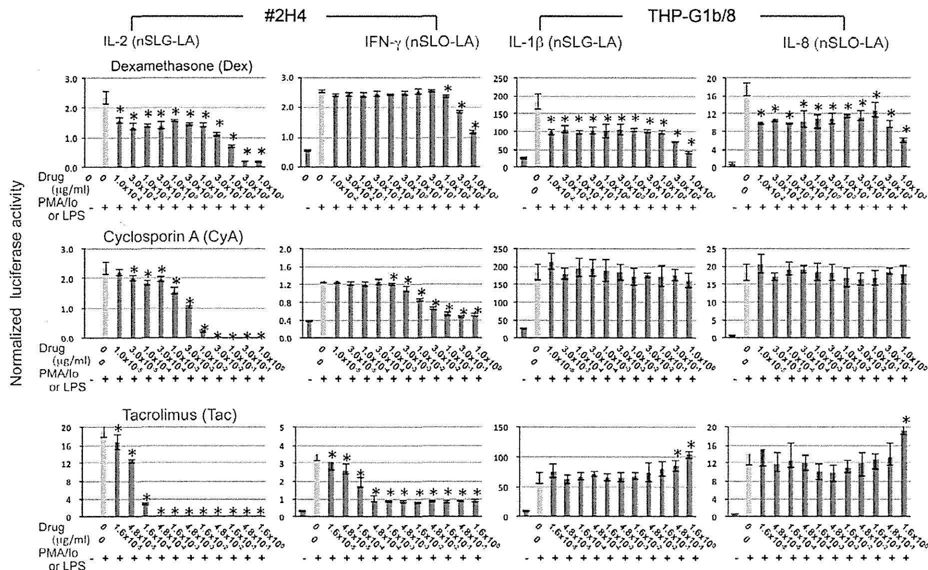

Fig. 2. The effects of Dex, CyA, and Tac on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 6 h. Luciferase 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

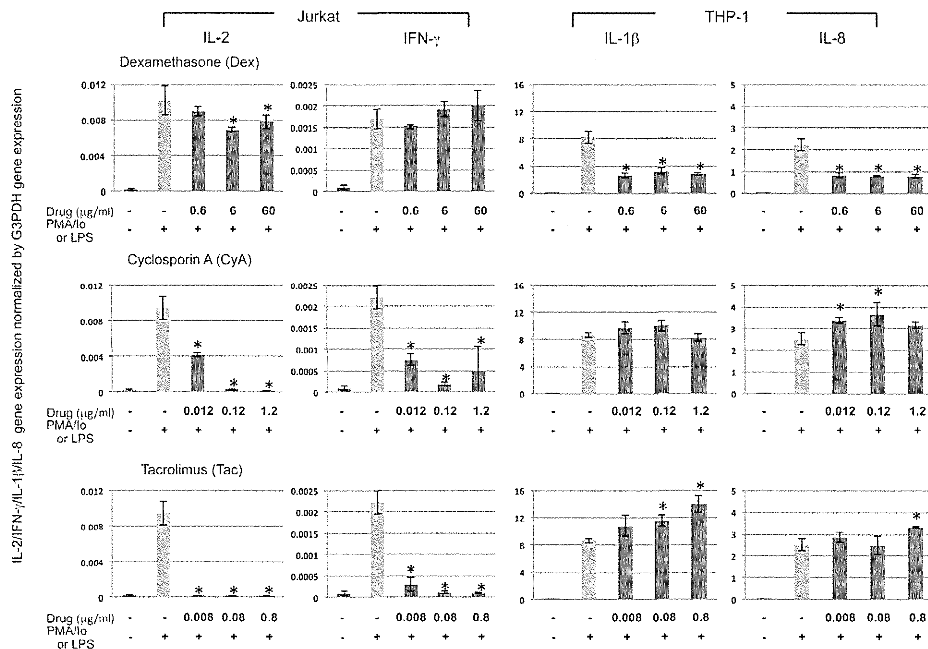

Fig. 3. The effects of Dex, CyA, and Tac on mRNA expression of IL-2 and IFN-γ by Jurkat cells and that of IL-1β and IL-8 by THP-1 cells after stimulation. Jurkat cells or THP-1 cells (3×10^6 cells/well) in 6-well plates were pretreated with different concentrations of drugs for 1 h, followed by stimulation 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 ⩽5 × Cmax, the observed effects can be expected in clinical use. On the other hand, when the effects are recognized only at >5 × 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 × 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 × 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 ⩽5 × 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 ⩽5 × Cmax in 2 of 3 experiments and IL-1β reporter activity at >5 × 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 ⩽5 × 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

