regular 28-day general toxicity tests. Namely, pre-screening for direct immunotoxicity starts with the evaluation of myelotoxicity. Compounds that are capable of damaging or destroying the bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they are tested for lymphotoxicity. Then, they are tested for immunotoxicity by approaches such as human whole-blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction, natural killer cell assay, T-cell-dependent antibody response, dendritic cell maturation, and fluorescent cell chip. Among these assays, HWBCRA has undergone formal prevalidation, although other techniques are being examined or have been previously examined in a rigorous prevalidation effort by ECVAM and other groups.

The principle of HWBCRA, described by Langezaal et al. (2002), is based on the well-known human whole-blood method for pyrogen testing (Hartung, 2002). In brief, human blood is treated with lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB),

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which causes monocytes and Th2 lymphocytes to produce IL-1 $\beta$  and IL-4, respectively. After incubation for 40 h in the presence or absence of immunotoxic and non-immunotoxic test compounds, the levels of IL-1 $\beta$  and IL-4 in the supernatant are quantified, and the 50% inhibitory concentration (IC50) and the fourfold stimulating concentration (SC4) are calculated to establish the immunotoxic potency (Langezaal et al., 2002). According to the EC-VAM workshop, this method has several advantages, such as the avoidance of species differences between humans and animals, employment of human primary cells, simple culture techniques, and reduced expense and time requirements as compared to animal experiments. The interindividual variation in leukocyte numbers and their response to stimuli is a major concern when using HWBCRA. Although cryopreservation techniques for human whole blood can overcome these problems (Schindler and Hartung, 2002), this method is not suitable as a high-throughput assay to evaluate vast numbers of chemicals.

In the present study, to develop a high-throughput screening system to evaluate chemical immunotoxicity, we first established 3 stable reporter cell lines transfected with luciferase genes under the control of IL-2, IFN- $\gamma$ , IL-8, and IL-1 $\beta$  promoters. We selected these 4 cytokines because IL-2 and IFN- $\gamma$  are mainly produced by T cells and reflect T-cell function, while IL-8 and IL-1 $\beta$  are mostly produced by monocytes or dendritic cells and correspond with their activity. Next, we examined the effects of 3 well-characterized immunosuppressive drugs, dexamethasone (Dex), cyclosporine A (CyA), and tacrolimus (Tac), on luciferase activities of these three cell lines stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) or lipopolysaccharide (LPS). Then, we compared the results with their effects on mRNA expression by the mother cell lines, Jurkat cells or THP-1 cells, under the relevant stimulation. Furthermore, we also compared their effects on luciferase activities with mRNA expression by human whole blood cells stimulated with PMA/Io or LPS in the presence of these immunosuppressive drugs. Finally, we treated these cell lines with immunosuppressive drugs, immunomodulatory drugs, or drugs without known immunomodulatory effects and estimated the performance of our screening system for immunotoxicity.

## 2. Materials and methods

### 2.1. Reagents

Water-soluble dexamethasone (Dex), cyclosporin A (CyA), tacrolimus (FK-506), rapamycin, cyclophosphamide (CP), azathioprine (AZ), mycophenolic acid (MPA), mizoribine (MZR), methotrexate (MTX), sulfasalazine (SASP), colchicine, chloroquine (CQ), minocycline (MC), nicotinamide (NA), acetaminophen (AA), digoxin, warfarin, phorbol 12-myristate 13-acetate (PMA), ionomycin (Io), and lipopolysaccharides from *E. coli* O26:B6 (LPS) were purchased from Sigma–Aldrich (St. Louis, MO).

### 2.2. Cell lines and reporter cell lines

The human acute T lymphoblastic leukemia cell line Jurkat and the human acute monocytic leukemia cell line THP-1 (ATCC, Manassas, VA) were cultured in RPMI-1640 (Gibco, Carlsbad, CA) with antibiotic–antimycotic (Invitrogen, Carlsbad, CA) and 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) (Growth medium) at 37 °C with 5% CO<sub>2</sub>. We previously established 2 reporter cell lines, #2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by IL-2 promoter, stable luciferase orange (SLO) regulated by IFN- $\gamma$  promoter, and stable luciferase red (SLR) regulated by G3PDH promoter (Saito et al., 2011) and THP-G8 cells derived from THP-1 cells containing SLO

regulated by IL-8 promoter and SLR regulated by G3PDH promoter (Takahashi et al., 2011).

In the present study, we further established THP-G1b cells derived from THP-1 cells containing SLG regulated by IL-1 $\beta$  promoter and SLR by G3PDH promoter. Full details are available in Supplementary Methods.

### 2.3. Chemical treatment

Based on the previous reports (Saito et al., 2011; Takahashi et al., 2011), #2H4 cells ( $2 \times 10^5$  cells/50  $\mu$ l/well), THP-G1b cells, or THP-G8 cells ( $5 \times 10^4$  cells/50  $\mu$ l/well) in 96-well black plates (Greiner bio-one GmbH, Frickenhausen, Germany) were pretreated with different concentrations of chemicals for 1 h. The optimum cell numbers at seeding were based on the previous reports. Afterwards, #2H4 cells were stimulated with 25 nM of PMA and 1  $\mu$ M of ionomycin (PMA/Io) for 6 h, while THP-G1b cells or THP-G8 cells were stimulated with 100 ng/ml of LPS for 6 h. In some experiments, we changed the stimulation time to determine the optimum incubation period for the luciferase assay. Three luciferase activities, SLG luciferase activity (SLG-LA), SLO luciferase activity (SLO-LA), and SLR luciferase activity (SLR-LA), were simultaneously determined by using a microplate-type luminometer with a multi-color detection system, Phelios (Atto Co., Tokyo, Japan), and the Tripluc luciferase assay reagent (TOYOBO) according to the manufacturer's instructions. To rule out the variation of cell number or cell viability after chemical treatment, we obtained normalized luciferase activity as follows:

Normalized SLG-LA (nSLG-LA) or normalized SLO-LA (nSLO-LA) = SLG-LA or SLO-LA/SLR-LA.

We also calculated percent suppression as follows:

% suppression =  $(1 - \text{nSLG-LA or nSLO-LA of the reporter cells treated with drugs/nSLG-LA or nSLO-LA of non-treated reporter cells}) \times 100$ .

To eliminate the data affected by cytotoxic effects of drugs or cell death, we also defined the inhibition index of SLR-LA (II-SLR-LA) as follows:

II-SLR-LA = SLR-LA of reporter cells that were treated with chemicals/SLR-LA of untreated reporter cells.

Since our previous study has reported that, in the treatment showing more than 5% in II-SLR-LA, more than 75% of cells are PI-excluding living cells (Takahashi et al., 2011), we presented only the data that demonstrated more than 5% in II-SLR-LA in this study.

