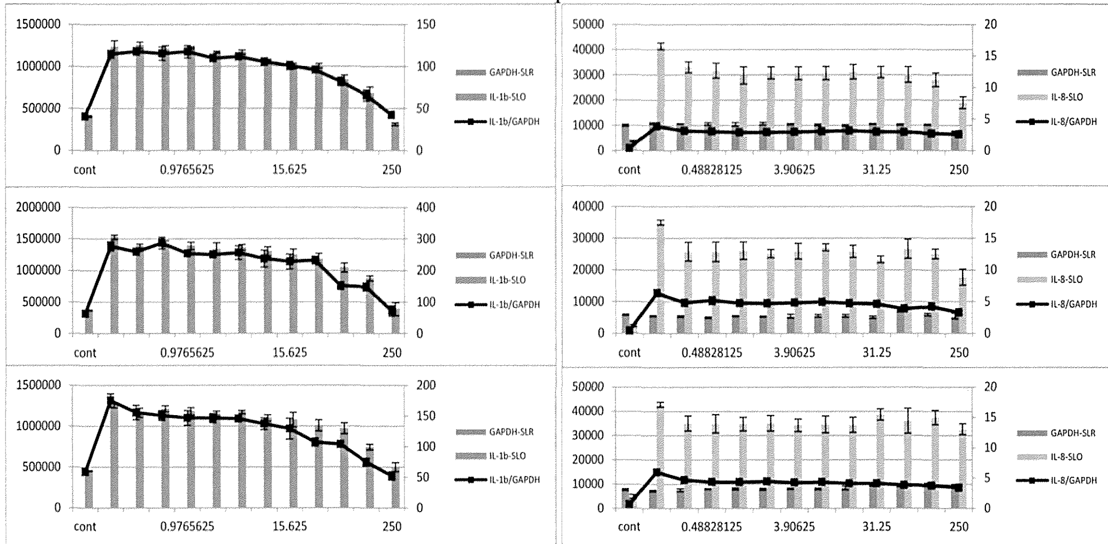
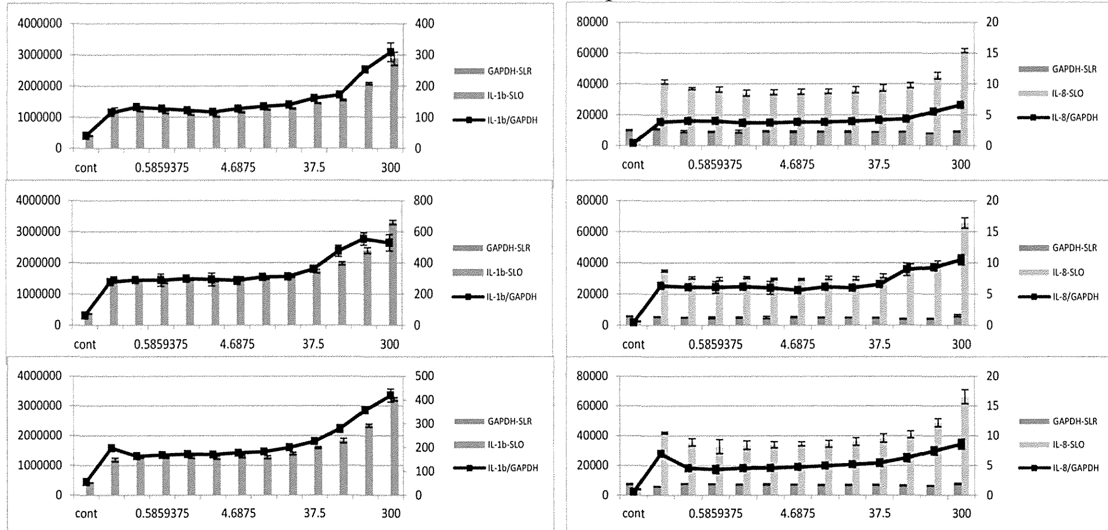
図４　#2H4 細胞（IL-2 および INF-γ）を用いた７物質の施設内再現性（続き）