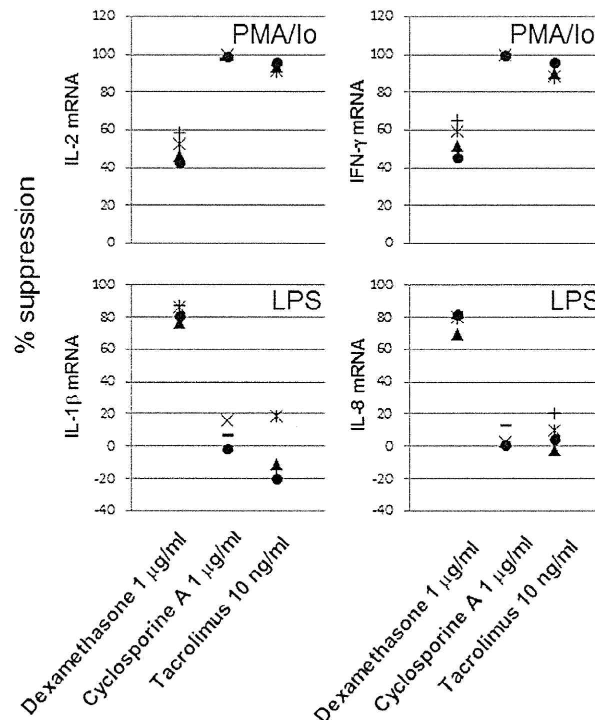

Fig. 4. The effects of Dex, CyA, and Tac on IL-2, IFN-γ, IL-1β, or IL-8 mRNA expression of whole blood cells stimulated with PMA/Io or LPS. Whole blood cells (WBC) from 6 healthy volunteers were collected using sodium heparin anti-coagulant and then diluted 1:2 with RPMI 1640 medium. Two ml of aliquots 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.

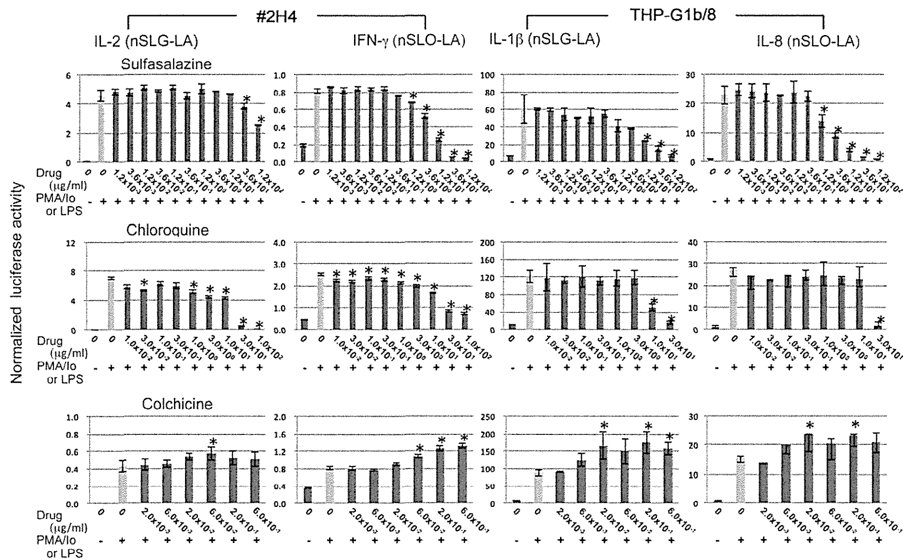

Fig. 5. The effects of SASP, CQ, and colchicine on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for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

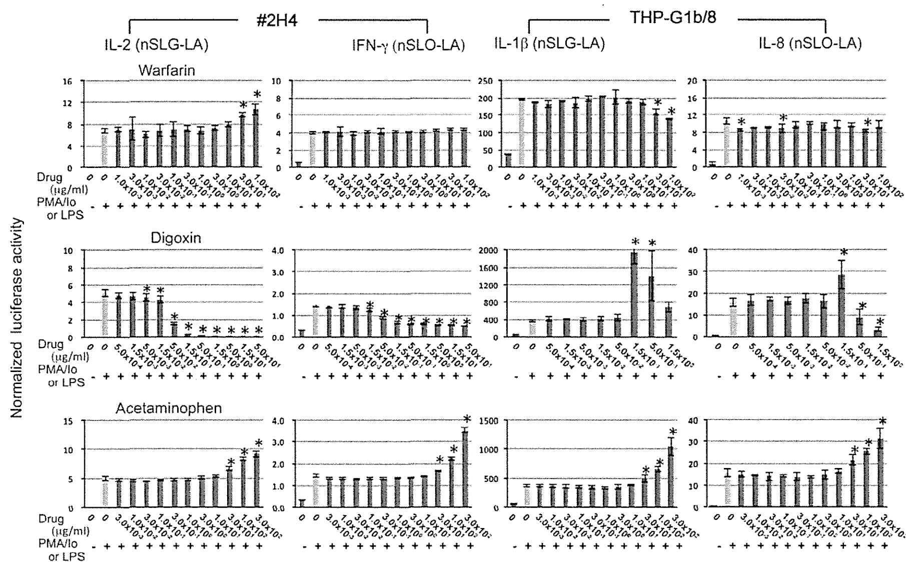

Fig. 6. The effects of warfarin, digoxin, and AA on IL-2, IFN-γ, IL-1β, or IL-8 reporter activity of reporter cell lines after relevant stimulation. #2H4 cells, THP-G1b cells, or THP-G8 cells were pretreated with the indicated concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 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).