### 2.4. Human whole-blood cytokine mRNA expression test (HWBCMET)

The human whole-blood cytokine mRNA expression test (HWBCMET) was performed by modifying the HWBCRA protocol by Langezaal et al. (2002) and Thurm and Halsey (2005). The following studies were approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan, and conducted according to the Declaration of Helsinki principles. Full details are available in Supplementary Methods.

### 2.5. mRNA expression by Jurkat and THP-1 cells

Jurkat or THP-1 cells ( $3 \times 10^6$  cells) in 6-well plates were pretreated with different concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 6 h, respectively. Total RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The total RNA concentration was measured by using a NanoDrop spectrophotometer.

## 2.6. Quantitative RT-PCR

Complementary DNAs (cDNAs) were synthesized by using the TaKaRa RNA PCR Kit (AMV) (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed by using the Mx3000p QPCR System (Stratagene; Agilent Technologies Division, Santa Clara, CA). Sequences for each target gene were obtained from GenBank. Forward and reverse primers and TaqMan probes were selected by Primer Express 1.0 (Applied Biosystems) and synthesized by SIGMA GENOSYS (Ishikari, Japan). Each primer and TaqMan probe set used is described in our previous publication (Saito et al., 2011). qPCR reaction mixtures (25  $\mu$ l) contained 10 ng of template cDNA, 400 nM of forward and reverse primers, 60 nM TaqMan probe, 30 nM ROX, and Brilliant II Fast QPCR Master Mix (Stratagene; Agilent Technologies Division). The thermal cycling conditions were 2 min for polymerase activation and cDNA denaturation at 95 °C and 45 cycles of 95 °C for 5 s and 60 °C for 20 s. Constitutively expressed G3PDH served as a normalization control by using the  $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). In the examination of mRNA from whole blood cells, percent suppression was calculated as follows:

% suppression =  $(1 - \frac{\text{normalized mRNA expression of WBC in the presence of drugs}}{\text{normalized mRNA expression of WBC in the absence of drugs}}) \times 100$ .

## 2.7. Statistics

Representative data from at least three independent experiments for each analysis is shown. For each experiment, a one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. For comparison of three independent experiments, the Student's *t*-test was used to evaluate statistical significance. *p* values <0.05 were considered statistically significant.

## 3. Results

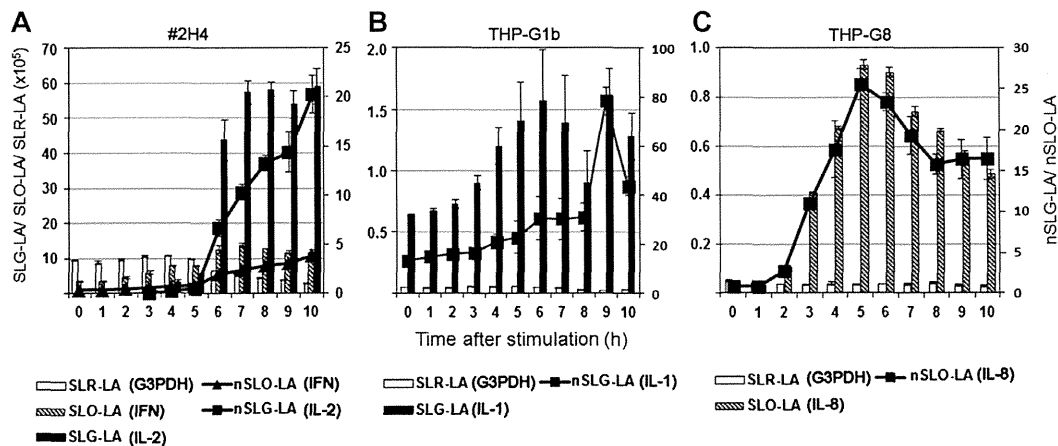
### 3.1. Three reporter cell lines, #2H4, THP-G1b, and THP-G8, responded with relevant stimulations by augmenting their SLG-LA or SLO-LA

First, we stimulated #2H4 cells with PMA/Io and THP-G1b or THP-G8 cells with LPS and measured SLG-LA, SLO-LA, and SLR-LA

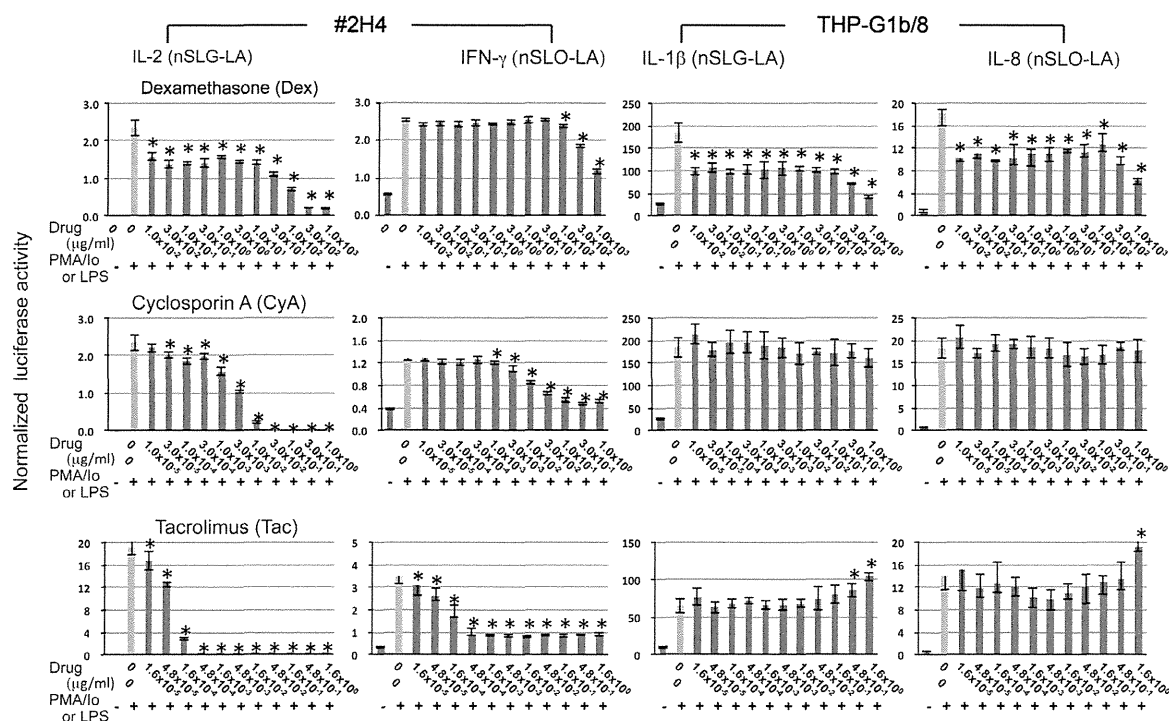
after stimulation. PMA/Io significantly augmented SLG(IL2)-LA and SLO(IFN)-LA of #2H4 cells corresponding with IL-2 and IFN- $\gamma$  promoter activities, respectively, from 6 h after stimulation, while it suppressed SLR(G3PDH)-LA corresponding with G3PDH promoter activity (Fig. 1A). Similarly, LPS significantly augmented SLG(IL1)-LA of THP-G1b cells corresponding with IL-1 $\beta$  promoter activity and SLO(IL8)-LA of THP-G8 cells corresponding with IL-8 promoter activity without affecting SLR(G3PDH)-LA of both cell lines from 3 h after stimulation (Fig. 1B and C). In Fig. 1A, B, and C, we also presented nSLG(IL2)-LA and nSLO(IFN)-LA of #2H4 cells, nSLG(IL1)-LA of THP-G1b cells, and nSLO-LA(IL8) of THP-G8 cells at various time periods after stimulation. PMA/Io significantly and time-dependently augmented both nSLG(IL2)-LA and nSLO(IFN)-LA of #2H4 cells from 6 to 10 h after stimulation. On the other hand, LPS significantly and time-dependently augmented nSLG(IL1)-LA of THP-G1b cells from 4 to 9 h after stimulation, while it significantly augmented nSLO(IL8)-LA of THP-G8 cells from 3 to 10 h after stimulation, with maximum induction at 5 h.

