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Cytokine Secretion and Proliferative Capacity of CD4⁺ T Cells in Delayed Type Hypersensitivity Reactions due to Ciprofloxacin

Siprofloksasine Bağlı Geç Tip Aşırı Duyarlılık Reaksiyonlarında CD4⁺ T Hücrelerinin Sitokin Salınımları ve Proliferatif Kapasiteleri

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Abstract

Introduction: Ciprofloxacin (CPFX), a frequently prescribed quinolone, may induce cutaneous adverse drug reactions. Delayed type hypersensitivity reactions (DTHR) are often difficult to deal with, therefore, *in vitro* testing for DTHR is the long-anticipated method for their management. This study aimed to evaluate potential value of lymphocyte transformation test (LTT) and intracellular cytokine secretion of drug stimulated CD4⁺ T cells in patients with DTHR against ciprofloxacin.

Material and Methods: Patients experienced DTHR with CPFX (n=8) and healthy subjects (n=10) were enrolled. CPFX skin prick, patch and intradermal tests were performed. LTT by flow cytometry aimed to determine CPFX-specific CD4⁺ T cell proliferation. Intracellular IL-4, IL-10, IL-2 & IFN-γ levels were analysed by flow cytometry in CPFX-specific CD4⁺ T cells. Cytokine contents of cell culture supernatants were evaluated by ELISA.

Results: In patients with DTHR, 5 and 10 μg/mL CPFX induced significant CD4⁺ T cell proliferation (p=0.014 and p=0.05, respectively). IL-2 (p=0.02, p=0.001 and p=0.001, respectively) and IL-4 (p=0.001) secreting CD4⁺ T cell percentages were increased, while IFN-γ⁺ (p=0.001, p=0.011 and p=0.012, respectively) and IL-10⁺ (p=0.001, p=0.001 and p=0.002, respectively) CD4⁺ T cells were decreased. The cell culture supernatants revealed downregulated IL-10 (p<0.000, p=0.004, p=0.001 and p=0.0001, respectively) and upregulated IL-4 levels (p=0.003, p=0.013 and p=0.0001, respectively) in patients, regardless of CPFX stimulation. Intradermal test was positive in only one patient while all patch tests remained negative.

Conclusion: Our findings suggest that the increase of IL-2 and IL-4-secreting CD4⁺ T cells together with the decrease of IL-10 and IFN-γ-secreting CD4⁺ T cells is related to DTHR seen in patients with delayed-type CPFX allergy. Intracellular cytokine measurement, together with LTT could ease the management of CPFX hypersensitivity when *in vivo* tests are non-available, remain inconclusive or negative.

Keywords: Allergy, CD4⁺ T cells, ciprofloxacin, hypersensitivity, intracellular cytokine, LTT

Öz

Giriş: Siprofloksasin (SPFX), en yaygın kullanılan kinolon grubu antibiyotiklerden olup, kutanöz ters ilaç reaksiyonları oluşturabilme kapasitesine sahiptir. Gecikmiş tip aşırı duyarlılık reaksiyonlarında (ADR) kesin tanı konulması zor olmakla beraber *in vitro* tanı testlerinin geç tip ilaç ADR'de kullanımı önemlidir. Çalışmamızda SPFX'e bağlı geç tip ADR geçiren hastalarda, ilaçlar ile uyarılmış CD4⁺ T hücrelerinde, hücre içi sitokin üretimleri ve lenfosit transformasyon (LTT) yanıtları araştırılmıştır.

Gereç ve Yöntemler: Çalışmaya SPFX ile geç tip hipersensitivite reaksiyonu (n=8) geçiren hastalar ve sağlıklı kontroller (n=10) dâhil edilmiş, intradermal testler ve yama testleri uygulanmıştır. LTT metodu ile SPFX'e özgül CD4⁺ T hücre proliferasyonu belirlenmiştir. SPFX'e özgül CD4⁺ T hücrelerinde hücre içi IL-4, IL-10, IL-2 ve IFN-γ düzeyleri akan hücre ölçer kullanımı ile değerlendirilmiştir. Kültür üst sıvılarındaki sitokin düzeyleri ELISA aracılığı ile ölçülmüştür.

Bulgular: Geç tip ADR geçiren hastalarda 5 ve 10 μg/ml SPFX uyarımı CD4⁺ T hücre proliferasyonunu arttırmıştır (p=0.014 ve p=0.05, sırasıyla). IL-2 (p=0.02, p=0.001 ve p=0.001, sırasıyla) ve IL-4 (p=0.001) salgılayan CD4⁺ T hücre yüzdesi artmasına rağmen IFN-γ⁺ (p=0.001, p=0.011 ve p=0.012, sırasıyla) ve IL-10⁺ (p=0.001, p=0.001 ve p=0.002, sırasıyla) CD4⁺ T hücre oranları azalmıştır. Hücre kültür üst sıvılarında SPX uyarımından bağımsız olarak, hastalarda IL-10 düzeyi (p<0.000, p=0.004, p=0.001 ve p=0.0001, sırasıyla) azalırken, IL-4 düzeyi (p=0.003, p=0.013 ve p=0.0001, sırasıyla) artmıştır. İntradermal test uygulanan hastaların sadece birinde pozitif yanıt saptanır iken, yama testlerinde pozitif sonuç elde edilmemiştir.

Sonuç: Bulgularımız, IL-2 ve IL-4 üreten CD4⁺ T hücrelerindeki artışla birlikte IL-10 ve IFN-γ üreten CD4⁺ T hücrelerindeki azalmanın, gecikmiş tipte CPFX alerjisine sahip hastalarda DTHR ile ilişkili olduğunu düşündürmektedir. LT ile birlikte hücre içi sitokin ölçümleri, *in vivo* testlerin negatif kaldıkları, karar verdirici olamadıkları ve hatta uygulanamadıkları durumlarda, gecikmiş tipte CPFX hipersensitivitesinin değerlendirilmesinde fayda sağlayabilir.