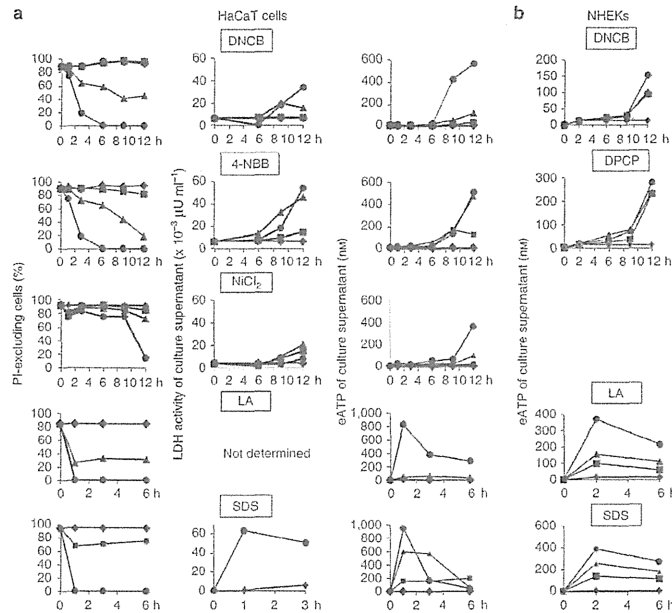

Figure 1. Haptens and irritants cause cell death of HaCaT cells and induce adenosine 5′-triphosphate (ATP) release with different time courses. (a) HaCaT cells or (b) normal human epidermal keratinocytes (NHEKs) cultured in 24-well plates were treated with graded concentrations of haptens dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), diphenylcyclopropenone (DPCP), or $NiCl_2$, or irritants lactic acid (LA) or SDS, for various time periods. After incubation, propidium iodide (PI) exclusion, lactate dehydrogenase (LDH) activity, and ATP release were examined to assess cell viability. The mean LDH activity and extracellular ATP (eATP) release of triplicate cultures were calculated for each chemical. The symbols ●, ▲, ■, and ♦ correspond to the highest, medium, and lowest concentrations and vehicle control of each chemical, respectively. Chemicals and their concentrations were as follows: DNCB, 4-NBB, and DPCP—100, 50, and 25 μM; $NiCl_2$—6.0, 3.0, and 1.5 mM; SDS—250, 125, and 62.5 μM; and LA—34 and 17 mM. Representative data from three independent experiments are shown.

Next, to clarify the source of ROS in HaCaT cells treated with haptens, we examined whether haptens induce mitochondrial superoxide anion generation using MitoSOX, a mitochondria-targeted ROS-specific fluorescent probe. DNCB, DPCP, and 4-NBB, although weakly, induced mitochondrial superoxide anion production 2 hours after cell exposure (Figure 3c), suggesting that mitochondria contribute to ROS production in HaCaT cells exposed to nonmetal haptens. Treatment with H_2O_2 also induced mitochondrial superoxide anion production. Pretreatment with NAC significantly attenuated mitochondrial superoxide anion production in HaCaT cells exposed to DNCB, 4-NBB, or DPCP, although its inhibitory effect on superoxide anion production by HaCaT cells exposed to 4-NBB was minimum (Figure 3c).

TEMPOL and apocynin do not rescue hapten-treated HaCaT cells from cell death but suppress ROS production and reduce ATP release

Although ROS production after hapten stimulation in the mitochondria of dendritic cells (Migdal *et al.*, 2010) and in the cytosol of keratinocytes (Mehrotra *et al.*, 2005) has been documented, the source of ROS production that causes keratinocyte cell death and ATP release has not yet been determined. Therefore, we examined whether the following reagents affect ROS production in hapten-exposed HaCaT cells: TEMPOL, a whole-cell antioxidant (Wilcox and Pearlman, 2008); MnTBAP, a superoxide dismutase mimetic, catalase mimetic, and peroxynitrite scavenger (Konorev *et al.*, 2002; Batinic-Haberle *et al.*, 2009); allopurinol, a xanthine oxidase inhibitor (Borges *et al.*, 2002); and apocynin, an NADPH oxidase inhibitor (Bedard and Krause, 2007). Results showed that TEMPOL, MnTBAP, and apocynin suppressed ROS production by HaCaT cells exposed to DNCB, 4-NBB, and DPCP, but their inhibitory effect on ROS production by 4-NBB-treated HaCaT cells was not statistically significant (Figure 4a). In contrast, allopurinol failed to suppress ROS production in HaCaT cells exposed to haptens.

