IN VITRO SYSTEMS

An in vitro coculture system of human peripheral blood mononuclear cells with hepatocellular carcinoma-derived cells for predicting drug-induced liver injury

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Abstract

Preventing clinical drug-induced liver injury (DILI) remains a major challenge, because DILI develops via multifactorial mechanisms. Immune and inflammatory reactions are considered important mechanisms of DILI; however, biomarkers from in vitro systems using immune cells have not been comprehensively studied. The aims of this study were (1) to identify promising biomarker genes for predicting DILI in an in vitro coculture model of peripheral blood mononuclear cells (PBMCs) with a human liver cell line, and (2) to evaluate these genes as predictors of DILI using a panel of drugs with different clinical DILI risk. Transcriptome-wide analysis of PBMCs cocultured with HepG2 or differentiated HepaRG cells that were treated with several drugs revealed an appropriate separation of DILI-positive and DILI-negative drugs, from which 12 putative biomarker genes were selected. To evaluate the predictive performance of these genes, PBMCs cocultured with HepG2 cells were exposed to 77 different drugs, and gene expression levels in PBMCs were determined. The MET proto-oncogene receptor tyrosine kinase (*MET*) showed the highest area under the receiver-operating characteristic curve (AUC) value of 0.81 among the 12 genes with a high sensitivity/specificity (85/66%). However, a stepwise logistic regression model using the 12 identified genes showed the highest AUC value of 0.94 with a high sensitivity/specificity (93/86%). Taken together, we established a coculture system using PBMCs and HepG2 cells and selected biomarkers that can predict DILI risk. The established model would be useful in detecting the DILI potential of compounds, in particular those that involve an immune mechanism.

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Introduction

Drug-induced liver injury (DILI) is an adverse drug reaction that presents with hepatitis, cholestasis, jaundice, steatosis, nodular regenerative hyperplasia, or sinusoidal obstruction syndrome (Kleiner 2017). DILI is a frequent cause of acute liver failure, accounting for 20–40% of all cases of hepatic failure (Bell and Chalasani 2009; Björnsson 2014; Ostapowicz et al. 2002). While hepatotoxicity due to acetaminophen overdose is the most common, that is predictable and dose-related, liver injury associated with several other drugs appears to be of an idiosyncratic nature with no explicit dose–toxicity relationship, and is largely host-dependent (Funk and Roth 2017). Although idiosyncratic DILI is relatively rare with an incidence of 1 in 1000–100,000 treated patients (de Abajo et al. 2004), its occurrence has a significant impact on the pharmaceutical

industry and regulatory agencies, because it can be a reason for discontinuation of clinical trials and postmarketing regulatory actions, including withdrawal from the market and labeling restrictions (Kaplowitz 2005). A survey reported that 462 drugs were withdrawn from the worldwide market between 1953 and 2013, and hepatotoxicity (81 drugs; 18%) was the most common adverse drug reaction that led to withdrawal, followed by immune-related reactions (79 drugs; 17%) (Onakpoya et al. 2016). For example, alatrofloxacin, bromfenac, troglitazone, and trovafloxacin were all approved by the U.S. Food and Drug Administration (USFDA) in 1997, but later withdrawn from the market because of severe liver toxicity or liver failure (Senior 2014). Therefore, it is one of the most important challenges to predict the toxicity of candidate compounds at the early nonclinical stage of drug development.

It is believed that DILI develops via multifactorial mechanisms involving both drug properties (dose, lipophilicity, reactive metabolites, mitochondrial liability, enzyme, and transporter inhibition) and host-specific factors (genetic variation, age, sex, microbiota, diet, and polypharmacy) (Weaver et al. 2020). Therefore, these risk factors can be applied to develop test platforms to evaluate the potential DILI risk of a compound. To date, researchers in the pharmaceutical industry, academia, and regulatory agencies have made great efforts in establishing DILI platforms for the detection of the toxic potential of compounds. Cell death, ATP, reactive oxygen species (ROS), mitochondrial membrane potential, bile salt export pump (BSEP) inhibition, and covalent binding in vitro have been examined using liver cell lines and primary hepatocytes. However, the problem is that these approaches largely identify the intrinsic toxicity of drugs, that are usually detected in experimental animals, and may not predict compounds at risk for idiosyncratic reactions.

Accumulating in vivo experimental evidence suggests that innate immunity and adaptive immunity and their interaction with drugs are important mechanisms of idiosyncratic DILI (Liguori et al. 2010; Song et al. 2019; Uetrecht 2019). These achievements suggest the possibility of in vitro platforms that incorporate immune factors for DILI risk assessment. A few such studies have been conducted by adapting coculture or media transfer methods using primary cells or cell lines of human hepatocytes and monocytic cells (Edling et al. 2009; Granitzny et al. 2017; Kim et al. 2017). We recently reported a cell-based assay in which human promyelocytic leukemia HL-60 cells were treated with conditioned media of HepG2 or HepaRG cells that were exposed to drugs (Oda et al. 2016). The study found that the expression levels of S100A9, IL-1B, and CXCL8 (IL-8) mRNA in HL-60 cells may be useful in predicting DILI potential to some extent. However, there is room for improvement to establish a more physiologically relevant assay.

In this study, we established an in vitro model by coculturing PBMCs as an immune cell source and hepatocellular carcinoma-derived HepG2 cells to predict the DILI potential of drugs. First, the transcriptome in PBMCs of the coculture that had been exposed to several DILI-positive and DILI-negative drugs was obtained to select candidate biomarker genes. Then, the identified genes were further evaluated for their predictivity of DILI using 77 drugs with different DILI potential.

Materials and methods

Test drugs

The supplier and the catalog number of the test drugs are provided in Table S1 and Table S2. A total of 10 and 77 drugs were examined by microarray and screening analyses, respectively. Information on the clinical DILI risk of the test drugs was collected from the USFDA-approved prescription drug labels cited in DailyMed (https://dailymed.nlm.nih.gov/dailymed/index.cfm) and the Liver Toxicity Knowledge Base Benchmark Dataset (LTKB-BD; https://www.fda.gov/ScienceResearch/BioinformaticsTools). USFDA-approved prescription drug labels classify DILI risk into the following four categories: boxed warning (BW), warnings and precautions (WP), adverse reactions (AR), or no mention (NM). Drug labeling balances the information of causality, incidence, and severity based on data from controlled trials, published literature reports, and spontaneous reports to AERS (adverse event reporting systems) (CDER 2006). LTKB-BD classifies DILI risks into four categories, namely, Most-DILI, Less-DILI, Ambiguous-DILI, and No-DILI concern, by considering the clinical severity of DILI and labeling approved by the USFDA (Chen et al. 2011). Specifically, a drug is classified into Most-DILI concern if the drug was withdrawn (WDN) from the market, had a BW-label regarding liver injury, or caused severe and moderate liver injury (fatal hepatotoxicity, acute liver failure, liver necrosis, jaundice, and hyperbilirubinemia) described in the WP section. A drug is classified into Less-DILI concern if it was associated with mild DILI as described in the WP and AR sections of the drug label. A No-DILI-concern drug had no mention regarding liver injury in its drug label. Among the drugs classified as Most-DILI and Less-DILI-concern previously, the drugs were recently reclassified into Ambiguous-DILI concern if the drugs were not verified as DILI cause (Chen et al. 2016). The classifications are shown as the "Drug label" and "LTKB annotation" in Table 1 and Tables S1–2. In this study, Most-DILI-concern drugs or withdrawn drugs were considered DILI-positive drugs, and Less-, No-, and Ambiguous-DILI-concern drugs were considered DILI-negative drugs ("DILI classification" in Table 1). Although

Table 1 Effects of drugs on mRNA expression levels in PBMCs cocultured with HepG2 cells and cell viability of PBMC or HepG2 in monoculture

No	Drug name	C_{max} (μM)	Treated conc. (× C_{max})	Drug label	LTKB annotation	DILI class	BMP6	CXCL8	EREG	IL-1A	IL-1B	IL-24	MET	PID1	PTGS2	SLC7A11	SLPI	TNFAIP6	PBM-CIC50 (× C_{max})	HepG2 viability (%)
1	Acetylsalicylic acid	133.00	50	NM	Less-DILI concern	Negative	3.3	47.6	19.8	2.1	4.6	15.9	6.5	1.1	5.0	2.9	3.0	0.6	100.0	46.0
2	Acyclovir	22.20	25	AR	Less-DILI concern	Negative	1.2	2.3	1.2	3.2	2.2	2.0	1.2	1.1	1.7	1.6	1.9	1.1	100.0	97.5
3	Albuterol	0.06	100	NM	No-DILI concern	Negative	1.3	0.8	1.3	0.6	0.6	1.5	1.2	1.7	0.6	0.8	0.6	1.1	100.0	97.4
4	Alpidem	0.67	100	WDN	Most-DILI concern	Positive	1.6	5.1	1.6	0.9	1.1	3.5	1.1	0.4	1.0	2.2	0.8	0.3	100.0	64.1
5	Amikacin	64.90	50	NM	No-DILI concern	Negative	0.7	0.6	0.8	0.6	0.4	0.4	0.8	1.6	0.5	0.4	2.7	1.0	100.0	105.9
6	Aminosalicylic acid	152.81	50	WP	Most-DILI concern	Positive	2.6	4.3	4.5	1.4	0.8	5.4	2.0	4.5	1.3	2.2	21.5	1.2	100.0	67.0
7	Amodiaquine	1.90	10	WDN	Not assigned	Positive	2.0	1.1	1.6	0.5	0.7	1.3	1.7	1.5	0.4	0.8	1.4	0.4	28.4	62.6
8	Atropine	0.03	100	NM	No-DILI concern	Negative	0.9	0.7	0.7	1.1	0.7	1.1	0.7	1.4	0.8	0.8	9.6	1.0	100.0	113.6
9	Benztropine	0.45	100	NM	No-DILI concern	Negative	1.8	1.5	1.2	0.6	0.5	3.5	1.6	3.3	0.5	1.1	5.0	1.2	100.0	71.6
10	Biperiden	0.32	100	NM	No-DILI concern	Negative	2.2	5.5	2.8	2.0	2.4	3.1	1.8	1.8	1.3	2.9	1.3	1.3	100.0	89.0
11	Bromfenac	14.40	50	WDN	Most-DILI concern	Positive	3.8	39.0	4.9	8.9	12.8	1.3	2.0	0.8	35.6	0.2	2.2	0.7	85.8	66.3
12	Brompheniramine	0.04	100	NM	No-DILI concern	Negative	1.0	1.4	0.9	1.2	1.4	0.9	0.7	1.0	1.4	1.1	1.1	1.0	100.0	91.2
13	Carbetapentane	0.35	100	NM	Not assigned	Negative	1.6	2.5	1.2	2.5	2.7	9.0	1.7	1.3	1.7	1.7	1.3	1.1	100.0	72.1
14	Chloroquine	0.48	100	AR	Less-DILI concern	Negative	2.1	2.3	1.3	1.5	1.7	2.3	1.6	1.7	0.9	1.5	2.4	1.1	100.0	70.3
15	Chlorpheniramine	0.52	100	NM	No-DILI concern	Negative	0.9	1.0	0.8	0.8	0.7	ND	0.9	1.4	0.6	0.9	1.3	1.0	100.0	91.9
16	Clavulanate	12.00	50	WP	Not assigned	Positive	1.4	16.5	9.1	2.2	2.9	2.2	4.3	0.7	0.7	3.8	2.3	1.7	100.0	93.7
17	Clemastine	0.00	100	NM	No-DILI concern	Negative	0.8	0.6	0.6	0.8	0.8	0.2	0.7	1.1	0.8	0.6	2.9	0.9	100.0	96.6
18	Deferoxamine	17.84	100	NM	Less-DILI concern	Negative	0.5	2.3	1.3	0.9	1.5	1.0	0.8	0.9	15.2	0.2	0.4	0.3	100.0	45.9
19	Diclofenac	10.10	25	WP	Most-DILI concern	Positive	5.6	6.4	1.7	2.5	2.1	29.1	5.2	3.1	2.9	1.7	1.9	1.4	73.4	91.9
20	Didanosine	3.90	100	WP	Most-DILI concern	Positive	0.9	0.7	0.7	0.9	0.7	0.3	0.8	1.3	0.9	0.8	2.9	1.0	100.0	113.2
21	Dihydroergotamine	0.02	100	NM	No-DILI concern	Negative	0.8	0.7	0.9	1.0	0.8	2.4	0.8	0.8	0.9	1.0	1.4	1.0	100.0	96.6