Anahtar Sözcükler: Alerji, CD4⁺ T hücresi, siprofloksasin, aşırı duyarlılık, hücre içi sitokin, lenfosit transformasyonu

Introduction

Quinolones have been used for over 30 years in treatment of severe infections and are usually well tolerated.[1] In recent years, along with the increased use of ciprofloxacin (CPFX), levofloxacin and moxifloxacin, an increase in the frequency of hypersensitivity reactions due to these drugs has been reported.[2] CPFX was found to be the most common quinolone which could induce cutaneous Delayed type hypersensitivity reactions (DTHR), by being the culprit agent among 0.37% of patients who had received a fluoroquinolone.[3,4]

Quinolones were reported to have the capacity to induce both immediate and delayed-type hypersensitivity reactions, mediated by IgE and T-cells, respectively. The most common reported IgE-type reactions due to quinolones are urticaria, angioedema and anaphylaxis.[5] T-cell mediated reactions are reported to be less frequent than IgE-mediated reactions and include maculopapular rash, fixed drug eruption, acute generalized exanthematous pustulosis, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).[6]

Few studies have been carried out on the mechanisms underlying the delayed type hypersensitivity reactions to quinolones. Schmid et al. revealed that these reactions could be associated with a strong T-cell mediated immune response against the relevant quinolone, providing evidence that the original compound was recognized by T cells without the necessity of drug metabolism or processing.[7] Most of the related non-immediate reactions were published as case reports.[8–11]

The purpose of an allergy work-up in drug hypersensitivity reactions is to confirm the symptoms caused by a drug hypersensitivity reaction and to identify the culprit drug.[12] *In vivo* assessments such as patch, prick and intradermal tests are basically used in daily clinical practice but they lack adequate sensitivity. Furthermore contradictory results with patch or intradermal testing of quinolones were published.[6] Drug provocation tests, which are thought to be the gold standard for immediate reactions, may cause serious consequences and are not standardized for delayed type reactions. Consequently, *in vitro* testing would be a safe procedure for patients, avoiding possible disadvantages of provocation tests and may provide a better understanding of the pathological mechanism of drug hypersensitivity reactions.[13]

Recent studies have shown that *in vitro* T-cell proliferation and activation tests could be used for the diagnosis of delayed type drug hypersensitivities.[14,15] The lymphocyte transformation test (LTT) is the most frequently used *in vitro* technique to identify the culprit drug in drug-induced non-immediate hypersensitivity reactions.[14] Several studies performed to date indicated that the general sensitivity of the LTT in well-defined DH may vary between 60% and 70% depending on the drug itself, the type of reaction and is superior to skin testing for non-immediate type reactions.[14] Furthermore, measurements of drug specific cytokine secretion have been shown to be useful for drug hypersensitivity diagnosis.[16,17]

Up to now, among cytokines, interferon-gamma (IFN-γ) which is mainly produced by cytotoxic T cells, has been measured in delayed type HR. Additionally, the roles of T helper (Th) 2 cells and related cytokines were reported in drug induced eruptions as well. Hence, an assay of multiple cytokines produced by both drug specific cytotoxic and helper T cells including IFN-γ and IL-4, IL-5, IL-13, respectively, showing the sensitization pattern can be more helpful in diagnosis of DTHR.[18]

In our study, we aimed to compare the value of *in vivo* and *in vitro* test results in the diagnosis of delayed type hypersensitivity reactions due to ciprofloxacin. *In vivo* assessments including intradermal tests with delayed reading, patch tests and oral provocation tests were performed in patients with delayed type hypersensitivity reactions due to CPFX. Additionally, intracellular Th1, Th2 and regulatory cytokine release of $CD4^+$ T helper cells, and LTT assay was performed in CPFX-stimulated $CD4^+$ T cells. Finally, cytokine levels in CPFX-induced culture supernatants were investigated in patients who experienced a delayed type hypersensitivity reaction to CPFX.

Material and Methods

Patient Selection

Eight patients, referred to Istanbul University, Istanbul Faculty of Medicine, Clinic of Adult Allergy and Immunology due to non-immediate type drug hypersensitivity reactions (DHR) to CPFX, and 10 healthy controls who tolerated CPFX were enrolled into this study. The mean age of patients and controls were 46±13 and 47±13 years, respectively.

Non-immediate type hypersensitivity reactions were considered as the DHRs which developed more than one hour after the last drug ingestion.[19] The patients who had comorbid diseases such as diabetes, asthma or hypertension, malignancy and cardiac diseases leading to deterioration in general health were excluded from the study. This study was approved by the Ethical Committee of Istanbul University and informed consent was taken from all patients and healthy controls.

Skin Testing

All patients were undergone skin prick test with 5 mg/ml CPFX (Cipro 400 mg/200 mL, Biofarma, Turkey) on the volar side of forearm. Tests were accepted as positive if a wheal greater than 3 mm in diameter was present after 20 minutes. Histamine (10 mg/mL) and saline were used as positive and negative controls, respectively. When the results were negative, intradermal tests were performed with CPFX in two incremental dilutions (1/1000 and 1/100).[20] Intradermal tests were assessed 24 hours later as well. Drug patch tests were performed with the commercialized forms of CPFX which were diluted in petrolatum with the drug being 30% of the mixture. The tests were evaluated on the second and fourth days according to guidelines.[21] Healthy controls were undergone the same skin test procedures.