We also examined whether MnTBAP or TEMPOL suppresses mitochondrial superoxide anion production by

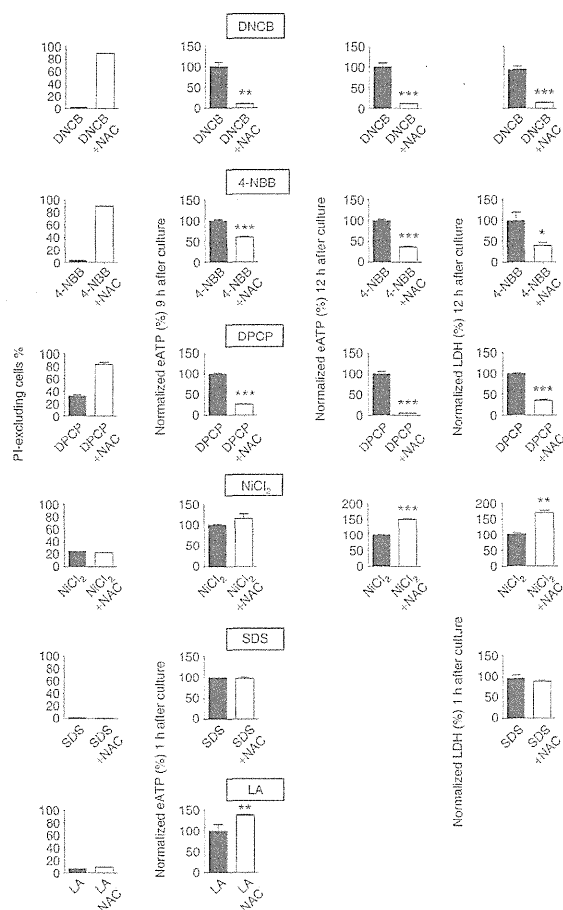

Figure 2. *N*-acetylcysteine (NAC) significantly attenuates cell death and adenosine 5′-triphosphate (ATP) and lactate dehydrogenase (LDH) release by HaCaT cells exposed to dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), or diphenylcyclopropenone (DPCP) but does not affect the death or ATP release of cells exposed to $NiCl_2$, SDS, or lactic acid (LA). HaCaT cells cultured in 24-well plates were either pretreated or not with 2.5 mM of NAC for 30 minutes, followed by exposure to 100 μM of DNCB, 4-NBB, or DPCP, 6 mM of $NiCl_2$, 250 μM of SDS, or 34 μM of LA for various time periods. The effects of NAC on cell death, extracellular ATP (eATP) levels, and LDH activity were assessed 6 hours after culture by propidium iodide (PI) exclusion assay, 9 and 12 hours after culture, and 12 hours after culture, respectively. The mean eATP or LDH activity of triplicate cultures was calculated for each chemical, and results were normalized to the data of hapten-exposed HaCaT cells without NAC. Bars represent mean ± SD. Significant differences between treatment groups: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Representative data from three independent experiments are shown.

hapten-exposed HaCaT cells. In contrast to its effects on ROS production in HaCaT cells, MnTBAP did not suppress mitochondrial superoxide anion production (Figure 4b). TEMPOL suppressed the MitoSOX fluorescence intensity of HaCaT cells exposed to 4-NBB, whereas it did not affect the fluorescence of cells exposed to DNCB or DPCP (Figure 4b).

Next, we examined whether ROS is involved in cell death or ATP release in HaCaT cells exposed to haptens. Assessment