Table 1 (continued)

No	Drug name	C_{max} (µM)	Treated conc. (×C_{max})	Drug label	LTKB annotation	DILI class	BMP6	CXCL8	EREG	IL-1A	IL-1B	IL-24	MET	PID1	PTGS2	SLC7A11	SLPI	TNFAIP6	PBM-CIC_{50} (×C_{max})	HepG2 viability (%)
22	Diphenhydramine	0.34	100	NM	No-DILI concern	Negative	0.5	0.6	0.5	0.7	0.5	0.4	0.7	1.1	0.6	0.6	2.0	0.8	100.0	100.4
23	Dobutamine	1.34	10	NM	No-DILI concern	Negative	0.7	2.9	1.1	0.7	0.6	1.2	0.8	1.2	0.5	1.3	1.2	0.4	28.7	94.1
24	Doxylamine	0.37	100	NM	No-DILI concern	Negative	0.9	1.0	0.8	1.2	0.9	0.5	0.7	1.0	0.9	1.0	1.4	0.9	100.0	103.3
25	Entacapone	4.30	25	NM	Less-DILI concern	Negative	1.3	15.5	4.2	3.2	2.7	0.8	1.8	0.3	2.6	0.6	0.4	0.3	87.4	49.4
26	Esmolol	6.80	100	NM	No-DILI concern	Negative	1.8	4.4	2.2	4.7	3.9	10.5	2.8	1.7	2.4	1.3	4.1	2.1	100.0	59.4
27	Ethambutol	31.80	100	WP	Most-DILI concern	Positive	0.4	1.9	0.9	1.6	1.2	1.3	2.1	1.6	1.1	2.1	3.9	1.4	100.0	87.2
28	Etodolac	68.50	10	WP	Most-DILI concern	Positive	7.3	7.6	6.5	1.5	1.5	39.0	10.8	4.2	1.1	1.1	2.8	1.0	27.5	76.1
29	Fexofenadine	0.98	100	NM	No-DILI concern	Negative	0.8	0.4	0.4	0.6	0.3	0.8	0.6	1.5	0.5	0.3	2.6	0.8	100.0	95.8
30	Flucloxacillin	72.60	50	WDN	Not assigned	Positive	12.0	278.1	93.4	29.3	44.3	766.3	21.0	1.5	277.5	1.9	2.3	1.0	100.0	94.6
31	Fludrocortisone	0.00	100	NM	No-DILI concern	Negative	0.6	0.4	0.4	0.5	0.2	0.9	1.7	2.1	0.2	0.0	1.6	0.4	100.0	95.8
32	Flutamide	5.40	25	BW	Most-DILI concern	Positive	9.7	9.7	5.0	1.1	2.3	29.2	14.2	5.5	1.7	1.9	2.2	2.3	95.3	21.6
33	Hydroxyzine	0.19	100	NM	No-DILI concern	Negative	1.1	0.8	0.8	0.7	0.5	1.8	0.9	1.6	0.7	0.9	3.7	1.0	100.0	83.0
34	Iproniazid	27.90	50	WDN	Most-DILI concern	Positive	2.7	1.6	1.7	1.1	0.9	5.8	1.7	3.0	0.7	1.3	5.8	1.0	100.0	95.1
35	Isoniazid	76.56	50	BW	Most-DILI concern	Positive	1.2	1.4	0.8	1.7	1.9	11.1	1.2	1.7	2.9	1.0	3.5	0.7	100.0	76.6
36	Isoproterenol	2.40	25	NM	No-DILI concern	Negative	1.2	8.3	4.2	0.5	0.5	1.8	1.4	0.9	0.4	2.6	2.2	0.4	62.7	87.3
37	Ketoconazole	11.30	2.5	BW	Most-DILI concern	Positive	2.6	11.2	4.4	2.0	3.5	334.5	7.6	1.1	2.4	5.0	0.3	1.1	3.9	56.2
38	Ketorolac	7.00	50	WP	Less-DILI concern	Negative	0.7	1.3	2.5	0.8	0.8	3.9	0.9	0.9	2.2	1.0	2.4	1.0	100.0	72.8
39	Levofloxacin	25.70	25	WP	Most-DILI concern	Positive	0.5	2.5	1.1	1.5	1.1	4.4	1.3	0.5	0.4	1.6	0.3	0.6	100.0	81.1
40	Liothyronine	0.11	100	NM	No-DILI concern	Negative	1.1	3.2	2.0	3.3	3.4	4.8	1.1	0.9	1.5	2.0	1.5	1.0	100.0	90.8
41	Loratadine	0.07	100	NM	No-DILI concern	Negative	0.9	1.1	0.7	1.2	1.0	1.2	0.8	1.1	1.0	1.0	2.5	1.1	100.0	91.6
42	Lumiracoxib	25.50	10	WDN	Most-DILI concern	Positive	3.5	4.6	2.4	1.1	1.6	72.1	4.7	1.4	1.1	2.0	0.3	1.0	29.1	92.6
43	Mebendazole	12.60	100	WP	Less-DILI concern	Negative	2.2	7.0	6.0	5.8	5.2	129.9	6.7	4.0	6.4	2.1	3.2	0.7	100.0	75.7

Table 1 (continued)

No	Drug name	C_{max} (μM)	Treated conc. (×C_{max})	Drug label	LTKB annotation	DILI class	BMP6	CXCL8	EREG	IL-1A	IL-1B	IL-24	MET	PID1	PTGS2	SLC7A11	SLPI	TNFAIP6	PBM-CIC_{50} (×C_{max})	HepG2 viability (%)
44	Metaproterenol	0.06	100	NM	No-DILI concern	Negative	0.9	0.8	1.3	0.6	0.5	1.4	0.8	2.1	0.6	0.7	4.7	0.8	100.0	87.5
45	Metronidazole	237.21	25	NM	Less-DILI concern	Negative	1.2	1.0	2.1	0.9	0.8	1.6	1.3	1.2	1.0	1.3	1.4	1.3	100.0	88.9
46	Minoxidil	0.96	100	NM	No-DILI concern	Negative	0.7	0.8	0.6	1.1	0.9	0.2	0.6	0.9	0.9	0.8	2.5	0.9	100.0	90.1
47	Nefazodone	4.30	5	BW	Most-DILI concern	Positive	1.6	3.2	1.9	0.6	0.4	5.6	6.9	2.0	0.8	1.1	3.7	1.2	8.4	55.0
48	Nevirapine	24.40	25	BW	Most-DILI concern	Positive	6.3	5.9	11.9	0.7	2.2	66.7	7.7	3.4	1.2	1.8	0.9	2.1	100.0	65.6
49	Nimesulide	21.08	25	WDN	Most-DILI concern	Positive	13.4	5.0	7.8	0.7	0.6	4.5	9.9	1.0	2.1	0.8	2.4	1.2	100.0	9.1
50	Nomifensine	0.38	100	WDN	Most-DILI concern	Positive	0.8	1.0	0.9	0.9	0.7	3.0	0.6	2.0	0.6	0.7	11.3	1.0	100.0	90.9
51	Oseltamivir	0.21	100	AR	Ambiguous-DILI concern	Negative	0.8	0.7	0.9	0.7	0.7	1.2	0.8	1.2	0.8	0.5	1.1	1.2	100.0	103.2
52	Oxybutynin	0.02	100	NM	No-DILI concern	Negative	0.8	1.0	0.7	1.1	1.0	0.2	0.6	1.1	1.0	0.9	4.5	0.9	100.0	88.8
53	Paliperidone	0.03	100	NM	No-DILI concern	Negative	1.0	3.0	0.8	2.0	2.1	5.8	0.9	1.1	1.4	1.4	1.0	1.1	100.0	95.5
54	Pamabrom	14.90	50	NM	No-DILI concern	Negative	2.6	3.2	1.4	1.5	0.9	1.7	4.0	2.1	1.6	1.2	2.5	1.8	100.0	75.1
55	Penbutolol	0.96	25	NM	No-DILI concern	Negative	2.4	6.9	2.2	5.2	5.5	42.8	2.9	4.1	3.3	2.1	6.4	1.4	41.6	74.9
56	Phenoxybenzamine	0.20	100	NM	No-DILI concern	Negative	0.7	0.7	0.6	1.0	0.8	0.3	0.6	0.8	0.7	0.9	1.1	1.0	100.0	96.0
57	Phentolamine	0.09	100	NM	No-DILI concern	Negative	0.8	0.6	0.6	0.7	0.7	0.6	0.4	1.1	0.8	0.7	0.8	0.9	100.0	85.7
58	Pioglitazone	2.90	100	WP	Less-DILI concern	Negative	6.5	106.7	61.1	122.9	75.5	459.7	13.7	2.3	178.3	4.5	1.4	1.5	100.0	87.0
59	Pirprofen	91.38	10	WDN	Most-DILI concern	Positive	1.9	5.6	1.5	1.3	1.5	5.3	3.9	1.6	1.3	0.7	1.7	2.3	31.4	76.3
60	Primaquine	1.08	100	NM	No-DILI concern	Negative	1.0	3.3	1.6	0.6	0.4	0.5	1.2	1.3	1.3	1.4	5.0	0.8	100.0	0.1
61	Primidone	4.67	100	NM	No-DILI concern	Negative	0.7	1.3	1.4	1.5	1.1	1.6	1.0	0.8	0.8	0.9	0.6	0.9	100.0	100.5
62	Procyclidine	2.00	50	NM	No-DILI concern	Negative	2.4	16.1	7.3	16.0	14.0	88.7	4.9	2.0	4.5	5.4	2.9	1.7	81.4	71.5
63	Promethazine	0.07	100	AR	Less-DILI concern	Negative	1.1	1.0	1.1	1.6	1.3	1.1	0.8	1.5	1.0	1.1	1.2	1.1	100.0	97.6
64	Protriptyline	0.78	25	NM	No-DILI concern	Negative	2.2	1.4	1.7	1.3	1.0	5.1	3.4	4.0	1.8	0.6	5.4	1.1	44.2	78.0