Drug Provocation Tests (DPTs)

To confirm the diagnosis, patients were challenged with single blind placebo controlled drug provocation tests (SBPCDPT) as suggested for drug hypersensitivity.[22] SBPCDPTs were performed with the culprit drug under close medical supervision of well trained personnel in patients with negative skin test results and if their initial reactions were not serious life-threatening non-immediate type hypersensitivity reactions such as Stevens Johnson syndrome, toxic epidermal necrolysis or acute generalized exanthematous pustulosis. On the first day, 5 doses of placebo and on the second day 5 doses of the CPFX starting with 1/100 of the drug and targeting the total dose of the drug were administered.[12,22]

Determination of Lymphocyte Transformation by CFSE Dilution Method

Proliferative responses of peripheral blood mononuclear cells (PBMC) from patients and healthy controls were investigated by flow cytometry with 5(6)-Carboxyfluorescein N-succinimidyl ester (CFSE) (Life Technologies, USA) dilution method. PBMCs from heparinized blood samples were purified by Ficoll (Sigma Chem. Co., St. Louis, MA) concentration gradient centrifugation, under sterile conditions. Cells were re-suspended in RPMI-1640 (Sigma Chem. Co., St. Louis, MO) enriched with penicillin, streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate and MEM vitamins (All from Gibco, Paisley, UK). Cells were incubated with 5 μM CFSE for 6 minutes in 4°C, at dark and were washed twice with RPMI-1640, following incubation. PBMCs were seeded to 48 well flat bottom culture plates at a concentration $1x10^6$ cells/ml, in 500 ml. 4 conditions were set in each experiment; unstimulated (US), 5 and 10 μg/ml CPFX and positive control (10ml/ml PHA). After 5 days of cell culture, PBMCs were labelled with anti-human CD4-PE (BD Biosciences, San Diego, USA), analyzed with a FACSCalibur (BD Bioscience, San Jose, CA) flow cytometer, and data analysis was performed using a FlowJo (version 8.7.1) (TreeStar, San Carlos, CA) software. Results were evaluated by the stimulation index (SI), which represents the ratio of drug-specific $CD4^+$ T cell proliferation to proliferation levels of US $CD4^+$ T cells.

Evaluation of Cytokine Production of $CD4^+$ T Cells By Flow Cytometry

Following cell culture, cells were collected to flow tubes, washed and re-suspended in 100 ml of PBS. 10 ml of anti-human-CD4-Allophycocyanin (APC) (BD Biosciences, San Diego, USA) was added to relevant conditions and incubated at dark for 30 minutes. PBMCs were washed once and resuspended in 250 μl Cytofix solution (BD Biosciences, San Diego, USA) for cell membrane fixation. After 20 min incubation in +4°C at dark, cells were washed once at 2000 RPM and resuspended in 100 ml Perm/Wash solution (BD Biosciences, San Diego, USA). For determination of intracellular cytokines, anti-human-IFN-γ-PE, anti-human-IL-2-PE, anti-human-IL4-PE and anti-human-IL-10-PE monoclonal antibodies (all from Diaclone, France) were added to relevant tubes and incubated for 20 minutes, in +40°C at dark. An unstained tube was used for determination of auto-fluorescence. Following incubation, cells were washed at 2000 RPM for 10 minutes, resuspended with 500 μl 2% paraformaldehyde PBS solution and were analyzed with a BD Facs Calibur flow cytometry, running CELLQuest software. Data was analysed by FlowJo (version 8.7.1) (TreeStar, San Carlos, CA).

Determination of IL-2, IL-4, IL-10 and IFN-γ Levels in CPFX-stimulated Cultures

Culture supernatants of lymphocyte transformation cell cultures (with stimulations CPFX (0, 5 and 10 μg/ml) and PHA) were used for determination of IL-2, IL-4, IL-10 and IFN-γ cytokine contents by sandwich ELISA kits (Invitrogen Corporation, CA). The tests were conducted according to the manufacturer's instructions. 96-well plates were pre-coated with antibodies against IL-2, IL-4, IL-10 and IFN-γ. Supplied standards were used to generate the standard curves. Samples and standards were pipetted to relevant wells in plates and plates were washed 4 times in ELX50 plate washer (Biotec Instruments, USA) with washing buffer supplied with kits. Following washing step, biotin conjugate followed by horseradish peroxidase-conjugated streptavidin were added in a stepwise manner. Stop solution was added to wells in order to terminate the reaction. Optical density of each well was determined at 450 nm wave length, by ELX800 Elisa reader (Biotec Instruments, USA). Minimal detection limits according to manufacturer were as follows: For IL-2 and IFN-γ, <4 pg/mL, for IL-4, <2 pg/mL and for IL-10, <1 pg/mL.

Statistical Analyses

SPSS 15 was utilized for statistical evaluation of data obtained. Wilcoxon test was used for evaluation of paired data and Mann Whitney U test was used for evaluation of unpaired data. P<0.05 was accepted as statistical significance level.

Results

Demographic Features, *in vivo* Test and LTT Results of the Patients

Of the 8 patients, 7 were female (the mean age was 46±13 years). The mean reaction time and the mean time between the reaction and the evaluation were 13.2 hours and 29.3±25.2 months, respectively. Reaction types were late onset urticaria in 3 patients, maculopapular eruptions in 4 patients and fixed drug eruption in 1 patient. Delayed reading of intradermal tests were negative in 5 patients and positive in 1 patient. Patch tests were performed in 5 patients and all were negative. DPTs were not performed in 5 patients due to history of severe reactions or unwillingness. DPTs were negative in 2 patients and was positive in 1 patient. LTT was performed in 7 patients (Table 1). Four patients were 'non-responsive' to CPFX LTT, while 3 patients had increased proliferation in response to 5 and 10 μg/ml of CPFX stimulation (p=0.014 and p=0.05, respectively). (Figure 1A and B).

Analysis of Intracellular IFN-γ, IL-2, IL-4, IL-10 Secretion of CD4+ T cells

In US, 5 μg/ml and 10 μg/ml CPFX stimulated conditions, intracellular content of IL-2 was significantly increased in patients compared with healthy controls (p=0.02, p=0.001 and p=0.001, respectively). Similar results were obtained for CD4+ T intracellular IL-4 (p=0.001, p=0.001 and p=0.001, respectively). Whereas, IL-10 was