Dapsone



Acetaminophen



Ethanol

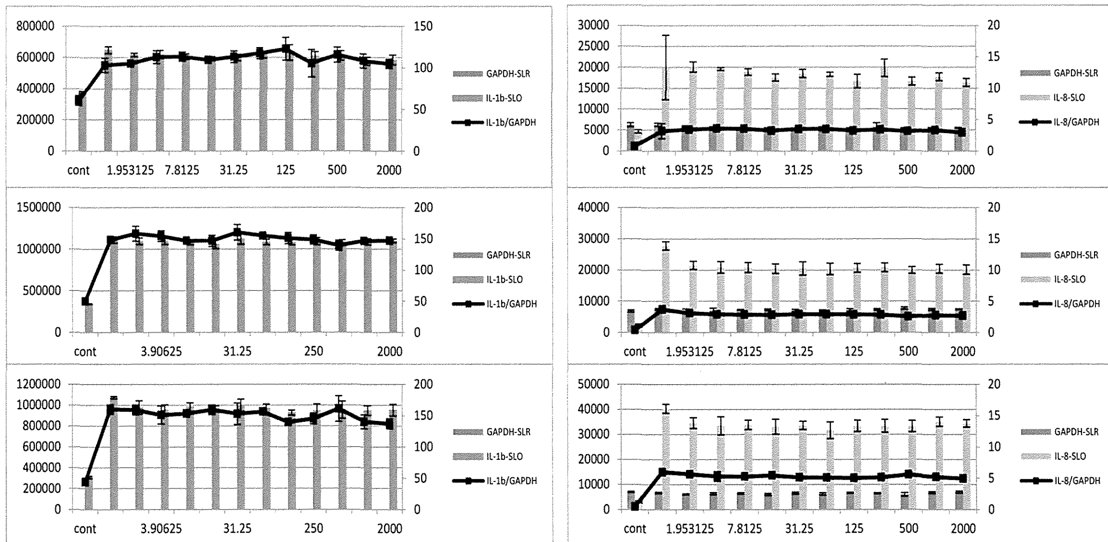


図５　THP-G1β 細胞(左)および THP-G8 細胞(右)を用いた７物質の施設内再現性

Sodium bromate

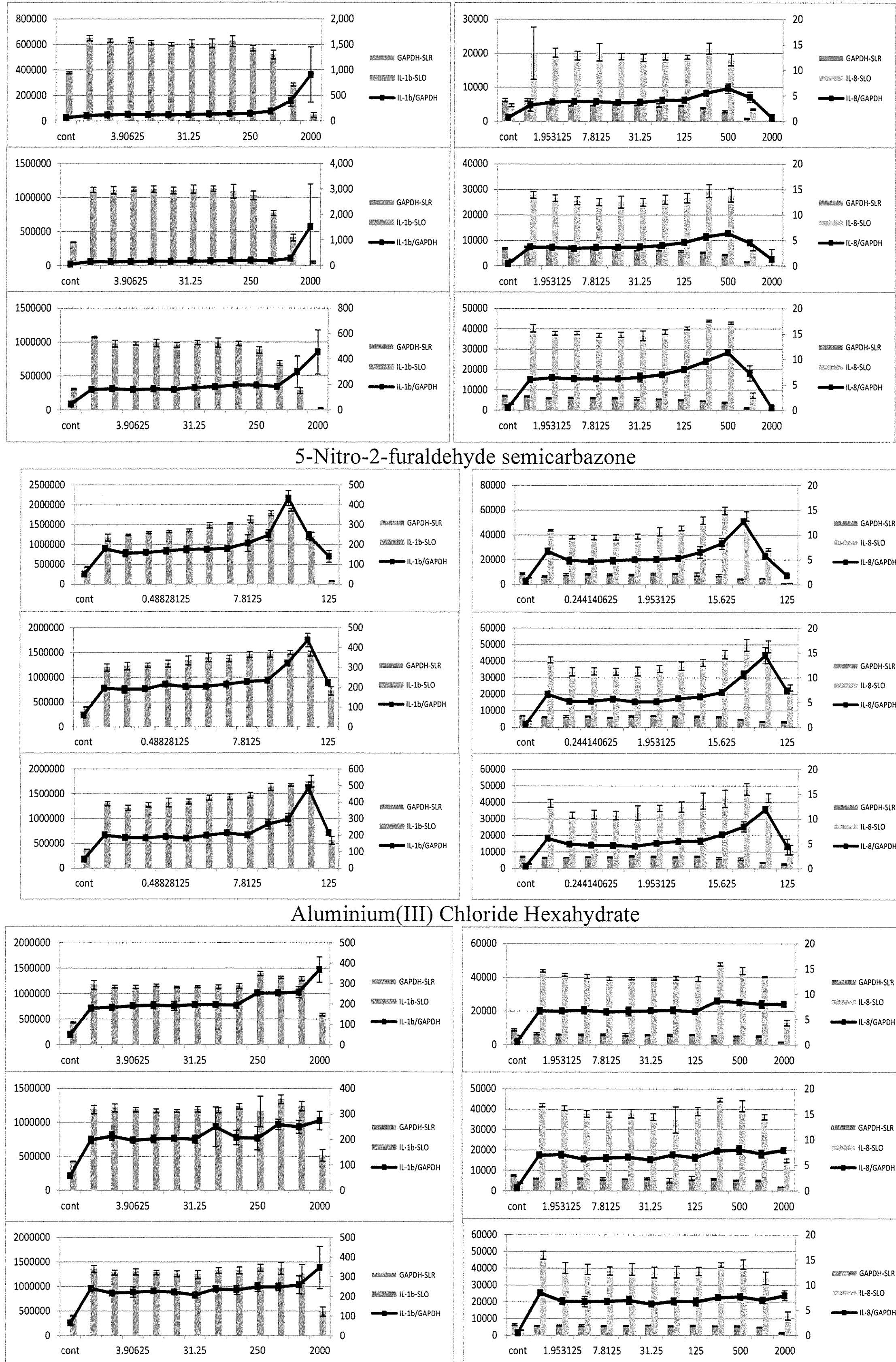


図５　THP-G1β 細胞(左)および THP-G8 細胞(右)を用いた７物質の施設内再現性(続き)

4-Nitroaniline

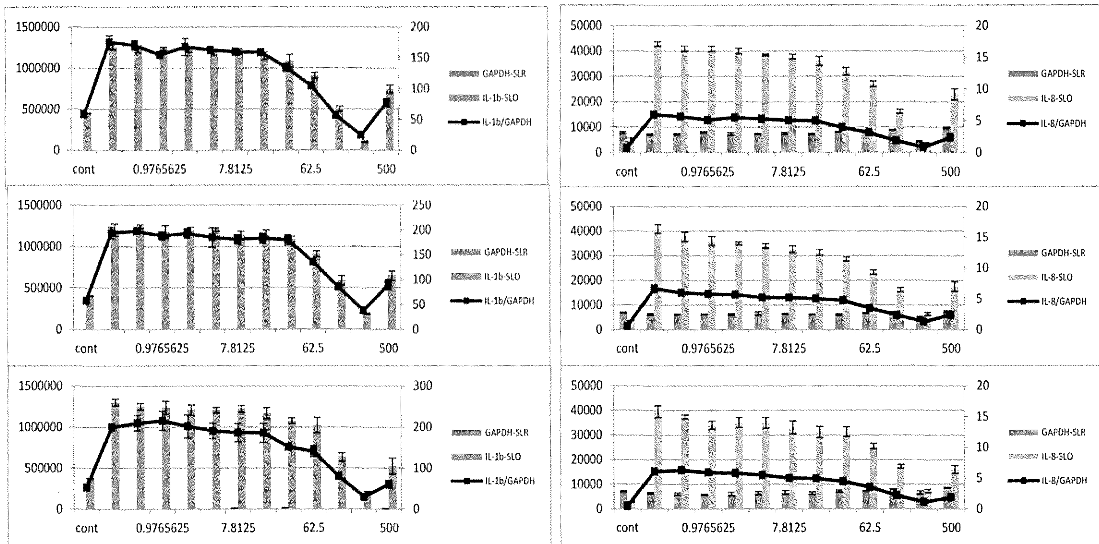
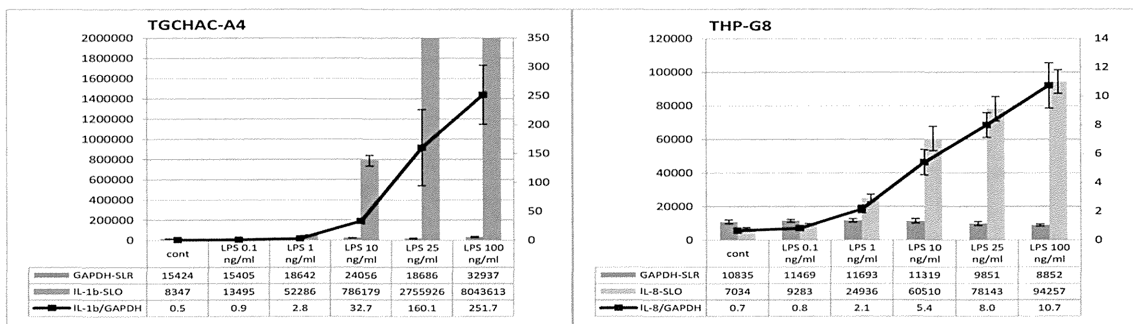


図５　THP-G1β 細胞(左)および THP-G8 細胞(右)を用いた７物質の施設内再現性(続き)



	cont	LPS 0.1 ng/ml	LPS 1 ng/ml	LPS 10 ng/ml	LPS 25 ng/ml	LPS 100 ng/ml
GAPDH-SLR	15424	15405	18642	24056	18686	32937
IL-1b-SLO	8347	13495	52286	786179	2755926	8043613
IL-1b/GAPDH	0.5	0.9	2.8	32.7	160.1	251.7

	cont	LPS 0.1 ng/ml	LPS 1 ng/ml	LPS 10 ng/ml	LPS 25 ng/ml	LPS 100 ng/ml
GAPDH-SLR	10835	11469	11693	11319	9851	8852
IL-8-SLO	7034	9283	24936	60510	78143	94257
IL-8/GAPDH	0.7	0.8	2.1	5.4	8.0	10.7

