

In Vitro Immunotoxicity Testing of Pesticides using Human Cytokine Promoter Based Reporter Cell Lines

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Abstract

Pesticides including fungicides, herbicides and insecticides are the class of chemicals deliberately released into the environment because of their utility. Worldwide, an estimated, 3 million cases of pesticide exposure are reported per year. It has been established that pesticide chemicals can alter immune function and causes immunotoxicity. We have developed a model for immunotoxicity testing of chemicals *in vitro* by analyzing cytokine gene expression. The changes in cytokine gene expression observed *in vitro* was used as an endpoint for studying perturbation of the immune system *in vivo*. This system employs cell lines that have been transformed using constructs that have promoter region of Th1, Th2 cytokines and pro-inflammatory cytokines. These promoter regions regulate the expression of a reporter gene, Green Fluorescent Protein (GFP), and the activity of promoter to the external stimuli was compared with the expression of endogenous cytokine genes. CyclosporinA, FK506 (tacrolimus) and pentoxifylline, have been tested on this model and they showed specific pattern of suppression of reporter gene expression treated with PMA/Ionomycin and LPS. These immunosuppressants also suppressed the PMA/Ionomycin and LPS induced endogenous gene expression of these cytokines. Thus our model has potential for application as a predictive screening test system for immunomodulatory activities of chemicals. The validation of the model was carried out using three pesticides chlorpyrifos, cypermethrin and captan that were known to have immunosuppressive activity on the expression of Th1 and Th2 cytokines. Following exposure to above pesticides, we also observed decrease in Th1 and Th2 cytokines promoter activity based GFP expression in our transformed cells line. Thus our model efficiently predicts the immunosuppressant nature of pesticides and therefore may serve as a tool for preliminary high throughput immunotoxicity screening of test agents such as pesticides and other environmental pollutants.

Keywords: Immunotoxicity; GFP; *In vitro*; T cells; RT PCR; Immunosuppressants; Pesticides

Introduction

Pesticides are the chemical substances used for preventing, destroying, repelling or mitigating any pest ranging from insects (i.e., insecticides), rodents (i.e., rodenticides) and weeds (herbicides) to microorganisms (i.e., algicides, fungicides or bactericides) [1]. Pesticides can be toxic to humans and lower animals based on concentration and mode of action [2-4]. Worldwide, an estimated three million cases of pesticide poisoning occur every year, resulting in an excess of 250 000 deaths, primarily in developing countries [5]. Pesticides have been found to reduce the numbers of white blood cells and disease-fighting lymphocytes and impair the ability to respond against bacteria and viruses. Pesticides alter the function of the thymus and spleen, key immune organs. Methoxychlor and Endosulfan causes aberrant IgA and IgG production [6,7]. Pesticides associated immunomodulatory consequences may include immune enhancement, such as hypersensitivity and autoimmunity or immune suppression, which may increase the incidence of infectious disease or neoplastic transformation [8-10]. General toxicity of compounds could be assessed *in vitro* by evaluating degree and extent of test substance exposure induced cell death. However, immunotoxicity is quite difficult and it cannot be accomplished with a single test [11-13]. Understanding the complexity of immune system, multiple molecular and cellular targets of immunotoxicants have to be taken into account for immunotoxicity testing. The cytokines are the important components of immune system that connect disperse elements of the immune system. Since the dysregulation of cytokines gene expression explicitly represents the dysregulation of the immune system, it has become the valuable markers to study the alteration of immune system. Consequently, for immunotoxicity testing, i.e. evaluating the toxic effects of chemical and pesticides on immune system, the analysis of cytokine gene expression has been widely employed [14-16].

The Propanil 3,4 dichloropropioanilide, a commonly used herbicides, to which agriculture workers were are frequently exposed, has been found to increase the plasma IgG1 levels and regulate IL-6, IL-10 and IFN release. This was also associated with a reduced IFN/IL-4 ratio [17]. Studies conducted in children reveal that environmental toxicants induced adverse health effects are closely associated with altered Th1/Th2 cytokine profile [18,19]. The effect of chemical mixture of polychlorinated biphenyls (PCB), organochlorine pesticides (OCs) and methylmercury (MeHg) was studied in children and results revealed that early exposures to these toxicants manifests itself as aberrant development of children's Th1/Th2 immune response. Studies conducted in rats reveal that perinatal exposure to environmental toxicants alters cytokine profile of rats [20]. Similarly amino acid derivative induce upregulation of IL-5 expression in lymphocytes *in vivo* [21] and chemokines in keratinocytes respectively [20,21]. Thus Assessment of cytokine expression could be a tool to evaluate chemical exposure effects on the immune system [22,23].

The cytokines gene expression could be efficiently measured by analyzing the promoter activity in response to external stimuli; therefore a reporter construct is made where the expression of reporter

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gene is regulated by cytokine cis- regulatory regions (promoters). Fluorescent proteins such as GFP (green fluorescent protein) as reporter genes allows for very efficient measure of translated reporter gene product in cell lines [24]. In present study, the GFP reporter constructs were made where reporter gene were regulated by Th1 and Th2 and inflammatory cytokine regulatory regions. The Th1 and Th2 regulatory region constructs were transfected in Jurkat T cell line and the inflammatory regulatory region was transfected in THP-1 cell line. The transformed cell lines reporter gene of Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) were made to respond to the exposure of immunostimulant PMA (Phorbol Myristate Acetate)/Ionomycin and LPS (Lipopolysaccharide) and immuno suppressants like cyclosporine A (CsA) and FK506 on PMA/Ionomycin. Further, three pesticides; chlorpyrifos, cypermethrin and captan have been tested on this cytokine promoter based assay system so as to establish its applicability as a rapid and effective means to evaluate the pesticides/chemicals induced immunotoxicity.

Materials and Methods

Construction of GFP reporter vectors

To develop reporter cell lines for *in vitro* assay we constructed a set of reporter constructs, in which expression of GFP, was driven by regulatory sequences derived from the human IL-2, IFN- γ (Interferon γ), IL-4, IL-10 and TNF- α genes. We made constructs of each cytokine promoters that included the main regulatory sites required for functional control of transcription. The IL-2 upstream region (-341 to +47), IFN- γ upstream region (-565 to +65), IL-4 upstream region (-582 to +50) upstream region (-582 to +50), IL-10 upstream region (-2087 to +12) and TNF- α upstream region (-1131 to +158) relative to the transcription start site were amplified from human genomic DNA using the primers listed in table 1. The PCR products were cloned into the pGlow Topo promoter less vector and sequenced for confirmation.

Cell culture and stable transfection

Jurkat T cells and THP-1 cells were obtained from in-house cell and tissue culture facility of Central Drug Research Institute. The Jurkat T and THP-1 cell lines were grown in RPMI 1640 complete media (20 mM L-Glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS) at 37°C in CO₂ incubator (5% CO₂). During transfection 4 \times 10⁶ Jurkat T and THP-1 cells were suspended in incomplete culture medium (without FBS and antibiotics) mixed with 20 μ g plasmid DNA in a total volume of 0.4 ml, transferred to cuvette (4mm gap) and electroporated at 250V (Jurkat T) and 300V (THP-1) using the electroporator (BTX). After 2hrs 30min of incubation in incomplete medium the cells were replenished with 20% FBS containing medium and incubated for 48 h. The cells were selected with G418 (1mg/ml) by replacing medium with G418 every second day until the untransfected cells were dead. Limiting dilution cloning was performed and resulting

single cell derived clones were expanded. The presence of the integrated reporter plasmids were verified by PCR for the EGFP coding region on genomic DNA from the clones using the following primer pair GFP fwd 5' ACT ACT GGA AAA CTA CCT GTT C 3' GFP rev 5' ATA CGA TTA ACA AGG GTA TCA C 3' which binds to the GFP gene of the pGlowTopo vector.