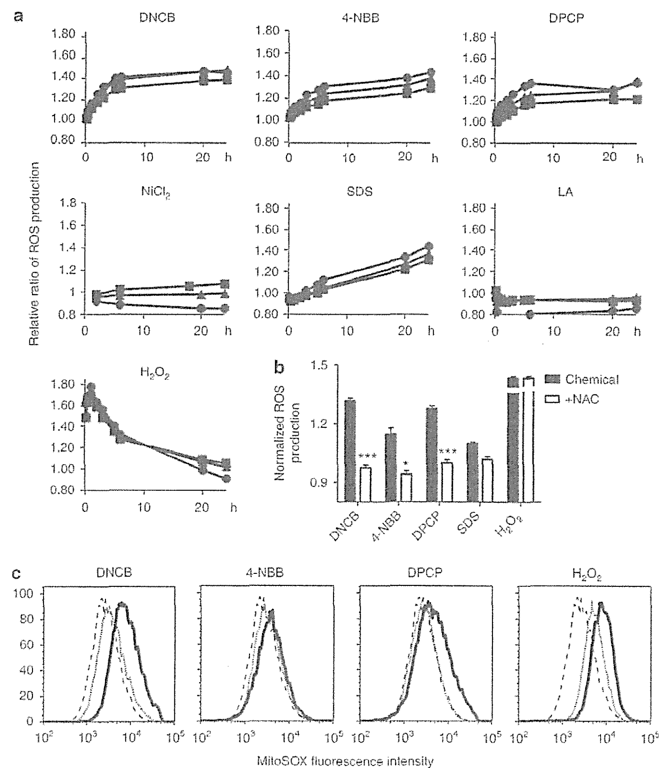

Figure 3. HaCaT cells treated with haptens generate reactive oxygen species (ROS) and mitochondrial superoxide anion in a dose-dependent manner that are significantly reduced by *N*-acetylcysteine (NAC). (**a**) HaCaT cells were treated with graded concentrations of haptens, irritants, or H_2O_2 as a positive control for various time periods. After culture, intracellular ROS were measured using a CM-H_2DCFDA probe. The mean fluorescence intensity of triplicate cultures was calculated for each chemical and expressed as percentage change. The symbols ■, ▲, and ● correspond to the lowest, medium, and highest concentrations, respectively. Chemicals used and their concentrations were as follows: dinitrochlorobenzene (DNCB), 4-nitrobenzylbromide (4-NBB), and diphenylcyclopropenone (DPCP)—25, 50, and 100 μM; $NiCl_2$—1.5, 3, and 6 mM; SDS—62.5, 125, and 250 μM; lactic acid (LA)—17 and 34 mM; and H_2O_2—44, 88, and 176 μM. (**b**) HaCaT cells either pretreated or not with NAC were exposed to haptens, SDS, or H_2O_2 for 6 hours. After culture, intracellular ROS were measured with a CM-H_2DCFDA probe. The mean fluorescence intensity of triplicate cultures was calculated for each chemical, and the data were normalized to the intensity of nontreated HaCaT cells. Bars represent mean ± SD. Significant differences between treatment groups: $^{*}P<0.05$, $^{***}P<0.001$. Representative data from three independent experiments are shown. (**c**) MitoSOX-preloaded HaCaT cells were either treated or not treated with NAC, followed by exposure to haptens or H_2O_2. The MitoSOX fluorescence was measured using a flow cytometer. The solid, dotted, and ruptured lines and the shaded area of the histograms represent HaCaT cells treated with hapten alone, hapten + NAC, vehicle control + NAC, and vehicle control alone, respectively.

of cell death by the PI exclusion assay showed that TEMPOL, MnTBAP, apocynin, and allopurinol could not rescue HaCaT cells from cell death after hapten treatment (Supplementary Figure S1 online). TEMPOL, MnTBAP, and apocynin reduced LDH activity and ATP release from DNCB- and 4-NBB-exposed HaCaT cells (Figure 4c and d), and TEMPOL attenuated LDH activity and ATP release from DPCP-exposed HaCaT cells (Supplementary Figure S2 online).

Inhibition of Panx-1 channels significantly suppresses ATP release from hapten-treated HaCaT cells

Multiple pathways other than cell lysis are involved in ATP release (Lohman *et al.*, 2012). It has been demonstrated that ATP release into the extracellular space by dying cells during apoptosis depends on the Panx channel (Chekeni *et al.*, 2010). The release of ATP through Panx hemichannels has also been reported in the setting of ischemia-induced oxidative stress

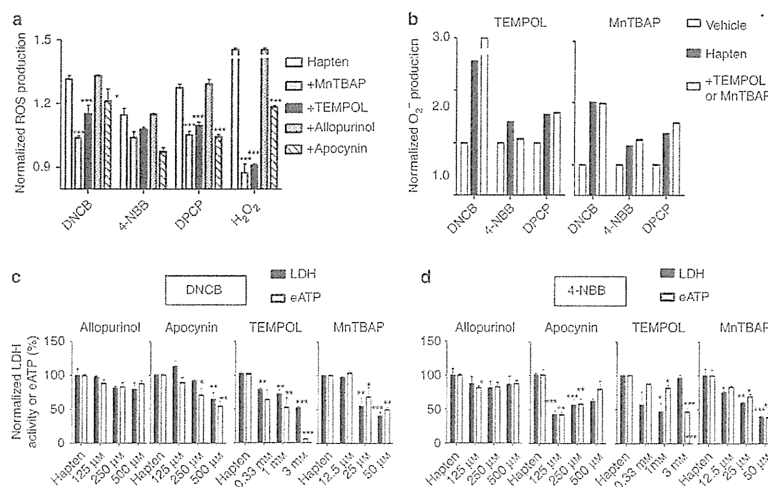

Figure 4. Antioxidants significantly suppress reactive oxygen species (ROS) production by hapten-treated HaCaT cells, and reduce lactate dehydrogenase (LDH) activity and adenosine 5′-triphosphate (ATP) release without decreasing the number of propidium iodide (PI)-positive cells. (**a**) HaCaT cells were either pretreated or not with antioxidants for 30 minutes, followed by exposure to haptens or H_2O_2. At 6 hours after culture, intracellular ROS were measured using a CM-H_2DCFDA probe. (**b**) MitoSOX-preloaded HaCaT cells were pretreated with TEMPOL or MnTBAP for 30 minutes, followed by exposure to haptens for 2 hours. The MitoSOX fluorescence was measured using a flow cytometer. Mean fluorescence intensity was calculated for each chemical, and the data were normalized to the intensity of nontreated HaCaT cells. (**c**, **d**) HaCaT cells were either pretreated or not with antioxidants for 30 minutes, followed by exposure to haptens. At 12 hours after culture, the LDH activity and ATP release in the culture supernatants were measured by colorimetric and luciferase assays, respectively. The mean LDH activity and ATP release of triplicate cultures were calculated for each chemical, and results were normalized to the data of hapten-treated HaCaT cells without inhibitors. Bars represent mean ± SD. Significant differences between treatment groups: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