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

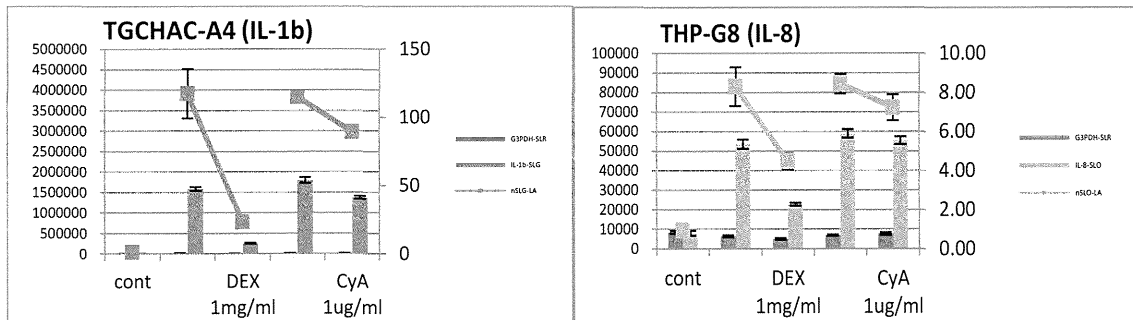
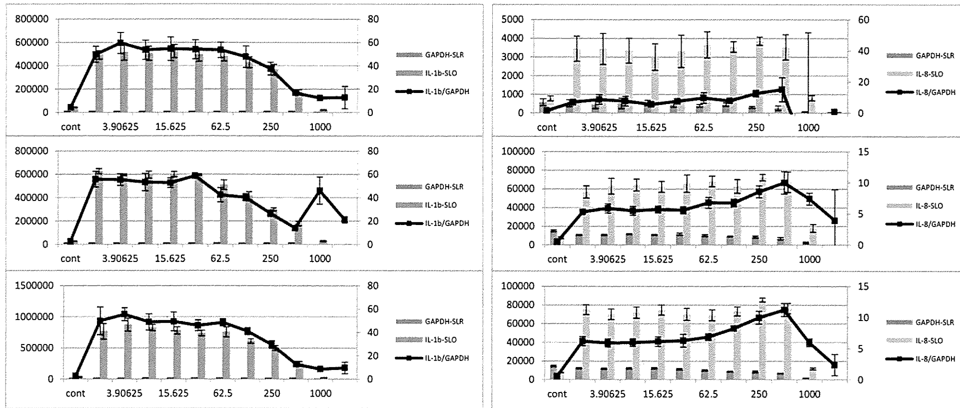
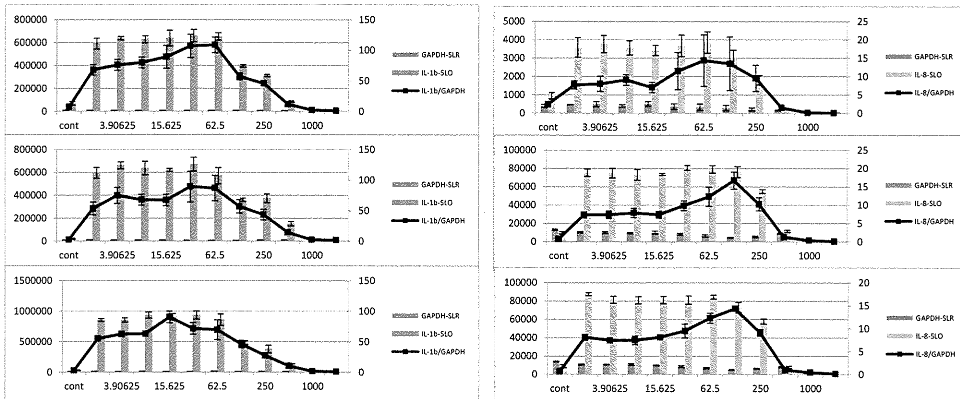


図７　TGCHAT-A4 細胞(左)および THP-G8 細胞(右)の DEX および CyA に対する反応性

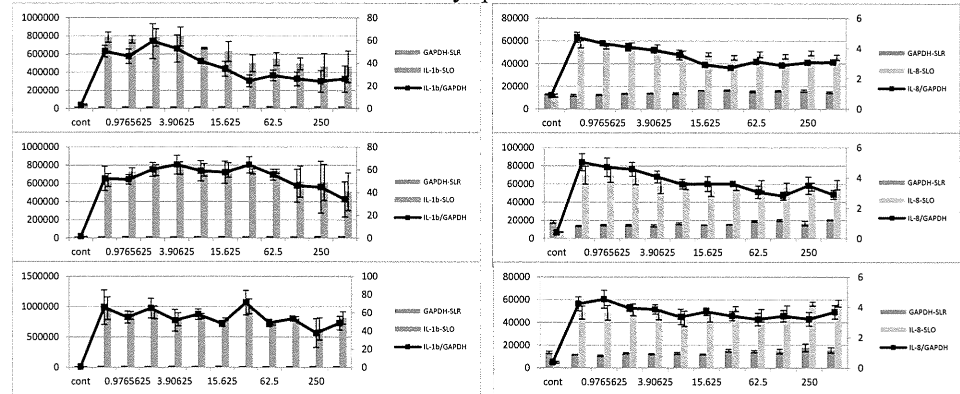
Sodium bromate



Nickel sulfate



Dibutyl phthalate



2-MBT

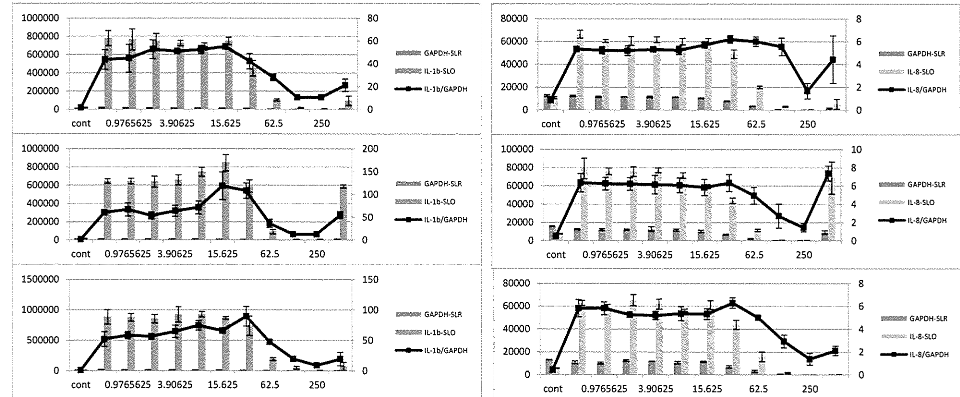


図８　TGCHAT-A4 細胞(左)および THP-G8 細胞(右)における４物質の施設内再現性

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。前年度、*in vitro*免疫毒性評価を目的に開発されたMITAの施設内および施設間再現性を確認するため実施した10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。実施したデータを再検討したところ、3ないし4ウェル間隔で値が低下するという傾向が認められたことから、連続分注している細胞賦活化試薬の添加操作に原因があると考えられた。そこで、同一プレート内で連続分注する群と単回で分注する群を設定して同時測定を実施した。その結果、連続分注した場合、分注回数が増えると値が低下する傾向が再現された。これは、細胞賦活化試薬を添加する際にはチップの先を培地につけて確実に添加するというプロトコルに従っていることから、複数ウェル分の細胞賦活化試薬を吸引する際にチップ外側に付着した微量の細胞賦活化試薬が添加の際にウェル内に持ち込まれるが、添加回数が増す毎にウェル内に持ち込まれる細胞賦活化試薬の量が減少することに起因していると考えられた。細胞賦活化剤は微量で効果を示すことから、目視で確認できない微量の持ち込み量がウェル間で異なるという結果になったものと考えられる。

この点を改善して再試験を実施した7物質については、多少のバラツキは認められるものの明らかな傾向としては認められず、手技としては改善されたと考えられる。

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

微量で強い反応を示す細胞賦活化剤の添加には注意が必要であることが明らかとなった。また、新たに開発されたTGCHAC-A4細胞は非常に安定した反応性を示したことから、今後のMITA用細胞として使用することで安定した結果が期待される。

F. 参考文献

なし

G. 研究発表

なし

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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研究成果の刊行物・別刷