Cytotoxicity assessment of compounds

Cytotoxicity of compounds on Jurkat T and THP-1 cell lines were analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reflecting cell viability. For MTT assay 1 \times 10⁵ cells were treated with compounds at various concentrations. After 48hrs of seeding in 96-well plate, 25 μ l of MTT solution (2.5 mg/ml) was added to each well and incubated for 4hrs. Media was removed and 200 μ l DMSO was added to each well and incubated for 30 min at room temperature with shaking to dissolve the Formazon crystal completely. The optical density was measured in microplate reader (Biotek instruments, USA) at 570 nm and 690 nm.

Exposure to immunomodulators

Transformed Jurkat T cell (0.5 \times 10⁶ cells/ml) activated with or without PMA (20ng/ml Sigma) and Ionomycin (1 μ M, Sigma) were exposed to different immunosuppressive chemicals viz. cyclosporine A (CsA), Tacrolimus and FK506 for 48 h. Differentiated THP-1 cells activated with or without LPS (1 μ g/ml) were treated with different chemicals for 48 hrs. After incubation cells were harvested and washed twice with pre-chilled PBS and then lysed in 1X Passive Lysis Buffer (100 μ l) by keeping cells on ice for 10 mins with intermittent vortexing. After centrifugation at 13000 rpm for 10mins, supernatant was collected/protein concentration was estimated. For normalization, an aliquot containing total 100 μ g protein was transferred to dark colored 96 well of plates (Costar) and fluorescence intensity was recorded at excitation 395nm and detection 520 nm in fluorimeter (Omega) which in turn served as a measure of GFP expression

RT-PCR and Real-Time PCR analysis

Control and treated cells were harvested and washed with PBS twice. Total RNA was isolated from 5X10⁶ fresh cells using Pure link RNA extraction kit (Invitrogen) The RNA yield was quantified by UV spectrophotometer (Powerwave XS Biotek Germany) by recording absorbance at 260/280 and 260/230. Further purity/integrity check was performed by subjecting one microgram of total RNA to electrophoresis through 1% formamide agarose gels. Preservation of 28S and 18s rRNA was used to assess RNA integrity. RNA Samples without detection of 28S/18S RNA were excluded from the further examination. RNA was treated with DNase (2unit/10 μ g of RNA) to degrade the residual genomic DNA. cDNA was synthesized from 2 μ g of total RNA using SuperScript[®] III One-Step RT-PCR System with Platinum[®]Taq (Invitrogen). The one-step format enables easy

Cytokines	Primer Sequence(5'.....3')	Region amplified
IL-2	Forward primer 5' TAT GAC AAA GAA AAT TTT CTG AGT TAC 3' Reverse primer 5' TGT GGC AGG AGT TGA GGT TAC TGT GAG 3'	-341 to +47
IFN- γ	Forward primer 5' GTA TTT CTA CTG GGC AGT GCT GAT CTA G 3' Forward primer 5' GGT CCA AAG GAC TTA ACT GAT CTT TCT C 3'	-565 to +65
IL-4	Forward primer 5' GGC CTC ACC TGA TAC GAC CTG TCC TTC T 3' Forward primer 5' TTG CAG TGA CAA TGT GAG GCA ATT AGT T 3'	-582 to +50
IL-10	Forward primer 5' ATG CTG AGT CCA CTT CTG GAA CAC CCA GCT 3' Forward primer 5' GCC TTC TTT TGC AAG TCT GTC TTG TG 3'	-2087 to +12
TNF- α	Forward primer 5' AGG GCT CAG AGA GCT TCA GGG ATA TGT GAT G 3' Forward primer 5' CAG GGG AGA GAG GGT GGA GCC GTG GGT CAG 3'	-1131 to +158

Table 1: Regulatory region of cytokines used for study and primer sequence used for amplification of these regions.

analysis of gene expression or detection of RNA. The cDNA and primer efficiency was confirmed by PCR amplification using Taq polymerase with pairs of primers as (Table 2). Quantitative real time PCR analysis was carried out in using same primers (0.5 pM/reaction) (Table 2). Quantitative PCR was performed on a Light Cycler 480 System (Roche) with cDNA (25ng/reaction) and duplicate primers (0.5pM/reaction) (Table 2) in 96-well plates using SYBR Green qPCR Master Mix (Invitrogen) in accordance with manufacturer's protocol. Data were analyzed using the Roche LightCycler 480 software (Version 1.5). Relative quantification of cDNA was done using δ CT method. The amount of the target mRNA was examined and normalized to the β -actin gene mRNA/GAPDH gene mRNA. Results represent data from three separate experiments.

Statistical analysis

Data represents Mean \pm SE of 3 independent experiments wherein individual experiments contained three replica sets. Statistical analyses were performed using Graph pad prism 5 software package. The differences between classes were determined by one-way ANOVA followed by Bonferroni's *t* test. *P* < 0.05 were considered significant.

Results

Effect of immunosuppressants on expression of reporter and endogenous cytokines gene

The reporter cell lines were tested for their response to different known immunosuppressive compounds. Reporter cell lines were treated with immunosuppressors (cylosporinA, CsA and Tacrolimus, FK506) and pentoxifylline in the presence of specific stimulators of cytokine expression and the effect was assessed by analyzing both GFP reporter and endogenous cytokine gene expression. We determined the IC-10 concentration (concentration that caused 10% toxicity), for all the test agents viz CsA (10 μ g/ml) FK506 (8 μ g/ml) and pentoxifylline (27.8 μ g/ml) (data not shown). Based on this, the studies were conducted on the proposed model system at IC-10 dosage/concentration and two subsequent dilutions of IC-10 viz IC-10/10 and IC-10/100.

PMA/Ionomycin induced GFP expression in IL-2, IFN- γ , IL-4 and IL-10 reporter jurkat T cell lines was significantly inhibited when activated cells were treated with cyclosporine A (CsA) at all the tested concentrations (Figure 1A) whereas the inhibition was in evident in LPS stimulated, TNF- α reporter THP-1 cells (Figure 1B). It also inhibited the PMA/Ionomycin IL-2, IFN- γ , IL-4 and IL-10 mRNA expression

of endogenous cytokine genes in the reporter cell lines (Figure 1C) whereas the inhibitory effect was in evident in case of TNF- α mRNA expression in LPS activated THP-1 cell line(Figure 1D).

Another immunosuppressant FK506, significantly inhibited the PMA/Ionomycin induced GFP expression in IL-2, IFN- γ , IL-4 and IL-10 reporter jurkat T cell lines (Figure 2A) at all the tested concentrations whereas no inhibitory effect was observed in LPS stimulated, TNF- α reporter THP-1 cells (Figure 2B). PMA/ Ionomycin induced endogenous IL-2, IFN- γ , IL-4 and IL-10 mRNA expression was also found to be inhibited upon treatment with this compound (Figure 2C) whereas it failed to alter TNF- α mRNA expression in LPS induced THP-1 cells (Figure 2D).