Table 1 (continued)

No	Drug name	C_{max} (μM)	Treated conc. (×C_{max})	Drug label	LTKB annotation	DILI class	BMP6	CXCL8	EREG	IL-1A	IL-1B	IL-24	MET	PID1	PTGS2	SLC7A11	SLPI	TNFAIP6	PBM-CIC_{50} (×C_{max})	HepG2 viability (%)
65	Rofecoxib	1.00	100	WDN	Not assigned	Negative	1.4	1.2	1.5	0.6	0.7	0.7	1.0	1.2	0.9	1.0	1.8	1.3	100.0	99.9
66	Terazosin	0.13	100	NM	No-DILI concern	Negative	0.9	0.9	1.2	0.8	0.7	5.3	1.1	0.9	0.9	0.7	1.3	0.8	100.0	100.2
67	Tienilic acid	172.10	5	WDN	Most-DILI concern	Positive	1.6	6.6	2.4	1.5	1.6	1.0	2.6	1.4	1.5	2.3	1.7	1.0	15.1	82.0
68	Tolcapone	47.60	2.5	BW	Most-DILI concern	Positive	0.7	1.7	0.6	1.3	1.1	0.6	1.7	0.7	2.0	0.6	0.6	1.0	8.8	51.1
69	Tolterodine	0.04	100	NM	No-DILI concern	Negative	1.2	0.9	0.8	0.7	0.6	0.8	1.2	1.0	0.8	0.8	1.5	1.0	100.0	95.8
70	Trazodone	5.06	25	AR	Less-DILI concern	Negative	2.0	4.3	2.5	2.2	1.6	2.9	2.2	1.6	1.9	2.1	1.8	1.5	57.1	72.5
71	Trihexyphenidyl	0.90	100	NM	No-DILI concern	Negative	3.1	10.6	5.5	8.8	8.6	19.1	4.0	2.1	6.1	3.8	2.4	2.0	100.0	65.8
72	Triprolidine	0.02	100	NM	No-DILI concern	Negative	1.5	1.7	1.5	1.3	1.4	2.2	1.1	0.9	0.7	1.3	0.3	0.8	100.0	82.9
73	Troglitazone	6.40	10	WDN	Most-DILI concern	Positive	8.7	203.7	35.5	59.2	147.4	30610.0	35.9	4.2	410.7	7.9	0.1	1.8	16.5	81.8
74	Trovafloxacin	7.00	50	WDN	Most-DILI concern	Positive	6.2	4.1	1.2	1.2	0.1	4.8	4.1	8.2	0.3	0.0	9.8	0.0	100.0	74.4
75	Valproic acid	693.40	10	BW	Most-DILI concern	Positive	2.5	0.5	0.5	0.2	0.1	7.3	1.5	0.9	0.2	0.0	3.2	0.1	41.6	86.2
76	Zafirlukast	1.21	50	WP	Most-DILI concern	Positive	2.9	4.2	2.4	1.3	0.6	29.0	8.4	2.0	1.8	0.5	2.6	0.5	80.1	54.1
77	Zolpidem	0.39	100	AR	Ambiguous-DILI concern	Negative	0.9	0.9	1.1	0.8	0.9	0.4	0.8	1.1	1.0	0.9	1.2	1.2	100.0	97.1

Cocultures of PBMCs (lot: LP226) and HepG2 cells were exposed to the drugs at the indicated concentrations, and the mRNA expression levels of each gene in PBMCs were determined by qPCR and normalized to GAPDH mRNA levels. The levels are expressed as relative to the DMSO control. Data are the mean of $n = 2$ biological replicates. Cell viability was determined in PBMCs (lot: LP226) or HepG2 monoculture ($n = 3$ to 4 biological replicates)

AR adverse reactions, *ND* not detected, *NM* no match, *WDN* withdrawn, *WP* warnings and precautions

rofecoxib was withdrawn from the market, due to cardiovascular events, it was considered a DILI-negative drug.

All drugs, except amikacin sulfate, chloroquine diphosphate, and valproate sodium, were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) as a stock solution. Amikacin sulfate, chloroquine diphosphate, and valproate sodium were dissolved in distilled water.

Peripheral blood mononuclear cells

Blood was collected from a healthy male donor aged 63 years into Vacutainer® CPT™ 8-mL tubes containing sodium citrate (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and centrifuged at 1800×g at room temperature for 20 min. The PBMC layer was transferred into a 50-mL tube and washed with 1×PBS twice. Freshly isolated PBMCs were used for a coculture experiment with HepG2 or HepaRG cells for microarray analysis as described below. Informed consent for participation in this study was obtained from the donor, and all protocols were designed in accordance with the Declaration of Helsinki and approved by the ethics committee of Nagoya University School of Medicine.

Cryopreserved PBMCs were purchased from Cellular Technology Limited (Cleveland, OH). Cryopreserved PBMCs were thawed according to the manufacturer's protocols. Briefly, cryopreserved PBMCs were thawed in RPMI 1640 medium (Nissui Pharmaceutical) supplemented with 2-mM L-glutamine and CTL Anti-Aggregate Wash™ solution (Cellular Technology Limited) and centrifuged at 330×g at room temperature for 20 min. The cells were then switched to CTL-Test™ medium (Cellular Technology Limited) supplemented with 2 mM L-glutamine and centrifuged at 330×g at room temperature for 20 min. The cryopreserved PBMCs were used for a coculture experiment with HepG2 for screening assays as described below. All cells were cultured in a humidified atmosphere of air containing 5% CO_2 at 37 °C.

Liver cell line

The human hepatocellular carcinoma cell line HepG2 was purchased from the American Type Culture Collection (Manassas, VA). HepG2 cells were grown in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1-mM nonessential amino acids, 10% fetal bovine serum (FBS; Sigma-Aldrich), and 2-mM L-glutamine. The medium was renewed every 2 days.

Cryopreserved human hepatoma HepaRG® cells (HRP116), which are terminally differentiated hepatic cells, were purchased from BioPredic International (Rennes, France). The cryopreserved differentiated HepaRG cells were seeded on a 24-well collagen-coated plate at a density of 4.8×10^5 cells/well in Medium 670 (HepaRG Thawing/

Plating/General purpose Medium; BioPredic International), and the media were shifted to Medium 620 (HepaRG Maintenance and Metabolism Medium; BioPredic International) 1 day after seeding. The cells were used 7 days after seeding for a coculture experiment with PBMCs.

Assessment of PBMC and HepG2 cell viability

Freshly isolated PBMCs (a healthy male donor aged 63 years) and commercial cryopreserved PBMCs (LP226; a healthy female donor, 55 years old, Cellular Technology Limited) were seeded onto a 96-well plate at 5.0×10^5 cells/well and treated with drugs at various concentrations corresponding to 100-, 50-, 25-, 10-, 5-, 2.5- or 1-fold (×) human plasma maximum concentration (C_{max}). The final DMSO concentration was 0.4%. After a 24-h incubation period, Cell Counting Kit-8 (Dojindo molecular technologies, Kumamoto, Japan) assays for the freshly isolated PBMCs and CellTiter-Glo®2.0 (Promega, Madison, WI) assays for the commercial cryopreserved PBMCs were conducted to determine cell viability. The maximum drug concentration that yielded cell viability of more than 70% was used for the coculture experiments of microarray and screening as described below. In addition, a drug concentration producing a 50% decrease in cell viability (IC_{50}) was determined from a plot of the concentration of the test drugs (X) versus the percentage (Y) of cell viabilities relative to the control by nonlinear regression analysis according to the following Hill equation with GraphPad Prism 8 software (Version 8.3.0, GraphPad Software, San Diego, CA):

$$Y = \frac{100}{1 + \left(\frac{IC_{50}}{X}\right)^{\text{Hillslope}}}.$$

IC_{50} values were expressed as the fold-over C_{max}. For compounds for which IC_{50} values could not be calculated correctly due to low toxic effects to the cells, 100×C_{max} was assigned as their IC_{50} values.

HepG2 cells were seeded onto a 96-well plate at 2.5×10^4 cells/well and treated with drugs at a single concentration for each drug. The tested concentration (Table 1) was the maximum drug concentration that yielded PBMC viability of more than 70% determined above. The final DMSO concentration was 0.4%. After a 24-h incubation period, CellTiter-Glo®2.0 assays were conducted to determine cell viability.

Drug treatment for coculture and monoculture of PBMCs and HepG2 or HepaRG cells

HepG2 (2.5×10^5 cells/well) and HepaRG (4.8×10^5 cells/well) cells were seeded onto a 24-well plate using 0.5 mL of the corresponding normal growth media as described

above. After 1 day (HepG2) or 7 days (HepaRG), media were removed, and 0.75 mL of CTL-Test Medium containing test drugs was added. Immediately, Falcon® culture inserts (0.4-µm pore size; catalog number: 353095, Corning; Corning, NY) were placed in each well, and PBMCs were seeded into the inserts at 5.0×10^5 cells/ well suspended in 0.125 mL of CTL-Test Medium. Then, 0.125 mL of CTL-Test Medium containing test drugs (at twofold of the final concentration) was added to the inserts. The drug concentration was set at a concentration for each drug based on PBMC viability. After 24 h of incubation (5% CO_2 at 37 °C), PBMCs were collected and used for RNA extraction. These procedures are illustrated in Fig. 1.

For microarray analysis, both HepG2 and HepaRG cells were used as liver cells, and freshly isolated PBMCs (a healthy male donor, 63 years old) were used as test cells. For these experiments, test drugs included amodiaquine, diclofenac sodium, ketoconazole, levofloxacin, tienilic acid, and trovafloxacin mesylate (DILI-positive) and chloroquine phosphate, ethacrinic acid, ketorolac tromethamine, and mebendazole (DILI-negative) as well as 0.4% DMSO as a control (Table S1).

For screening assays, only HepG2 cells were used as liver cells, commercial cryopreserved PBMCs (lot: LP226; a healthy female donor, 55 years old) were used, and 77 drugs were examined (Table S2) followed by qPCR analysis. To investigate donor-to-donor differences in predictive performance, cryopreserved PBMCs from another donor (lot: LP316; a healthy male donor, 25 years old, Cellular Technology Limited) were used, and 77 drugs were examined followed by the qPCR analysis.

For monoculture of PBMCs, commercial cryopreserved PBMCs (LP226; a healthy female donor, 55 years old) were cultured in the absence of liver cells using culture inserts as described above, and 77 drugs were examined

followed by qPCR analysis at a concentration shown in Table S2.

