Chapter 4: Effects of Moxifloxacin on PHA-Activated Human Mononuclear Cells

4.1 INTRODUCTION

Activation of lymphocytes involves a complex, and finely regulated cascade of events that results in the expression of cytokine receptors, production and secretion of cytokines and expression of several surface molecules that eventually lead to divergent immune responses. The laboratory research presented in this chapter was designed to evaluate the effect of moxifloxacin on lymphocyte proliferation, expression of the activation marker CD25 (a hallmark of cellular activation, present on peripheral blood lymphocytes with density increasing *in vitro* upon activation) and cytokine production by PHA-activated lymphocytes.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Moxifloxacin was used, as described in 3.2.1 (page 44) of this dissertation. Bio-Plex™ cytokine assay kits were purchased from Bio-Rad, Hercules, CA, USA. Monoclonal antibodies were purchased from Beckman Coulter.

4.2.2 Isolation of mononuclear leukocytes

Purified human MNL were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy adult volunteers and separated from granulocytes by centrifugation on Histopaque®-1077 cushions at 400 g for 25 min at room temperature. Due to the various densities of the different types of cells, differential sedimentation velocity resulted in the formation of four cell fractions; plasma containing thrombocytes, mononuclear leukocytes, ficoll and the mixture of erythrocytes and granulocytes. The mononuclear leukocyte (MNL) layer was removed and cells were washed with PBS containing ethylene glycol-bis (beta-amino-ethyl-ether)-N, N, N', N'- tetraacetic acid (EGTA, 1 mM) to prevent aggregation of the cells. After centrifugation at 250 g for 10 minutes, residual erythrocytes were removed by selective lysis with 0.84% NH_4Cl for 10 minutes at 4˚C. The resultant pellet was then washed with PBS/EGTA. The MNLs which were routinely of high purity and viability (>90%), were then resuspended to 1 x 10^7 cells/ml in RPMI 1640 tissue culture medium and held on ice until use. Purity of isolated lymphocytes was assessed microscopically and assessment of viability was done by dye-exclusion using 0.1% methylene blue.

4.2.3 Lymphocyte proliferation assay (LPA)

This procedure measures the ability of lymphocytes to undergo polyclonal/clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. Cells were resuspended at 1 x 10^6 cells/ml in RPMI 1640 culture medium. Using 96 well flat-bottomed microtitre plates, lymphocytes (50 µl of a 1 x 10^6 cells/ml MNL suspension i.e. 5 x 10^4 cells/well) were supplemented with fetal calf serum (FCS; 10% final, 20µl/well) in the presence and absence of moxifloxacin (2.5, 5 and 10 µg/ml; 20 µl/well). The plates were then incubated for 24hrs at 37˚C in a humidified CO_2 incubator (5% CO_2) before the addition of the mitogen phytohemagglutinin (PHA 2.5 and 5 µg/ml; 20 µl/well). The final volume in each well was 200 µl. The plates were agitated gently on a microplate agitator for 5 seconds and incubated for a further 48 hrs. Proliferation was assessed radiometrically according to the magnitude of uptake of tritiated thymidine (^{3}H, specific activity 0.2 µCi/well, Du Pont-NEN, Research Products, Boston, MA, USA) for 18 hrs, into the newly synthesized DNA of the dividing cells. Cells were then harvested on glass fiber filters using the PHD multi-well cell harvester (Cambridge Technology, USA). The disks were dried using methanol, placed in a glass vials, followed by the addition of 4 ml scintillation fluid (Packard Bioscience, USA). The amount of radioactivity incorporated into DNA in each well was measured using a liquid scintillation counter (TRI-CARB – 2100TR, Packard, Canberra Co, USA) and expressed as counts per minute.

