Effect of gender , aging , smoking and urinary tract infections on level of CD4+ T lymphocytes in hypertensive patients treated with propranolol and captopril drugs .

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Abstract :

Forms of adaptive response may be useful markers of endothelial activation and local or systemic inflammation. In this study the CD4+ T lymphocytes were evaluated during vascular disease like hypertension and the effect of other factors like sex, aging , smoking , anti hypertension drugs treatment (mainly with some β-blockers or angiotensin converting enzyme inhibitors) and urinary tract infections on expression of the CD4+ T lymphocytes were also studied . The results showed that mean level of CD4+ T lymphocytes was increased in hypertensive patients ($p \leq 0.01$) and this increase is more in female compared to male . Level of CD4+ T lymphocytes was significantly elevated in hypertensive subjects with age group of 30-40 years and the level started to decreased with aging. However, upon treatment with antihypertensive drugs (mainly β-blocker or angiotensin enzyme inhibitors) the level of CD4+ T lymphocytes was reported to decreased . CD4+T lymphocytes level was significantly increased in hypertensive smoking patients($P \leq 0.01$) and in patients suffering from urinary tract infections (UTIs) .

تأثير الجنس , العمر , التدخين والتهاب المسالك البولية على مستوى الخلايا اللمفية عند المرضى المصابين بارتفاع ضغط الدم والمعالجين ببعض الادوية المضادة لارتفاع ضغط الدم .

المفاتيح : جزيئات الخلايا اللمفية (CD4+ T cell) , ارتفاع ضغط الدم , الجنس , العمر , الشيخوخة , التدخين , التهاب المسالك البولية .

الخلاصه :

أشكال المناعة التكيفية قد تكون من العلامات المفيدة الناتجة عن تنشيط الخلايا البطانية أو تنشيط الالتهابات التي قد تكون شامله أو ضمن أطار محدد . في هذه الدراسة وجد أن نسبة جزيئات الخلايا اللمفية (CD4+ T cell) المرتبطة في الخلايا اللمفاوية مع بعض الأمراض الوعائية مثل ارتفاع ضغط الدم قد ارتفع بشكل معنوي . كما وجد أن نسبة ارتفاع جزيئات الخلايا اللمفية (CD4+ T cell) في النساء المصابات بارتفاع ضغط الدم أكثر من الرجال . كما أظهرت النتائج زيادة في نسبة جزيئات الخلايا اللمفية (CD4+ T cell) في حالات التدخين و عند المرضى أللذين يعانون من التهاب المسالك البولية . في حين انخفضت بشكل معنوي في حالات الشيخوخة وحالات العلاج بأحد أدوية علاج الضغط مثل (β-blocker or Angiotensin converting enzyme inhibitor) .