Microarray analysis

For microarray analysis, total RNA was extracted from freshly isolated PBMCs, which were cocultured with HepG2 or HepaRG cells, using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and integrity of total RNA were determined with a 2100 Bio Analyzer (Agilent Technologies, Santa Clara, CA), and the RNA integrity number (RIN) was confirmed to be greater than 7, that is of sufficient quality for use in microarray experiments. Samples were analyzed by a microarray platform of Sure Print G3 Human 8×60 K ver.3.0 (Agilent Technology). The data were analyzed using Subio Platform ver. 1.21 (Subio, Amami, Japan). Raw signal intensities were normalized by a 75th percentile shift for either PBMCs/HepG2 or PBMCs/HepaRG groups, and fold changes in the drug-treated samples against each DMSO-treated group were calculated. Principal component analysis (PCA) of the expression levels of all genes was conducted using the "prcomp" function of R software version 3.6.0 (R Core Team 2019). Hierarchical clustering analysis of 12 predictor genes was performed by Euclidean distance and the "ward. D2" clustering method of the R software. A heatmap was generated using the "heatmap.2" function of the R package "gplots." Microarray data were deposited in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov) under accession number GSE147866.

RNA extraction and real-time reverse transcription (RT)-PCR

For qPCR analysis of RNA samples from screening assays, total RNA was extracted from the PBMCs using RNAiso Plus (Takara Bio, Kusatsu, Japan) according to the

Fig. 1 Illustration of the research flow. Human PBMCs were cocultured with HepG2 or HepaRG cells using culture inserts. PBMCs were seeded into culture inserts and placed on 24-well plates containing HepG2 or HepaRG cells, and the tested drugs were added to both parts. For microarray analysis, the coculture was treated with 10 drugs, and RNA from PBMCs was extracted. For qPCR analysis, the coculture of PBMCs and HepG2 cells was treated with 77 drugs, and RNA from PBMCs was extracted
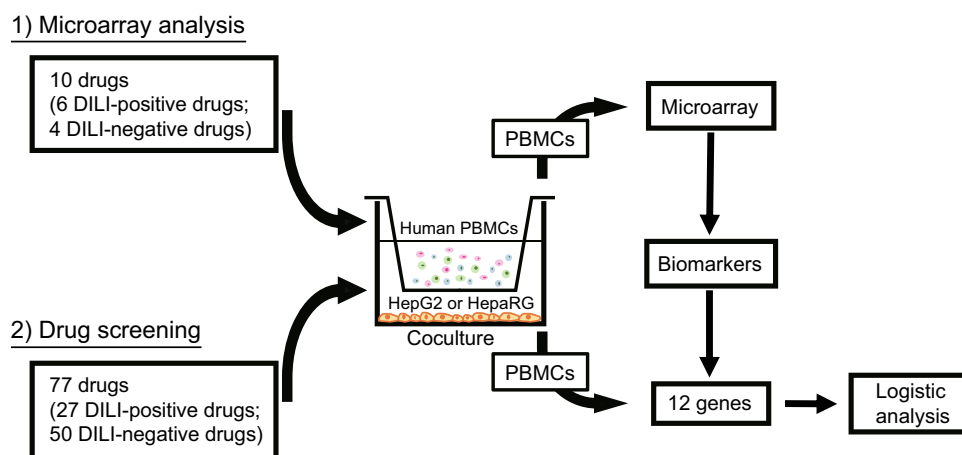
manufacturer's instructions. Five hundred nanograms of total RNA were reverse transcribed to cDNA using a Rever-Tra Ace® qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with total volumes of 10 μL containing 0.5 μL of cDNA, 5 μL of $2 \times$ TB Green® Premix Ex Taq (Tli RNaseH Plus, Takara Bio, Kusatsu, Japan) solution, 0.2 μL of 50-fold ROX reference dye, and 4 pmol each of the forward and reverse primers. Sequences of the primers that were commercially synthesized (Hokkaido System Sciences, Sapporo, Japan) are shown in Table S3. Real-time PCR was performed using an Mx3000P instrument (Agilent Technologies) with an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Each assay included 1:10 serial dilution points (10^4–10^9-fold) of PCR amplicons of each gene, and a calibration curve was constructed by plotting the PCR threshold cycle (Ct) value against the $\log_{10}$ (template dilution value). The relative mRNA expression levels in experimental samples were calculated from the standard curve and normalized to GAPDH mRNA levels.

Statistical analysis

A comparison of the two groups was performed with an unpaired Student's t test. Correlation analyses were performed using Pearson's test.

Logistic regression analyses were performed for each biomarker individually and in combination with simple logistic model and multiple logistic model, respectively, to evaluate the discriminatory ability of biomarkers between DILI-positive and DILI-negative drugs. In the evaluation of each biomarker, we used the simple logistic regression model with the mRNA expression level x_g of each biomarker g and the probability of DILI-positive drug P_g for each biomarker g:

$$\operatorname{logit}\left(P_g\right) = \beta_{0,g} + \beta_g x_g.$$

In case of evaluating the combination of biomarkers, the multiple logistic regression model was used with the probability of DILI-positive drug P:

$$\operatorname{logit}(P) = \beta_0 + \sum_g \beta_g x_g.$$

The best combination of biomarkers was selected by minimizing the Akaike information criterion (AIC) through a backward stepwise selection. For each logistic regression model, receiver-operating characteristic (ROC) curves were drawn by plotting the true positive fraction (sensitivity) as a function of the false-positive fraction (1-specificity) for all possible thresholds. The optimal cut-off values were determined according to the maximum value of Youden's index $[J]([J] = \text{Sensitivity} + \text{Specificity} - 1)$. The overall

discriminatory performance was estimated based on the area under the ROC curve (AUC). Sensitivity, specificity, and accuracy were calculated using the following formulas:

$$\text{Sensitivity} = TP/(TP + FN)$$

$$\text{Specificity} = TN/(TN + FP)$$

$$\text{Accuracy} = (TP + TN)/(TP + FN + FP + TN),$$

where TP, FN, FP, and TN denote the numbers of true positives, false negatives, false positives, and true negatives, respectively. Statistical analyses were performed using JMP Pro 14 Software (Version 14. 2. 0, SAS Institute, Cary, NC) or GraphPad Prism 8 software. In all tests, a p value < 0.05 was considered statistically significant.

Results

Selection of biomarkers for predicting DILI potential by microarray analysis

For gene expression analysis, we considered that treatment with the tested drugs should not cause a significant reduction in PBMC viability. Freshly isolated PBMCs (a healthy male donor aged 63 years) alone were exposed to ten drugs, including amodiaquine (AMQ), chloroquine (CLQ), diclofenac (DCF), ethaclinic acid (ETA), ketoconazole (KTZ), ketorolac (KTL), levofloxacin (LVF), mebendazole (MBZ), tienilic acid (TNA), and trovafloxacin (TVF), at concentrations corresponding to 100, 50, 25, 10, 5, 2.5, or $1 \times C_{\max}$ of each drug, and viability was determined after 24 h using the Cell Counting Kit-8, which measures intracellular dehydrogenase activities (Table S4). Since cell death-dependent decrease in intracellular RNA content hinders a successful transcriptome-wide expression analysis, we chose a maximum concentration for each drug up to $100 \times C_{\max}$ at which viability was over 70% of the 0.4% DMSO vehicle-treated control. The selected concentrations are shown in bold in Table S4. Since TNA at $25 \times C_{\max}$ was insoluble in media, $10 \times C_{\max}$ was selected.

There is increasing evidence that idiosyncratic DILI is in part immune-mediated (Andrade et al. 2019; Fontana 2014) and that drugs can initiate immune reactions by activating innate immune reactions via the release of DAMPs from hepatocytes (Holt and Ju 2006). Therefore, an in vitro model that allows communication between immune cells and hepatic parenchymal cells may mimic the in vivo circumstance. In this study, we established an in vitro coculture system of PBMCs together with the liver-derived cell lines HepaRG and HepG2 using culture inserts (Fig. 1).

For microarray analysis, freshly isolated PBMCs (a healthy male donor aged 63 years) were used. The coculture system (both insert and transwell sides) was treated with ten separate drugs mentioned earlier at the selected concentration, and transcriptome-wide expression analysis in PBMCs was performed. PCA of all detected genes showed an overall discrimination between DILI-positive and DILI-negative drugs, although the separation was not perfect (Fig. 2a). This separation was attributable to PC2, which accounts for 15.6% of the variance. To uncover the genes contributing to the PC, factor loading was calculated for each gene. Factor loadings range from − 1 to 1, and values close to − 1 and 1 indicate a strong association. Here, we selected genes with factor loadings below the 1st quartile (25th percentile) or more than the 4th quartile (75th percentile), some of which were further subjected to validation analysis between microarray and RT-qPCR data (not shown). The following 12 genes showing a significant correlation between microarray and RT-qPCR were selected as biomarkers (Fig. 2b): bone morphogenetic protein 6 (*BMP6*), C-X-C motif chemokine ligand 8 (*CXCL8*), epiregulin (*EREG*), interleukin 1 alpha (*IL-1A*), interleukin 1 beta (*IL-1B*), interleukin 24 (*IL-24*), MET proto-oncogene, receptor tyrosine kinase (*MET*), phosphotyrosine interaction domain containing 1 (*PID1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), solute carrier family 7 member 11 (*SLC7A11*, also known as *xCT*), secretory leukocyte peptidase inhibitor (*SLPI*), and TNF alpha-induced protein 6 (*TNFAIP6*). Hierarchical clustering of the 12 genes indicated that DILI-positive and DILI-negative

drugs were mostly clustered separately regardless of whether the cocultured cells were HepG2 or HepaRG cells (Fig. 2b). Thus, HepG2 cells were used as a counterpart of PBMCs in the subsequent study.

Evaluation of viabilities of PBMC and HepG2 cells as biomarkers for discriminating between DILI-negative and DILI-positive drugs