Pentoxifylline did not alter the PMA/Ionomycin induced up-regulation of GFP mediated fluorescence in IL-2, IFN- γ , IL-4 and IL-10 reporter jurkat T cell lines (Figure 3A), whereas it inhibited the LPS induced GFP expression in TNF- α reporter THP-1 cells at all the doses in a characteristic dose dependent manner (Figure 3B). Pentoxifylline showed no effect on endogenous mRNA expression of IL-2, IFN gamma, IL-4 and IL-10 in PMA/Ionomycin activated Jurkat T cells and showed inhibitory effect on TNF- α reporter gene and endogenous TNF- α mRNA expression in LPS activated THP-1 cells (Figures 3C and 3D).

These results demonstrated that CsA and FK-506 specifically targeted endogenous cytokine genes in jurkat T cells and these were able to regulate GFP reporter genes that were under the transcription control of cytokine regulatory regions. The specificity of regulating reporter genes was shown by pentoxifylline which is TNF- α activator (Table 3).

Effect of pesticides on expression of reporter and endogenous cytokine genes

The Reporter cell lines responded specifically to known immunosuppressants. Further, different pesticides were analysed on these cell lines alone as well as in the presence of activators at IC-10 (chlorpyrifos, 10 μ g/ml; cypermethrin 10 μ g/ml and captan 1 μ g/ml) and two subsequent 1/10 and 1/100 times dilutions of the IC-10 concentration. These pesticides on their own could not alter the basal GFP expression in reporter cell lines (data not presented) but they caused significant alterations in activated jurkat T cells and THP-1 cells.

Chlorpyrifos: PMA/Ionomycin induced GFP expression from

Cytokine	Primer Sequence (5'.....3')
IL-2	Forward primer 5' GAA AAC ACA GCT ACA ACT GGA GCA T 3' Reverse primer 5' ATT TAG CAC TTC CTC CAG AGG TTT G 3'
IFN- γ	Forward primer 5' TTC AGA TGT AGC GGA TAA TGG AAC 3' Reverse primer 5' CCG AAT AAT TAG TCA GCT TTT CGA AG 3'
IL-4	Forward primer 5' GAG ATC ATC AAA ACT TTG AAC AGC CT 3' Reverse primer 5' CGT TTC AGG AAT CGG ATC AGC TG 3'
IL-10	Forward primer 5' CAA CCT GCC TAA CAT GCT TCG AG 3' Reverse primer 5' ATG ACA GCG CCG TAG CCT CAG 3'
TNF- α	Forward primer 5' GAC CTC TCT CTA ATC AGC CCT CTG 3' Reverse primer 5' CAG CCT TGG CCC TTG AAG AGG AC 3'
β -Act	Forward primer 5' ATC TGG CAC CAC ACC TTC TAC AAT G 3' Reverse primer 5' ATC TTC ATG AGG TAG TCA GTC AGG TC 3'
gfp	Forward Primer 5' ATGGCTAGCAAAGGAGAAGAAGCTTTTCAC 3' Reverse Primer 5'ACACATGGCATGGATGAGCTCTACAAATAA 3'
GAPDH	Forward Primer 5'CAT GTT CGT CAT GGG TGT GAA CCA 3' Reverse Primer 5' AGT GAT GGC ATG GAC TGT GGT CAT 3'

Table 2: Primers used for amplification of endogenous cytokines by RT PCR.

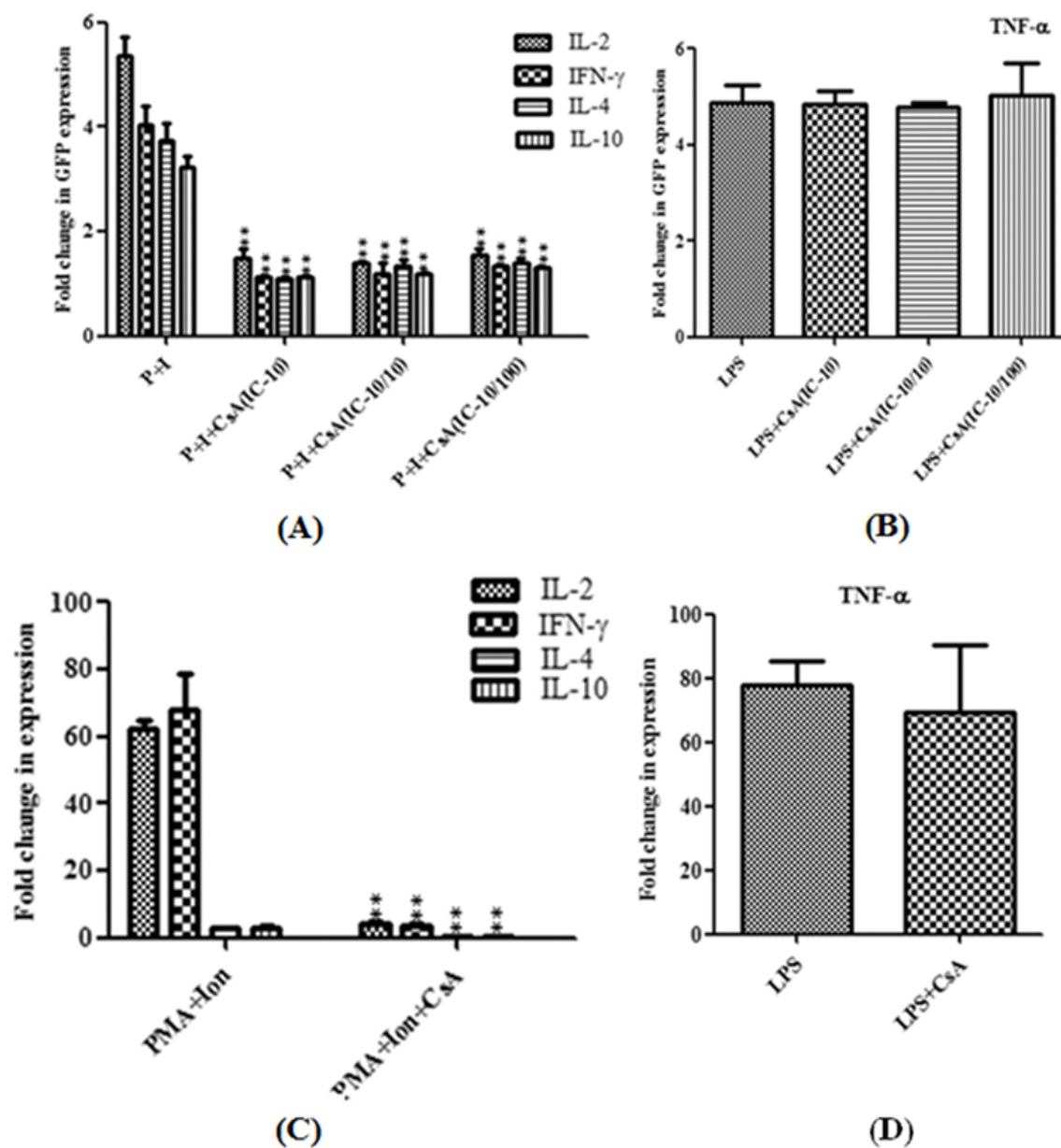


Figure 1: Cyclosporine A (CsA) effect on GFP and Cytokine gene expression.. (A) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with CsA activated by PMA/Ionomycin (B) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (10 μ g/ml) and two concentration of IC10/10 and IC10/100 for 48 h. Relative fold change in expression of GFP from these cell lines was calculated in comparison to expression of GFP in untreated control reporter cells. (C) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with CsA activated by PMA/Ionomycin (D) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (10 μ g/ml) and two concentration of IC10/10 and IC10/100 for 24 h. Real time PCR was done on RNA extracted from these treated cells. Relative fold change in expression of cytokine's gene was calculated in comparison to their expression in untreated control cell. Results are shown as mean fold change \pm SEM (n=3) and change was considered significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

IL-2, IFN- γ , IL-4 and IL-10 reporter jurkat T cell lines was significantly suppressed by chlorpyrifos treatment at all concentration (Figure 4A). LPS induced GFP expression from TNF- α reporter THP-1 cell line was not altered significantly on treatment with chlorpyrifos at IC-10 as well as at lower concentrations (Figure 4B).