Introduction

Hypertension or high blood pressure is a cardiac chronic medical condition in which the systemic arterial blood pressure is elevated. Hypertension is classified as either primary (essential) hypertension or secondary hypertension; About 90–95% of cases are categorized as "primary hypertension," which means high blood pressure with no obvious medical cause (*1)* . The remaining 5–10% of cases (Secondary hypertension) are caused by other conditions that affect the kidneys, arteries, heart or endocrine system (*2*). In contrast to the innate immune response, the adaptive immune response is highly selective and can recognize specific pathogens via humoral (B-cell–mediated antibody production) and cellular (T-cell–mediated) responses . The classical concept of T-cell function is that viral or bacterial antigens are taken up at peripheral sites by antigen-presenting cells (APCs), including dendritic cells, that migrate to regional lymph nodes. Antigens are processed into peptides that are presented on the cell surface of APCs in the MHC. T-helper cells (Th) (CD4 + lymphocytes) recognize antigenic peptides in the context of MHC-II molecules, whereas cytolytic or cytotoxic CD8 + Lymphocytes recognize MHC-I molecule–peptide complexes. *Wu (3*) indicated that the fat adjacent to blood vessels is particularly prone to T-cell infiltration and that during angiotensin II infusion, this tissue begins to express large RANTES amounts. The perivascular adipose tissue is rich in sympathetic nerves, which could contribute to signaling an increase in chemokines, such as RANTES. Thus, by acting as a reservoir of inflammatory cells, the perivascular fat is potentially an important bridge linking the central nervous system, inflammation, the vasculature, and the kidney in hypertension . Within the large pool of different immune effector cells, the recently rediscovered regulatory T cells (Tregs) play an important role in controlling immune responses and silencing self-reactive T cells (*4)* . Different Tregs subsets are now subdivided based on expression of cell surface markers, production of cytokines, and mechanisms of action. Naturally occurring thymic-derived $CD4^+CD25^+$ Tregs are a T cell population with immunosuppressive properties that constitutes 5–10% of the total peripheral $CD4^+$ T cells (*5*). Besides the expression of CD25, they constitutively express other several activation markers, such as the glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein (GITR), OX40 (CD134), L-selectin (CD62 ligand (CD62L)), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152). However, it should be noted that none of these markers exclusively identifies Tregs as they can also be expressed to various degrees on activated T cell subsets and various antigen-presenting cells (APCs). More recent studies have identified the transcription factor forkhead box P3 (FOXP3) as a more exclusive intracellular marker for the identification of Tregs (*6).*

Patients and Methods

Selection of patients and control :

Sixty Patients attending the cardiovascular and urology unit from March to December 2011 were eligible for this study ,with a mean age of 50 years (range 30-70 years) were chosen .They were all suffering from clinical manifestation of hypertension. The diagnosis in each case was established by specialist doctor and the patients were divided as follow :

1 –Twenty five patients (15 male and 10 female) were presented with only hypertension disease (hypertension for the first time) .

2-While the rest fifteen patients (10 male and 5 female) were presented with hypertension (mainly treated by either angiotensin converting enzyme inhibitor (captopril) or β blocker (propranolol)) .

3-Twenty hypertensive patients (10 male and 10 female) were suffering from UTIs . 4-Twenty apparently healthy volunteers (10 male and 10 female) with the mean age 42 years and age range (24-60) years were enrolled as control.

Sample collection:

Blood samples were obtained after an overnight fast for measurement of CD4+T lymphocytes. From each patient and control ,five ml venous blood was aspirated from a suitable vein efficient

disinfecting over the injection site. blood samples were immediately transferred to sterile heparin-ised vacutainer tubes for lymphocyte separation.

Lymphocyte separation :

The Isopaque-ficol technique originally described by boyum(7) was used for lymphocyte separation.

Direct immune flourescence staining technique for the detection of CD4 +T lymphocytes:

The principle :

Flourescen isothiocynate (FTIC) and Rhodamin- phycorethine (RPE) labeled monoclonal antibodies directly react with antigens on the surface of separated lymphocytes . FTIC and RPE are molecules that have the ability to absorb invisible, ultraviolet radiation, and then have the capability of emitting an apple green and orange red fluorescence colored light respectively after exposure to UV light .

Direct immunoflourescence procedure :

The method of IF-labeling of fixed cells was done as described by (*9*) as following :

Slides were prepared as described then, the pre-coated slides with lymphocytes were removed from freezer , allowed to reach room temprature , unwrapped and washed with PBS by dipping the slides into PBS- containing jar for about 5 minutes at room temperature. Slides were lied flat , smear-slide up, in humidity chamber then 10ul of 1/75 diluted fluorochrom (FTIC) conjugated monoclonal antibodies were added to each smear , cover the chamber and slide were left undisturbed in incubator at 37C for 50 minutes. Slides then transferred to staining jar filled with PBS at room temperature and PBS replaced twice at 5 minutes intervals , then drained and blotted gently. Two drops of mounting media [(nine parts glycerol to one part of o.2 M carbonate buffer , pH=9 (9) to enhance fluorescence and retard fading on exposure to UV- light (8)] placed on each smear of slides. Then cover slips were lowered into place slowly to avoid bubbles ; cover slips may be sealed around edges with clear nail polish . (Slides were examined then with fluorescence microscope at 490 nm ; positive cells give green- apple when stained with FTIC and Rhodamin- phycorethine (RPE) labeled antibodies and exposed to UV light). In each smear , the countable field was located and the total number of 100 cells was counted , the percentage of positive cells was calculated as following :

Percentage of positive cells = (No. of positive cells / total No. of cells) × 100 % .