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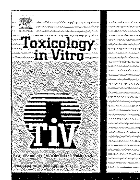

Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs

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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-γ, IL-1β, 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, prescreening 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-1β 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-1β 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 ECVAM 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-γ, IL-8, and IL-1β promoters. We selected these 4 cytokines because IL-2 and IFN-γ are mainly produced by T cells and reflect T-cell function, while IL-8 and IL-1β 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 026: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_2. 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-γ 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-1β 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 (2×10^5 cells/50 μl/well), THP-G1b cells, or THP-G8 cells (5×10^4 cells/50 μl/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 μM 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 multicolor 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 − nSLG-LA or nSLO-LA of the reporter cells treated with drugs/nSLG-LA or nSLO-LA of non-treated reporter cells) × 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 (3×10^6 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 μl) 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 ΔΔCt method (Livak and Schmittgen, 2001). In the examination of mRNA from whole blood cells, percent suppression was calculated as follows:

% suppression = (1 − the normalized mRNA expression of WBC in the presence of drugs/the normalized mRNA expression of WBC in the absence of drugs) × 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-γ 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-1β 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 μg/ml and greater (⩾0.01 μg/ml) and nSLO(IFN)-LA at ⩾100 μg/ml, while CyA suppressed nSLG(IL2)-LA at ⩾0.03 ng/ml and nSLO(IFN)-LA at ⩾0.001 μg/ml, and Tac suppressed nSLG(IL2)-LA and nSLO(IFN)-LA at ⩾0.016 ng/ml. Although Dex suppressed nSLO(IFN)-LA, the concentration to decrease nSLO(IFN)-LA was greater than 100 μg/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 ⩾0.01 μg/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 ⩾0.01 μg/ml.

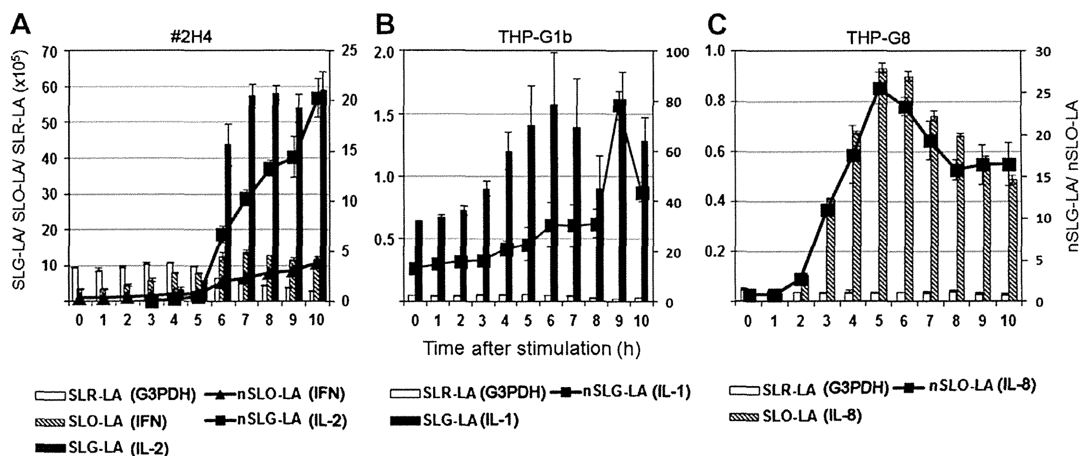

Fig. 1. Time course of IL-2, IFN-γ, IL-1β, and IL-8 reporter activities in PMA/Io-stimulated #2H4 cells and LPS-stimulated THP-G1b or THP-G8 cells. #2H4 cells (2 × 10^5 cells/100 μl/well) (A) in 96-well black plates were stimulated with PMA/Io, while THP-G1b (B) or THP-G8 cells (5 × 10^4 cells/100 μl/well) (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 +/− SD (*n* = 4). SLG-LA (IL-2), SLO-LA (IFN-γ), and SLR-LA (G3PDH) in A, SLG-LA (IL-1β) and SLR-LA (G3PDH) in B, and SLO-LA (IL-8) and SLR-LA (G3PDH) in C.

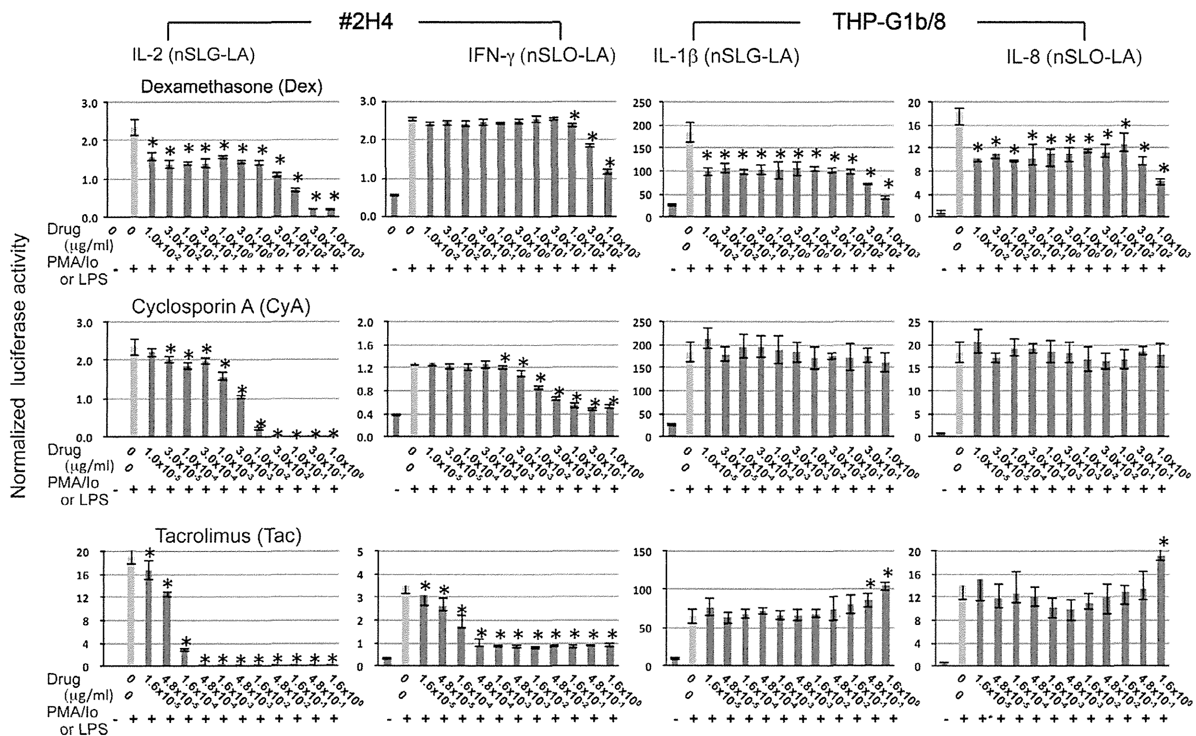

Fig. 2. The effects of Dex, CyA, and Tac on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 6 h. Luciferase activity was determined by using a microplate-type luminometer with a multi-color detection system. IL-2 and IFN-γ promoter activities are represented as nSLG-LA and nSLO-LA of #2H4 cells, respectively. IL-1β and IL-8 promoter activities are represented as nSLG-LA of THP-G1b and nSLO-LA of THP-G8 cells, respectively. 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.