### 3.2. The effects of 3 immunosuppressive drugs on the reporter activity of the three reporter cells correlate with their effects on mRNA expression by Jurkat or THP-1 cells

Next, we examined whether the effects of 3 well-characterized immunosuppressive drugs, Dex, CyA, and Tac, on nSLG-LA or nSLO-LA of 3 reporter cells stimulated with PMA/Io or LPS correlate with their effects on the corresponding mRNA expression by Jurkat or THP-1 cells (Fig. 2). When we stimulated #2H4 cells with PMA/Io in the presence of Dex, CyA or Tac, Dex significantly suppressed nSLG(IL2)-LA at concentrations of 0.01  $\mu$ g/ml and greater ( $\geq 0.01$   $\mu$ g/ml) and nSLO(IFN)-LA at  $\geq 100$   $\mu$ g/ml, while CyA suppressed nSLG(IL2)-LA at  $\geq 0.03$  ng/ml and nSLO(IFN)-LA at  $\geq 0.001$   $\mu$ g/ml, and Tac suppressed nSLG(IL2)-LA and nSLO(IFN)-LA at  $\geq 0.016$  ng/ml. Although Dex suppressed nSLO(IFN)-LA, the concentration to decrease nSLO(IFN)-LA was greater than 100  $\mu$ g/ml, and the magnitude of the suppression was small. When we stimulated THP-G1b cells with LPS in the presence of Dex, CyA, or Tac, Dex significantly suppressed nSLG(IL1)-LA at  $\geq 0.01$   $\mu$ g/ml, but CyA and Tac did not. Similarly, when THP-G8 cells were stimulated with LPS in the presence of Dex, CyA, or Tac, only Dex significantly suppressed nSLO(IL8)-LA at  $\geq 0.01$   $\mu$ g/ml.



**Fig. 1.** Time course of IL-2, IFN- $\gamma$ , IL-1 $\beta$ , and IL-8 reporter activities in PMA/Io-stimulated #2H4 cells and LPS-stimulated THP-G1b or THP-G8 cells. #2H4 cells ( $2 \times 10^5$  cells/100  $\mu$ l/well) (A) in 96-well black plates were stimulated with PMA/Io, while THP-G1b (B) or THP-G8 cells ( $5 \times 10^4$  cells/100  $\mu$ l/well) (C) were treated with LPS. Then, SLG-LA, SLO-LA, and SLR-LA were measured after stimulation by using a microplate-type luminometer with a multi-color detection system. To rule out the variation of cell number or cell viability after chemical treatment, normalized SLG luciferase activity (nSLG-LA) or SLO luciferase activity (nSLO-LA) was obtained by dividing SLG-LA or SLO-LA with SLR-LA. Data represent means  $\pm$  SD (*n* = 4). SLG-LA (IL-2), SLO-LA (IFN- $\gamma$ ), and SLR-LA (G3PDH) in A, SLG-LA (IL-1 $\beta$ ) and SLR-LA (G3PDH) in B, and SLO-LA (IL-8) and SLR-LA (G3PDH) in C.



**Fig. 2.** The effects of Dex, CyA, and Tac on IL-2, IFN- $\gamma$ , IL-1 $\beta$ , or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 6 h. Luciferase activity was determined by using a microplate-type luminometer with a multi-color detection system. IL-2 and IFN- $\gamma$  promoter activities are represented as nSLG-LA and nSLO-LA of #2H4 cells, respectively. IL-1 $\beta$  and IL-8 promoter activities are represented as nSLG-LA of THP-G1b and nSLO-LA of THP-G8 cells, respectively. Data represent means  $\pm$  SD ( $n = 4$ ). \* Means statistical significance ( $p < 0.05$ ). Gray bars indicate the value for stimulation without drugs. These results are representative of three independent experiments.

Next, we stimulated the mother cell line of #2H4 cells, Jurkat cells, with PMA/Io in the presence of Dex, CyA, or Tac and the IL-2 or IFN- $\gamma$  mRNA expression was examined by qPCR (Fig. 3). Dex suppressed only IL-2 mRNA expression at  $\geq 6 \mu\text{g/ml}$ , while both CyA and Tac suppressed IL-2 as well as IFN- $\gamma$  mRNA expression at concentrations of  $\geq 0.012 \mu\text{g/ml}$  and  $0.008 \mu\text{g/ml}$ , respectively. When the mother cell line of THP-G1b and THP-G8 cells, THP-1 cells, was stimulated with LPS in the presence of the three inhibitors, Dex significantly suppressed both IL-1 $\beta$  and IL-8 mRNA at the concentration of  $\geq 0.6 \mu\text{g/ml}$ , but CyA and Tac did not (Fig 3). These data indicate that the suppression profiles obtained by three reporter cell lines correlate closely with those obtained by qPCR analysis of mRNA expression by Jurkat cells or THP-1 cells. Thus, we designated the immunotoxicity assay using 3 reporter cell lines as Multi-ImmunoTox Assay (MITA).