To set an optimal drug concentration used for coculture of PBMCs and HepG2 cells for the screening assay, commercial cryopreserved PBMCs (LP226; a healthy female donor, 55 years old) alone were exposed to 77 test drugs at concentrations corresponding to 100, 50, 25, 10, 5, 2.5, or $1 \times C_{max}$ of each drug, and viability was determined after 24 h using CellTiter-Glo®2.0 reagent, which measures the intracellular ATP content (Table S5). A maximum concentration for each drug up to $100 \times C_{max}$ at which viability was over 70% of the 0.4% DMSO-treated control was selected for coculture of PBMCs and HepG2 cells. The selected concentrations are shown in Table S5. In addition, the IC_{50} values expressed as the fold above C_{max} of each drug were calculated (Table S5). When IC_{50} values could not be calculated due to low toxicity up to $100 \times C_{max}$, they were set at $100 \times C_{max}$. Next, we calculated the predictive performance of the IC_{50} value for DILI. For this analysis, "No-DILI" and "Less-DILI-concern" drugs annotated at LTKB-BD were considered to be DILI-negative drugs, while "Most-DILI-concern" drugs at LTKB-BD were
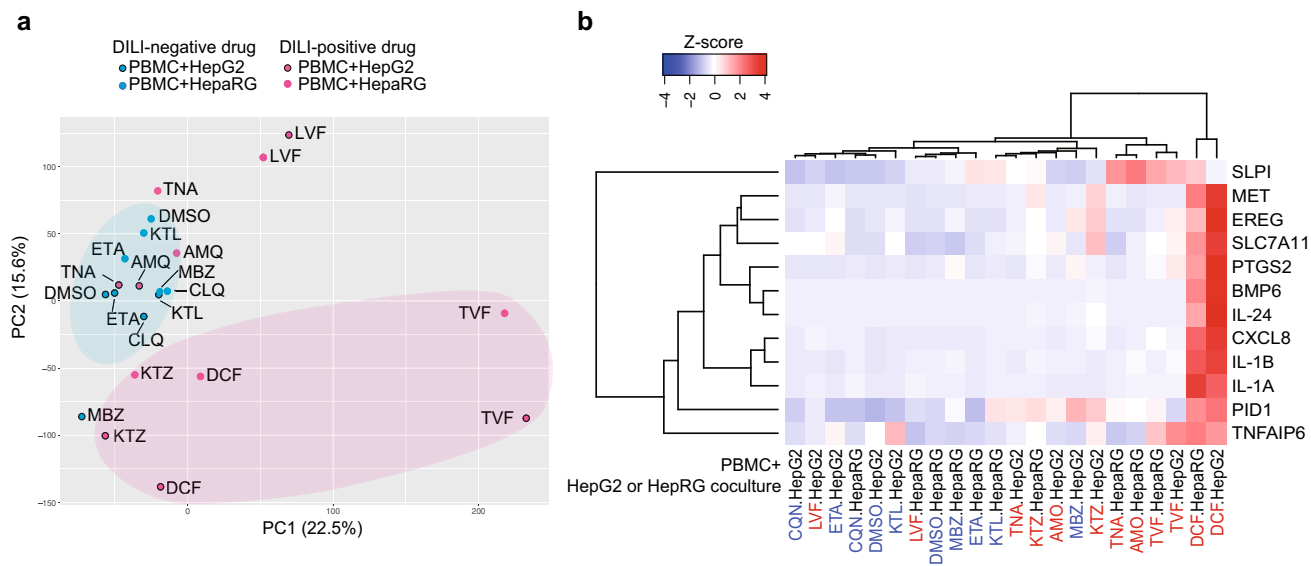

Fig. 2 Principal component analysis and hierarchical clustering of gene expression. Microarray analysis was performed using RNA from PBMCs that had been cocultured with HepG2 or HepaRG cells exposed to ten drugs as well as 0.4% DMSO vehicle-treated control ($n=1$). **a** Principal component analysis of transcriptome-wide analy-

sis. **b** Hierarchical clustering of selected genes. DILI-negative and DILI-positive drugs are shown in blue and red, respectively. *AMQ* amodiaquine, *CLQ* chloroquine, *DCF* diclofenac, *ETA* ethaclinic acid, *KTL* ketorolac, *KTZ* ketoconazole, *LVF* levofloxacin, *MBZ* mebendazole, *TNA* tienilic acid, *TVF* trovafloxacin

considered DILI-positive drugs. "Most-DILI-concern" drugs (DILI-positive) exhibited significantly lower IC_{50} values in comparison to "No-DILI" and "Less-DILI-concern" drugs (DILI-negative drugs) (Fig. 3a, top and Table 1), suggesting that DILI-positive drugs were cytotoxic to some extent. Logistic regression analysis followed by ROC analysis yielded an AUC of 0.70 with 52%/86% (sensitivity/specificity) (Fig. 3a, bottom; Table 2), suggesting that cytotoxicity can flag approximately half of DILI-positive drugs.

To evaluate the cytotoxicity of drugs to HepG2 cells, the selected single concentration for each drug was treated to HepG2 cells in a monoculture condition (Table 1). DILI-positive drugs significantly reduced viability ($p = 0.0212$) of HepG2 cells compared to those treated with DILI-negative drugs (Fig. 3b, top; Table 1). The predictive performance of HepG2 cell viability for DILI resulted in an AUC of 0.69 with 67%/68% (sensitivity/specificity) (Fig. 3b, bottom and Table 2), which was almost equal to that of PBMC viability (AUC = 0.70) (Fig. 3a, bottom).
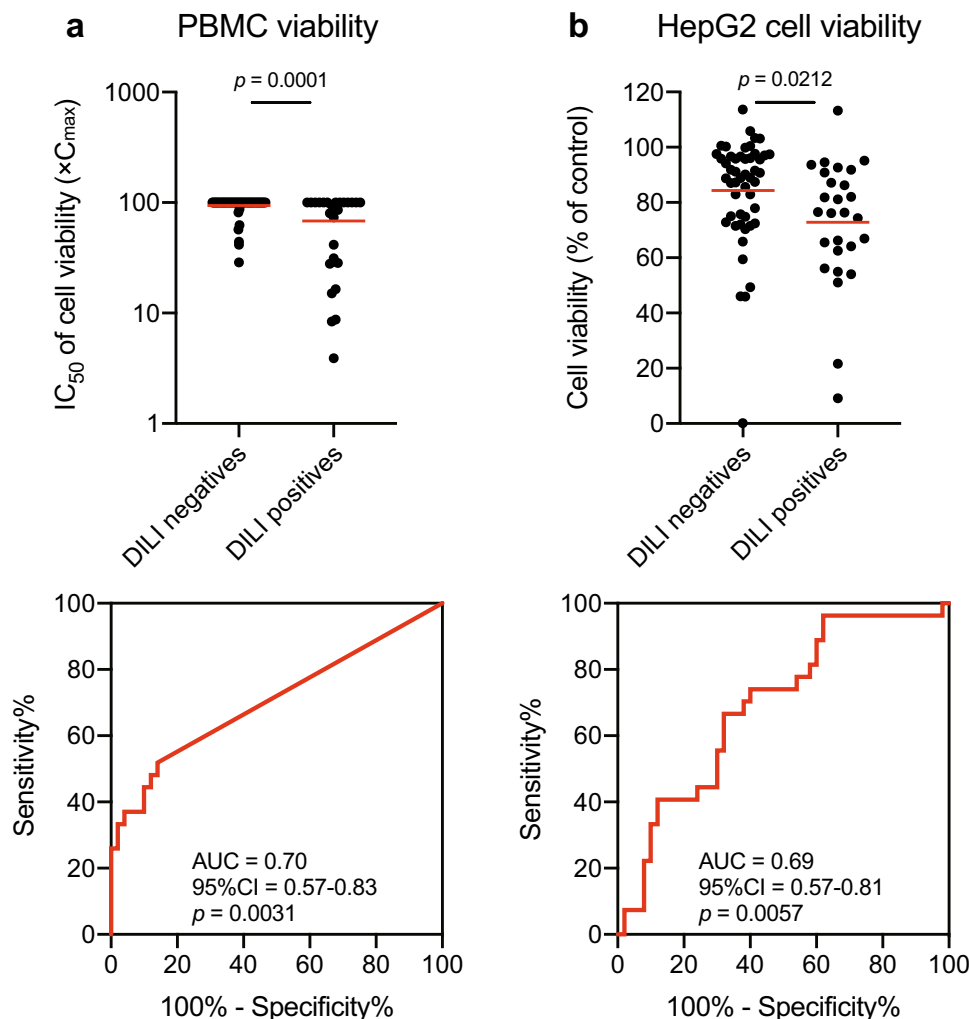
Fig. 3 Effects of drugs ($n = 77$) on PBMC and HepG2 cell viability and their discriminatory abilities for DILI. **a** PBMCs (lot: LP226) in monoculture were treated with various concentrations (100, 50, 25, 10, 5, 2.5, 1, 0.5, or $0.25 \times C_{max}$) of DILI-positive ($n = 27$) and DILI-negative drugs ($n = 50$), and cell viabilities were determined based on cellular ATP levels ($n = 3$ or 4 biological replicates). Top. IC_{50} values of test drugs for the inhibition of PBMC viability. IC_{50} values are expressed as the fold change ($\times$) of the human plasma maximum concentration (C_{max}) and are shown as dots. The red horizontal line represents the mean of all drugs. Bottom. ROC curve for the discrimination of DILI-positive drugs from DILI-negative drugs using the IC_{50} values. **b** HepG2 cells in monoculture were treated with a single concentration of DILI-positive ($n = 27$) and DILI-negative drugs ($n = 50$), and cell viabilities were determined based on cellular ATP levels ($n = 3$ or 4 biological replicates). The tested concentration shown in Table 1 was a maximum concentration for each drug up to $100 \times C_{max}$ at which PBMC viability was over 70% of the 0.4% DMSO vehicle-treated control. Top. HepG2 cell viability expressed as the percentage of the DMSO vehicle-treated control is shown as dots. Bottom. ROC curve for the discrimination of DILI-positive drugs from DILI-negative drugs using the values of HepG2 cell viability. *CI* confidence interval

Table 2 ROC analysis of the mRNA expression levels of each gene for discriminating DILI-positive and DILI-negative drugs

Predictors	Sensitivity	Specificity	Cutoff	TP	TN	FP	FN	Accuracy
BMP6	0.78	0.70	1.45	21	35	15	6	0.73
CXCL8	0.85	0.56	1.45	23	28	22	4	0.66
EREG	0.70	0.68	1.53	19	34	16	8	0.69
IL-1A	0.81	0.40	0.86	22	20	30	5	0.55
IL-1B	0.63	0.60	1.08	17	30	20	10	0.61
IL-24	0.74	0.69	2.98	20	34	15	7	0.71
MET	0.85	0.66	1.35	23	33	17	4	0.73
PID1	0.70	0.58	1.35	19	29	21	8	0.62
PTGS2	0.63	0.60	1.09	17	30	20	10	0.61
SLC7A11	0.48	0.78	1.61	13	39	11	14	0.68
SLPI	0.70	0.48	1.73	19	24	26	8	0.56
TNFAIP6	0.19	0.94	1.74	5	47	3	22	0.68
PBMC viability (IC_{50})	0.52	0.86	95.25	14	43	7	13	0.74
HepG2 cell viability (%)	0.67	0.68	82.01	18	34	16	9	0.68

Gene expression levels were determined in PBMC (lot: LP226) + HepG2 coculture. Cell viability of PBMCs (lot: LP226) or HepG2 was determined in monoculture. The 27 DILI-positive drugs and 50 DILI-negative drugs were used to predict the DILI potential by logistic analysis of each mRNA expression level (Table S6). The cut-off values were chosen to maximize Youden's index ([J] = sensitivity + specificity—1). TP, FN, FP, and TN denote the numbers of true positives, false negatives, false positives, and true negatives, respectively

Evaluation of gene expression levels as biomarkers for discriminating between DILI-negative and DILI-positive drugs

We next compared the coculture system of cryopreserved PBMCs with HepG2 cells to the PBMC monoculture system in terms of the inducibility of selected genes to evaluate the utility of the coculture system (Fig. 4). The PBMC (lot: LP226)/HepG2 coculture system and PBMC (lot: LP226) monoculture were treated with DILI-positive drugs, including diclofenac, etodolac, flucloxacillin, flutamide, nevirapine, nimesulide, troglitazone, trovafloxacin, and zafirlukast, at each concentration that yielded more than 70% cell viability (Table S5), and mRNA expression in PBMCs was determined. Treatment with most DILI-positive drugs highly induced BMP6, CXCL8, and MET mRNA expression levels in the PBMC/HepG2 coculture system compared to the PBMC monoculture (Fig. 4). These results indicate that the PBMC/HepG2 coculture system has a higher reactivity than the PBMC monoculture system, suggesting that marker gene expression in the PBMC/HepG2 coculture system could be applied to DILI assessment. Therefore, we next investigated the utility of the selected biomarker genes for predicting the DILI potential of 77 randomly selected marketed or withdrawn drugs with different DILI risks.