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-γ mRNA expression was examined by qPCR (Fig. 3). Dex suppressed only IL-2 mRNA expression at ⩾6 μg/ml, while both CyA and Tac suppressed IL-2 as well as IFN-γ mRNA expression at concentrations of ⩾0.012 μg/ml and 0.008 μg/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-1β and IL-8 mRNA at the concentration of ⩾0.6 μg/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-1β and IL-4 to characterize the immunotoxicity of chemicals. In contrast, MITA uses PMA/Io and LPS as stimulants and IL-2, IFN-γ, IL-1β, and IL-8 promoter activities as outputs. Therefore, we stimulated whole blood cells (WBC) from healthy volunteers with 25 nM of PMA and 1 μM 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-γ, IL-1β, and IL-8 mRNA by q PCR (HWBCMET). Fig. 4, which shows the % suppression of 3 drugs on the induction of mRNA for 4 cytokines in each individual, indicates that 1 μg/ml of Dex, 1 μg/ml of CyA, or 0.01 μg/ml of Tac significantly suppressed both IL-2 and IFN-γ 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 μg/ml of Dex significantly suppressed IL-1β 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-γ 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 × 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 × Cmax (⩽5 × Cmax) and greater than 5 × Cmax (>5 × Cmax). Cmax is defined as the peak plasma concentration of each drug after administration. Cmax of each drug

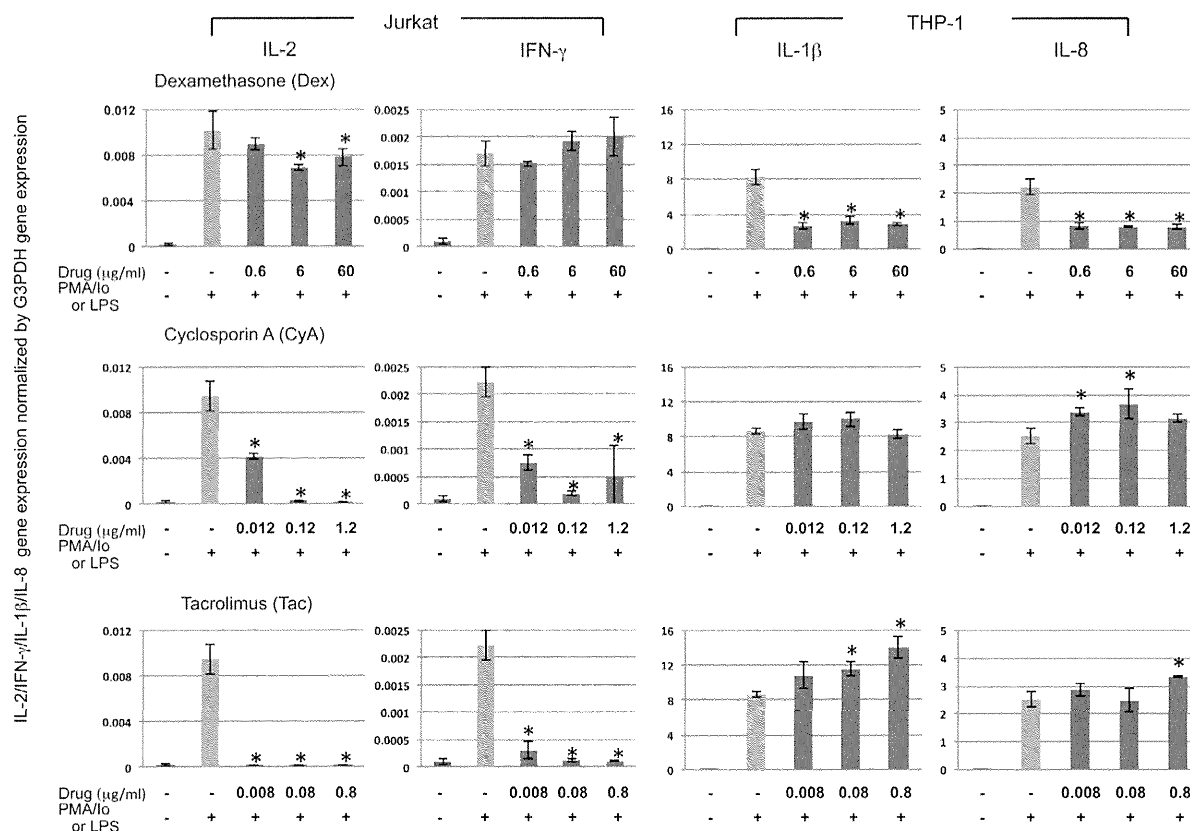

Fig. 3. The effects of Dex, CyA, and Tac on mRNA expression of IL-2 and IFN-γ by Jurkat cells and that of IL-1β and IL-8 by THP-1 cells after stimulation. Jurkat cells or THP-1 cells (3×10^6 cells/well) in 6-well plates were pretreated with different concentrations of drugs for 1 h, followed by stimulation with PMA/Io 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 +/− 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 Cmax 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 $\leqslant 5 \times$ Cmax, the observed effects can be expected in clinical use. On the other hand, when the effects are recognized only at $>5 \times$ Cmax, 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 $5 \times$ Cmax 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 immunoaugmentation, and N for no effect.

As mentioned above, Dex significantly suppressed IL-2, IL-1β, and IL-8 reporter activities at any concentration ranges in at least 2 of 3 experiments and that of IFN-γ at $>5 \times$ Cmax in 2 of 3 experiments (Table 1). Among 3 kinase or phosphatase inhibitors, CyA and Tac suppressed IL-2 and IFN-γ reporter activities at $\leqslant 5 \times$ Cmax in 3 experiments, while rapamycin did not show any inhibitory effects on IL-2 or IFN-γ reporter activity, but rather augmented IL-2 reporter activity at $\leqslant 5 \times$ Cmax in 2 of 3 experiments and IL-1β reporter activity at $>5 \times$ Cmax 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-1β reporter activity by Dex at $\leqslant 5 \times$ Cmax 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