Urine collection :

Proper collection of urine specimen is the most important step in urine culture , and prevention of contamination is the most important consideration for collection of urine sample *(10).* Patients were instructed to wash their outer genitalia with water which was consider satisfactory and preferable than other antiseptic in the form of soap or other chemicals .Mid stream urine (MSU)samples were collected in clean and sterile screw capped containers .

Cultivation and microscopic examination of urine specimens :

The urine specimens were cultured immediately (*10)* . The urine specimens were inoculated on both blood and MacConkey agar plates by direct streaking method using calibrated loop to deliver 0.01 ml of the urine specimen (diameter of the loop is 4mm) . The plates were incubated at 37C for 18-24 hours then examined for bacterial growth . If there was no growth ,the plates were

reincubated for another 24 hours before they were discarded as negative culture (11). When the bacterial growth appeared after the incubation period, the presence of each colony represented a single bacterium from the original specimen and this was called colony forming unit (CFU). A growth equal to or more than 10^5 CFU/ml of a single pathogen was considered significant .A gram stained smear of uncenterifuged well mixed urine specimen was done and examined microscopically to reveal the gram negative bacilli. Then the urine specimens were centrifuged at 2000 rpm for 5 minutes , and drop of the deposit was examined under high power magnification for the presence of pus cells, RBCs, casts, crystals , epithelial cells, ova, and others (11).

Statistical Analysis

Statistical analysis were conducted to describe different variables and parameters in this research and to describe relationships with each other as well. Calculation of means ± standard errors was done for quantitative data. Sensitivity and specificity were also calculated to specify and compare the efficiency of different diagnostic tests which was done by using SAS computer analysis program (12).

Results

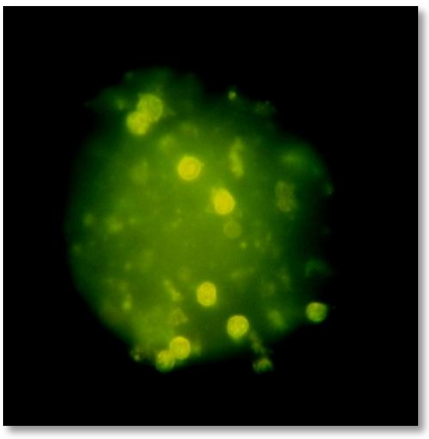
CD4+ T lymphocytes and hypertension :

Table-1 showed a significant increase ($p \leq 0.01$) in hypertensive patients with mean value of (20.95±1.43) in hypertensive patients compared to normal control value (14.20±1.08) (figure-1).

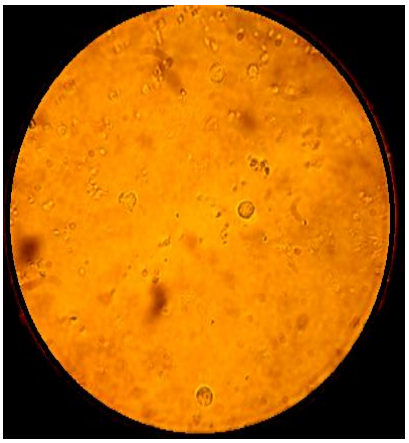
Table -1: Differences in CD4+ T lymphocytes between normal and hypertensive patients .

Patients	CD4+ T lymphocytes
Hypertension	20.95 ±1.43 A
Normal control	14.20 ± 1.08 B
Significance	$P \leq 0.01$

Note : Means with the same letter are not significantly different.