culminating in the cell death of oligodendrocytes (Domercq *et al.*, 2010). We therefore examined whether CBX, a nonspecific Panx inhibitor (Suadicani *et al.*, 2006; Ma *et al.*, 2009), can suppress ATP release from hapten-treated HaCaT cells (Figure 5a). Interestingly, CBX significantly decreased ATP release from hapten-treated but not irritant-treated HaCaT cells in a dose-dependent manner. Furthermore, CBX suppressed LDH release from DNCB-treated but not 4-NBB- or DPCP-treated HaCaT cells (data not shown). Evaluation of cell death by the PI exclusion assay showed that CBX was unable to rescue hapten-exposed HaCaT cells from cell death.

To exclude the possibility that CBX reduced levels of intracellular ATP, thereby decreasing ATP release from hapten-treated cells, we examined the concentration of intracellular ATP in DNCB-exposed HaCaT cells. Results showed that CBX at concentrations of 7.8 to 31 μM increased intracellular ATP levels and decreased ATP release, whereas CBX at 62 μM slightly decreased the intracellular ATP level and significantly decreased ATP release (Figure 5b). These findings exclude the possibility that CBX reduces ATP release by depleting intracellular ATP.

To further examine the role of Panx hemichannels in ATP release from hapten-treated HaCaT cells, we examined the effects of another Panx-1 inhibitor, probenecid (Silverman *et al.*, 2008), and a Panx-1 mimetic blocking peptide (Pelegrin and Surprenant, 2006) on ATP release from hapten-treated HaCaT cells. Probenecid significantly suppressed ATP release from DNCB- or 4-NBB-treated HaCaT cells, and Panx1-blocking peptide also significantly inhibited ATP release from DNCB-treated HaCaT cells (Figure 5c and d). We also examined the effect of small interfering RNA (siRNA) against Panx1. Attenuation of Panx1 mRNA expression in HaCaT cells by Panx1 siRNA significantly suppressed ATP release from cells exposed to either DNCB or DPCP (Figure 5e and f).

Inhibition of Panx1 by CBX significantly reduces CHS induced by DNCB

Finally, to explore the role of Panx1 in the induction of CHS, we administered CBX by intraperitoneal injection and induced CHS using DNCB. After challenge with 0.5% DNCB, the ear swelling of mice pretreated with CBX was significantly reduced compared with those of saline-injected control mice, suggesting that CBX attenuated the CHS response (Figure 6). In contrast, CBX treatment did not affect the ear swelling induced by 0.5% DNCB without sensitization.