4.2.4 Analysis of CD25 expression

The effects of moxifloxacin on the functional response of lymphocytes were also assessed according to the expression of the surface activation marker CD25 (IL-2αR), which is an alternative method to evaluate T-cell proliferation. Lymphocytes (1 x 10^6 cells/ml) were resuspended in RPMI 1640 in the presence and absence of moxifloxacin (0.625 - 10 µg/ml) and incubated for 24 hrs at 37˚C in a CO_2 incubator (5% CO_2) before the addition of the mitogen, PHA (2.5 and 5 µg/ml). The tubes, which contained a final volume of 2 ml were incubated for a further 24 hrs, and CD25 was detected flow cytometrically using an anti-CD25 FITC-conjugate. Briefly, 500 µl of cultured lymphocyte suspension were diluted with 500 µl HBSS. The cells were then incubated for 15 min at room temperature in the dark with anti-CD25 FITC monoclonal antibodies (mAb), or anti-IgG FITC conjugate for detection of

nonspecific background staining. The Epics Altra (Beckman Coulter, Miami, FL, USA) equipped with a water-cooled coherent Enterprise laser, was used to detect the CD25 positive cells. Expo 32 software (Beckman Coulter) was used to analyze the results obtained. Both the percentage CD25 positive cells and the density of the activation marker expression (mean fluorescence intensity, MFI) were calculated and represented as the normalized mean fluorescence intensity (NMFI) value, which is the percentage of positive lymphocytes multiplied by the corresponding MFI values (Oxenhandler *et al.*, 1984).

4.2.5 Cytokine production by PHA=activated mononuclear leukocytes

4.2.5.1 Suspension array system for cytokine assay

This assay employs a bead-based sandwich enzyme immunoassay technique. A monoclonal antibody specific for the cytokine of interest is coupled onto a designated bead with a known internal fluorescence. Cytokine standards, provided as a lyophilized cocktail, were reconstituted with 500 µl RPMI 1640 to obtain stock concentrations of 50 000 pg/ml of each cytokine. Serial 4 fold dilutions (1.95 - 32 000 pg/ml) for control cytokines were used in all the experiments. The conjugated beads and detection antibodies were diluted 25-fold using assay buffer, while streptavidin-PE was diluted 100-fold using detection diluent.

4.2.5.2 Cytokine detection

Experiments were performed to measure the effects of moxifloxacin on the cytokine profiles (both pro-inflammatory and anti-inflammatory) of PHA-activated MNLs using suspension bead protein array technology (Bio-Plex™). Mononuclear cells (1 x 10^6 cells/ml) were resuspended in RPMI 1640 in the presence and absence of moxifloxacin (0.625 - 10 µg/ml) and incubated for 24 hrs at 37˚C with 5% CO_2 before the addition of the mitogen, PHA (2.5 and 5 µg/ml). The tubes, which contained a final volume of 2 ml, were incubated for a further 24 hrs, and culture supernatants were collected prior to the cytokine assay. Briefly, the assay was performed using 96 well filter plates that were first saturated with 200 µl of assay buffer. The plates were then covered with plastic lids and incubated for 1 hr at room temperature. The assay buffer was removed by using a vacuum manifold apparatus, followed by gentle blotting of the plates on paper. The conjugated beads (50 µl/well) were added, and

deposited on the filter by exposing the plate to a vacuum, after which the plate was washed twice with 100 µl of wash buffer. In each designated well, 50 µl of standard, control, or culture supernatant were added in duplicate. The plates were covered with a sealing tape and aluminium foil, agitated for 30 sec on the plate agitator and for 60 min at 300 rpm on the orbital shaker at room temperature to promote bead-cytokine binding. After incubation, the plates were vacuum filtered as before, followed by the addition of the detection biotinylated antibodies (25 µl/well). The plates were again covered with a sealing tape and aluminium foil, agitated for 30 sec and incubated for 30 min at room temperature on the orbital shaker at 300 rpm. The plates were drained and washed three times with 100 µl/well wash buffer. For the detection and quantification of each captured cytokine, 50 µl of streptavidin-PE (a fluorescently labelled reporter molecule that specifically binds to the analyte), was added to each well. The plates were sealed again, as before, and mixed for 10 min at 300 rpm on the orbital shaker at room temperature, followed by a triplicate wash step, with 100 µl/well wash buffer. After the third wash, assay buffer (125 µl/well) was added into each well, and the plates agitated for 30 sec. The contents of each well were analyzed using Bio-Plex™ plate reader software (version 3.0) and the concentration of each cytokine (pg/ml) calculated from standard curve generated. The following cytokines were assayed: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, and TNF-α.