Table 1. Patients' characteristics and diagnostic test results

Patient	Sex	Age (year)	Diagnosis	Time* (hour)	Time from the reaction to test (months)	Reaction type	Delayed reading of IDT	PATCH TEST	DPT	LTT (SI) (5 mg/ml)	LTT (SI) (10 mg/ml)
Case 1	F	44	Urinary tract infection	24	25	Urticarial angioedema	+	-	X	1.8 (+)	1.5 (+)
Case 2	F	29	Urinary tract infection	6	60	MPE	-	-	-	0.63 (-)	0.54 (-)
Case 3	F	30	Cystitis	8	6	MPE	-	-	+	X	X
Case 4	M	64	Gastroenteritis	24	23	Urticaria	X	X	X	0.8 (-)	0.4 (-)
Case 5	F	55	Gastroenteritis	4	36	FDE	-	X	X	3.1 (+)	3.5 (+)
Case 6	F	57	Urinary tract infection	6	7	MPE	X	-	X	0.5 (-)	0.4 (-)
Case 7	F	42	Gastroenteritis	10	6	MPE	-	-	X	2 (+)	1.6 (+)
Case 8	F	50	Gastroenteritis	24	72	Urticaria	-	X	-	0.36 (-)	0.3 (-)

*Time interval between drug intake and reaction, M: male, F: female, MPE: maculopapular eruption, FDE: fixed drug eruption, IDT: intradermal test, DPT: drug provocation test, LTT: lymphocyte transformation test, SI: stimulation index, X: not performed

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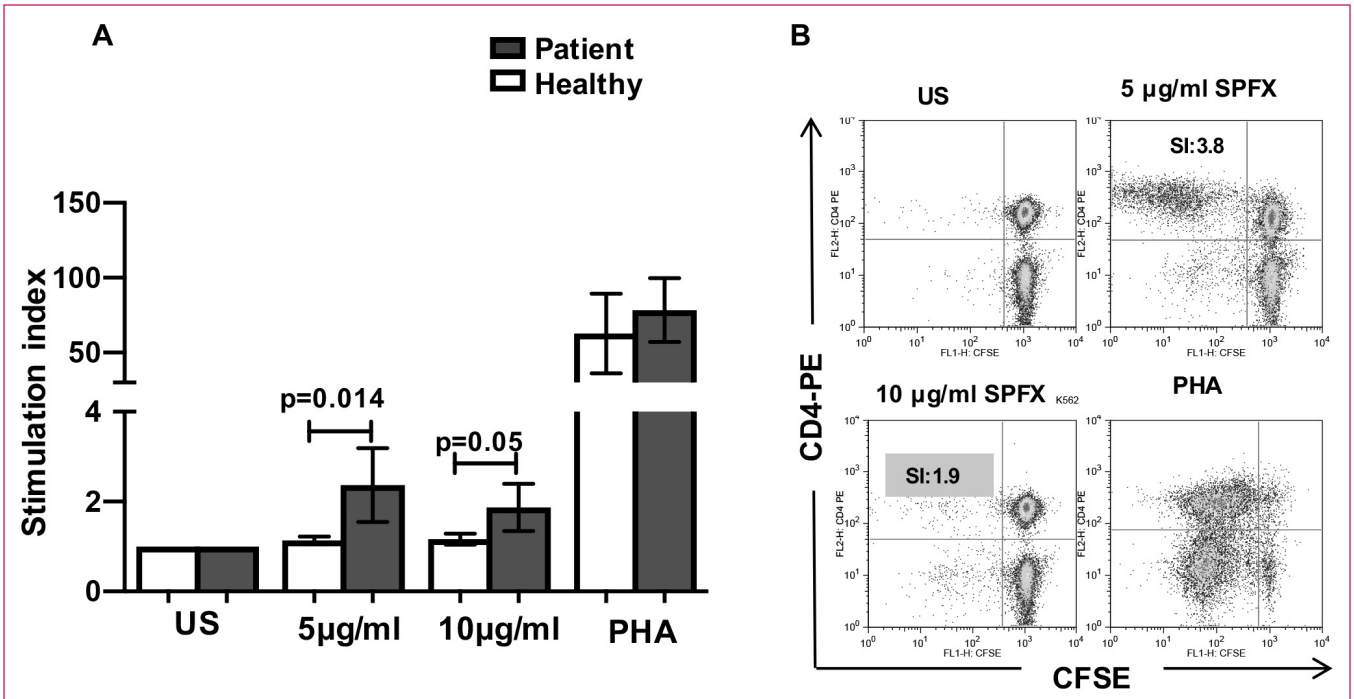

Figure 1. Proliferative response of CD4⁺ T cells in response to ciprofloxacin stimulation. Peripheral blood mononuclear cells isolated from patients and healthy controls were stained with CFSE and were cultured with the absence and existence of ciprofloxacin of 5 and 10 µg/ml doses for 120 hours. Proliferative responses of CD4⁺ T cells were evaluated by flow cytometry. Bar graphs demonstrate stimulation indices evaluated as the ratio of relevant condition to the US condition (Filled bars: patients, empty bars: healthy controls, figures show median ± range, Mann Whitney U test was used for statistical comparison of the groups, Wilcoxon paired ranks test was used for investigation of effects of conditions, $p<0.05$ was accepted as statistical significance level) **(A)**. FlowJo program images, shown are one representative dot plots from ciprofloxacin responsive patient proliferative responses to 0, 5 and 10 µg/ml doses of ciprofloxacin and PHA as positive control and SI values are also indicated **(B).** US: unstimulated, PHA: phytohemagglutinin, SI: stimulation index.

decreased in these above-mentioned three conditions in patients, in comparison with healthy controls (p=0.001, p=0.001 and p=0.002, respectively). CPFX (10 µg/ml) induced an increase of IL-2 content within CD4⁺ T cells (p=0.022) in patient group. IFN-γ secreting CD4⁺ T cell ratio was found to be reduced in patients compared to healthy controls in all conditions (p=0.001, p=0.011 and p=0.012, respectively) (Figure 2).