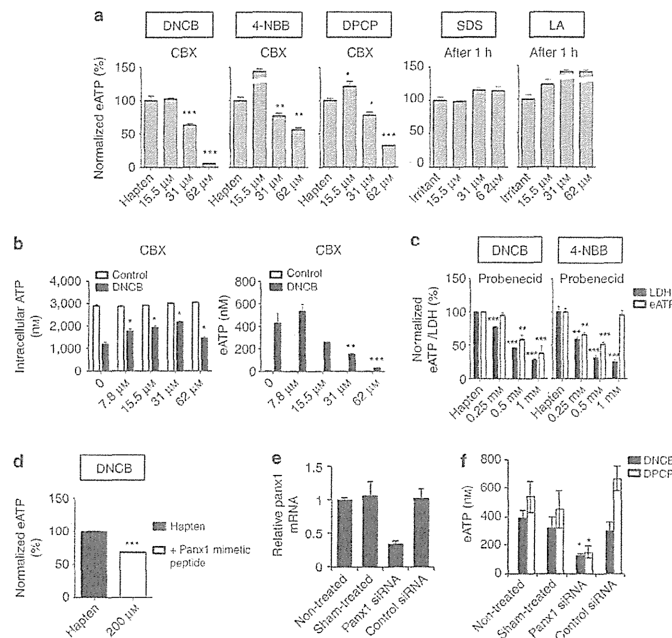

Figure 5. Carbenoxolone (CBX), probenecid, and a pannexin-1 (Panx1) mimetic blocking peptide suppress adenosine 5′-triphosphate (ATP) release from hapten-treated HaCaT cells. (**a**) HaCaT cells were either pretreated or not pretreated with graded concentrations of CBX, followed by exposure to haptens for 12 hours. After culture, ATP in the culture supernatants was measured by luciferase assay. (**b**) The intracellular ATP of HaCaT cells and ATP in the culture supernatants from the same culture were measured by luciferase assay. HaCaT cells were pretreated with (**c**) probenecid or a (**d**) Panx1 mimetic blocking peptide for 30 minutes, followed by exposure to haptens for 12 hours. After culture, ATP or lactate dehydrogenase (LDH) activity in the culture supernatants was measured. The mean extracellular ATP (eATP) and LDH activity of triplicate cultures was calculated for each chemical and results were normalized to the data of hapten-exposed HaCaT cells without inhibitors. Bars represent mean ± SD. Significant differences between treatment groups: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. HaCaT cells were either treated or not with 10 nM of Panx1 siRNA or control siRNA in transfection reagent. (**e**) After 51 hours of culture, Panx1 mRNA expression in each treatment group was measured by quantitative real-time PCR. (**f**) Cells were subsequently treated with dinitrochlorobenzene (DNCB) or diphenylcyclopropenone (DPCP) for 12 hours and the recovered supernatants were used to measure eATP. Significant differences between treatment groups: $^{*}P<0.05$.

DISCUSSION

In this study, we demonstrated that the haptens DNCB, 4-NBB, DPCP, and $NiCl_2$, and the irritants killed keratinocytes and induced ATP release from keratinocytes with different time courses. This suggests that the mechanism of hapten-induced keratinocyte cell death leading to ATP release is different from that of irritants. Furthermore, keratinocyte cell death caused by nonmetal haptens DNCB, 4-NBB, and DPCP, but not cell death caused by the metal hapten $NiCl_2$ or by irritants, was abrogated by NAC. The fact that NAC is a thiol-containing compound that interferes with thiol redox transitions (Parasassi *et al.*, 2010), and that haptens exhibit a strong affinity toward thiol groups (Becker *et al.*, 2003), suggests that nonmetal haptens kill keratinocytes via reactivity to thiol residues in keratinocytes. In contrast, the mechanism of cell death induced by $NiCl_2$ or irritants was not dependent on this.

Next, we demonstrated that only nonmetal haptens induced ROS production by HaCaT cells that was significantly attenuated by NAC treatment. Again, this suggests that thiol modification by haptens has a crucial role in ROS production. Apart from NAC, MnTBAP, TEMPOL, and apocynin significantly suppressed ROS production by hapten-treated HaCaT cells. However, the three antioxidants did not decrease cell death as evaluated by PI exclusion that suggests that ROS generated by hapten-treated HaCaT cells does not cause membrane disruption.

On the other hand, the three antioxidants suppressed ATP and LDH release from hapten-treated HaCaT cells. We found that ATP and LDH release from hapten-treated HaCaT cells

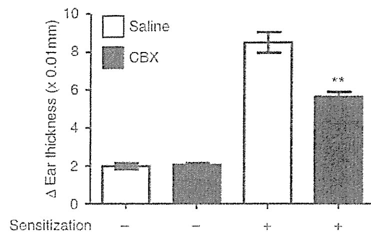

Figure 6. Inhibition of pannexin-1 (Panx1) by carbenoxolone (CBX) significantly reduces contact hypersensitivity (CHS) induced by dinitrochlorobenzene (DNCB). To examine the role of extracellular adenosine 5′-triphosphate (eATP) in sensitization, C57Bl/6 mice were given an intraperitoneal injection of CBX or saline on day 0 and then sensitized with 1% DNCB (or without sensitization) on days 0, 1, and 2, followed by epicutaneous application of 20 μl of 0.5% DNCB on the dorsum of both ears on day 4. Ear measurement was taken on days 4 and 6. The data represent the mean increase in ear thickness for groups of seven mice ± SD. Representative data from three independent experiments are shown. $^{**}P<0.01$ (vs. saline control, Student's *t*-test).

was evident 6 hours after hapten treatment, whereas they started to incorporate PI from 1 hour after exposure. As the molecular weights of LDH and PI are ~140,000 and 688 Da, respectively, we speculate that considerable time is required for sufficient membrane disruption to occur that permits the passage of large molecules. However, molecular size alone cannot fully explain the delay in ATP release as the molecular weight of ATP is lower than that of PI.

It has been reported that ischemia-related oxidative stress culminating in the cell death of oligodendrocytes induced ATP release through the opening of Panx hemichannels (Domercq *et al.*, 2010). As Panx1 is ubiquitously expressed in human tissues including the skin (Baranova *et al.*, 2004), we hypothesized that ROS produced by hapten-treated HaCaT cells may open Panx hemichannels. Indeed, Panx inhibitors as well as Panx1 siRNA significantly attenuated ATP release from HaCaT cells exposed to DNCB, 4-NBB, or DPCP. In addition, significant suppression of ATP release by antioxidants in a dose-dependent manner suggests that ROS production by hapten-treated HaCaT cells has a role in the opening of Panx hemichannels. Combined, our findings suggest that irritants induce ATP release from keratinocytes by disrupting cell membranes, whereas nonmetal haptens such as DNCB, 4-NBB, and DPCP induce ATP release from keratinocytes via ROS-mediated opening of Panx1 channels. Therefore, it is conceivable that Panx hemichannels have a crucial role in sensitization, just as eATP and $P2X_7$ are essential in the mouse CHS model (Weber *et al.*, 2010). This is supported by observation of the attenuated CHS response after CBX pretreatment in mice.