Chlorpyrifos treatment caused suppression of PMA/Ionomycin induced expression of IL-2, IFN- γ , IL-4 and IL-10 cytokine genes at transcript level (Figure 4C) whereas LPS induced TNF- α gene expression was not suppressed significantly (Figure 4D).

Cypermethrin: moderate suppression of PMA/Ionomycin induced GFP expression from IL-2, IFN- γ , IL-4 and IL-10 reporter jurkat T cell line was obtained upon cypermethrin treatment at IC-10 and IC-10/10 concentration. Descending the test concentration further down to IC 10/100 failed to cause any significant suppression (Figure 5A). LPS induced GFP expression from TNF- α reporter THP-1 cell line was not altered significantly at IC-10 as well as at the lower tested concentrations (Figure 5B).

Cypermethrin treatment caused moderate suppression of PMA/

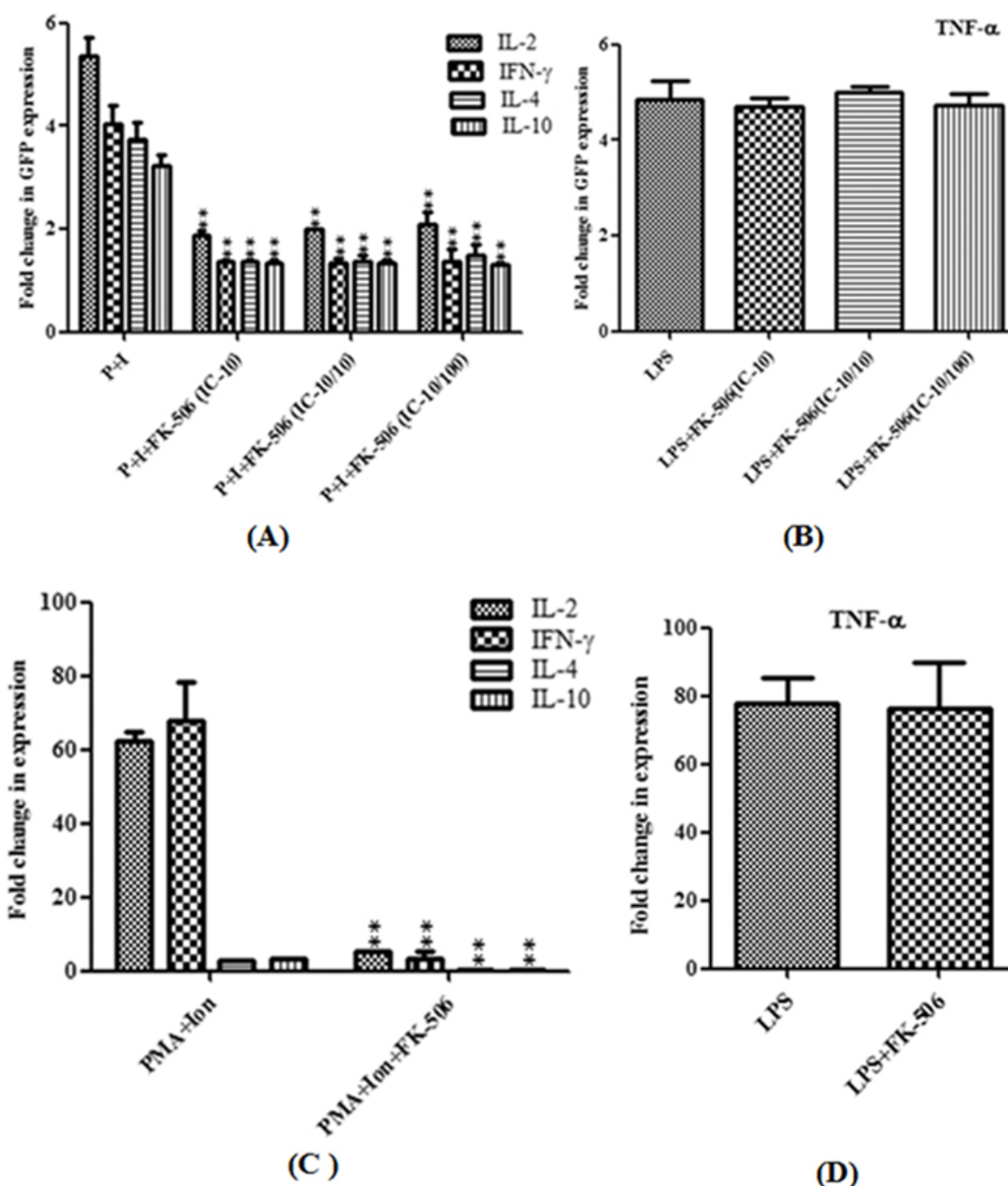


Figure 2: FK506 effect on GFP and Cytokine expression. (A) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with FK506 activated by PMA/Ionomycin (B) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (8 μ g/ml) and two concentration of IC10/10 and IC10/100 for 48 h. Relative fold change in expression of GFP from these cell lines was calculated in comparison to expression of GFP in untreated control reporter cells. (C) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with FK506 activated by PMA/Ionomycin (D) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (8 μ g/ml) and two concentration of IC10/10 and IC10/100 for 24 h. Real time PCR was done on RNA extracted from these treated cells. Relative fold change in expression of cytokine's gene was calculated in comparison to their expression in untreated control cell. Results are shown as mean fold change \pm SEM (n=3) and change was considered significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

Ionomycin induced expression of IL-2, IFN- γ , IL-4 and IL-10 cytokine genes at transcript level (Figure 5C) whereas LPS induced TNF- α gene expression was not suppressed significantly (Figure 5D).

Captan: A moderate suppression of PMA/Ionomycin induced GFP expression from IL-2, IFN- γ , IL-4 and IL-10 reporter jurkat T cell lines was obtained on captan treatment at IC-10 concentration. On further

decreasing the treatment dose to IC10/10 and IC 10/100 only significant suppression was obtained in case of IL-2 and IFN- γ (Figure 6A). LPS induced GFP expression from TNF- α reporter cell line was not altered significantly on treatment with captan at IC-10 concentration as well as at all the lower tested concentrations (Figure 6B).