4.2.6 Statistical analysis

Statistical analyses were performed as described in 3.2.7 (page 48)

4.3 RESULTS

4.3.1 Effects of moxifloxacin on lymphocyte proliferation and expression of CD25

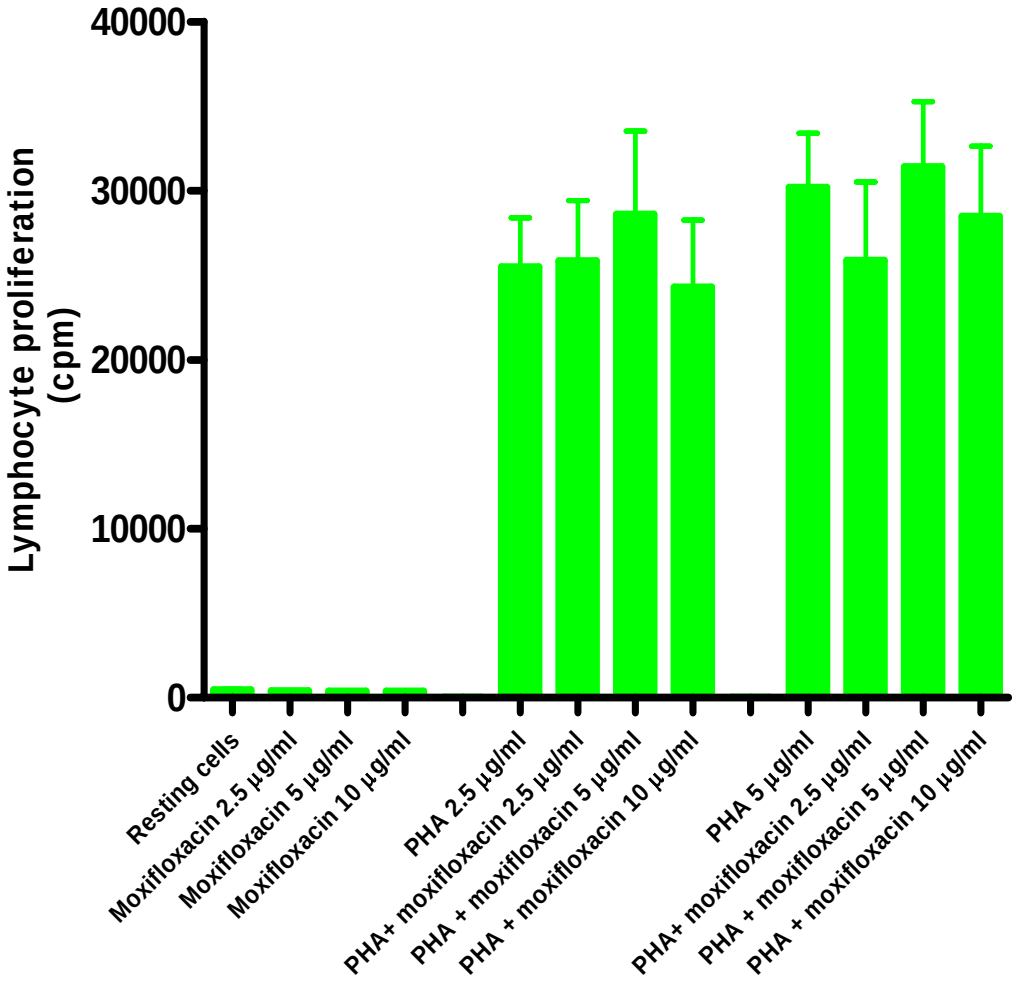
To investigate the effect of moxifloxacin on lymphocyte proliferation, ^{3}H-thymidine incorporation into newly synthesized DNA of T-cells was measured after treatment of cells with moxifloxacin followed by activation with PHA, while expression of CD25 was measured by flow cytometry. As shown in Figures 4.1 and 4.2 moxifloxacin at

concentrations 0.625, 1.25, 2.5, 5 and 10 μg/ml did not affect either lymphocyte proliferation or CD25 expression.