Interleukin-2, IL-4, IL-10 and IFN-γ Levels in CPFX-Stimulated Culture Supernatants

To understand the effect of two doses of CPFX on Th1 or Th2 type cytokine secretions of PBMCs in patients and in healthy subjects, IL-2, IL-4, IL-10 and IFN-γ levels were analysed in cell culture supernatants by ELISA. IL-10 levels were found to be reduced (p<0.0001, p=0.004, p=0.001, p=0.0001, respectively), in US, 5 µg/mL and 10 µg/mL CPFX- and PHA-stimulated conditions of patients in comparison with that of healthy controls. While comparing the different conditions among them, CPFX has induced a decrease in IL-10 levels in comparison with US condition in patients and in controls (p=0.019 and p=0.008, respectively). No considerable differences in IL-2 levels between the allergic and healthy individuals were observed. IL-4 levels were increased in the patient group, under US, 5 µg/ml CPFX stimulated and PHA stimulated conditions (p=0.003, p=0.013 and p=0.0001, respectively). PHA stimulation has also resulted in a reduction of IFN-γ levels in the patient group (p=0.0001) (Figure 3).

Discussion

The ultimate need for validating standard methods and determining the sensitivity, and specificity for *in vitro* tests in diagnosis of delayed type drug hypersensitivity reactions is increasing, for enabling their utilization into routine clinical practice.[23] Drug allergy labelling rather than performing safe and validated tests in patients with suspected delayed antibiotic reactions can have detrimental

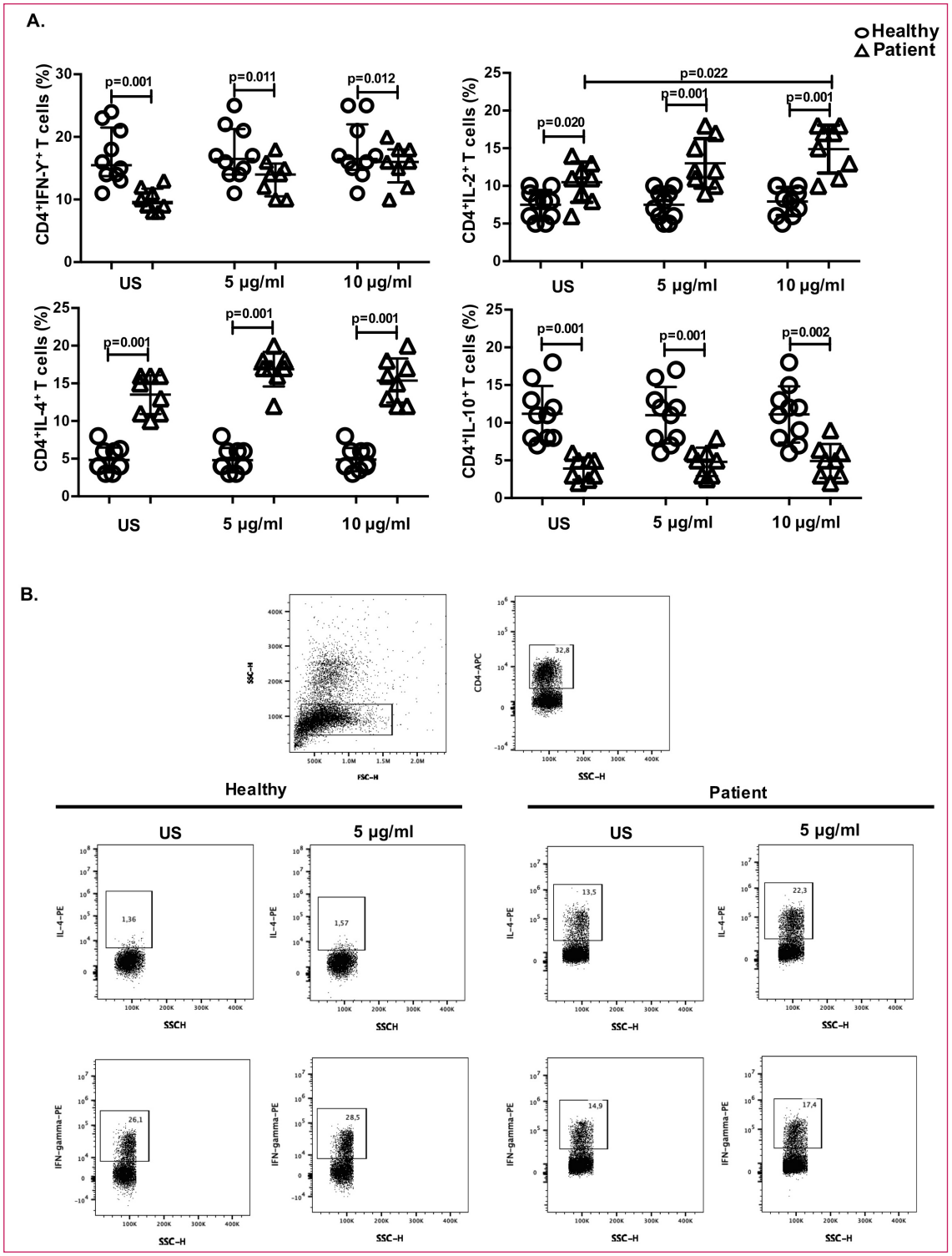

Figure 2. Intracellular IFN-γ, IL-2, IL-4 and IL-10 levels of CD4⁺ T lymphocytes following ciprofloxacin encounter in cell culture conditions. Peripheral blood mononuclear cells isolated from blood samples of patients with delayed hypersensitivity to ciprofloxacin, and healthy controls were cultured with the absence and existence of ciprofloxacin of 5 and 10 µg/ml doses, for five days. Following cell culture, intracellular IFN-γ, IL-2, IL-4 and IL-10 contents of CD4⁺ T cells were investigated by flow cytometry. Scatter plot graphs demonstrate CD4⁺IFN-γ⁺, CD4⁺IL-2⁺, CD4⁺IL-4⁺ and CD4⁺ IL-10⁺ T cell percentages. Figures show median ± quartile values, Mann Whitney U test was used for statistical comparison of the groups, Wilcoxon paired ranks test was used for investigation of effects of conditions, $p<0.05$ was accepted as statistical significance level. Cells were acquired by a BD FACSCalibur and were analyzed by Cell Quest Software. Values indicate expression percentages of relevant intracellular cytokines in CD4⁺ T cells **(A)**. The gating strategy to identify IFN-γ⁺CD4⁺ and IL-4⁺CD4⁺ T cell subsets from patient and healthy subject by flow cytometry is shown. Results are shown as dot plots; percentages of cells within the quadrants are given. **(B)**. Circles indicate healthy controls, triangles indicate patients. US: unstimulated,