Captan treatment suppressed, PMA /Ionomycin induced

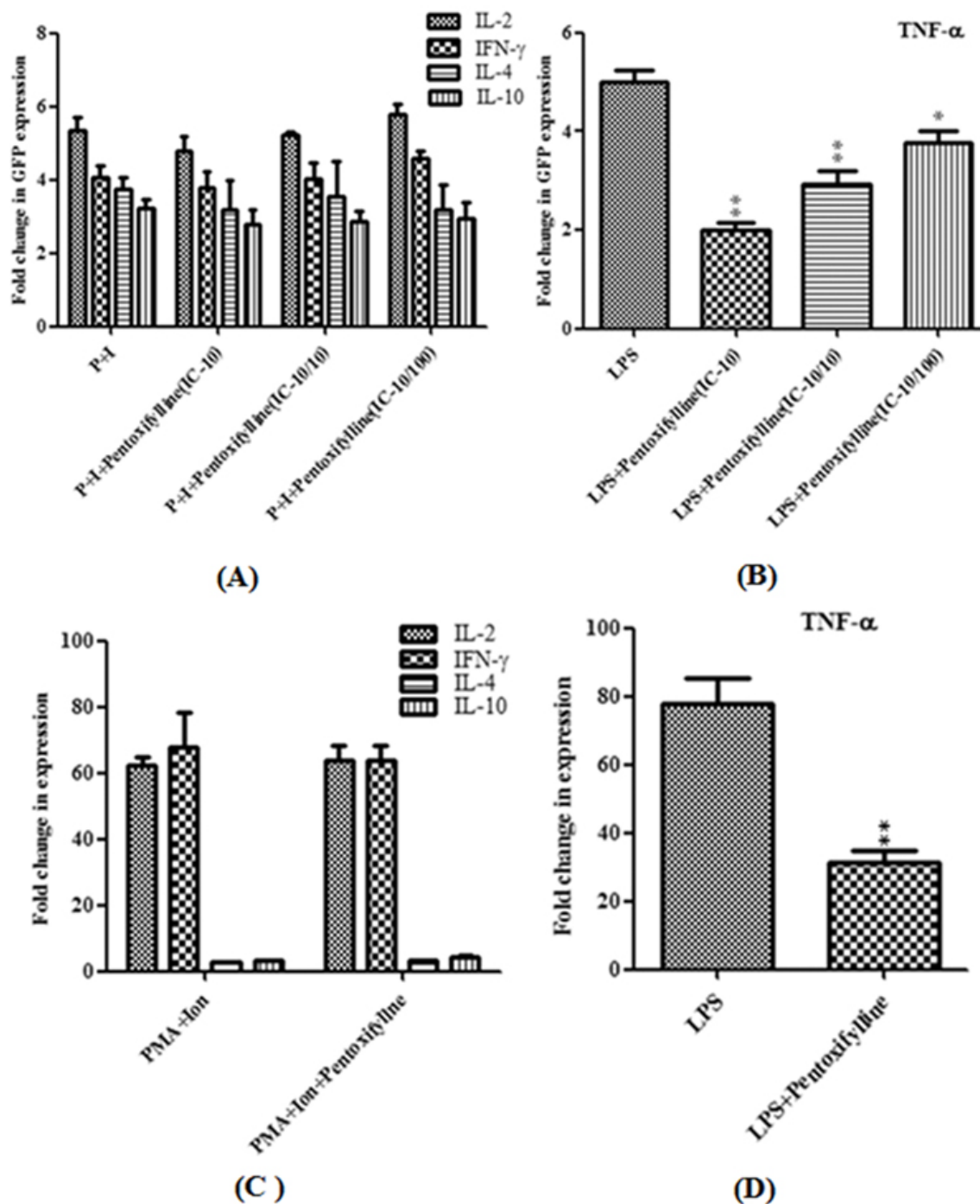


Figure 3: Pentoxifylline effect on GFP and Cytokine expression. (A) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with **Pentoxifylline** activated by PMA/Ionomycin (B) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (27.8 μ g/ml) and two concentration of IC10/10 and IC10/100 for 48 h. Relative fold change in expression of GFP from these cell lines was calculated in comparison to expression of GFP in untreated control reporter cells. (C) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with **Pentoxifylline** activated by PMA/Ionomycin (D) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (27.8 μ g/ml) and two concentration of IC10/10 and IC10/100 for 24 h. Real time PCR was done on RNA extracted from these treated cells. Relative fold change in expression of cytokine's gene was calculated in comparison to their expression in untreated control cell. Results are shown as mean fold change \pm SEM (n=3) and change was considered significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

endogenous expression of IL-2, IFN- γ , IL-4 gene, suppression of IL-10 was however not statistically significant (Figure 6C). LPS induced TNF- α gene expression was neither suppressed nor stimulated to a significant level on captan treatment (Figure6D).

These results show that the pesticides showed immunosuppressor activity on activated Jurkat T cells and have no effect on activated THP-1 cells (Table 3). Therefore, our model has potential to screen xenobiotic compounds that influence immune system by analyzing expression of

Treatment at IC-10 Concentration	IL-2		IFN-γ		IL-4		IL-10		TNF-α	
	Reporter GFP	mRNA exp.	Reporter GFP	mRNA exp.	Reporter GFP	mRNA exp.	Reporter GFP	mRNA exp.	Reporter GFP	mRNA exp.
PMA+ Ion	5.35 ± 0.498	62.22 ± 3.98	4.05 ± 0.47	67.66 ± 15.36	3.75 ± 0.44	2.85 ± 0.27	3.21 ± 0.33	3.180 ± 0.29		
P+I + CSA(10µg/ml)	1.48 ± 0.26	4.46 ± 0.48	1.13 ± 0.02	3.60 ± 0.58	1.11 ± 0.004	0.62 ± 0.10	1.13 ± 0.03	0.36 ± 0.05		
P+I +FK506 (8 µg/ml)	1.87 ± 0.12	5.39 ± 0.40	1.37 ± .021	3.4 ± 2.8	1.38 ± 0.01	0.259 ± 0.002	1.33 ± 0.084	0.57 ± 0.005		
P+I+Pentoxifylline (27.8µg/ml)	4.77 ± 0.57	63.7± 6.63	3.791 ± 0.59	63.82 ± 6.519	3.205 ± 1.11	3.12 ± 0.49	2.80 ± 0.54	4.41 ± 0.685		
P+I+Chlorpyriphos (10µg/ml)	2.16 ± 0.21	9.27 ± 0.424	1.49 ± 0.12	6.79 ± 3.88	1.38 ± 0.21	0.268 ± 0.01	1.54 ± 0.413	0.622 ± 0.04		
P+I+Cypermethin (10µg/ml)	3.00 ± 0.26	41.87 ± 5.38	12.19 ± 0.22	37.24 ± 11.92	2.08 ± 0.38	1.42 ± 0.278	1.240 ± 0.169	0.857 ± 0.160		
P+I+Captan (1µg/ml)	3.49 ± 0.28	43.7 ± 5.69	2.54 ± 0.26	45.8 ± 2.69	2.52 ± 0.54	1.44 ± 0.28	2.11 ± 0.05	2.9 ± 0.28		
LPS									4.87 ± 0.51	77.81±10.65
LPS+CSA (10µg/ml)									4.86 ± 0.36	69.37 ± 29.51
LPS+FK506 (8 µg/ml)									4.710 ± 0.25	76.24 ± 19.17
LPS+Pentoxifylline (27.8µg/ml)									2.01 ± 0.24	30.51 ± 3.58
LPS+Chlorpyriphos (10µg/ml)									4.85 ± 0.8	79.96 ± 12.65
LPS+Cypermethrin (10µg/ml)									4.9 ± 0.32	80.94 ± 7.9
LPS+Captan (1µg/ml)									5.27 ± 0.7	82.28 ± 5.57

Table 3: Reporter gene and endogenous cytokine gene expression after treatment with immunosuppressant and chemicals at IC-10 concentration in activated cells.

reporter gene regulated by regulatory regions of cytokines. This model helps for fast screening of compounds of all class based on cytokine gene response which is implicated in Th1 and Th 2 type of immune response.