Cryopreserved PBMCs (lot: LP226) were cocultured with HepG2 cells exposed to the test drugs at the selected drug concentration that yields more than 70% cell viability, and mRNA expression was determined by qPCR. The mRNA expression levels of BMP6, MET, and PID1 were

significantly higher in the cocultured PBMCs treated with DILI-positive drugs than in those treated with DILI-negative drugs (Fig. 5a). DILI-positive drugs increased the expression levels of CXCL8, EREG, IL-24, and SLPI mRNA, although they were not statistically significant. Then, logistic regression analyses were conducted for each gene to evaluate how accurately these genes discriminate DILI-positive drugs versus DILI-negative drugs (Table S6). The ROC analysis revealed that the AUC values were the highest for MET (AUC: 0.81), followed by BMP6 (0.76), IL-24 (0.73), CXCL8 (0.72), and EREG (0.67), with significance (Fig. 5b). The AUC values of the four genes (MET, BMP6, IL-24, and CXCL8) were higher than that of the IC_{50} of PBMC viability (Fig. 3b). MET and BMP6 showed sensitivity/specificity of 85%/66% and 78%/70%, respectively (Table 2). Thus, marker gene expression levels could be of utility for the prediction of DILI potential.

We next investigated whether the marker gene levels reflect the DILI potential of drugs within the same pharmacological class. For this purpose, the MET expression levels were compared among drugs within the same pharmacological categories, because its AUC was the highest (Fig. 5c). Using the cut-off value which maximizes Youden's index, eight NSAIDs were successfully predicted as DILI-positive or DILI-negative by MET expression, as were antibacterials except the DILI-positive drug levofloxacin, which was falsely predicted as DILI-negative. However, the antidiabetic thiazolidinedione pioglitazone and dopaminergic entacapone were cases of false-positive prediction. Thus, the single parameter analysis of marker gene expression levels
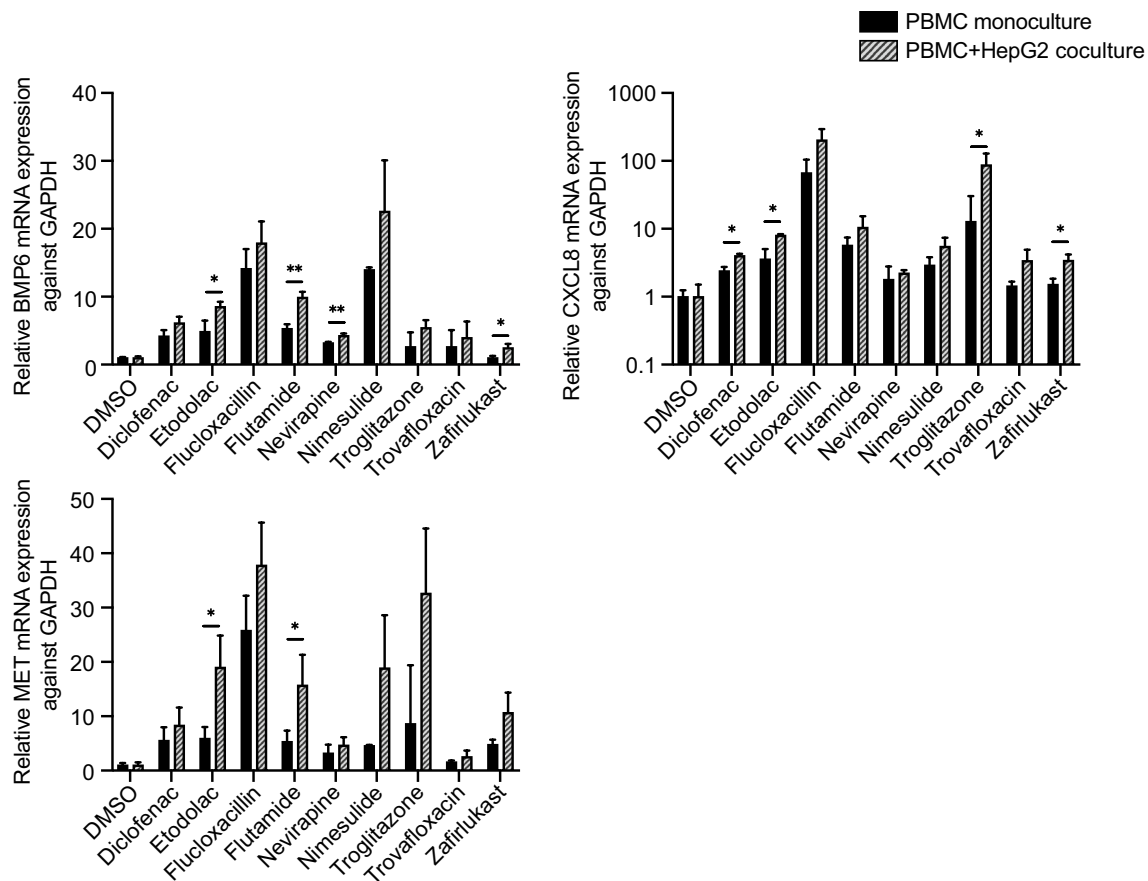
Fig. 4 Effects of coculture with HepG2 cells on the induction of gene expression in in vitro culture of PBMCs exposed to DILI-positive drugs. Human PBMCs (lot: LP226) were cultured with (PBMC + HepG2 coculture) or without (PBMC monoculture) HepG2 cells using the culture insert and exposed to DILI-positive drugs (diclofenac, etodolac, flucloxacillin, flutamide, nevirapine, nime- sulide, troglitazone, trovafloxacin, and zafirlukast) at the concentrations shown in Table 1. BMP6, CXCL8, and MET mRNA expression levels were determined by qPCR and normalized to that of GAPDH. Data are the mean ± SD ($n = 3$ biological replicates). $*p < 0.05$ and $**p < 0.01$ by two-tailed unpaired Student's t test

in the PBMC/HepG2 coculture system showed superiority to PBMC viability.

The combination of biomarker genes better predicts DILI potential

To investigate whether the combination of marker gene expression levels affects prediction, multiple logistic analysis followed by ROC analysis was performed. By the stepwise procedure, we found that a combination of BMP6, EREG, IL-1A, MET, PID1, PTGS2, SLC7A11, SLPI, and TNFAIP6 (Table S7) provided the highest AUC value of 0.94 (Fig. 6a, top). The estimated probability of being a DILI-positive drug was calculated and shown with the cut-off value, which maximizes Youden's index (Fig. 6a, bottom). As a result, the sensitivity and specificity were calculated as 93% and 86%, respectively, with an accuracy of 88% (Fig. 6a, bottom; Table 3). Two out of 27 DILI-positive drugs were mispredicted as false negatives, including didanosine and tolcapone, while 7 out of 50 DILI-negative drugs were mispredicted as false positives, including atropine, chloroquine, deferoxamine, isoproterenol, mebendazole, pamabrom, and primaquine (Fig. 6a, bottom).

Evaluation of donor-to-donor differences and comparison of PBMC monoculture with coculture

To investigate donor-to-donor differences in the predictive performance of gene expression levels, another donor-derived PBMC (lot: LP316) were cocultured with HepG2 and the 77 drugs were treated. mRNA expression levels of the nine genes used for the multiple logistic regression analysis were measured in PBMCs (Supplementary Fig. S1). In addition to BMP6, MET, and PID1, the mRNA expression levels of EREG were highly induced by DILI-positive drugs in this lot (Supplementary Fig. S1A and Fig. 5a). AUC values of BMP6, MET, PID1, and SLPI mRNA levels
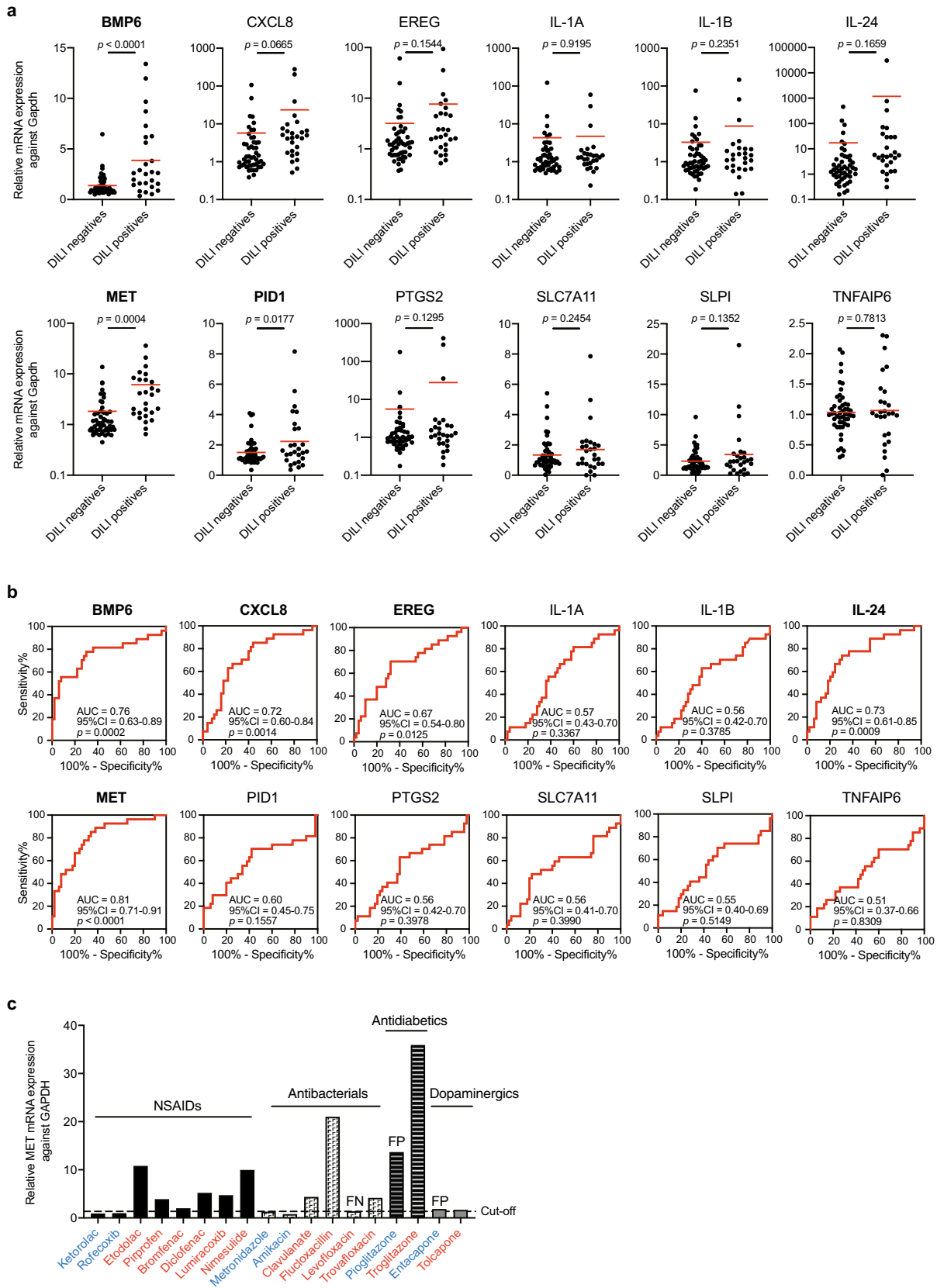
◀**Fig. 5** Effects of drugs ($n = 77$) on the mRNA expression of biomarkers and their discriminatory ability for DILI in PBMC and HepG2 cell coculture. Human PBMCs (lot: 226) were cultured with HepG2 cells and exposed to DILI-positive ($n = 27$) and DILI-negative drugs ($n = 50$) at the concentrations shown in Table 1. **a** The mRNA expression levels in the PBMCs were normalized to that of GAPDH and are shown as relative to 0.4% DMSO vehicle-treated control. Each dot represents the mean of each drug ($n = 2$ biological replicates), and the red horizontal line represents the mean of all drugs. *P* values determined by two-tailed unpaired Student's t test are shown. **b** ROC curves for the discrimination of DILI-positive drugs from DILI-negative drugs using the mRNA expression levels. *CI* confidence interval. **c** MET gene expression levels in PBMCs cocultured with HepG2 cells exposed to drugs. Pharmacological categories are shown above the columns. Each column represents the mean of each drug ($n = 2$). Blue: DILI-negative drug; red: DILI-positive drug; *FN* false negative, *FP* false positive (color figure online)