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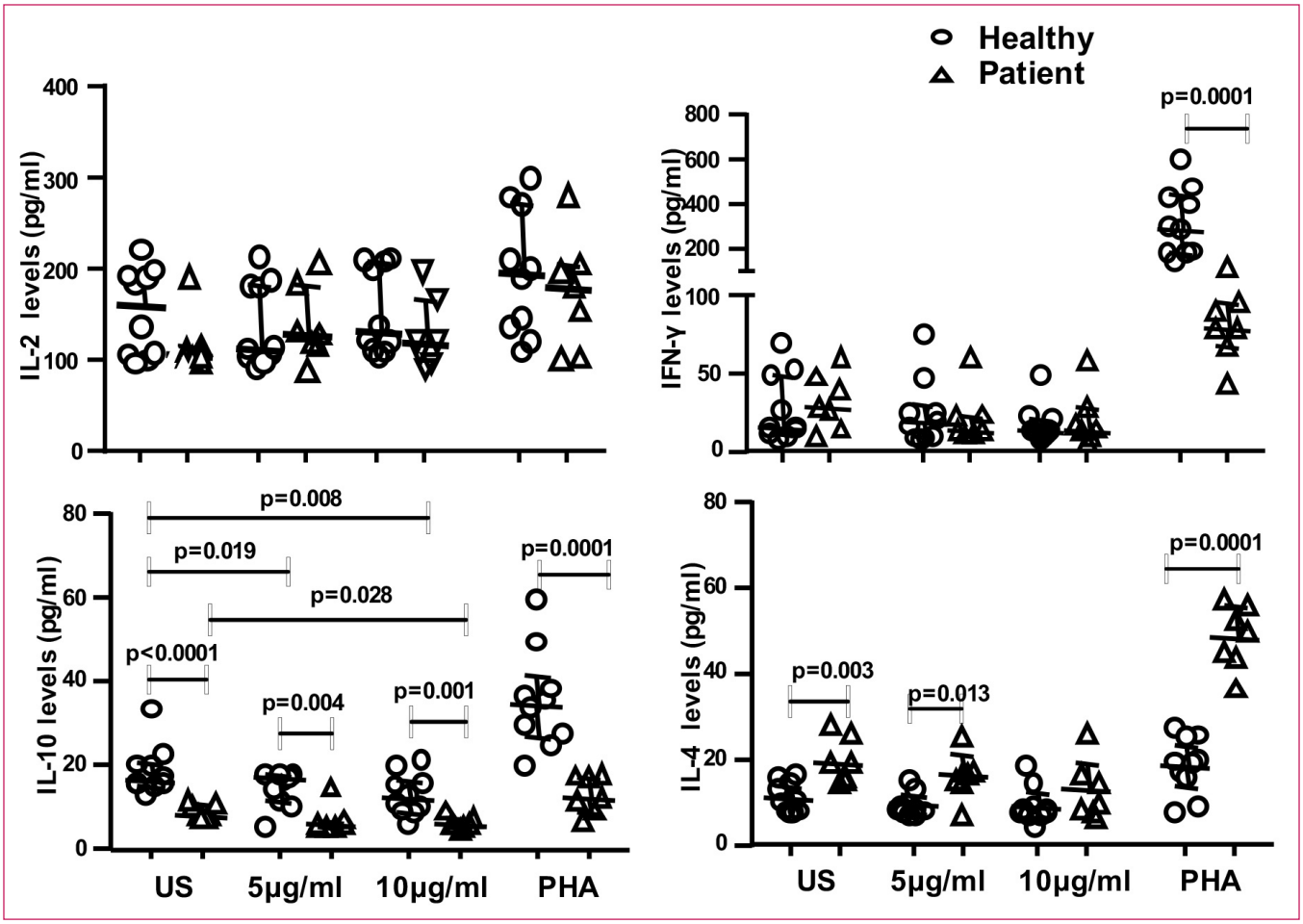

Figure 3. IL-2, IL-4, IL-10 and IFN-γ cytokine levels in ciprofloxacin stimulated culture supernatants. Peripheral blood mononuclear cells isolated from patients and healthy controls were cultured with the absence and existence of ciprofloxacin of 5 and 10 µg/ml doses and with PHA for 120 hours. Cell culture supernatants were collected during the termination of culture. Scatter plot graphs represent values of each individual with median and quartile values (Triangles represent patients and circles represent healthy controls, Mann Whitney U test was used for statistical comparison of the groups, p<0.05 was accepted as statistical significance level). US: unstimulated, PHA: phytohemagglutinin.

effects on general health, antibiotic resistance and also on health economics.[24] Therefore different methods for improving the validity of *in vitro* tests in the diagnosis of delayed drug hypersensitivities need to be reported and discussed. In our study we have analysed cytokine contents in T cells and cell culture supernatants following stimulation with CPFX in patients who experienced non-immediate type hypersensitivity reactions due to CPFX in conjunction with i*n vivo* diagnostic tests.

Different T cell subgroups play roles in T cell mediated delayed type hypersensitivity reactions. Studies claim utilization of *in vitro* T cell proliferation and activation tests for diagnose of drug hypersensitivity reactions.[14,15] LTT is a well-known *in vitro* method used to detect sensitized T cells against the drug and drug-induced cytokine production by circulating lymphocytes has also been proposed for the identification of drug-sensitized individuals.[18,25] Many researchers have focused on the correlation of Th1 or Th2 type immune reactions with clinical picture in various drug allergies.[26,27] While some demonstrated predominance of Th1-type cytokine pattern in the non-immediate type hypersensitivity reactions, many studies have indicated a mixed Th1/Th2 pattern.[28] Thus, in many studies, IFN-γ has been shown to be a predominant cytokine in cutaneous drug reactions according to drug-induced cytokine measurements and has been determined as a possible *in vitro* marker for responsible drug identification initiating non-immediate type hypersensitivity reactions.[17,29–31] Additionally other cytokines such as, IL-5, IL-2 and IL-13 have been evaluated in culture supernatants of PBMC isolated from patients with non-immediate type hypersensitivity