This study also demonstrated differences in the mechanism of ATP release among haptens. ATP release from HaCaT cells treated with the metal hapten $NiCl_2$ was independent of thiol reactivity of $NiCl_2$, whereas nonmetal hapten-induced ATP release from HaCaT cells was dependent on reactivity to thiol residues and ROS production. It has been reported that Ni can stimulate human TLR4 (Schmidt *et al.*, 2010) that suggests that Ni utilizes the TLR4 pathway to activate the innate immune response instead of generating ROS in allergic sensitization. In addition, there was quantitative difference in ROS and superoxide anion production among nonmetal haptens. It is conceivable that different nonmetal haptens generate ROS by different mechanisms dependent on their own chemical properties. Further studies are required to examine the precise mechanism by which nonmetal haptens generate ROS and/or superoxide anion and open Panx channels.

In this study, we attempted to determine the source of ROS in keratinocytes after hapten exposure. Although superoxide anion production by mitochondria occurred after hapten exposure, antioxidants such as TEMPOL and MnTBAP did not attenuate mitochondrial superoxide anion production despite their suppression of ROS production, LDH activity, and ATP release. This suggests that ROS production by mitochondria does not have a significant role in ATP release from hapten-treated keratinocytes, consistent with the observation by Mehrotra *et al.* (2005). However, our study could not determine which cytosolic compartment or enzyme was responsible for ROS production that led to the release of ATP. Although Kim *et al.* (2012) and Esser *et al.* (2012) demonstrated ROS production and mitochondrial superoxide anion production by hapten-treated keratinocytes, neither group succeeded in identifying the source of ROS production that influenced IL-1α production, ICAM-1 expression, or induction of hyaluronidase activity.

Our study provides an insight into the mechanism by which haptens kill keratinocytes and cause a large release of ATP. These findings provide additional evidence of the crucial role of keratinocytes in the sensitization of CHS. In addition, the results of this study suggest that Panx1 may be targeted to protect humans from sensitization by haptens. The Panx1 inhibitor CBX has already been approved as a cosmetic ingredient and may be useful as a topical agent in inflammatory or immune skin diseases by modulating innate immunity.

MATERIALS AND METHODS

Test chemicals and preparation of chemicals

Four contact sensitizers (DNCB, 4-NBB, $NiCl_2$, and DPCP) and two irritants (SDS and LA) were used. The following antioxidants were used in experiments: NAC, allopurinol, MnTBAP, and apocynin. Panx was inhibited using carbenoxolone disodium salt (CBX), probenecid, or Panx-1 mimetic blocking peptide. Full details are available in the Supplementary Methods online.

Keratinocyte culture

HaCaT cells, a gift from Norbert Fusenig in Heidelberg, Germany, and neonatal foreskin NHEKs purchased from Kurabo (Osaka, Japan) were used in this study. Full details regarding cell culture are available in the Supplementary Methods online.

Chemicals exposure of keratinocytes

HaCaT cells or NHEKs were cultured in 24-well plates, washed twice 48 hours later, and incubated with DMEM without phenol red at

37 °C in 10% CO_2 for 1 hour. Afterwards, they were pretreated with or without graded concentrations of antioxidants or Panx inhibitors for 30 minutes, followed by treatment with graded concentrations of haptens or irritants for various time periods at 37 °C in 10% CO_2.

Knockdown of Panx1 by stealth siRNA

In some experiments, HaCaT cells were treated with siRNA against Panx1 as described previously (Hirakawa *et al.*, 2011), followed by hapten exposure. Full details are available in the Supplementary Methods online.

Cell viability

Cell viability was determined by either a PI exclusion assay using flow cytometry or LDH release. Full details are available in the Supplementary Methods online.

Measurement of intracellular ROS

Intracellular ROS were measured fluorometrically using a CM-H_2DCFDA probe (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Full details are available in the Supplementary Methods online.

In vitro detection of mitochondrial superoxide anion

Mitochondrial superoxide anion was detected by MitoSOX RED (Invitrogen). Full details are available in the Supplementary Methods online.

Measurement of ATP

The extracellular ATP level was measured with a commercially available kit (ENLITEN, rLuciferase/Luciferin Reagent; Promega, Madison, WI). Full details are available in the Supplementary Methods online.

Murine model of CHS

Female C57Bl/6 mice were sensitized by painting the shaved abdominal skin with 100 μl of 1% DNCB in 4:1 (v/v) acetone/olive oil on days 0, 1, and 2. For elicitation, 20 μl of 0.5% DNCB was applied to the dorsum of both ears on day 4. To examine the role of eATP in sensitization, we injected $20\,mg\,kg^{-1}$ of CBX into the peritoneum on day 0. Full details are available in the Supplementary Methods online.

Statistical analysis

At least three independent experiments were performed for each analysis and representative data from one experiment are shown. A one-way or two-way analysis of variance test was used to evaluate statistical significance. The *P*-values of <0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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