4.3.2 Effects of moxifloxacin on cytokine production by PHA-stimulated mononuclear leukocytes cultures

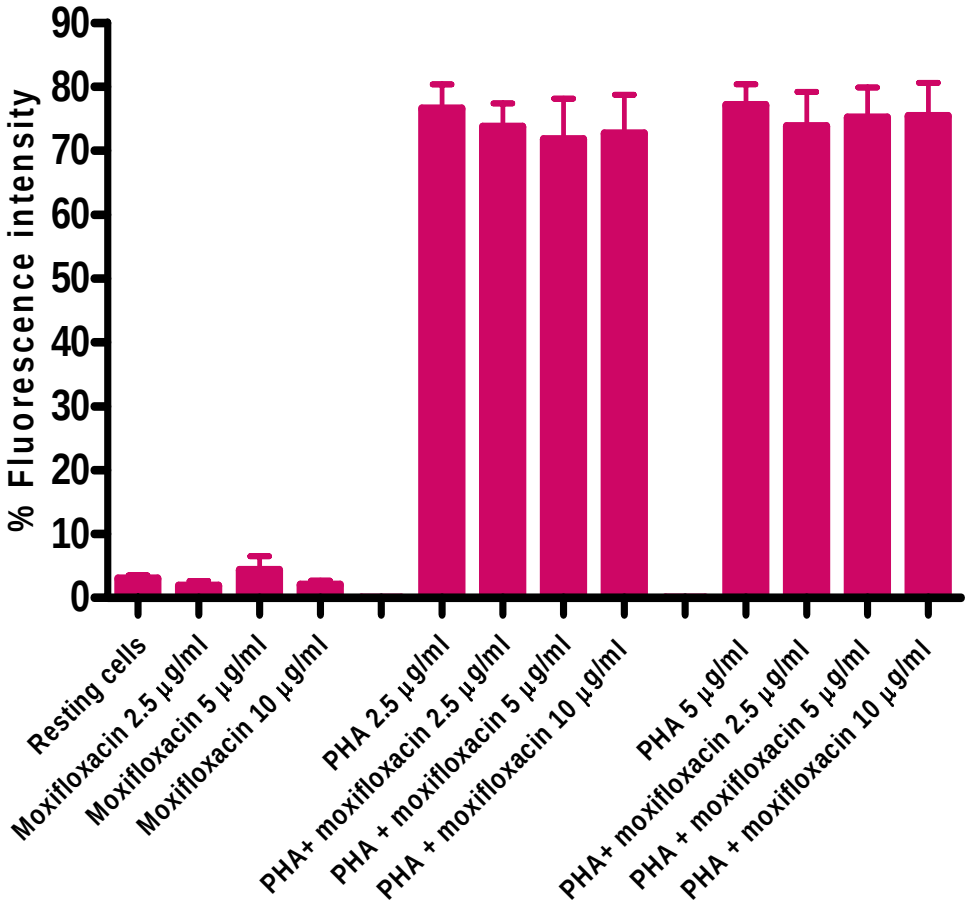
Stimulation of lymphocytes with PHA (2.5 and 5μg/ml) resulted in a significant increase in the production of most of the cytokines in the cell culture supernatants, which was more pronounced with PHA 5μg/ml. As shown in Table 4.1, exposure of PHA-stimulated lymphocytes to moxifloxacin (0.625 - 10 μg/ml) did not have a significant effect on the production of the following cytokines: IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL13, IL-17, IFN-γ, GM-CSF, G-CSF, TNF-α, and MCP-1. Although levels of IL-6 and IL-12 were decreased following exposure of MNL to moxifloxacin, the magnitudes of inhibition did not achieve statistical significance (Table 4.1 and Figures 4.3 and 4.4).

Fig 4.1 Effects of moxifloxacin on proliferation of PHA-activated MNL



The results of 15 experiments are presented as the mean values ± SEMs for uptake of radiolabelled thymidine by unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