High power feild



low power feild

Figure -1 : Immunoflourescent CD4+ T lymphocytes from hypertensive patients.

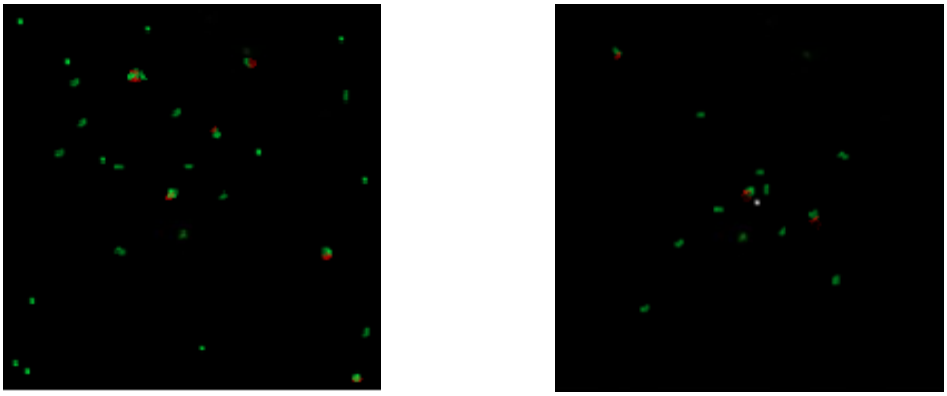
CD4+ T lymphocytes and gender :

Regarding the role of gender on level of CD4+ T lymphocytes in patients suffering from hypertension . Table (2) showed the frequency distribution of CD4+ T lymphocytes among male and female which showed a significant difference ($p \leq 0.01$) with mean values of (18.54±1.76) in male and (22.65±2.46) for female (figure 2).

Table -2: Effect of gender on level of CD4+ T lymphocytes in hypertensive patients .

SEX	CD4+ T lymphocytes
Male	18.54± 1.76 B
Female	22.65±2.46 A
Significance	P≤0.01

Note : Means with the same letter are not significantly different.



Female Male

Figure-2 : Differences between hypertensive male and female CD4+ T lymphocytes .

CD4+T lymphocytes and aging :

Table-3 elucidate the level of CD4+ T lymphocytes in patients suffering from hypertension with different age groups . The table showed a significant difference in CD4+ T lymphocytes level ($p \leq 0.01$) with highest mean value of (22.69±3.3) at age group of (30-40 y) and the value started to decrease reaching the lowest level at age group of ≥ 61 year.

Table -3 : Distribution of CD4+ T lymphocytes in hypertensive patients with different age groups.

Age /year	CD4+ T lymphocytes
30 – 40 y	22.69±3.3 A
41 – 50 y	20.18±2.42 AB
51 – 60 y	18.45±2.2 B
≥ 61 y	17.81±3.0 B
Significance	P≤ 0.01

Note : Means with the same letter are not significantly different.

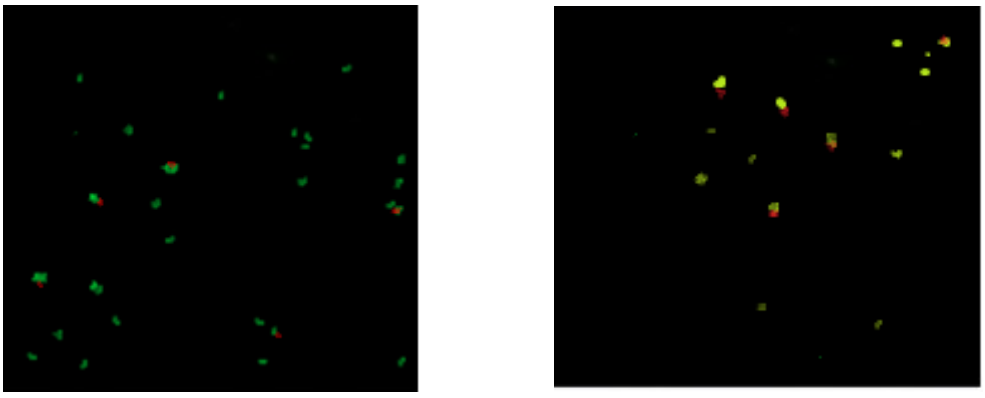
CD4+ T lymphocytes and smoking :