### 3.3. The effects of 3 immunosuppressive drugs on mRNA expression by whole blood cells stimulated with PMA/Io or LPS also corresponded with their effects on the reporter activities of the 3 reporter cell lines

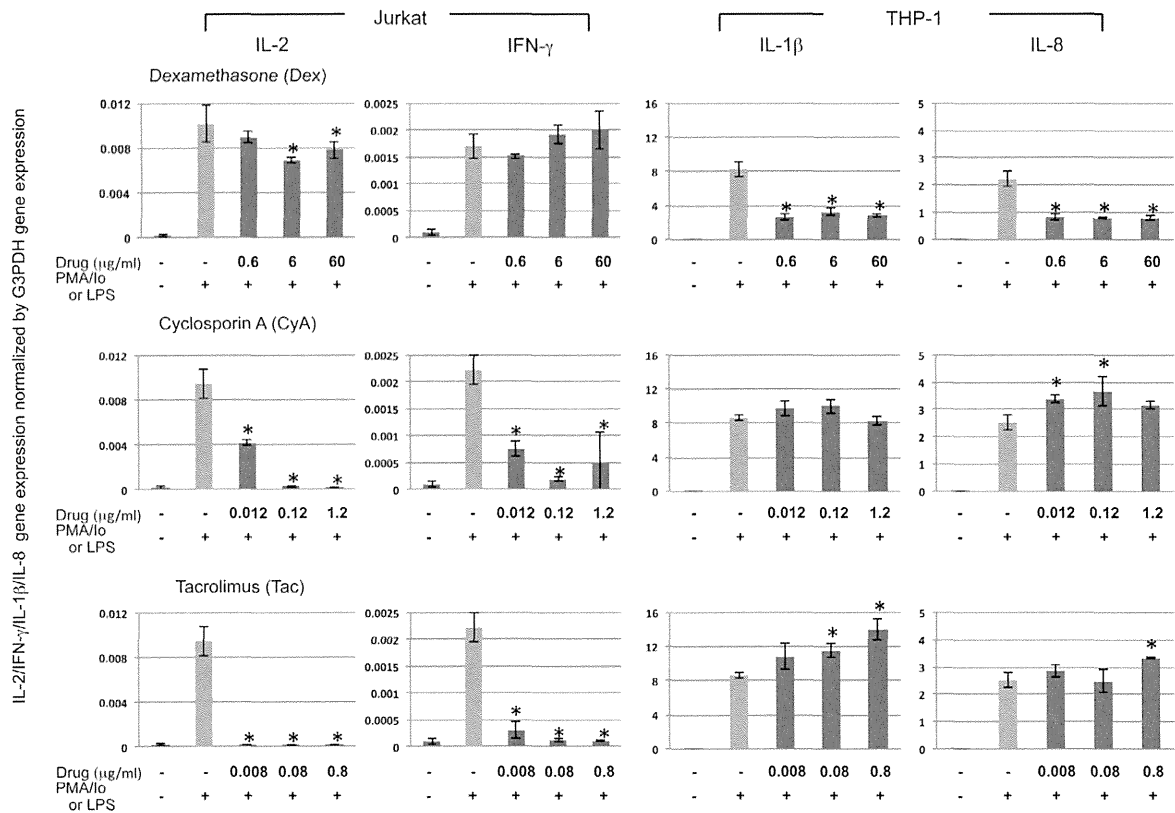
Next, we examined the correlation between HWBCRA and MITA. However, HWBCRA used SEB and LPS as stimulants and quantified IL-1 $\beta$  and IL-4 to characterize the immunotoxicity of chemicals. In contrast, MITA uses PMA/Io and LPS as stimulants and IL-2, IFN- $\gamma$ , IL-1 $\beta$ , and IL-8 promoter activities as outputs. Therefore, we stimulated whole blood cells (WBC) from healthy volunteers with 25 nM of PMA and 1  $\mu\text{M}$  of Io or 100 ng/ml LPS for 6 h in the presence or absence of 3 representative immunosuppressing drugs and analyzed IL-2, IFN- $\gamma$ , IL-1 $\beta$ , and IL-8 mRNA by qPCR (HWBCMET). Fig. 4, which shows the % suppression of 3 drugs

on the induction of mRNA for 4 cytokines in each individual, indicates that 1  $\mu\text{g/ml}$  of Dex, 1  $\mu\text{g/ml}$  of CyA, or 0.01  $\mu\text{g/ml}$  of Tac significantly suppressed both IL-2 and IFN- $\gamma$  mRNA induction after stimulation with PMA/Io, although the suppression by Dex was much smaller than that by CyA or Tac. On the other hand, only 1  $\mu\text{g/ml}$  of Dex significantly suppressed IL-1 $\beta$  and IL-8 mRNA induction by WBC stimulated with LPS, while CyA and Tac did not. These data suggest that the evaluation of 3 immunosuppressive drugs by MITA corresponded well with that by HWBCMET, although the statistical analysis on MITA results could not detect the effects of Dex on IFN- $\gamma$  reporter activity.

### 3.4. Several immunosuppressive drugs suppressed the reporter activity of the 3 reporter cell lines at concentrations equal to or less than $5 \times \text{Cmax}$

Based on these results, we evaluated other immunosuppressive or immunomodulatory drugs by using the 3 reporter cell lines. In Table 1, we present the results of immunosuppressive drugs classified by their principal mechanism (reviewed by Allison (2000)). Dex regulates gene expression; CyA, Tac, and RPM inhibit kinase or phosphatase; CP alkylates DNA; AZ, MPA, and MZR inhibit de novo purine synthesis; and MTX inhibits pyrimidine and purine synthesis.

We conducted 3 independent experiments for each drug and determined in each experiment whether chemicals induce statistically significant suppression or augmentation at two concentration ranges, equal or less than  $5 \times \text{Cmax}$  ( $\leq 5 \times \text{Cmax}$ ) and greater than  $5 \times \text{Cmax}$  ( $> 5 \times \text{Cmax}$ ).  $\text{Cmax}$  is defined as the peak plasma concentration of each drug after administration.  $\text{Cmax}$  of each drug



**Fig. 3.** The effects of Dex, CyA, and Tac on mRNA expression of IL-2 and IFN- $\gamma$  by Jurkat cells and that of IL-1 $\beta$  and IL-8 by THP-1 cells ( $3 \times 10^6$  cells/well) in 6-well plates were pretreated with different concentrations of drugs for 1 h, followed by stimulation with PMA/lo or LPS for 6 h, respectively. Then, mRNA expression of the indicated genes was analyzed by qPCR. The gene expressions were normalized by G3PDH gene expression. Data represent means  $\pm$  SD ( $n = 3$ ). \* Means statistical significance ( $p < 0.05$ ). Gray bars indicate the value for stimulation without drugs.

is shown along with dose and route of administration in Table S1. Since the drug concentration of interstitial fluid can be higher than that of blood in some drugs (Kiang et al., 2012; Wiskirchen et al., 2011) and  $C_{max}$  is variable among subjects, we evaluated drugs in these two different concentration ranges, considering that if a drug suppresses or augments cytokine reporter activities at  $\leq 5 \times C_{max}$ , the observed effects can be expected in clinical use. On the other hand, when the effects are recognized only at  $>5 \times C_{max}$ , the drug potentially has immunomodulatory effects, which are not expected in clinical use. Statistically significant suppression is shown as  $-$ , statistically significant augmentation as  $+$ , and no significance as  $0$ ; and a lack of data due to the inability to dissolve chemicals in solvents at the concentration of  $5 \times C_{max}$  is shown as ND.