reactions and then was proposed as a promising method of diagnosis for these cases.[26,27,32] Polak et al. tested the utility of the lymphocyte proliferation assays (LPA) versus IFN-γ and IL-4 drug enzyme-linked immunosorbent spot (ELISpot) assays for delayed-type DHR and found a combined sensitivity of 77–83% and a specificity of 83–95%.[18] These findings revealed a potential diagnostic value in the acute setting as they allow safe testing for multiple drugs.[18,25] Although contradictory results have also been reported.[33] Interestingly, in our study, cytokines other than IFN-γ were also found to be related with CPFX induced delayed hypersensitivity reactions. Our findings showed that stimulation with CPFX increased $CD4^+IL\text{-}2^+$ and $CD4^+IL\text{-}4^+$ T cell percentages and decreased $CD4^+IL\text{-}10^+$ and $CD4^+IFN\text{-}\gamma^+$ T cell ratio in patients with CPFX hypersensitivity. When intracellular cytokine contents of $CD4^+$ T cells were investigated, increase of IL-2 and IL-4 in patients may reveal the activated status of Th2 cells which contributes in allergic inflammation in response to CPFX. Decrease of IFN-γ is a general expectation in allergic inflammation while down-regulated IL-10 responses may underline a dysregulated immune response in patients. Additionally, in LTT culture supernatants, a decrease in IL-10 and an increase in IL-4 levels occurred only in the patient group following CPFX stimulation, whereas no difference was observed in the levels of IFN-γ and IL-2.

In the first study showing the interaction of T cells with various quinolones in delayed drug hypersensitivity reactions, Schmid et al. investigated 6 patients with late reaction to CPFX, moxifloxacin and norfloxacin.[7] The LTT test was positive in all patients with various concentrations (0.1 μg/ml-20 μg/mL) of the drugs, while positive patch test results were obtained in only half of the patients. A quinolone concentration of 20 μg/ml was determined as toxic dose and the 10 μg/mL as an optimal concentration.[7] In our preliminary experiments, the optimal concentrations for CPFX were found to be 5 and 10 μg/ml. The proliferative capacity of CPFX-stimulated T cells at concentrations of 5 and 10 μg/mL were significantly higher in the patient group when compared to the control group, whereas LTT assay gave a positive result in 3 of 8 patients enrolled to this study.

One limitation of our study is the presence of a long time between the reaction date and the evaluation. LTT and drug provocation tests of two patients who previously described a delayed hypersensitivity reaction to CPFX occurred 5-6 years ago, were negative and therefore their negative results do not seem to exclude their diagnosis. The negative provocation test may be due to the long time-gap between the reaction and the test day. Eventually the statistical evaluation of intracellular cytokine levels in these LTT-negative patients would not be reliable. The second limitation of our study was the limited number of patients due to hardness of obtaining patients which have drug allergies specific to CPFX. Further studies with increased number of patients may support the findings of the present study and better elucidate the effect of time interval between the reaction time and the evaluation.

In conclusion, our results suggest that an increase in IL-2 and IL-4 secreting $CD4^+$ T cells in association with a reduction in $IL\text{-}10^+CD4^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells could be expected in patients with non-immediate type hypersensitivity reactions to CPFX may be helpful for *in vitro* testing of this group of patients where skin tests may be inadequate and DPT may be contraindicated to diagnose hypersensitivity reactions to CPFX. Taking account the risk of having allergy labels without definite diagnosis or attempting to perform a drug challenge test without knowing what reaction our patient may develop, LTT and intracellular cytokine assays might be very beneficial steps to be implemented, hopefully, in our clinical practice, in a near future, as a result of more reports shared in this area.

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References

1. Van Bambeke F, Tulkens PM. Safety profile of the respiratory fluoroquinolone moxifloxacin: comparison with other fluoroquinolones and other antibacterial classes. Drug Saf 2009;32:359–78. [Crossref]
2. Dona I, Blanca-Lopez N, Torres MJ, Garcia-Campos J, Garcia-Nunez I, Gomez F, et al. Drug hypersensitivity reactions: response patterns, drug involved, and temporal variations in a large series of patients. J Investig Allergol Clin Immunol 2012;22:363–71. http://www.jiaci.org/issues/vol22issue5/7.pdf
3. Kulthanan K, Chularojanamontri L, Manapajon A, Dhana N, Jongjarearnprasert K. Cutaneous adverse reactions to fluoroquinolones. Dermatitis 2011;22:155–60. [Crossref]