Fig 4.2 Effect of moxifloxacin on CD25 expression by PHA-activated MNL



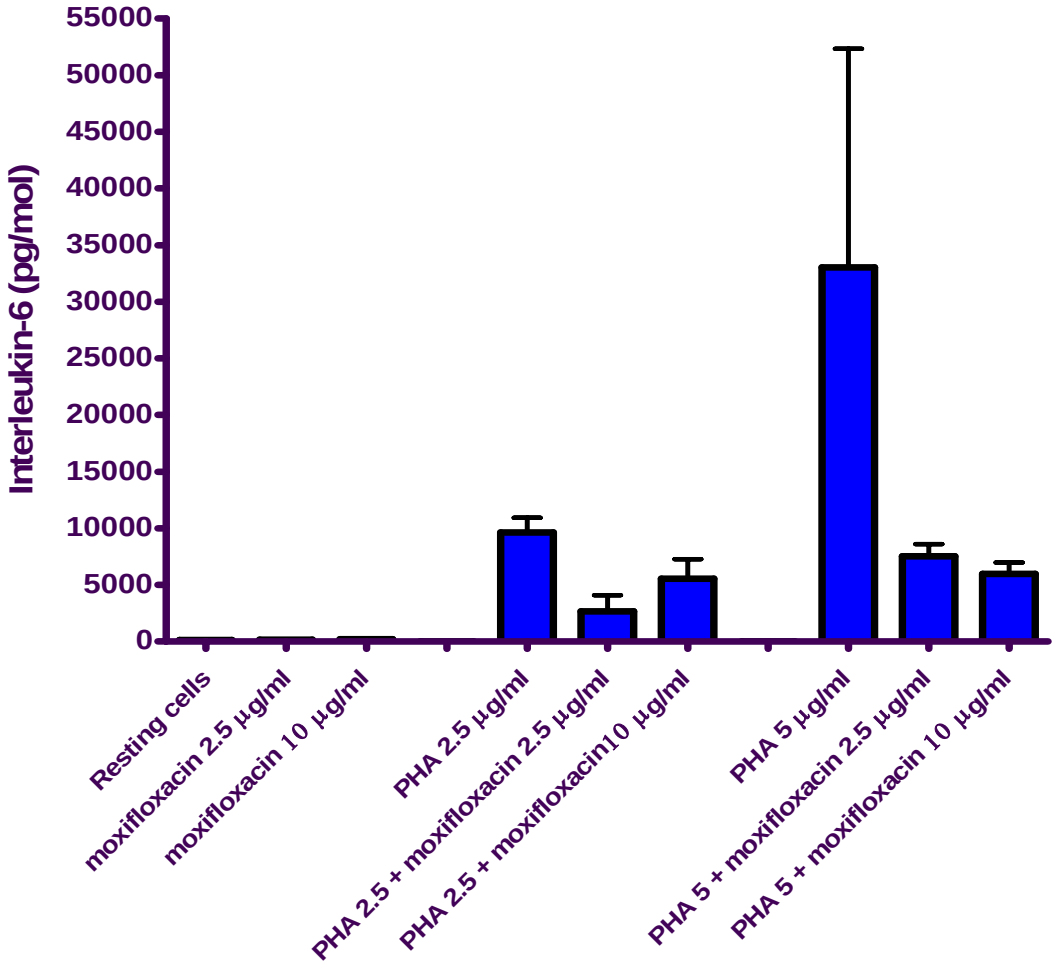
The results of 7 experiments are presented as the mean values ± SEMs for CD25 expression of unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

Table 4.1 Effects of Moxifloxacin on IL-6 and IL-12 production by PHA-activated MNLs

CYTOKINE	IL-6 (pg/ml)	IL-12 (pg/ml)
Resting cells	154.5 ± 9.3	1.8 ± 0.5
Moxifloxacin (2.5 µg/ml)	193.3 ± 21.8	1.6 ± 0.7
Moxifloxacin (10 µg/ml)	206.3 ± 38.4	1.2 ± 1.1
PHA (2.5 µg/ml)	9646.0 ± 1278.1	15.6 ± 5.4
PHA(2.5 µg/ml) + Moxifloxacin (2.5 µg/ml)	2672.1 ± 1433.7	8.2 ± 2.0
PHA (2.5 µg/ml) + Moxifloxacin (10 µg/ml)	5565.6 ± 1699.0	5.6 ± 1.0
PHA (5 µg/ml)	33048.3 ± 19325.0	36.7 ± 22.6
PHA (5 µg/ml) + Moxifloxacin (2.5 µg/ml)	7547.5 ± 1069.8	11.3 ± 3.8
PHA (5 µg/ml) + Moxifloxacin (10 µg/ml)	5982.4 ± 997.4	4.1 ± 0.4