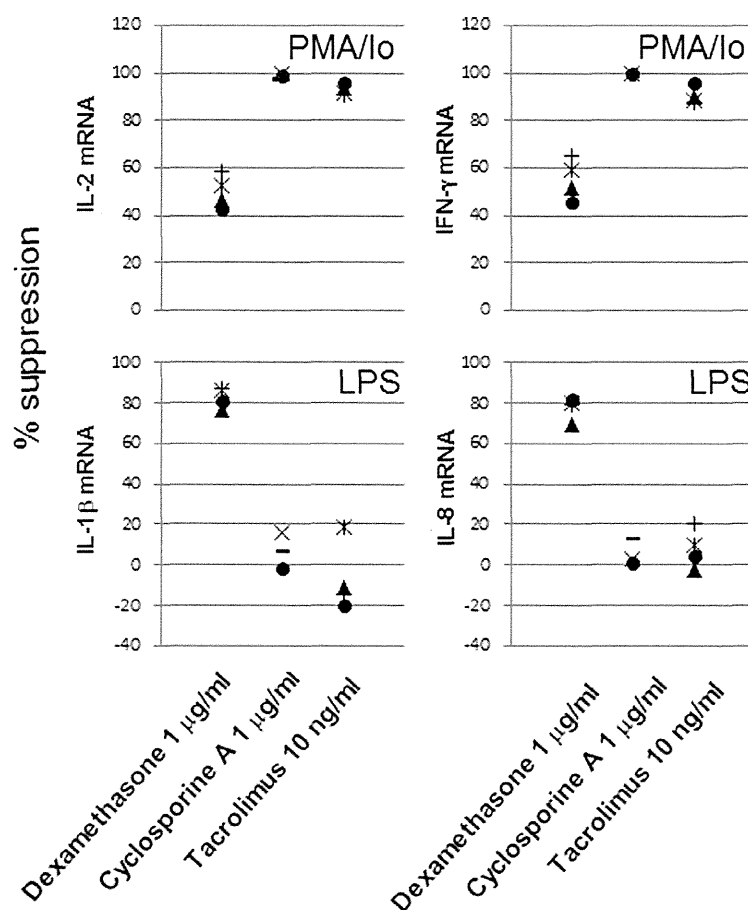
Furthermore, since the statistical evaluation of the chemicals in each experiment was not necessarily consistent among three independent experiments, we conducted statistical analysis on the results of 3 independent experiments. If chemicals showed statistically significant immunosuppression or immunostimulation 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 three experiments that

showed the most remarkable change, calculated their percent suppression or percent augmentation, and statistically compared suppression or augmentation of chemicals with that of vehicle control in three different experiments by the Student's  $t$ -test. Only when chemicals demonstrated statistical significance, they were judged as immunosuppressive or immunostimulatory, respectively. In Table 1, the final judgment of immunotoxicity of chemicals by MITA was indicated as S for immunosuppression, A for immunostimulation, and N for no effect.

As mentioned above, Dex significantly suppressed IL-2, IL-1 $\beta$ , and IL-8 reporter activities at any concentration ranges in at least 2 of 3 experiments and that of IFN- $\gamma$  at  $>5 \times C_{max}$  in 2 of 3 experiments (Table 1). Among 3 kinase or phosphatase inhibitors, CyA and Tac suppressed IL-2 and IFN- $\gamma$  reporter activities at  $\leq 5 \times C_{max}$  in 3 experiments, while rapamycin did not show any inhibitory effects on IL-2 or IFN- $\gamma$  reporter activity, but rather augmented IL-2 reporter activity at  $\leq 5 \times C_{max}$  in 2 of 3 experiments and IL-1 $\beta$  reporter activity at  $>5 \times C_{max}$  in 3 experiments (Table 1). In addition to the evaluations in which 3 out of 3 experiments demonstrated consistent results, the statistical analysis on the examinations in which only 2 out of 3 experiments demonstrated consistent results showed significant suppression in IL-1 $\beta$  reporter activity by Dex at  $\leq 5 \times C_{max}$  and significant augmentation in IL-2 reporter activity by RPM at both concentrations.

Among an alkylating agent, inhibitors of de novo purine synthesis, and an inhibitor of pyrimidine and purine synthesis (Table 1), none of the drugs except for AZ demonstrated immunosuppressive effects at any concentration ranges. Only AZ significantly suppressed



**Fig. 4.** The effects of Dex, CyA, and Tac on IL-2, IFN- $\gamma$ , IL-1 $\beta$ , or IL-8 mRNA expression of whole blood cells stimulated with PMA/Io or LPS. Whole blood cells (WBC) from 6 healthy volunteers were collected using sodium heparin anti-coagulant and then diluted 1:2 with RPMI 1640 medium. Two ml of aliquots was either untreated or treated with 1  $\mu$ g/ml of Dex, or 1  $\mu$ g/ml of CyA or 10 ng/ml of Tac for 1 h. Then, the WBC were stimulated with PMA/Io or LPS for 6 h at 37 °C. The mRNA expression of indicated genes was analyzed by qPCR. The gene expressions were normalized by G3PDH gene expression. Percent suppression was calculated as described in Section 2. The percent suppression of drugs in each individual is plotted. Each symbol indicates a different individual.

IL-8 reporter activities at  $>5 \times C_{max}$  in 2 of 3 experiments. On the other hand, MPA significantly augmented IL-2 and IFN- $\gamma$  reporter activities at  $\leq 5 \times C_{max}$  in 3 experiments. MZR increased all reporter activities at any concentration ranges in at least 2 of 3 experiments. In addition, CP augmented IFN- $\gamma$  reporter activity at  $>5 \times C_{max}$  in 3 experiments, while MTX augmented IL-2 and IFN- $\gamma$  reporter activities at any concentration ranges in 2 of 3 experiments. The statistical analysis on the examinations in which only 2 out of 3 experiments demonstrated consistent results showed significant augmentation in IL-1 $\beta$  reporter activity by AZ at  $>5 \times C_{max}$ , IL-2 reporter activity by MTX at  $>5 \times C_{max}$ , and IFN- $\gamma$  reporter activity by MTX at both concentrations.