Discussion

We have developed an alternative in vitro model based on transformed human cell lines for the purpose of immunotoxicity testing of xenobiotics. This model was based on the expression of signature cytokines of Th1 and Th2 type of immune responses and inflammatory cytokine expression by macrophages. Lymphoid and myeloid cells represent two distinct lineages that are integral to functional immune system. Therefore, so as to ascertain the lineage specificity of the response we selected two different cell lines, with each one representing one particular lineage of cells. While Jurkat T cells represent lymphoid lineage cells (T cells), THP-1 cells represent myeloid lineage cells (Macrophages/momocytes). The Jurkat T cells were stably transformed with GFP reporter constructs regulated by IL-2, IFN-γ, IL-4 and IL-10 gene regulatory regions. THP-1 cells were stably transformed with GFP reporter regulated by TNF-α gene regulatory region. This model was validated using immunosuppressant CsA and FK506. Our model successfully predicted immunosuppressant activity of known immunosuppressant CsA and FK506 in jurkat transformed cell lines stimulated with immunostimulators PMA/Ionomycin and pentoxifylline induced suppression in LPS activated THP-1 cells. PMA/Ionomycin has been reported to induce T cell activation and cytokine secretion; PMA activates protein kinase C (PKC), leading to initiation of the signaling cascades responsible for the nuclear localisation of transcription factor AP-1 and NFκB, while ionomycin causes a release of intracellular calcium stores resulting in increased nuclear localisation of transcription factor NFAT. Thus, treatment with both PMA and ionomycin leads to increased nuclear localisation of these transcription factors which regulates expression of IL-2, IFN-γ, IL-4 and IL-10 cytokines resulting in increased expression of these cytokines [25]. Induction of reporter gene expression on PMA/

Ionomycin treatment, from IL-2, IFN-γ, IL-4 and IL-10 reporter Jurkat T cell lines is in accordance with previous reports and established the functionality of the cloned regulatory region. Lipopolysaccharides (LPS) a known macrophage activator, activates a complex signalling network including the activation of nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pathways ultimately leading to the expression of TNF and other inflammatory factors [26]. LPS treatment induced GFP upregulation in THP-1 cells (monocytic cell line) that were stably expressing regulatory region of TNF-α and was in agreement with previous report thus established the functionality of the cloned regulatory region [27].

In our model, immunosuppressive drug cyclosporine (CsA) and FK506 were found to suppress PMA/Ionomycin induced GFP expression in IL-2, IFN-γ, IL-4 and IL-10 reporter cell lines. CsA has been reported to bind with cytoplasmic protein cyclophilin, forming a complex that blocks the phosphatase activity of calcineurin which in turn prevents NFAT activation and its translocation to nucleus thus preventing NFAT regulated expression of cytokines. Similarly FK506 has been reported to bind with cytoplasmic proteins called FKBP, forming a complex that blocks the phosphatase activity of calcineurin which in turn prevents NFAT activation and translocation to nucleus thus preventing NFAT regulated expression of cytokines [28]. Suppression of PMA/Ionomycin induced GFP expression from IL-2, IFN-γ, IL-4 and IL-10 reporter cell line on CsA and FK506 treatment is thus justified. LPS induced GFP expression from TNF-α reporter cell line was not suppressed by CsA as well as FK506. This was in agreement with previous studies wherein they have demonstrated that LPS induced TNF-α stimulation remains unaffected by CsA and FK506 treatment [29,30].

Immunomodulatory activity of pesticides has been reported previously in different animal model and human case studies. Immunological parameters in pesticide applicators exposed to a mixture of organophosphates (OPs), carbamates, phenoxy herbicides and pyrethroids was examined [10].