Smoking versus expression of CD4+ T lymphocytes in hypertensive patients was explained in (Table-4) in which high significant differences ($p \leq 0.01$) was seen in CD4+ T lymphocytes with mean value of (24.8 ±2.14) in smoking patients compared to (20.95±1.43) for control (figure-3).

Table -4: Effect of smoking on level of CD4+ T lymphocytes in hypertensive patients .

Hypertensive Patients	CD4+ T lymphocytes
Control	20.95 ± 1.43 B
Smoking	24.8± 2.14 A
Significance	P ≤ 0.01

Note : Means with the same letter are not significantly different.



Hypertensive smoker Control

Figure-3: Immunoflourescent CD4+ T lymphocytes in hypertensive smoker compared to control.

CD4+ T lymphocytes level and anti hypertensive treatment :

The other group which was measured is the role of some anti hypertensive drugs (mainly β-blocker (propranolol) and angiotensin converting enzyme inhibitor (captopril)) on level of serum CD4+ T lymphocytes (Table-5). The anti hypertensive treatment showed a significant effect on expression level of CD4+ T lymphocytes ($p \leq 0.01$), with mean value of (16.48±1.01) for beta blocker and (14.17±0.8) for angiotensin converting enzyme inhibitor compared to mean value of (20.95±1.43) for positive control and (14.20 ± 1.08) for negative control.

Table -5: Role of antihypertensive drugs (mainly beta blocker and Angiotensin converting enzyme inhibitor) on level of CD4+ lymphocytes .

Treatment	CD4+ T lymphocyte
β.blocker	16.48±1.01 B
ACE inh.*	14.17±0.8 C
Positive control	20.95±1.43 A
Negative control	14.20 ± 1.08 C
Significance	P≤ 0.01

Note : Means with the same letter are not significantly different.

*ACE inh : Angiotensin converting enzyme inhibitor .

Effect of urinary tract infections on CD4+ T lymphocytes level :

In the present study we investigate the level of expression of CD4+ T lymphocytes in hypertensive patients suffering from UTI . Table-6 showed a significant increase in CD4+ T lymphocytes level ($p \leq 0.05$) with mean value of (26.59±2.21) for patients having bacterial UTI compared to mean value of (20.95±1.43) for positive control and 14.20 ± 1.08 for negative control .

Table -6: Effect of bacterial UTI on level of CD4+ in hypertensive patients .

Hypertensive Patients	CD4+ T cell
With UTI	26.59± 2.21 A
Positive control	20.95±1.43 B
Negative control	14.20 ± 1.08 C
Significance	P≤ 0.05

Note : Means with the same letter are not significantly different.

DISCUSSION :

Hypertension and CD4+ T lymphocytes :

Our finding indicate that level of CD4+ lymphocytes were increase in hypertensive patients ($p \leq 0.01$) and such increase was similar to (13) who authorized that for almost half a century there has been evidence that the immune system is involved in hypertension. Inflammatory cells, including T cells and macrophages, have been observed in the kidneys of hypertensive animals and humans and have been implicated in renal damage. . In addition to receptors and legends within the CD4+ T-cell receptor complex, T cells possess accessory receptors outside of the immunologic synapse that modulate their activation, differentiation, and function, which could be relevant to hypertension. Of importance to hypertension, T cells express angiotensin type 1 (AT 1) receptors that affect lymphocyte function . These investigators also found that angiotensin II promoted T-cell activation and the recruitment of effecter-like T cells into the fat adjacent to blood vessels and hypothesized that these cells released cytokines that affected vascular tone and renal function. *Agrewala* (14) showed that recently activated, but not rested, Th1 and Th2 CD4 + cells home to visceral fat. The study also demonstrated that infiltration of T cells was much greater in visceral compared with subcutaneous fat .Taken together, these studies indicate that activated effector T cells have a propensity to non specifically invade visceral adipose tissue and have implications for the metabolic syndrome, in that visceral obesity would provide a reservoir for activated T cells to reside and release cytokines that could have untoward effects on adjacent vessels and organs. Our results was also agreed with *(13)* study who reported that cells of the monocyte/macrophage line have been identified in the kidney and the vasculature in hypertension for many years .T cells have been reported to infiltrate the kidney in various hypertensive models , and efforts to decrease this have proven effective in lowering blood pressure. However , studies