### 3.5. Sulfasalazine and nicotinamide significantly suppressed IL-1 $\beta$ and IL-8 reporter activities

Next, we examined the effects of drugs that are not classified as immunosuppressive or immunomodulatory drugs, but are currently in off-label use for the treatment of autoimmune or inflammatory disorders (Capell and Madhok, 2008; Chaiamnuay and Alarcon, 2008; Sturrock, 2008; Surjana and Damian, 2011) (Fig. 5 and Table 1). Among 5 off-label immunosuppressive drugs, SASP significantly suppressed 4 reporter activities at any concentration

ranges in at least 2 of 3 experiments. CQ significantly suppressed IL-2 and IFN- $\gamma$  reporter activities at any concentration ranges in at least 2 of 3 experiments, while it significantly suppressed IL-1 $\beta$  and IL-8 at  $>5 \times C_{max}$  in 3 experiments. MC significantly suppressed IL-2 and IFN- $\gamma$  reporter activities at any concentration ranges in 3 experiments, while it did not affect IL-1 $\beta$  and IL-8 reporter activities. NA significantly suppressed IL-1 $\beta$  and IL-8 reporter activities at any concentrations in 3 experiments, while it augmented IL-2 and IFN- $\gamma$  reporter activities at any concentrations in at least 2 of 3 experiments. Colchicine increased IL-1 $\beta$  reporter activity at  $\leq 5 \times C_{max}$  in 2 of 3 experiments and all reporter activities at  $>5 \times C_{max}$  in 3 experiments. The statistical analysis of the examinations in which only 2 out of 3 experiments demonstrated consistent results showed significant augmentation in IL-1 $\beta$  reporter activity by colchicine at  $\leq 5 \times C_{max}$ .

### 3.6. Two non-immunological drugs, warfarin and digoxin, suppressed some reporter activities at concentrations of $\leq 5 \times C_{max}$

We examined the effects of 3 non-immunological drugs on the reporter activities of the three reporter cell lines (Fig. 6 and Table 1). Warfarin suppressed IL-1 $\beta$  and IL-8 reporter activities at  $\leq 5 \times C_{max}$  in 2 of 3 experiments, while it augmented IL-2 and

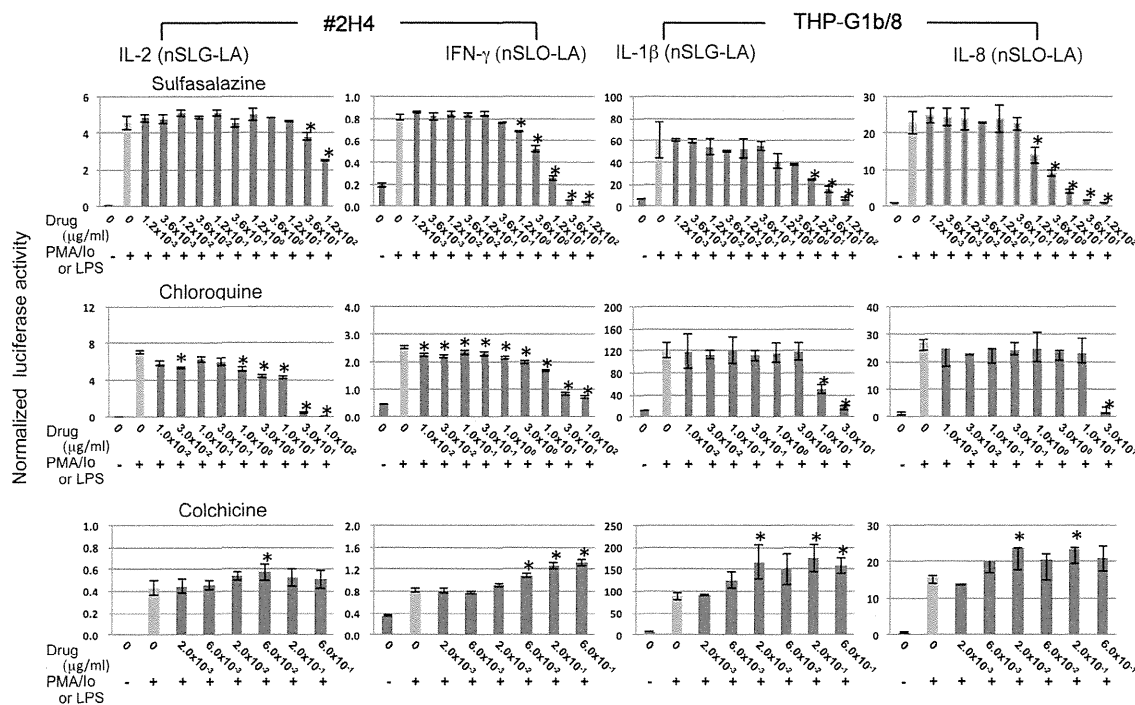


**Table 1**  
Summary of MITA for 9 immunosuppressive drugs, 5 off-label immunosuppressive drugs, and 3 non-immunomodulatory drugs.