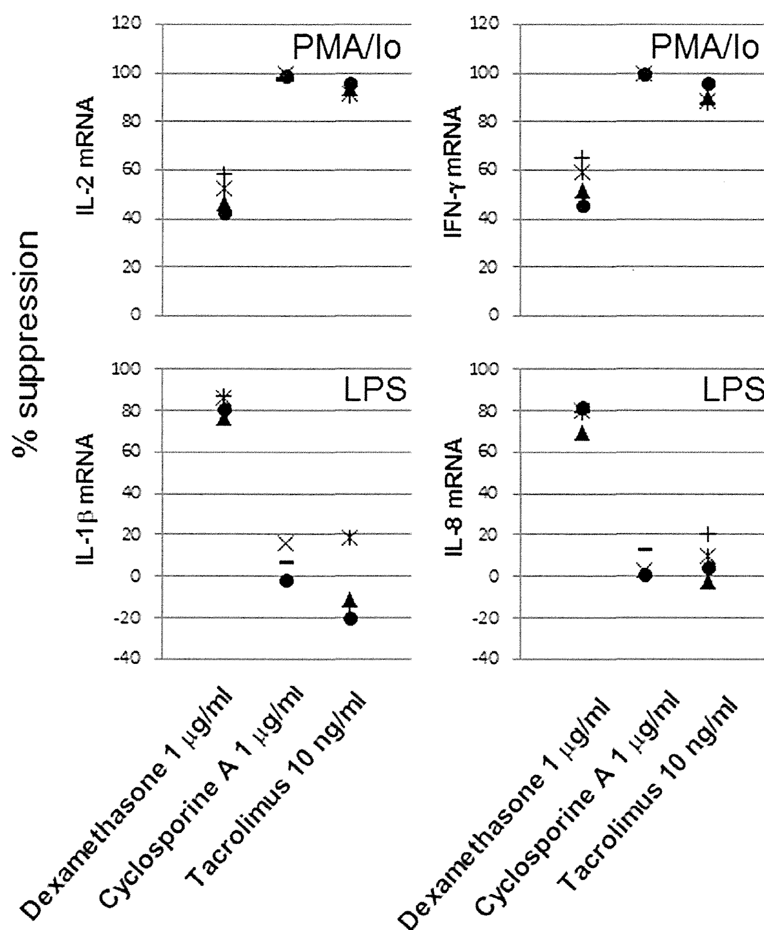

Fig. 4. The effects of Dex, CyA, and Tac on IL-2, IFN-γ, IL-1β, or IL-8 mRNA expression of whole blood cells stimulated with PMA/Io or LPS. Whole blood cells (WBC) from 6 healthy volunteers were collected using sodium heparin anti-coagulant and then diluted 1:2 with RPMI 1640 medium. Two ml of aliquots was either untreated or treated with 1 μg/ml of Dex, or 1 μg/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 × Cmax in 2 of 3 experiments. On the other hand, MPA significantly augmented IL-2 and IFN-γ reporter activities at ⩽5 × Cmax in 3 experiments. MZR increased all reporter activities at any concentration ranges in at least 2 of 3 experiments. In addition, CP augmented IFN-γ reporter activity at >5 × Cmax in 3 experiments, while MTX augmented IL-2 and IFN-γ 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-1β reporter activity by AZ at >5 × Cmax, IL-2 reporter activity by MTX at >5 × Cmax, and IFN-γ reporter activity by MTX at both concentrations.

3.5. Sulfasalazine and nicotinamide significantly suppressed IL-1β 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-γ reporter activities at any concentration ranges in at least 2 of 3 experiments, while it significantly suppressed IL-1β and IL-8 at >5 × Cmax in 3 experiments. MC significantly suppressed IL-2 and IFN-γ reporter activities at any concentration ranges in 3 experiments, while it did not affect IL-1β and IL-8 reporter activities. NA significantly suppressed IL-1β and IL-8 reporter activities at any concentrations in 3 experiments, while it augmented IL-2 and IFN-γ reporter activities at any concentrations in at least 2 of 3 experiments. Colchicine increased IL-1β reporter activity at ⩽5 × Cmax in 2 of 3 experiments and all reporter activities at >5 × Cmax 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-1β reporter activity by colchicine at ⩽5 × Cmax.

3.6. Two non-immunological drugs, warfarin and digoxin, suppressed some reporter activities at concentrations of ⩽5 × Cmax

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-1β and IL-8 reporter activities at ⩽5 × Cmax 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-γ				IL-1β				IL-8			
			⩽5 × Cmax		>5 × Cmax		⩽5 × Cmax		>5 × Cmax		⩽ × Cmax		>5 × Cmax		⩽5 × Cmax		>5 × Cmax	
Immunosuppressing drugs																		
Regulation of gene expression	Dexamethasone (Dex)	88 ng/ml	–/–/–	S	–/–/–	S	–/0/0	N	–/+/–	N	–/–/–	S	–/–/–	S	–/–/0*	S	–/–/–	S
Kinase and phosphatase inhibitors	Cyclosporin A (CyA)	2144 μg/ml	–/–/–	S	ND/ND/ND		–/–/–	S	ND/ND/ND		0/0/0	N	ND/ND/ND		0/–/0	N	ND/ND/ND	
	Tacrolimus (Tac)	44.6 ng/ml	–/–/–	S	–/–/–	S	–/–/–	S	–/–/–	S	+/0/0	N	0/+/0	N	–/0/0	N	0/+/0	N
	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
Alkylation	Cyclophosphamide (CP)	6.36 μg/ml	+/0/–	N	0/0/–	N	+/0/–	N	+/+/+	A	0/0/–	N	0/0/–	N	0/0/–	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
	Mycophenolic acid (MPA)	34.0 μg/ml	+/+/+	A	ND/ND/ND		+/+/+	A	ND/ND/ND		0/0/+	N	ND/ND/ND		0/0/0	N	ND/ND/ND	
	Mizoribine (MZR)	9.6 μg/ml	+/+/–	N	+/+/0	N	+/+/–	N	+/0/+	N	+/+/+	A	+/+/+	A	+/+/+	A	+/+/+	A
Inhibition of pyrimidine and purine synthesis	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
Off-label immunosuppressing drugs																		
	Sulfasalazine (SASP)	15.6 μg/ml	+/–/–	N	–/–/–	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	+/+/+	A
	Chloroquine (CQ)	555 ng/ml	–/–/–	S	–/–/–	S	–/–/0	N	–/–/–	S	0/0/0	N	–/–/–	S	0/0/0	N	–/–/–	S
	Minocycline (MC)	4.8 μg/ml	–/–/–	S	–/–/–	S	–/–/–	S	–/–/–	S	0/0/0	N	0/0/0	N	0/0/0	N	0/0/+	N
	Nicotinamide (NA)	22.4–26.3 μg/ml	+/–/+	N	+/+/+	A	0/+/+	N	+/+/+	A	–/–/–	S	–/–/–	S	–/–/–	S	–/–/–	S
Non-immunomodulatory drugs																		
	Acetaminophen (AA)	9.4 ng/ml	+/+/+	A	+/+/+	A	+/+/+	A	+/+/+	A	+/0/0	N	+/+/+	A	+/0/0	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
	Warfarin	685 μg/ml	+/+/+	A	ND/ND/ND		0/+/+	N	ND/ND/ND		–/0/*	S	ND/ND/ND		–/–/0*	S	ND/ND/ND	

– 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.

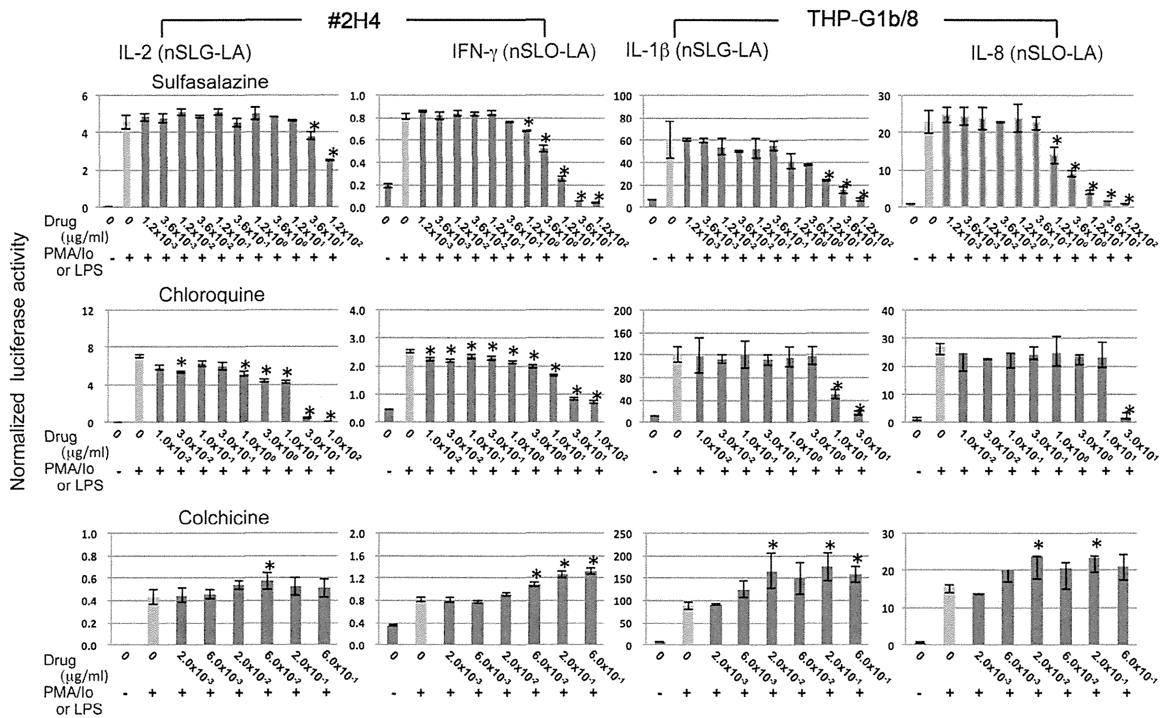

Fig. 5. The effects of SASP, CQ, and colchicine on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 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.