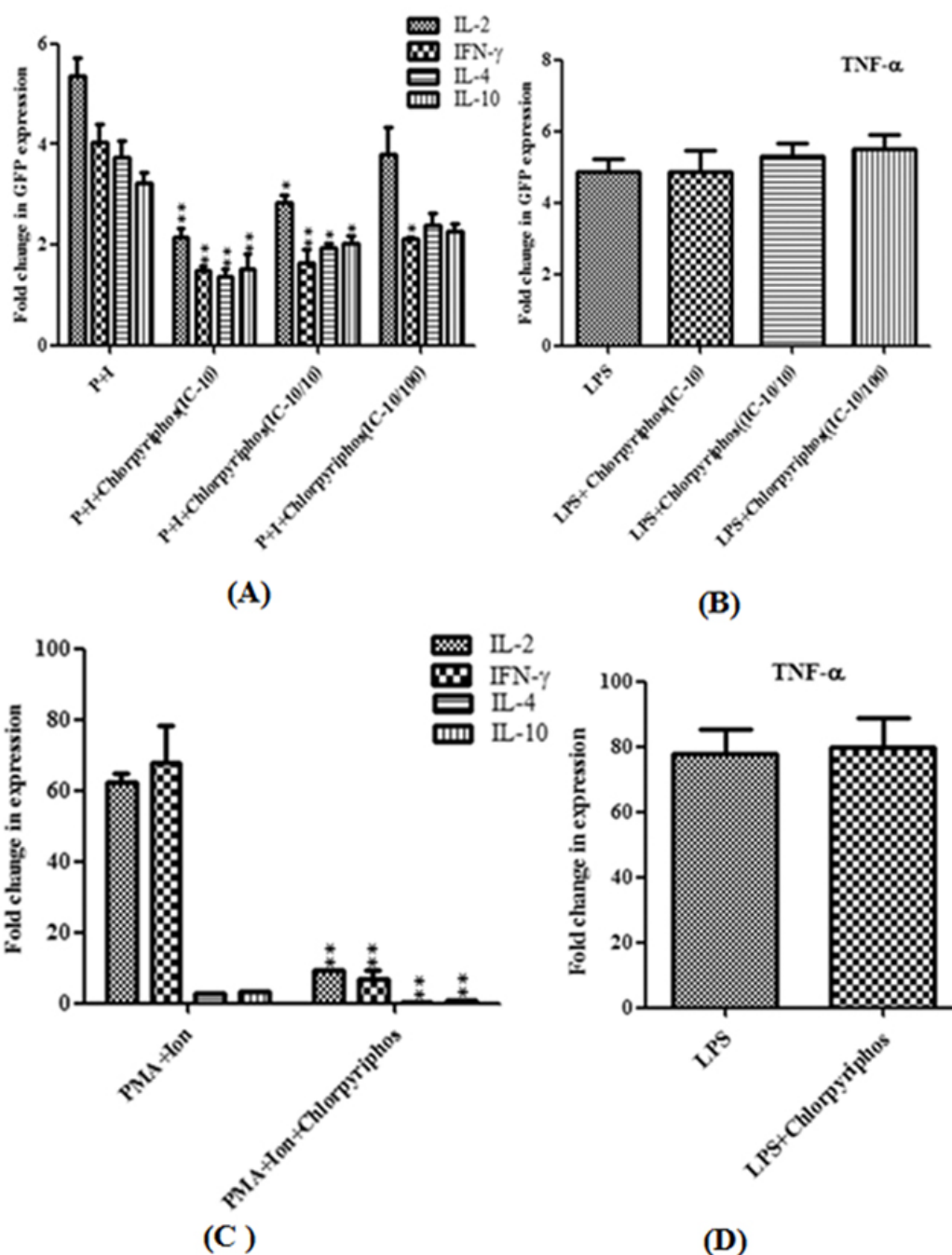


Figure 4: Chlorpyrifos effect on GFP and Cytokine expression. (A) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with Chlorpyrifos activated by PMA/Ionomycin (B) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (10 μ g/ml) and two concentration of IC10/10 and IC10/100 for 48 h. Relative fold change in expression of GFP from these cell lines was calculated in comparison to expression of GFP in untreated control reporter cells. (C) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with Chlorpyrifos activated by PMA/Ionomycin (D) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (10 μ g/ml) and two concentration of IC10/10 and IC10/100 for 24 h. Real time PCR was done on RNA extracted from these treated cells. Relative fold change in expression of cytokine's gene was calculated in comparison to their expression in untreated control cell. Results are shown as mean fold change \pm SEM (n=3) and change was considered significant at P \leq 0.05 (*) and P \leq 0.01 (**).

Chlorpyrifos, an organophosphorus (OP) insecticide is used to control a wide range of pests, such as cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. This compound is applied to different crops including cotton, nuts,

vegetables and ornamental plants, and may enter surface water by runoff, spraydrift, or accidental spills [31]. It exhibits moderate persistence in natural systems and has low solubility in water [32,33]. In our model, chlorpyrifos caused significant suppression of PMA/

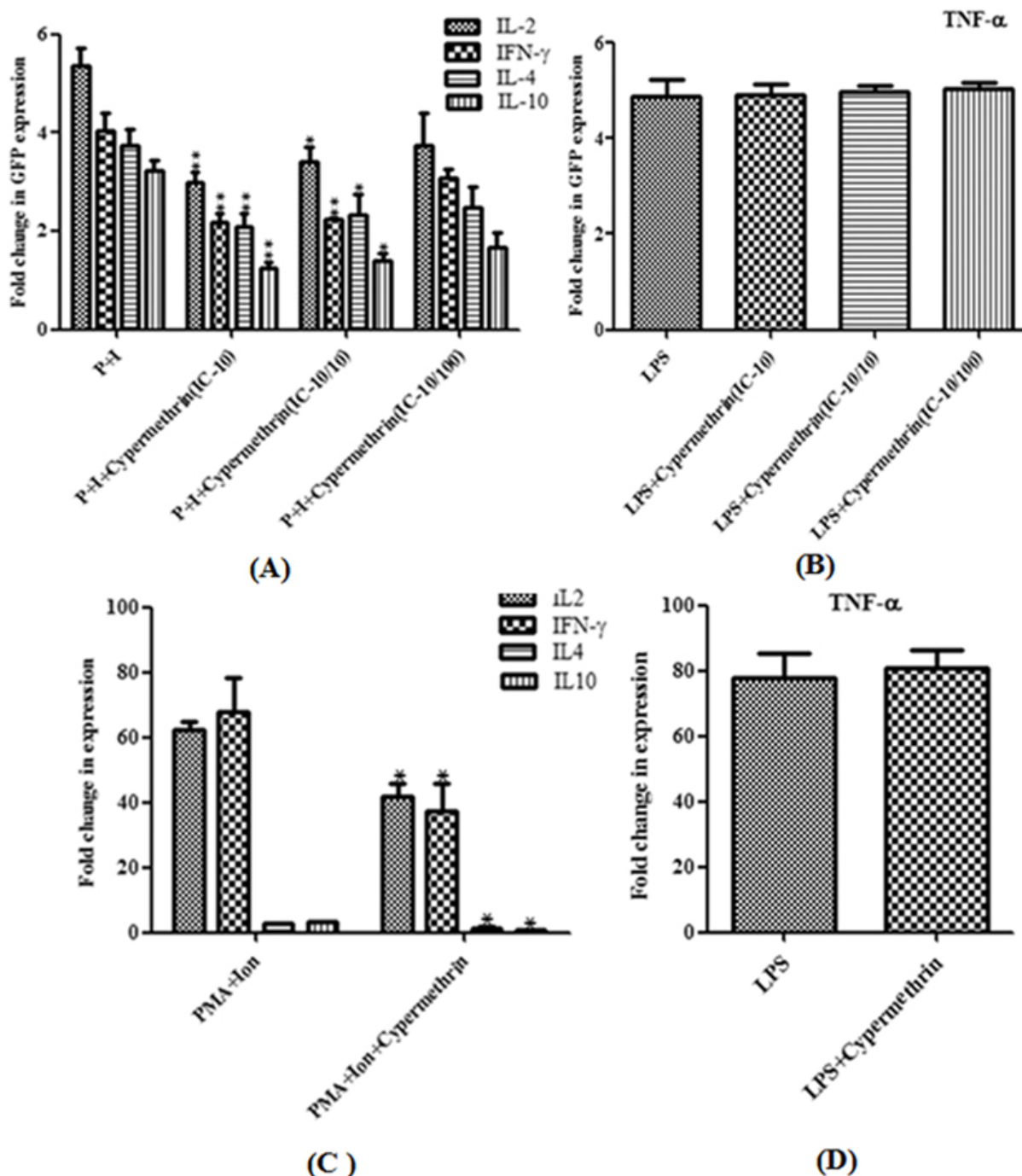


Figure 5: Cypermethrin effect on GFP and Cytokine expression. (A) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with **Cypermethrin** activated by PMA/Ionomycin (B) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (10 μ g/ml) and two concentration of IC10/100 and IC10/100 for 48 h. Relative fold change in expression of GFP from these cell lines was calculated in comparison to expression of GFP in untreated control reporter cells. (C) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with **Cypermethrin** activated by PMA/Ionomycin (D) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (10 μ g/ml) and two concentration of IC10/10 and IC10/100 for 24 h. Real time PCR was done on RNA extracted from these treated cells. Relative fold change in expression of cytokine's gene was calculated in comparison to their expression in untreated control cell. Results are shown as mean fold change \pm SEM (n=3) and change was considered significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

Ionomycin induced GFP expression from IL-2, IFN- γ , IL-4 and IL-10 reporter cell lines at IC-10 and IC-10/100 concentration which suggests immunosuppressive action of chlorpyrifos. Recent research has identified immune system abnormalities in individuals exposed to

chlorpyrifos. Higher than usual frequencies of allergies and sensitivities to antibiotics together with atypical abundances of certain types of lymphocytes (decrease in T cells and increase in CD26 cells) were found in patients exposed to chlorpyrifos for a period ranging from