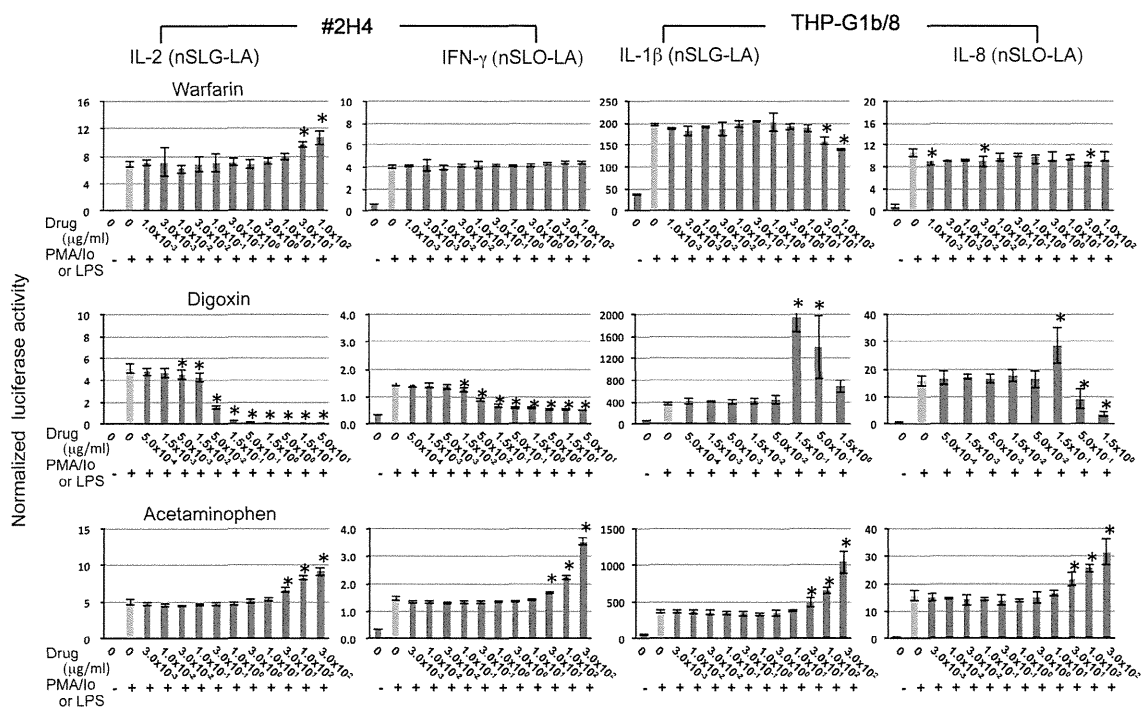
Principal mechanism of action	Cmax	IL-2		IFN- $\gamma$		IL-1 $\beta$		IL-8				
		$\leq 5 \times \text{Cmax}$	$> 5 \times \text{Cmax}$	$\leq 5 \times \text{Cmax}$	$> 5 \times \text{Cmax}$	$\leq \times \text{Cmax}$	$> 5 \times \text{Cmax}$	$\leq 5 \times \text{Cmax}$	$> 5 \times \text{Cmax}$			
<i>Immunosuppressing drugs</i>												
Regulation of gene expression	Dexamethasone (Dex)	88 ng/ml	-/-/	S -/-/	S -/0/0	N -/+/-	N -/-/	S -/-/	S 0	S -/-/	S -/-/	S
Kinase and phosphatase inhibitors	Cyclosporin A (CyA)	2144 $\mu\text{g/ml}$	-/-/	S ND/ND/ND	-/-/	S ND/ND/ND	0/0/0	N ND/ND/ND	0/-/0	N ND/ND/ND	ND/ND/ND	N
	Tacrolimus (Tac)	44.6 ng/ml	-/-/	S -/-/	S -/-/	S -/-/	S +/0/0	N 0/+/0	N -/0/0	N 0/+/0	N 0/+/0	N
Alkylation	Rapamycin (RPM)	4.0 ng/ml	0/+/*	A 0/+/*	A 0/-/0	N 0/0/0	N 0/0/0	N +/+/*	A 0/0/0	N 0/0/0	N 0/0/0	N
	Cyclophosphamide (CP)	6.36 $\mu\text{g/ml}$	+/0/-	N 0/0/-	N +/0/-	N +/+/*	A 0/0/-	N 0/0/-	N 0/0/-	N 0/+/-	N 0/+/-	N
Inhibition of de novo purine synthesis	Azathioprine (AZ)	73.7 ng/ml	0/0/0	N 0/-/	N 0/0/0	N +/+/*	A 0/0/0	N +/-/*	A 0/0/0	N +/-/	N +/-/	N
	Mycophenolic acid (MPA)	34.0 $\mu\text{g/ml}$	+/*/*	A ND/ND/ND	+/*/*	A ND/ND/ND	0/0/+	N ND/ND/ND	0/0/0	N ND/ND/ND	ND/ND/ND	N
Inhibition of pyrimidine and purine synthesis	Mizoribine (MZR)	9.6 $\mu\text{g/ml}$	+/*/-	N +/*/0	N +/*/-	N +/0/+	N +/*/+	A +/*/+	A +/*/+	A +/*/+	A +/*/+	A
	Methotrexate (MTX)	162.2 ng/ml	+/0/+	N +/0/+	A 0/+/*	A 0/+/*	A 0/0/-	N 0/0/-	N 0/0/-	N 0/0/-	N 0/0/-	N
<i>Off-label immunosuppressing drugs</i>												
	Sulfasalazine (SASP)	15.6 $\mu\text{g/ml}$	+/-/-	N -/-/	S -/-/	S -/-/	S -/-/	S -/-/	S -/-/	S -/-/	S -/-/	S
	Colchicine	5.64 ng/ml	0/0/0	N +/*/*	A 0/+/0	N +/*/*	A +/0/+	A +/*/*	A +/0/0	N +/*/*	N +/*/*	A
	Chloroquine (CQ)	555 ng/ml	-/-/	S -/-/	S -/-/0	N -/-/	S 0/0/0	N -/-/	S 0/0/0	N -/-/	S -/-/	S
	Minocycline (MC)	4.8 $\mu\text{g/ml}$	-/-/	S -/-/	S -/-/	S -/-/	S 0/0/0	N 0/0/0	N 0/0/0	N 0/0/0	N 0/0/+	N
	Nicotinamide (NA)	22.4-26.3 $\mu\text{g/ml}$	+/-/+	N +/*/*	A 0/+/*	N +/*/*	A -/-/	S -/-/	S -/-/	S -/-/	S -/-/	S
<i>Non-immunomodulatory drugs</i>												
	Acetaminophen (AA)	9.4 ng/ml	+/*/*	A +/*/*	A +/*/*	A +/*/*	A +/0/0	N +/*/*	A +/0/0	N +/*/*	N +/*/*	A
	Digoxin	2.92 ng/ml	-/0/-	N -/-/	S 0/0/-	N -/-/	S 0/0/0	N +/0/0	N 0/0/0	N +/0/0	N +/0/0	N
	Warfarin	685 $\mu\text{g/ml}$	+/*/*	A ND/ND/ND	0/+/*	N ND/ND/ND	-/0/	S ND/ND/ND	-/0/	S ND/ND/ND	0	N

- and + mean significant suppression and augmentation by one-way ANOVA followed by a Dunnett's post hoc test compared with the control group, respectively. 0 means no significant change.

\* Means statistical significance by Student's t test S, A, and N indicate immunosuppression, immunoaugmentation, and no effect in final judgment, respectively.



**Fig. 5.** The effects of SASP, CQ, and colchicine on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/lo or LPS for 6 h. Luciferase activity was determined by using a microplate-type luminometer with a multi-color detection system. Data represent means ± SD (n = 4). \* Means statistical significance (p < 0.05). Gray bars indicate the value for stimulation without drugs. These results are representative of three independent experiments.



**Fig. 6.** The effects of warfarin, digoxin, and AA on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/lo or LPS for 6 h. Luciferase activity was determined by using a microplate-type luminometer with a multi-color detection system. Data represent means ± SD (n = 4). \* Means statistical significance (p < 0.05). Gray bars indicate the value for stimulation without drugs. These results are representative of three independent experiments.

IFN- $\gamma$  reporter activities at  $\leq 5 \times C_{max}$  in at least 2 of 3 experiments. Digoxin suppressed IL-2 reporter activity at  $\leq 5 \times C_{max}$  in 2 of 3 experiments, while it suppressed IL-2 and IFN- $\gamma$  reporter activities at  $> 5 \times C_{max}$  in 3 experiments. AA augmented IL-2 and IFN- $\gamma$  reporter activities at  $\leq 5 \times C_{max}$  in 3 experiments and 4 reporter activities at  $> 5 \times C_{max}$ . The statistical analysis of the examinations in which only 2 out of 3 experiments demonstrated consistent results showed significant suppression in IL-8 and IL-1 $\beta$  reporter activities by warfarin at  $\leq 5 \times C_{max}$ .