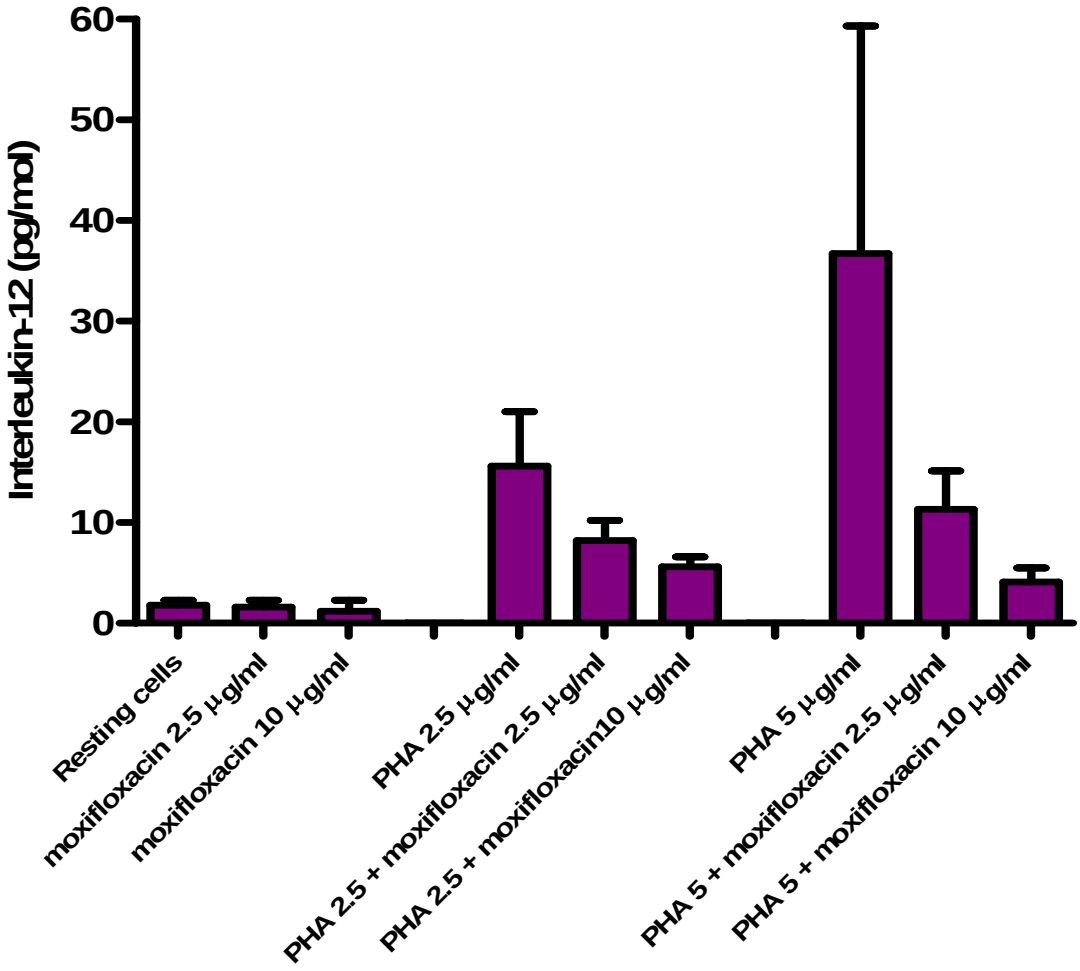
The results of 4 experiments are presented as the mean values ± SEMs as pg/ml for each cytokine.

Fig 4.3: Effects of moxifloxacin on IL-6 production by PHA-activated MNLs



The results of 4 experiments are presented as the mean values ± SEMs for IL-6 production by unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

Fig 4.4: Effects of moxifloxacin on IL-12 production by PHA-activated MNLs



The results of 4 experiments are presented as the mean values ± SEMs for IL-12 production by unstimulated and PHA-activated MNL in the absence and presence of moxifloxacin.