have suggested that central signals activate macrophages and T cell, which home to the kidney and vasculature and release cytokines , including IL-6 and IL-17, which in turn cause renal and vascular dysfunction and lead to blood pressure elevation .

Gender and CD4+ T lymphocytes :

There is a growing body of basic science literature supporting a role for inflammatory lymphocytes in the development and maintenance of hypertension in male experimental animals . Although there is little direct evidence linking immune cells with hypertension in females, women are more likely than men to develop inflammatory and immunological disorders. Our results was found similar to (*15*) who authorized that the mechanism responsible for the observed sex differences in T cells is currently unknown; however, it is tempting to speculate that sex hormones contribute. Sex hormones influence the immune system, although there are contradictory reports regarding the direction of the sex hormone effect. The majority of the evidence indicates that testosterone is immunosuppressive whereas estrogen has been shown to be both pro-inflammatory and an anti-inflammatory modulator . Consistent with infiltrating CD4+ T cells modulating blood pressure, male spontaneously hypertensive rats (SHR) have a higher blood pressure than female SHR at baseline conditions and male SHR have greater CD4+ T cell infiltration in the kidney compared to females (15).

Aging and CD4+ T lymphocytes :

Our results was in line with (*16*) showed that in hypertensive aged patients the composition of the CD4+ T cell compartment revealed a shift in the ratio of cells individuals is mainly composed of naïve cells, the proportion of memory cells increases with age in both mice and humans . This shift is thought to mainly result from thymic involution, which is the shrinking in size and function of the thymus that leads to the reduced output of new naïve T cells toward the periphery. The reduction of naïve T cell output from the thymus, the increase in memory T cells from multiple antigenic encounters and homeostatic proliferation, as well as the increase in regulatory T cells, are profound qualitative and quantitative changes that occur with secretion from both CD4 and CD8 T cells was decreases with age . Although the decline in T cell immunity is considered a key factor in the increased susceptibility to infections in the elderly, the impact of aging on the innate immune system is not well understood . Our results was also similar to (*17*) study which reported that decline in CD4+ T cell with aging may related to intrinsic defects in CD4+ T cell function .

Smoking and CD4+ T cell :

Our results was similar to (*18*) study which reported that with an emphasis on CD4+ subsets, a biological change hypothesized to be promoted by the chronic exposure to the tobacco constituents in the lung . It is possible that this acute increase in the percentage of naive T-cell subpopulations could be a consequence of cellular recruitment or redistribution as part of physiological response against the damaging agents present in the inhaled tobacco smoke. This would lead to the redistribution of T cells from the bronchial lining to the periphery. Active smoking on the other hand increases the CD4+ T-cell count, especially the CD4+ memory cells because of its more chronic and heavier character of exposure (*19*) . *Lidia (20)* Reported the effects of smoking on the different subsets of lymphocyte T cells are conflicting. The influence of cigarette smoking on lymphocyte T-cell subpopulations in the peripheral blood has been investigated by means of monoclonal antibodies. Light to moderate smokers (history of less than 50 pack years) were reported to have a significant increase in CD4+ counts and a trend toward increased CD8+ lymphocyte count. Our results was also in line with (*21*) study which showed that Interleukin (IL-16), previously known as lymphocyte chemoattractant factor, is a recently characterised cytokine produced in the airways mainly by CD8+ T cells and bronchial epithelial cells. Being a ligand for CD4+, IL-16 causes effects exclusively restricted to cells bearing the CD4 receptor including CD4+ T cells and subsets of macrophages and eosinophils. The correlation of airway levels of IL-16 with the percentage of CD4+ T cells and the responsiveness of peripheral blood lymphocytes

suggested that the increased IL-16 level may alter systemic immunomodulation by influencing the status of systemic T lymphocytes .