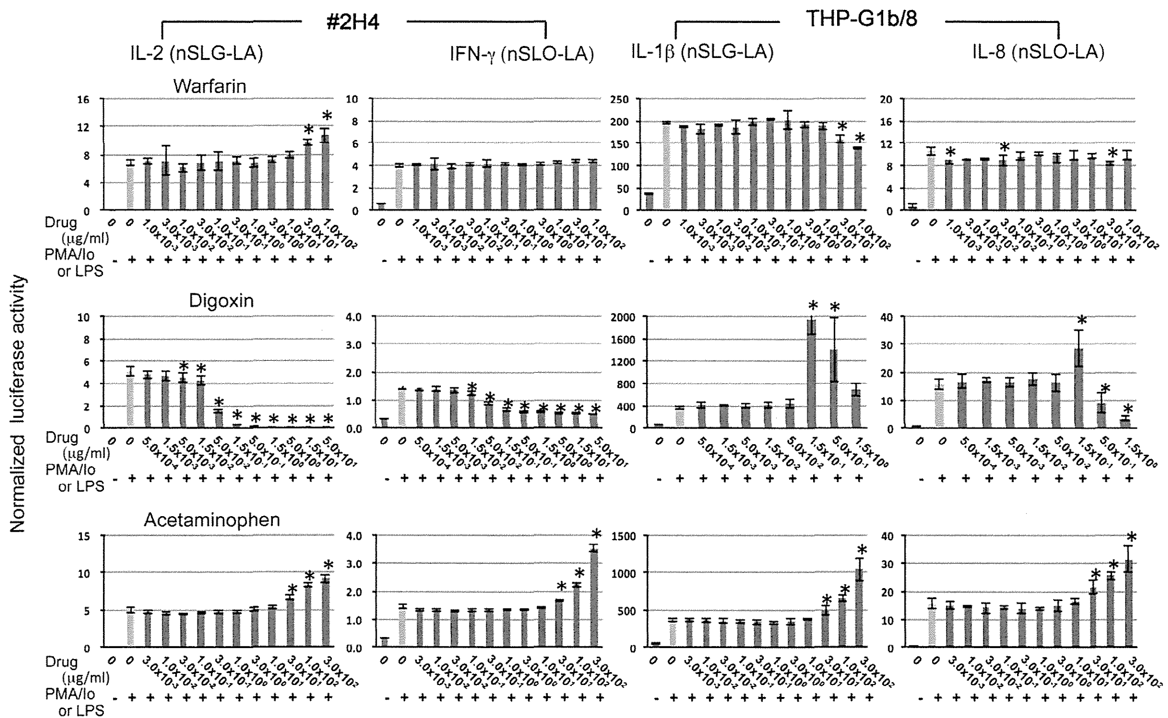

Fig. 6. The effects of warfarin, digoxin, and AA on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 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-γ reporter activities at ⩽5 × Cmax in at least 2 of 3 experiments. Digoxin suppressed IL-2 reporter activity at ⩽5 × Cmax in 2 of 3 experiments, while it suppressed IL-2 and IFN-γ reporter activities at >5 × Cmax in 3 experiments. AA augmented IL-2 and IFN-γ reporter activities at ⩽5 × Cmax in 3 experiments and 4 reporter activities at >5 × Cmax. 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-1β reporter activities by warfarin at ⩽5 × Cmax.

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-γ, IL-1β, 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-1β 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-γ mRNA induction after stimulation with PMA/Io, while only Dex significantly suppressed IL-1β 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 × Cmax. 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-1β and IL-4 by AA were greater than 1 mM and much higher than 5 × Cmax. MITA detected inhibitory effects of warfarin in IL-1β and IL-8 reporter activities, but not those in IL-2 or IFN-γ reporter activities. Likewise, HWBCRA revealed that warfarin suppressed IL-1β formation more strongly than IL-4 formation. Moreover, MITA demonstrated that digoxin suppressed IL-2 and IFN-γ 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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Nonmetal Haptens Induce ATP Release from Keratinocytes through Opening of Pannexin Hemichannels by Reactive Oxygen Species

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Although extracellular adenosine 5′-triphosphate (eATP) has a crucial role in the sensitization phase of contact hypersensitivity (CHS), the mechanism by which hapten causes keratinocyte cell death and ATP release is unknown. We examined the time course of cell death, reactive oxygen species (ROS) production, and ATP release in HaCaT cells and in normal human keratinocytes after exposure to nonmetal haptens, $NiCl_2$, or irritants. Both haptens and irritants caused cell death of keratinocytes but with different time courses. *N*-acetylcysteine (NAC) significantly reduced only nonmetal hapten-induced cell death as assessed by propidium iodide exclusion. We examined the effects of antioxidants and pannexin (Panx) inhibitors on cell death, ROS production, and ATP release by chemical-treated HaCaT cells. Nonmetal hapten-induced cell death, but not $NiCl_2$- or irritant-related cell death, was dependent on reactivity to thiol residues in the cells. NAC reduced cell death and ATP release, whereas antioxidants and Panx inhibitors did not inhibit cell death but significantly attenuated ATP release. Panx1 small interfering RNA (siRNA) also suppressed ATP release from hapten-exposed HaCaT cells. Intraperitoneal injection of a Panx1 inhibitor attenuated murine CHS. These findings suggest that nonmetal hapten reactivity to thiol residues causes membrane disruption of keratinocytes and ROS production that leads to ATP release through opening of Panx hemichannels.

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INTRODUCTION

Sensitization to contact allergens requires activation of the innate immune system that leads to dendritic cell (DC) activation. However, the mechanisms by which contact allergens activate innate immune signaling pathways are incompletely understood. It is possible that "endogenous danger signals" or "damage-associated molecular patterns" (reviewed in Willart and Lambrecht, 2009) are responsible for activation of the innate immune system in allergic sensitization. The following molecules have been identified as damage-associated molecular patterns: adenosine 5′-triphosphate (ATP), heat shock proteins, hyaluronic acid, monosodium urate, galectins, thioredoxin, adenosine, high-mobility group box protein 1, IL-1α, and IL-33 (reviewed in Hirsinger *et al.*, 2012).