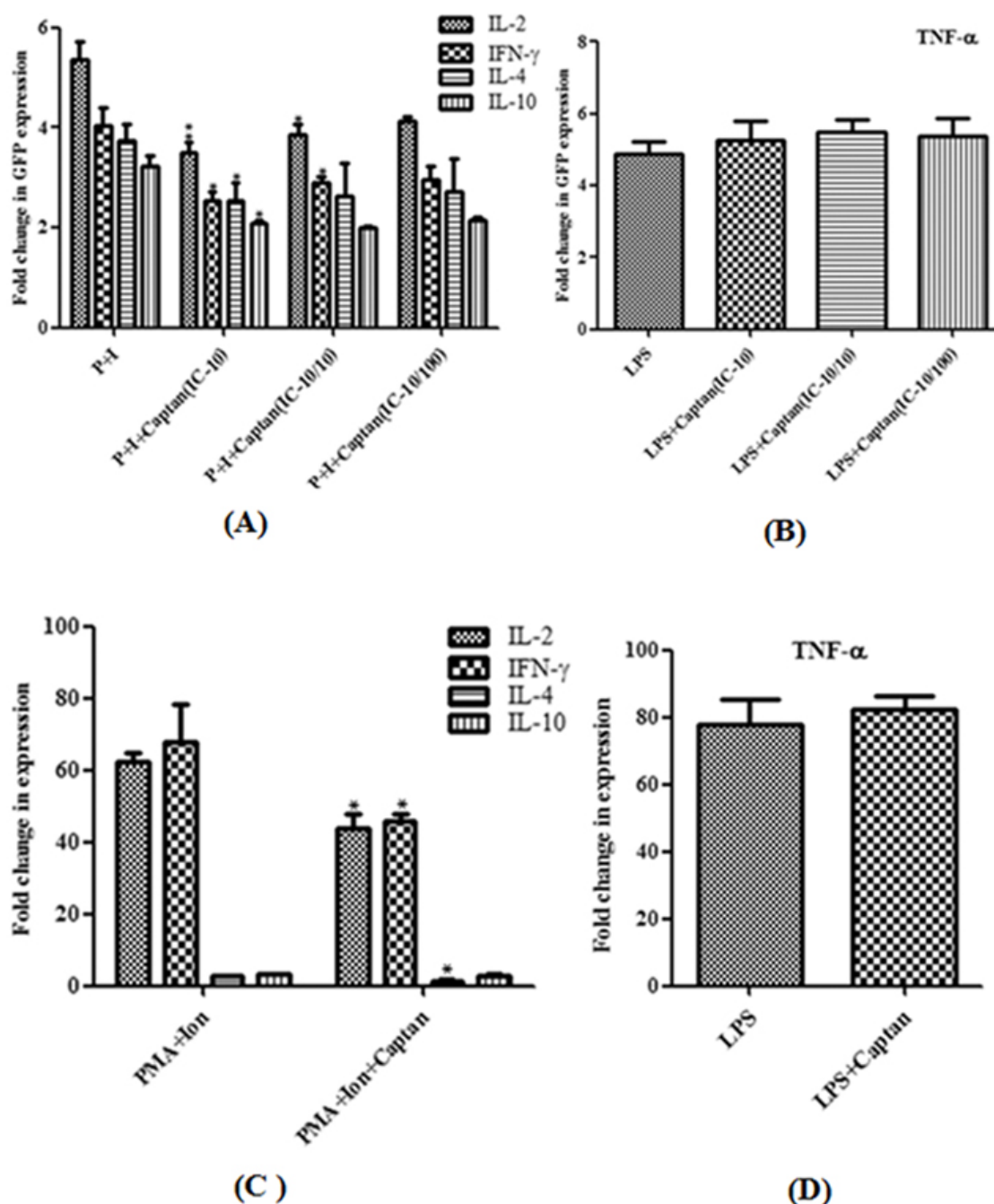


Figure 6: Captan effect on GFP and Cytokine expression. (A) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with Captan activated by PMA/Ionomycin (B) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (1 μ g/ml) and two concentration of IC10/10 and IC10/100 for 48 h. Relative fold change in expression of GFP from these cell lines was calculated in comparison to expression of GFP in untreated control reporter cells. (C) IL-2; IFN- γ ; IL-4; and IL-10 reporter jurkat cell lines were treated with Captan activated by PMA/Ionomycin (D) TNF- α reporter THP-1 cell line was treated in presence of LPS at IC-10 (1 μ g/ml) and two concentration of IC10/10 and IC10/100 for 24 h. Real time PCR was done on RNA extracted from these treated cells. Relative fold change in expression of cytokine's gene was calculated in comparison to their expression in untreated control cell. Results are shown as mean fold change \pm SEM (n=3) and change was considered significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

one to five years [34]. Chlorpyrifos was found to impair T-lymphocyte blastogenesis induced by concanavalin A and phytohemagglutinin but did not alter the B lymphocyte blastogenesis induced by lipopolysaccharide/dextran in rat [35]. These reports support the

immunosuppressive action of chlorpyrifos on T cell population as observed in our study.

Cypermethrin, a pyrethroid insecticide is a constituent of many household insecticides. In our model cypermethrin caused significant

suppression of PMA/Ionomycin induced GFP expression within IL-2, IFN- γ , IL-4 and IL-10 reporter cell lines at IC-10 and IC-10/10 concentration which suggests immunosuppressive action of this compound. There have been many reports of immunomodulation caused by this insecticide; recently Sankar et al. [36], reported a significant decline in antibody titre in cypermethrin treated rats suggesting cypermethrin-induced immunosuppression. Similarly, Stelzer and Gordon (1984) showed that cypermethrin inhibited the proliferation of mouse T and B cells at 10 to 50 μ M concentration range [37]. Tamang et al. reported *in vivo* immunosuppressive effect of cypermethrin in mice and goats [38]. Their results revealed significant suppression of cell mediated immunity in both the species, and humoral immune response in goats. Cypermethrin given in feed at a concentration of 100 ppm for 8 weeks was also found to cause significant depression in DTH reaction in broiler chickens [39].

Captan, a nonsystemic phthalimide fungicide is used to control diseases of many fruit, ornamental, and vegetable crops. In our model captan caused moderate but significant suppression of PMA/Ionomycin induced GFP expression from IL-2, IFN- γ , IL-4 and IL-10 reporter cell lines at IC-10 concentration, confirming immunosuppressive action of this compound. The effect of short-term oral administration of captan, on the immune response was studied in rats and mice [40]. The SRBC-antibody formation, lymphoblastic stimulation of splenic cells by PHA and by LPS was depressed in both species after 14 days of captan treatment. These results pointed out a clear depressive effect of captan diet on the immune response of the animals which goes in line with our results.

There have been previous reports of using cytokines promoter based reporter cell lines for immunotoxicity testing. Ulleras et al. have used cell lines of murine origin and EGFP as reporter gene; quantitation of reporter gene expression was done by flow cytometry [41]. Ootsingh et al. have used cell lines of human origin and luciferase as reporter gene [42]. We have used lymphoid and myeloid cell lines of human origin and GFP as reporter gene a combination of both the reports. Owing to this, our model has two advantages. Use of cell lines of human origin instead of murine origin makes it a better representative of responses likely to occur in humans. Secondly, employing GFP as a reporter gene makes our model a cost effective means of high throughput screening. The immunotoxicity occurring due to metabolic activation of test agents cannot be evaluated in current system. Our model showed the immunotoxic effect on Jurkat T cells expressing IL-2, IFN- γ , IL-4, and IL-10 reporter cells but showed no effect on THP-1 reporter cells which showed the same effect in humans on exposure of these pesticides. All these pesticides tested on this model were found to have immunosuppressive activity on expression of Th1 and Th2 cytokines which can be supported by previous immunotoxicity studies pertaining to these pesticides. So this model can be employed for rapid high throughput immunotoxicity screening of pesticides and other compounds for rapid screening to identify the immunotoxic potential of xenobiotics

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