Anti-hypertension(mainly Angiotensin converting enzyme inhibitor(captopril) and Beta blocker(propranolol)) treatment and CD4+ T lymphocytes level :

Our results was in line with (*22*) study which showed that ACE inhibitors and Angiotensin type 1 and type 2 receptors (AT1R and AT2R) antagonists might modulate the immune response via alteration of cytokine release . ACE inhibitors and AT1R and AT2R antagonists might therefore inhibit cytokine secretion by decreasing the amount or antagonizing the effects of angiotensin II(AII) . AII increases the transcription of IL-6 in macrophages, which can be inhibited by AT1R antagonists. This decrease in IL-6 production has also been observed in a model of chronic heart failure and myocardial infarction treatment . ACE inhibitor treatment in a model of hypertension reduced expression of IL-1β, IL-5, IL-6, and TNF-α . ACE inhibition was also shown to decrease the amount of IL-2, IL-12 and IFN-γ produced by T cells. This down regulation may be mediated in part by the simple fact that AII is not produced, as in the case of ACE inhibition, or its activity is inhibited, as in the case with receptor antagonism *(23)* . For β blocker our results was agreed with (*24*) study which investigated the presence of catecholamines receptor on T lymphocytes and the effect of L-dopa and dopamine on lymphocyte proliferation and differentiation, also the study indicated that catecholamines produced by lymphocytes act in an autocrine or paracrine way and are important regulatory molecules and, thus, are potentially important during an ongoing immune response. This hypothesis is consistent with findings that immune system cells carry (β-adrenergic and dopaminergic receptors),which are a prerequisite for subsequent interaction with catecholamines which lead to increase lymphocyte proliferation and differentiation. However the use of β blocker will decrease T lymphocytes activation and lowering hypertension rate .

Urinary tract infection and CD4+ T lymphocytes :

In this study a particular significant level of CD4+ T lymphocytes was observed in hypertensive patients having UTI , such results was found to be in line with (*25*) study which mentioned that TLR expression is predominantly studied on cells of the innate immune system. There is, however, also evidence for TLR expression on T cells. Moreover, numerous in vitro studies showed that engagement of TLR by their respective ligands modulate T cell activity, including up-regulation of interferon gamma synthesis . They found in the majority of patients CD4+ and CD8+ cells expressing of TLR, particularly TLR2, a receptor selective for lipoteichoic acid, a major component of the outer cell wall of Gram-positive bacteria. Terminally differentiated T cells are a rich source of numerous cytokines, and in our patients up to 90% of the infiltrated T cells synthesized interferon gamma , apparent as an accumulation of the cytokine within the cells. Interferon gamma is a potent activator of phagocytic cells. It prevents apoptosis of PMN and enhances their phagocytic and bactericidal activity and thus could support the local host defence (*26*). Evidence by (*27*) reported that TLR ligands directly enhance the survival of activated $CD4^+$ T cells. TLR-mediated survival had the net effect of increasing expansion and slowing contraction rates of activated $CD4^+$ T cells without accentuating proliferation. It has been hypothesized that adjuvant-induced activated $CD4^+$ T survival can be mediated through APCs by the secretion of proinflammatory cytokines . Moreover, in contrast to the indirect means by which TLR ligands control $CD4^+$ T cell responses through APCs, direct effects may allow Ag-specific $CD4^+$ T cells to respond to pathogen-associated molecular patterns (PAMPs) in situations where APC function is suboptimal, perhaps due to infection .

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