#### 4. Discussion

We developed an immunotoxicity assay system, the Multi-ImmunoTox Assay (MITA), with 3 reporter cell lines that can evaluate the effects of chemicals on the promoter activity of IL-2, IFN- $\gamma$ , IL-1 $\beta$ , and IL-8. Then, we demonstrated the tight correlation between the evaluation of the effects of 3 representative immunosuppressive drugs based on MITA and that based on mRNA expression of the mother cell lines, Jurkat cells and THP-1 cells. There was a minor discrepancy between the two assays. The assay based on qPCR demonstrated significant augmentation of IL-1 $\beta$  or IL-8 mRNA expression by THP-1 cells treated with CyA or Tac. Although we do not know the exact reason for the discrepancy, the results obtained by HWBCMET suggest that the data obtained by MITA are more appropriate.

Next, we demonstrated the correlation between the evaluation of the effects of these 3 immunosuppressive drugs based on MITA and that based on mRNA expression of whole blood cells. In the originally reported HWBCRA, the final output was determined by the amount of released cytokines. In contrast, our altered method of HWBCRA, HWBCMET, measured mRNA expression of cytokines by qPCR. When we compared the immunosuppressive effects of Dex, CyA, and Tac recognized by HWBCMET with those by the original HWBCRA, which were reported by Langezaal et al. (2002), the qualitative HWBCMET evaluation of these 3 immunosuppressive drugs was consistent with that of HWBCRA. Namely, in both assays, CyA and Tac were more potent in the suppression of T cell cytokine expression than Dex, while Dex was more potent in the suppression of monocyte cytokine expression than CyA and Tac. Next, we demonstrated that the qualitative evaluation of Dex, CyA and Tac was consistent between HWBCMET and MITA, which indicates that the qualitative evaluation by MITA was also consistent with that by the original HWBCRA. Furthermore, the results demonstrating that CyA or Tac significantly suppressed both IL-2 and IFN- $\gamma$  mRNA induction after stimulation with PMA/Io, while only Dex significantly suppressed IL-1 $\beta$  and IL-8 mRNA induction by LPS, are consistent with the previously reported effects of these drugs on human T cells or macrophages (reviewed in Saag (2008) and Furst and Clements (2008)).

Moreover, in general, when immunotoxicity of chemicals is examined by using human blood cells or murine spleen cells, it is not easy to determine whether the immunotoxicity is caused by their effects on T cells or antigen presenting cells such as monocytes and dendritic cells because of the difficulty in purifying each population. Indeed, HWBCRA cannot confidently determine whether the detected immunological effects of chemicals are due to their direct effects on T cells or antigen presenting cells. In contrast, since MITA uses established T cell and monocyte cell lines, it can separately determine immunotoxicity of chemicals on T cells and monocytes.

In the present study, although we demonstrated that MITA can correctly characterize the effects of 3 representative immunosuppressive drugs, Dex, CyA, and Tac, on the cytokine production by T cells as well as monocytes, it did not reveal the immunosuppressive effects of rapamycin, an alkylating agent or inhibitors of

purine or pyrimidine synthesis. Rapamycin inhibits the action of growth-promoting cytokines, while both alkylating agents and antimetabolites induce immunosuppressive effects through their inhibitory action on cell division (reviewed by Hardinger et al. (2004)). In general, immunotoxicity assays detecting the inhibitory effects of chemicals on cytokine expression may not be able to detect their immunosuppressive effects. Indeed, HWBCRA could not detect the immunotoxicity of CP, AZ, and MZR either since the log IC50 values against release of IL-4 by CP, AZA, and MZR were beyond their therapeutic plasma concentrations (Langezaal et al., 2002). The Fluorescent Cell Chip (FCC) could not detect immunotoxicity of cyclophosphamide (Wagner et al., 2006). Therefore, at present, to overcome the drawbacks of these assays, they may need to be combined with assays that can detect the inhibitory action of chemicals on cell division, such as the conventional 28-day subacute toxicity test (Investigators, 1998).

Unexpectedly, rapamycin, CP or inhibitors of purine or pyrimidine synthesis augmented some reporter activities mostly at  $> 5 \times C_{max}$ . Since these immunosuppressive drugs inhibit cell growth at much lower concentration than they augment reporter activities, these effects might be overlooked in an *in vivo* system. Further investigation is required to clarify their mechanism and the significance in detecting immunotoxicity *in vitro*.

In addition to AZ and MZR, we also examined immunosuppressive effects of drugs evaluated by HWBCRA, such as colchicine, AA, digoxin, and warfarin (Langezaal et al., 2002). MITA did not detect any suppressive effects of AA on the 4 reporter activities at any concentration. Similarly, the log IC50 values against release of both IL-1 $\beta$  and IL-4 by AA were greater than 1 mM and much higher than  $5 \times C_{max}$ . MITA detected inhibitory effects of warfarin in IL-1 $\beta$  and IL-8 reporter activities, but not those in IL-2 or IFN- $\gamma$  reporter activities. Likewise, HWBCRA revealed that warfarin suppressed IL-1 $\beta$  formation more strongly than IL-4 formation. Moreover, MITA demonstrated that digoxin suppressed IL-2 and IFN- $\gamma$  reporter activities. Similarly, HWBCRA showed that it suppressed IL-4 release. These data are also consistent with the recent publication demonstrating the immunological effects of digoxin (Huh et al., 2011). Therefore, although MITA detected some immunological effects in presumptive non-immunological drugs, these drugs might have unrecognized immunoregulatory activities.

Finally, in the present study, we presented the performance of MITA in evaluation of immunosuppressive or immunostimulatory activities of chemicals and demonstrated that MITA can distinguish which cells, either T cells or monocytes, were primary targets for immunological effects of chemicals. We have already reported that, without any additional stimuli, THP-G8 cells can predict skin sensitizers with test accuracies of greater than 80% (IL-8 Luc assay) (Takahashi et al., 2011). Thus, taking the present and previous studies into consideration, we believe that MITA combined with the IL-8 Luc assay can present a novel high-throughput assay to detect immunotoxicity of chemicals and provide insight into their mechanism in humans. The obtained information from these assays can be used to assess the risks from chemicals by industries as well as regulatory agencies. Needless to say, a larger number of chemicals must be evaluated by MITA to determine the potential and limits of this technique.

#### Conflict of Interest

The authors declare that there are no conflicts of interest.

#### Transparency Document

The Transparency document associated with this article can be found in the online version.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tiv.2014.02.013>.

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