were significant in the coculture, among which BMP6 and MET were common to both PBMC donors (Supplementary Fig. S1B and Fig. 5b). Furthermore, multiple logistic regression analysis of the nine genes resulted in an AUC of 0.91 (Fig. 6b) and sensitivity/specificity of 74%/100% (Table 3) in PBMC (LP316) + HepG2 coculture, which were almost equal to those of PBMC (LP226) + HepG2 coculture (AUC = 0.94, Fig. 6a; sensitivity/specificity 93%/86%, Table 3). Therefore, although there were some donor-to-donor differences in the predictive performance of each gene, the integrated analysis suggests that predictive performance was almost not affected by the donor difference.

Finally, we compared the predictive performance of mRNA expression levels of the nine genes between PBMC monoculture and PBMC/HepG2 coculture. For this purpose, PBMC (lot: LP226) was cultured on culture inserts and 77 drugs were treated in the absence of liver cells. The mRNA expression levels in PBMC monoculture were not different between DILI-positive drugs and DILI-negative drugs except SLC7A11 (Supplementary Fig. S2A), and AUCs of each gene ranges from 0.53 to 0.77 (Supplementary Fig. S2B). These values were lower than those of PBMC + HepG2 coculture (AUC range: 0.51 to 0.81) (Fig. 5b). Furthermore, multiple logistic regression analysis of the nine genes resulted in an AUC of 0.83 (Fig. 6c) and sensitivity/specificity of 74%/86% in PBMC monoculture (Table 3), which were lower than those of PBMC + HepG2 coculture (AUC = 0.94; sensitivity/specificity 93%/86%, Fig. 6a). These results demonstrate that PBMC + HepG2 coculture has a better predictive performance for DILI than PBMC monoculture, supporting the utility of PBMC + HepG2 coculture.

Discussion

Most cell-based assays for the assessment of DILI reported so far utilize cytotoxicity in hepatic parenchymal cells as readouts (Gómez-Lechón et al. 2016). However, their

predictabilities for DILI are not so high. It is important to note that clinical features of immune-mediated or hypersensitivity drug reactions can be observed in one-quarter of patients (Andrade et al. 2019). Thus, biological responses in immune cells could be applied to the risk assessment of DILI. However, there is a paucity of such studies that attempt to evaluate clinical DILI risks comprehensively by immune cell responses in vitro. In this study, we explored and evaluated marker genes in human PBMCs cocultured with a liver-derived HepG2 cell line by transcriptome-wide analysis and evaluated their predictability for DILI.

We previously reported a cell-based assay in which the human promyelocytic leukemia cell line HL-60 was incubated with conditioned media of HepG2 or HepaRG cells that had been treated with test drugs. In that study, immune- and inflammatory-related genes in HL-60 cells, including MCP-1, S100A9, IL-1B, CXCL8, and TNFA, were tested as biomarkers (Oda et al. 2016), because these genes were upregulated in the liver of mice treated with DILI-positive drugs compared to mice treated with DILI-negative drugs (Yano et al. 2014). Screening of 79 pharmaceuticals revealed that CXCL8 in HL-60 cells treated with conditioned media of HepaRG cells that had been treated with test drugs provided acceptable discriminatory performance for DILI with an AUC of 0.75. However, the previous study (Oda et al. 2016) subjectively selected biomarker genes from in vivo mouse studies, which motivated us to identify more suitable genes for DILI assessment in this study focusing on human cell-based assays that included an immune component (PBMCs). Therefore, this study implemented transcriptome-wide gene expression analysis to objectively select candidate biomarker genes. In addition to CXCL8 and IL-1B, which are the only common biomarkers between this and previous studies (Oda et al. 2016), we found several promising biomarkers.

We found that each of the mRNA expression levels of MET, BMP6, and IL-24 in PBMCs provided superior predictability (AUC 0.73–0.81) of DILI compared to CXCL8 (IL-8) and IL-1B (Fig. 5b). In particular, the MET level is promising, because it showed the highest AUC among all single parameters evaluated with a sensitivity of 85% and a specificity of 66% (Table 2). Furthermore, MET expression levels could differentiate some DILI-positive drugs from DILI-negative drugs within the same pharmaceutical categories (Fig. 5c). Thus, evaluation of DILI potential by MET levels in PBMCs may aid in selecting compounds at the lead optimization or early stage of drug development. Most in vitro assays for evaluating DILI potential established so far utilize cell viability, superoxide production, and mitochondrial membrane integrity in hepatic parenchymal cells as readouts (O'Brien et al. 2006; Wink et al. 2014; Xu et al. 2008). These conventional markers have no satisfactory predictive power (cell viability: 10% sensitivity and
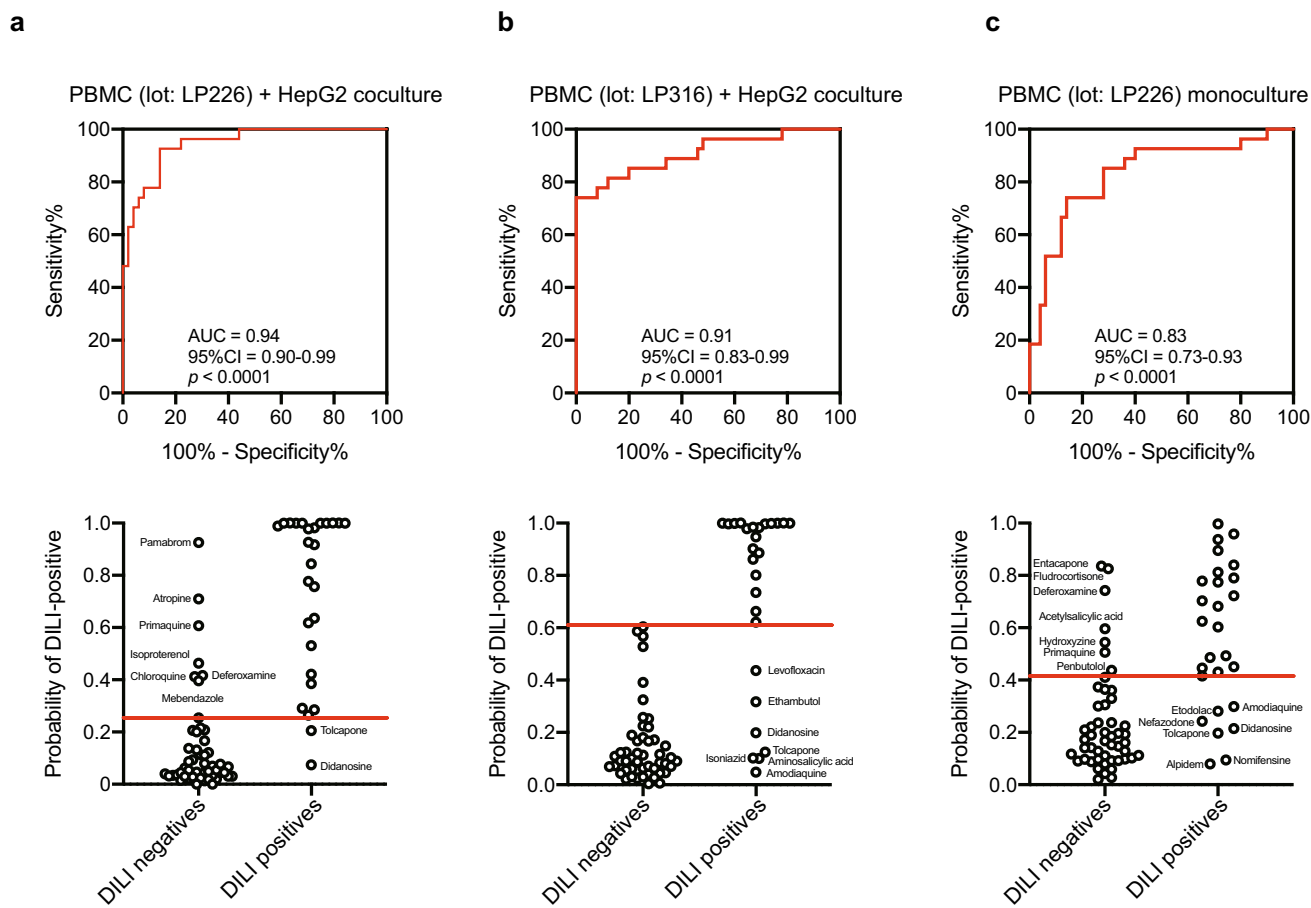
a

PBMC (lot: LP226) + HepG2 coculture

AUC = 0.94
95%CI = 0.90-0.99
$p < 0.0001$

b

PBMC (lot: LP316) + HepG2 coculture

AUC = 0.91
95%CI = 0.83-0.99
$p < 0.0001$

c

PBMC (lot: LP226) monoculture

AUC = 0.83
95%CI = 0.73-0.93
$p < 0.0001$

Fig. 6 Development of a multiple logistic regression model of gene expression levels for detecting DILI-positive drugs. Top. ROC curves for the discrimination of DILI-positive drugs ($n = 27$) from DILI-negative drugs ($n = 50$) using a multiple logistic regression model of mRNA expression levels of nine genes (BMP6, EREG, IL-1A, MET, PID1, PTGS2, SLC7A11, SLPI, and TNFAIP6) in **a** PBMC