4. Lapi F, Tuccori M, Motola D, Pugi A, Vietri M, Montanaro N, et al. Safety profile of the fluoroquinolones: analysis of adverse drug reactions in relation to prescription data using four regional pharmacovigilance databases in Italy. Drug Saf 2010;33:789–99. [Crossref]
5. Neuman MG, Cohen LB, Nanau RM. Quinolones-induced hypersensitivity reactions. Clin Biochem 2015;48:716–39. [Crossref]
6. Blanca-Lopez N, Ariza A, Dona I, Mayorga C, Montanez MI, Garcia-Campos J, et al. Hypersensitivity reactions to fluoroquinolones: analysis of the factors involved. Clin Exp Allergy 2013;43:560–7. [Crossref]
7. Schmid DA, Depta JP, Pichler WJ. T cell-mediated hypersensitivity to quinolones: mechanisms and cross-reactivity. Clin Exp Allergy 2006;36:59–69. [Crossref]
8. Fernandez-Rivas M. Fixed drug eruption (FDE) caused by norfloxacin. Allergy 1997;52:477–8. [Crossref]
9. Lozano Ayllon M, Gomez Martinez M, Mosquera MR, Laguna Martinez JJ, Orta Martiartu M, Fernandez de Miguel C. Fixed eruption caused by ciprofloxacin without cross-sensitivity to norfloxacin. Allergy 1995;50:598–9. [Crossref]
10. Garcia Rodriguez R, Galindo Bonilla PA, Feo Brito FJ, Gomez Torrijos E, Borja Segade J, Lara de la Rosa P, Jiménez AC. Chronic desensitization to quinolones in fixed drug eruption. J Investig Allergol Clin Immunol 2011;21:76–80. http://www.jiaci.org/issues/vol21issue01/13-16.pdf
11. Davila G, Ruiz-Hornillos J, Rojas P, De Castro F, Zubeldia JM. Toxic epidermal necrolysis induced by levofloxacin. Ann Allergy Asthma Immunol 2009;102:441–2. [Crossref]
12. Blanca-Lopez N, Andreu I, Torres Jaen MJ. Hypersensitivity reactions to quinolones. Current opinion in allergy and clinical immunology. 2011;11(4):285-91 [Crossref]
13. Lochmatter P, Zawodniak A, Pichler WJ. In vitro tests in drug hypersensitivity diagnosis. Immunol Allergy Clin North Am 2009;29:537–54. [Crossref]
14. Pichler WJ, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. Allergy 2004;59:809–20. [Crossref]
15. Beeler A, Zaccaria L, Kawabata T, Gerber BO, Pichler WJ. CD69 upregulation on T cells as an in vitro marker for delayed-type drug hypersensitivity. Allergy 2008;63:181–8. [Crossref]
16. Merk HF. Diagnosis of drug hypersensitivity: lymphocyte transformation test and cytokines. Toxicology 2005;209:217–20. [Crossref]
17. Halevy S, Cohen AD, Grossman N. Clinical implications of in vitro drug-induced interferon gamma release from peripheral blood lymphocytes in cutaneous adverse drug reactions. J Am Acad Dermatol 2005;52:254–61. [Crossref]
18. Polak ME, Belgi G, McGuire C, Pickard C, Healy E, Friedmann PS, Ardern-Jones MR. In vitro diagnostic assays are effective during the acute phase of delayed-type drug hypersensitivity reactions. Br J Dermatol 2013;168:539–49. [Crossref]
19. Demoly P, Adkinson NF, Brockow K, Castells M, Chiriac AM, Greenberger PA, et al. International Consensus on drug allergy. Allergy 2014;69:420–37. [Crossref]
20. Rouzaire P, Nosbaum A, Denis L, Bienvenu F, Berard F, Cozon G, Bienvenu J. Negativity of the basophil activation test in quinolone hypersensitivity: a breakthrough for provocation test decision-making. Int Arch Allergy Immunol 2012;157:299–302. [Crossref]
21. Barbaud A, Goncalo M, Bruynzeel D, Bircher A. Guidelines for performing skin tests with drugs in the investigation of cutaneous adverse drug reactions. Contact Dermatitis 2001;45:321–8. [Crossref]
22. Aberer W, Bircher A, Romano A, Blanca M, Campi P, Fernandez J, et al. Drug provocation testing in the diagnosis of drug hypersensitivity reactions: general considerations. Allergy 2003;58:854–63. [Crossref]
23. Mayorga C, Dona I, Perez-Inestrosa E, Fernandez TD, Torres MJ. The Value of In Vitro Tests to Diminish Drug Challenges. Int J Mol Sci 2017;18:1222. [Crossref]
24. Lucas M, Arnold A, Sommerfield A, Trevenen M, Braconnier L, Schilling A, et al. Antibiotic Allergy Labels in Children Are Associated with Adverse Clinical Outcomes. J Allergy Clin Immunol Pract 2019;7:975–82. [Crossref]
25. Porebski G. In Vitro Assays in Severe Cutaneous Adverse Drug Reactions: Are They Still Research Tools or Diagnostic Tests Already? Int J Mol Sci 2017;18:1737. [Crossref]
26. Yawalkar N, Shrikhande M, Hari Y, Nievergelt H, Braathen LR, Pichler WJ. Evidence for a role for IL-5 and eotaxin in activating and recruiting eosinophils in drug-induced cutaneous eruptions. J Allergy Clin Immunol 2000;106:1171–6. [Crossref]
27. Khalil G, El-Sabban M, Al-Ghadban S, Azzi S, Shamra S, Khalife S, Maroun R. Cytokine expression profile of sensitized human T lymphocytes following in vitro stimulation with amoxicillin. Eur Cytokine Netw 2008;19:131–41. [Crossref]
28. Posadas SJ, Torres MJ, Mayorga C, Juarez C, Blanca M. Gene expression levels of cytokine profile and cytotoxic markers in non-immediate reactions to drugs. Blood Cells Mol Dis 2002;29:179–89. [Crossref]
29. Naisbitt DJ, Britschgi M, Wong G, Farrell J, Depta JP, Chadwick DW, et al. Hypersensitivity reactions to carbamazepine: characterization of the specificity, phenotype, and cytokine profile of drug-specific T cell clones. Mol Pharmacol 2003;63:732–41. [Crossref]
30. Gaspard I, Guinnepain MT, Laurent J, Bachot N, Kerdine-Römer S, Bertoglio J, et al. Il-4 and IFN-gamma mRNA induction in human peripheral lymphocytes specific for beta-lactam antibiotics in immediate or delayed hypersensitivity reactions. J Clin Immunol 2000;20:107–16. [Crossref]
31. Goldberg I, Hanson M, Chodick G, Shirazi I, Brenner S. In vitro release of interferon-gamma from peripheral blood lymphocytes in cutaneous adverse drug reactions. Clin Dev Immunol 2012;2012:687532. [Crossref]
32. Lochmatter P, Beeler A, Kawabata TT, Gerber BO, Pichler WJ. Drug-specific in vitro release of IL-2, IL-5, IL-13 and IFN-gamma in patients with delayed-type drug hypersensitivity. Allergy 2009;64:1269–78. [Crossref]
33. Sedlackova L, Prucha M, Polakova I, Mikova B. Evaluation of IFN-gamma Enzyme-linked Immunospot Assay (ELISPOT) as a First-line Test in the Diagnosis of Non-Immediate Hypersensitivity to Amoxicillin and Penicillin. Prague Med Rep 2018;119:30–42. [Crossref]