Recent studies using gene targeting in mice demonstrated that Toll-like receptor 2 (TLR2)/TLR4 double-deficient mice (Martin *et al.*, 2008) or purinergic receptor $P2X_7$-deficient mice (Weber *et al.*, 2010) are resistant to allergic contact hypersensitivity (CHS), indicating an essential role of TLR2/TLR4 as well as of purinergic receptor $P2X_7$ in the mouse CHS model. Breakdown products of hyaluronic acid in the range of 1.2 to 500 kDa that are generated during inflammation or tissue damage have been demonstrated to stimulate TLR2 and/or TLR4 in immune cells such as macrophages or DCs (Termeer *et al.*, 2002; Scheibner *et al.*, 2006). Indeed, Esser *et al.* (2012) reported that haptens induce reactive oxygen species (ROS) production by keratinocytes *in vitro* as well as *in vivo*, thereby increasing hyaluronidase activity in the skin that results in the production of low-molecular-weight hyaluronic acid fragments. Combined, these observations suggest that hapten-exposed keratinocytes generate pro-inflammatory low-molecular-weight hyaluronic acid fragments that induce CHS via stimulation of TLR2/TLR4.

Similarly, extracellular ATP (eATP) released by stressed or damaged cells can also activate innate immune responses. The transmembrane ATP receptor $P2X_7$ has been implicated in the post-translational processing of pro-IL-1β and pro-IL-18 via activation of the NLRP3 inflammasome (reviewed by Vitiello *et al.*, 2012). Although Weber *et al.* (2010)

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Abbreviations: ATP, adenosine 5′-triphosphate; CBX, carbenoxolone; CHS, contact hypersensitivity; DC, dendritic cell; DNCB, dinitrochlorobenzene; DPCP, diphenylcyclopropenone; eATP, extracellular ATP; LA, lactic acid; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; 4-NBB, 4-nitrobenzylbromide; NHEK, normal human epidermal keratinocyte; Panx, pannexin; PI, propidium iodide; ROS, reactive oxygen species; siRNA, small interfering RNA; TLR, Toll-like receptor

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demonstrated eATP release in skin painted with hapten, they did not address the mechanism by which hapten-treated keratinocytes release ATP.

In this study, we compared the effects of haptens with irritants on keratinocyte cell death, ROS generation, and ATP release. Although the generation of ROS by hapten-exposed keratinocytes has been demonstrated in several studies (Mehrotra *et al.*, 2005; Esser *et al.*, 2012; Kim *et al.*, 2012), the effect of the generated ROS on cell death and ATP release from hapten-exposed keratinocytes has not been examined. First, we demonstrated that haptens and irritants caused the cell death of the human keratinocyte cell line HaCaT and normal human epidermal keratinocytes (NHEKs), and induced ATP release with different time courses. *N*-acetylcysteine (NAC) significantly reduced cell death of HaCaT cells exposed to haptens but did not affect the death of cells exposed to irritants. Three representative nonmetal haptens, dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), and diphenylcyclopropenone (DPCP), induced the generation of ROS in HaCaT cells that was significantly attenuated by pretreatment with NAC and several antioxidants. Despite the inhibitory effects of NAC and antioxidants on ROS generation, antioxidants suppressed lactate dehydrogenase (LDH) activity and ATP release but did not affect cell death that was assessed using propidium iodide (PI) exclusion. In addition, studies using pannexin (Panx) inhibitors revealed that ATP release from hapten-treated HaCaT cells was through Panx hemichannels. Furthermore, intraperitoneal injection of a pannexin inhibitor, carbenoxolone (CBX), significantly attenuated CHS induced by DNCB. Combined, these results provide an insight into the mechanism by which haptens cause keratinocyte death and ATP release in CHS.

RESULTS

Both haptens and irritants induce HaCaT and NHEK cell death and ATP release but with different time courses

Evaluation of cell death by PI exclusion using flow cytometry and LDH release assay showed that two nonmetal haptens DNCB and 4-NBB, one metal hapten $NiCl_2$, and two irritants SDS and lactic acid (LA) induced HaCaT cell death in a dose-dependent manner (Figure 1a). The minimum concentrations of reagents that were toxic to all cells were 100 μM for DNCB and 4-NBB, 6 mM for $NiCl_2$, 250 μM for SDS, and 34 mM for LA. Haptens and irritants induced cell death with different time courses: maximum cell death was evident 1 hour after irritant exposure but maximum cell death was only achieved after more than 6 hours of hapten exposure as assessed by PI staining. LDH activity in culture supernatants was increased 6 hours after hapten exposure, whereas maximum release of LDH was seen 1 hour after SDS exposure. The LDH activity of LA-treated HaCaT cells could not be measured, possibly because of disturbance of LDH enzyme activity due to the acidity of culture supernatants containing LA.

The time course of ATP release was also different between haptens and irritants. ATP release was evident 6 hours after hapten exposure, whereas maximum ATP release was seen 1 hour after exposure to irritants (Figure 1a). To test whether higher concentrations of hapten induce an earlier release of ATP, HaCaT cells were incubated with increased concentrations of DNCB (from 100 μM to 3.2 mM); however, ATP release earlier than 6 hours after DNCB exposure was not observed (data not shown). To clarify whether the delayed ATP release is limited to HaCaT cells, ATP release in culture supernatants of NHEKs exposed to either haptens or irritants was assessed. Similar to HaCaT cells, the time course of ATP release from NHEKs was also different between haptens and irritants (Figure 1b).

NAC attenuates HaCaT cell death caused by DNCB, 4-NBB, and DPCP but does not affect cell death caused by $NiCl_2$, SDS, or LA

We previously reported that haptens induce a redox imbalance in DCs that stimulates the phosphorylation of p38 mitogen-activated protein kinase and DC activation, and that pretreatment of DCs with NAC corrects the redox imbalance and abrogates the phosphorylation of p38 mitogen-activated protein kinase as well as DC activation (Mizuashi *et al.*, 2005). Therefore, we examined whether NAC could attenuate cell death caused by haptens and irritants. NAC significantly suppressed HaCaT cell death caused by nonmetal haptens DNCB, 4-NBB, and DPCP, as assessed by PI-positive cells, but did not affect cell death caused by a metal hapten $NiCl_2$, or irritants SDS or LA (Figure 2). Similarly, ATP release and LDH activity of HaCaT cells 12 hours after DNCB, 4-NBB, or DPCP exposure were significantly attenuated by NAC, but NAC had little effect on ATP release and LDH activity induced by $NiCl_2$, LA, or SDS (Figure 2).

HaCaT cells exposed to haptens and irritants produce ROS and mitochondrial superoxide anion depending on their thiol reactivity

Next, we examined whether exposure of HaCaT cells to haptens or irritants results in production of ROS using the CM-H_2DCFDA probe (Figure 3a). All nonmetal haptens but not the metal hapten $NiCl_2$ stimulated intracellular ROS production in a dose-dependent manner from 30 minutes to 6 hours after stimulation. ROS production in HaCaT cells exposed to DNCB or DPCP was greater than that in cells exposed to 4-NBB. SDS exposure resulted in the production of ROS in HaCaT cells from 30 minutes to 24 hours after exposure, but the amount of ROS production in the first 6 hours after exposure was much smaller than that caused by hapten exposure. The concentration of SDS that induced cell death of the majority of HaCaT cells did not result in a significant amount of ROS generation within 1 hour after exposure, suggesting that ROS did not contribute to SDS-related HaCaT cell death. LA did not induce ROS in keratinocytes. The positive control H_2O_2 triggered immediate production of ROS in HaCaT cells.

As pretreatment with NAC significantly attenuated HaCaT cell death as well as ATP release, we examined the effects of NAC on ROS production by hapten-exposed HaCaT cells. Pretreatment of HaCaT cells with NAC significantly attenuated ROS production by hapten-exposed HaCaT cells 6 hours after exposure, whereas it did not significantly affect ROS production by SDS- or H_2O_2-treated HaCaT cells (Figure 3b).