(lot: LP226) + HepG2 coculture ($n = 2$ biological replicates), **b** PBMC (lot: LP316) + HepG2 coculture ($n = 1$), and **c** PBMC (lot: LP226) monoculture ($n = 1$). Bottom. The probability of being DILI-positive for each test drug ($n = 77$) was calculated from the logistic model for each culture. The red horizontal line represents the cut-off probability. *CI* confidence interval

Table 3 ROC analysis of the mRNA expression levels of the selected nine genes for discriminating DILI-positive and DILI-negative drugs

Cell	Sensitivity	Specificity	Cutoff (probability)	TP	TN	FP	FN	Accuracy
PBMC (lot: LP226) + HepG2 coculture	0.93	0.86	0.264	25	43	7	2	0.88
PBMC (lot: LP316) + HepG2 coculture	0.74	1.00	0.622	20	50	0	7	0.91
PBMC (lot: LP226) monoculture	0.74	0.86	0.622	20	43	7	7	0.82

The 27 DILI-positive drugs and 50 DILI-negative drugs were used to predict the DILI potential by multiple logistic analysis of the expression levels of BMP6, EREG, IL-1A, MET, PID1, PTGS2, SLC7A11, SLPI, and TNFAIP6 (Table S7). The cut-off probability was chosen to maximize the Youden's index ([*J*] = sensitivity + specificity—1). TP, FN, FP, and TN denote the numbers of true positives, false negatives, false positives, and true negatives, respectively

92% specificity; glutathione depletion: 19% sensitivity and 85% specificity) in HepG2 cells when assayed at a single concentration (O'Brien et al. 2006). Recent studies, however, utilized long-term spheroid cultures of primary human hepatocytes for the evaluation of hepatotoxic liabilities

of drugs (Proctor et al. 2017; Vorrink et al. 2018). They reported improved sensitivities/specificities of 59%/80% in 110 drugs (Proctor et al. 2017) and 69%/100% in 123 drugs at $100 \times C_{max}$ concentration (Vorrink et al. 2018), suggesting that hepatocytes retaining physiological cellular phenotypes

can be translated into an improved prediction. However, it should be noted that the overall predictive performance of these studies was determined by a single donor of hepatocytes. More recently, with a support vector machine-based classifier using human whole blood C_{max} and EC_{10} of cytotoxicity to sandwich cultured primary human hepatocytes (corresponding to the drug concentration where the sigmoidal curve reaches the value of 90%), sensitivity and specificity were 88% and 100%, respectively, although the number of tested drugs was only 28 (Albrecht et al. 2019). Although the number of tested drugs and exposure concentrations as well as target cells (monoculture of hepatocytes versus coculture of PBMCs and HepG2 cells) are different between the two studies and our study, the predictability of DILI by MET expression levels is comparable to those of the previous studies. Taken together, single gene expression, such as MET, in PBMCs can be an alternative in vitro tool for detecting DILI potential.

Recently, the DILI potential of drugs has been evaluated by a hybrid evaluation system that includes several biological parameters (cytotoxicity, mitochondrial dysfunction, and bile salt export pump inhibition) and physicochemical properties (dose, lipophilicity, ionization state, and carbon bond saturation) (Aleo et al. 2020). These approaches take into consideration the nature of idiosyncratic DILI, which develops by multifactorial mechanisms (Kaplowitz 2005; Stephens et al. 2014). In this study, using the nine identified biomarker genes, a multiple logistic model of BMP6, EREG, IL-1A, MET, PID1, PTGS2, SLC7A11, SLPI, and TNFAIP6 expression levels was modeled (Table S7). This model provided the best prediction in distinguishing DILI-positive drugs from DILI-negative drugs, with the highest AUC value of 0.94 (Fig. 6a) with a sensitivity of 93% and a specificity of 86% (Table 3), which was higher than the 85% sensitivity of MET. This trend was also observed in the hierarchical clustering analysis, where the consideration of several genes rather than a single gene appears to discriminate between DILI-positive and DILI-negative drugs (Fig. 2b). Thus, the use of a combination of several biomarker genes is expected to improve the predictive performance of DILI potential. Two drugs, didanosine and tolcapone, were consistently predicted to be false negatives in PBMC+HepG2 cell coculture irrespective of PBMC donor (Fig. 6a, b). Tolcapone is reported to form reactive metabolites that are trapped by glutathione, whereas the structural analog entacapone, a non-DILI drug, is not (Dragovic et al. 2016; Smith et al. 2003; Stepan et al. 2011). Thus, it should be emphasized that DILI potential should be evaluated by considering multiple factors, including the formation of reactive metabolites, cytotoxicity, drug plasma/tissue concentration, and immune-related responses.

Of particular interest in this study was that the extent of induction of BMP6, CXCL8, and MET mRNA by DILI-positive drugs (diclofenac, etodolac, flutamide, nevirapine, troglitazone, and zafirlukast) was largely higher in the coculture of PBMCs with HepG2 cells than in the PBMC monoculture (Fig. 4). This suggests that humoral factors secreted or released from drug-exposed HepG2 cells may affect the inducibility of these genes in PBMCs. It is known that stressed hepatocytes have the potential to release DAMPs such as high mobility group box-1 (HMGB1) and heat shock proteins (HSPs) to activate the immune system through Toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGEs) (Holt and Ju 2006; Kono and Rock 2008). This study did not identify which DAMPs are released from HepG2 cells and which immune cells receive the stimuli and are activated. Other working groups also suggested that hepatocytes treated with DILI drugs (such as amodiaquine, diclofenac, ketoconazole, nevirapine, tolcapone, and troglitazone) release DAMPs to activate immune cells (Granitzny et al. 2017; Kato and Uetrecht 2017; Mak et al. 2018). This immune cell activation in vitro by DILI drugs would be linked with DILI, because some drugs discussed here are likely to cause liver injury via an immune-mediated mechanism as follows: flucloxacillin-induced liver injury is associated with the human leukocyte antigen (*HLA)*B5701* allele (Daly et al. 2009; Wuillemin et al. 2014); nevirapine-induced skin rash is associated with the *HLA-B3505* allele (Chantarangsu et al. 2009); flutamide-induced liver injury is associated with Th2 cytokines in wild-type mice (Higuchi et al. 2012); diclofenac (Deng et al. 2006) and trovafloxacin (Shaw et al. 2009) cause liver injury in rats or mice pretreated with lipopolysaccharide; and troglitazone and tolcapone cause liver injury only in PD1 knockout mice under anti-CTLA4 antibody treatment (Mak et al. 2018).

Although we found several genes that can predict a drug's potential to cause DILI, their association with the mechanism of DILI remains unknown. One such gene, MET, also known as hepatocyte growth factor receptor (HGFR), is a proto-oncogenic receptor tyrosine kinase that recognizes the endogenous ligand hepatocyte growth factor/scatter factor (HGF) (Trusolino et al. 2010). MET is expressed in monocytes, B cells, and natural killer (NK) cells but not in peripheral blood CD8^{+} or CD4^{+} T cells (Molnarfi et al. 2012), although the function of HGF-MET signaling in these cells remains unclear. Another study, however, demonstrated that MET is expressed in memory T cells in lymph nodes and that HGF–MET signaling facilitates T-cell recruitment to heart tissue to contribute to inflammation (Komarowska et al. 2015). BMP6 is a member of the TGF-β superfamily of BMPs and exhibits a broad spectrum of biological functions, including cell growth and differentiation (Miyazono et al. 2010). BMPs, including BMP6, are known to produce and activate naïve CD4^{+} T cells in response to T-cell receptor stimulation (Martinez et al. 2015). Although the

pathophysiological roles of MET and BMP6 in DILI are unknown, the significant upregulation of MET and BMP6 mRNA in PBMCs by DILI-positive drugs may reflect the activation of monocytes, B cells, NK cells, or T cells. Because of limited information on the association between these genes and DILI, further study is required.

Idiosyncratic DILI is thought to be associated with multifactorial mechanisms, including patient-associated factors (i.e., sex, genetic variation, and infectious status) as well as the compound potential to cause DILI. In fact, certain alleles of *HLA* are associated with the increased incidence of DILI caused by specific drugs (Usui and Naisbitt 2017). However, such an association can be identified only after the drug reaches the market under the current circumstance. Thus, predictive models for DILI are urgently required. With respect to in vitro models utilizing immune cells for the risk assessment of DILI, Kegel et al. established an in vitro culture of human Kupffer cells treated with conditioned media of hepatocytes that were exposed to diclofenac and acetaminophen (Kegel et al. 2015). They found a compound- and donor-dependent increase in proinflammatory cytokines produced by Kupffer cells. However, this system seems to be unsuitable for screening large numbers of compounds due to the limited availability of primary human liver cells. In addition, Kupffer cells alone may not be sufficient to capture potential immune activation induced by drugs. In this regard, our coculture model using PBMCs and HepG2 cells may be useful in that cells are readily available and PBMCs are comprised of several immune cell types, including subsets of lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells.

There are several limitations in this study. First, most of the DILI-positive drugs selected for our microarray experiments have been demonstrated to have immunoallergic features, including amodiaquine, diclofenac, levofloxacin, (LiverTox: https://www.ncbi.nlm.nih.gov/books/NBK547852/), tienilic acid (Lecoeur et al. 1996), and trovafloxacin (Ball et al. 1999). This would constitute a bias with respect to biomarker selection as many other DILI-positive drugs not having an immune component in their mechanisms may be excluded. Second, although we considered Less-DILI-concern drugs as DILI-negative in this study, other database reported some DILI concern for them. For example, acetylsalicylic acid and trazodone, which are classified as Less-DILI concern in LTKB-BD, are reported to cause clinically apparent liver injury in the LiverTox database. In addition, although rofecoxib is not assigned in LTKB-BD and, hence, is considered as DILI-negative in this study, it can cause clinically apparent, symptomatic drug-induced liver injury with jaundice in rare instance according to the LiverTox database. Third, although we used 77 drugs, the validity of this assay needs to be confirmed using another set of drugs. Finally, we used only two donors for investigating

donor-to-donor differences in the predictability of DILI, because we focused on the identification and evaluation of biomarker genes with a panel of drugs. PBMC composition, and preexisting inflammatory status are different between donors (Ter Horst et al. 2016); therefore, further study is warranted to elucidate the effects of individual differences in PBMCs on the predictability of DILI. However, we believe that information regarding the evaluation of DILI potential by gene expression provided from this study is important, even though the data were obtained using cells from one donor.

In conclusion, we developed an in vitro coculture model using PBMCs with HepG2 cells to discriminate DILI-positive drugs from DILI-negative drugs based on gene expression signatures. The MET mRNA level provided good sensitivity and specificity for predicting DILI, but a combination of biomarker genes showed superior predictivity. However, because the number of DILI-positive drugs in the dataset was limited to 27, additional studies using a large number of compounds are needed to confirm the usefulness of these coculture systems. Nevertheless, to the best of our knowledge, this study is the first to identify biomarkers from transcriptome-wide analysis of PBMCs cocultured with liver cells for predicting the DILI potential of drugs. The established coculture model could be useful in detecting the DILI potential of compounds, in particular those that are immune-mediated.

Compliance with ethical standards

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