Table 4.2 Effects of Moxifloxacin on cytokine production by PHA-activated MNLs

CYTOKINE	IL-1β	IL-2	IL-4	IL-5	IL-7	IL-10	IL-13
Resting cells	10.2 ± 3.5	31.1 ± 0.3	73.2 ± 22.8	1.5 ± 0.1	2.0 ± 0.5	11.0 ± 5.1	4.6 ± 1.3
Moxifloxacin (2.5 μg/ml)	22.0 ± 8.6	47.2 ± 5.0	85.3 ± 22.7	1.6 ± 0.2	2.5 ± 0.6	12.1 ± 5.2	5.7 ± 2.0
Moxifloxacin (10 μg/ml)	9.1 ± 3.1	33.9 ± 2.8	64.2 ± 24.1	1.8 ± 0.1	1.8 ± 0.6	9.1 ± 5.0	4.2 ± 2.0
PHA (2.5 μg/ml)	115.7 ± 68.3	139.3 ± 32	237.5 ± 23.9	26 ± 2.1	5.5 ± 1.4	165.3 ± 69.0	92.5 ± 25.1
PHA (2.5 μg/ml) + Moxifloxacin (2.5 μg/ml)	108.4 ± 64.7	158.5 ± 56.7	266.2 ± 48.3	22 ± 6.1	5.5 ± 1.0	190.2 ± 80.4	47.2 ± 16.9
PHA (2.5 μg/ml) + Moxifloxacin (10 μg/ml)	68.4 ± 25.7	149.3 ± 17.0	214.9 ± 54.7	18.5 ± 2.1	4.7 ± 1.0	133.0 ± 69.3	99.5 ± 25.3
PHA (5 μg/ml)	149.3 ± 69.8	113.1 ± 11.8	280.7 ± 28	37.1 ± 2.7	6.9 ± 1.5	323.4 ± 163.2	117.6 ± 30.0
PHA (5 μg/ml) + Moxifloxacin (2.5 μg/ml)	119.6 ± 61.2	171.3 ± 43.7	224.2 ± 45.4	38.5 ± 13.9	5.1 ± 1.0	534.4 ± 251	124.8 ± 42.1
PHA (5 μg/ml) + Moxifloxacin (10 μg/ml)	137.7 ± 61.2	128 ± 1.9	275.2 ± 60.5	38.7 ± 7.89	6.2 ± 1.7	451.6 ± 214.3	251.0 ± 91.4

Table continued (P.T.O)

Table 4.2 Effects of Moxifloxacin on cytokine production by PHA-activated MNLs

CYTOKINE	IL-17	TNF-α	IFN-γ	G-CSF	GM-CSF	MCP-1
Resting cells	6.2 ± 1.7	23.8 ± 7.3	107.3 ± 43.3	70.5 ± 26.2	56.0 ± 17.4	2909.6 ± 1672.7
Moxifloxacin (2.5 μg/ml)	6.3 ± 3.2	44.3 ± 16.4	172.0 ± 64.5	144 ± 58.3	61.4 ± 25.7	8209.6 ± 4290.4
Moxifloxacin (10 μg/ml)	6.9 ± 2.6	28.2 ± 9.1	114.3 ± 40.3	63.7 ± 20.8	38.1 ± 14.6	2230.1 ± 1298.1
PHA (2.5 μg/ml)	30.8 ± 8.0	456.0 ± 203.0	939.6 ± 373.9	509.6 ± 112.6	250.6 ± 48.9	25259.0 ± 11748.0
PHA (2.5 μg/ml) + Moxifloxacin (2.5 μg/ml)	25.5 ± 6.2	293.5 ± 169.3	796.8 ± 406.7	531.4 ± 148.8	233.6 ± 46.9	17030.4 ± 4565.1
PHA (2.5 μg/ml) + Moxifloxacin (10 μg/ml)	29.1 ± 6.6	243.6 ± 117.4	734.1 ± 334.1	466.0 ± 198.9	169.4 ± 47.0	26127.6 ± 9497.8
PHA (5 μg/ml)	34.0 ± 4.6	455.7 ± 177.5	1348.7 ± 346.2	842.0 ± 229.9	296.7 ± 38.0	32046.9 ± 9145.2
PHA (5 μg/ml) + Moxifloxacin (2.5 μg/ml)	33.3 ± 8.9	598.5 ± 274.7	1664.4 ± 607.3	743.2 ± 228.0	297.0 ± 82.8	23998.1 ± 8406.3
PHA (5 μg/ml) + Moxifloxacin (10 μg/ml)	46.7 ± 19.0	370.4 ± 245.5	1667.2 ± 528.8	767.3 ± 223.9	315.2 ± 99.9	24838.3 ± 1333.2

The results of 4 experiments are presented as the mean values ± SEMs as pg/ml for each cytokine.