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Research Article / Araştırma Makalesi

Flow Cytometric Analysis of T Lymphocyte Activation in CML Patients Under Imatinib Therapy

İmatinib Tedavisi Alan Hastalarda T Lenfosit Aktivasyonunun Akım Sitometrik Analizi

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Abstract: To analyze T cell functions by flow cytometry and to evaluate the possible functional changes that might occur under imatinib therapy in CML patients. A total of 29 patients and 9 healthy control subjects were enrolled. Newly diagnosed patients having no treatment (group 1), patients receiving imatinib for 1 year (group 2) and patients receiving imatinib more than 1 year (group 3), healthy control subjects (group 4). IL-4 and IFN gamma expression on CD4+ cells; how much percentage of CD3+ T cells were activated (CD3+CD69+); CD8+ T cells and the ratio and grade of expression of HLA-ABC and HLA-DR on those cells were evaluated, respectively. There was no significant difference in terms of mean number of CD4+ cells between the groups. However, there was a tendency towards higher CD4+ cells in control group. IL-4 and IFN gamma were found not to be statistically significant between the groups. Control group has lower IL-4 and IFN gamma expression values. Mean number of CD4+ cells, which did not express IL-4 and IFN gamma, were statistically higher in control group when compared to other groups. In control group, % activation was decreased when compared to that of other groups. CD8+ cell ratio was found to be statistically lower in all patient groups (p=0.001). The expression of HLA-ABC and HLA-DR on CD8+ cells were similar between the groups. We could not show any inhibitory effect of imatinib on T cell functions in concordance with clinical experience and safety profile.

Keywords: chronic myelogenous leukemia, flow cytometry, imatinib,T cell

Özet: İmatinibin in vivo ortamda T lenfosit fonksiyonları üzerine olan etkileri akım sitometrik olarak değerlendirilmiştir. Çalışmaya toplam 29 KML hastası ve 9 sağlıklı birey dahil edilmiştir. KML hastaları kendi aralarında sırasıyla, yeni tanı almış ve tedavi görmeyen, 1 yıldır imatinib alan ve 1 yıldan uzun süredir imatinib alan hastalar olarak 3 gruba ayrılmıştır. CD4+ hücreler ve bu hücrelerdeki IL-4 ve IFNg ifadeleri, CD3+ T lenfositlerin % kaçının aktive edilmiş olduğu (CD3+CD69+), CD8+ hücreler ve bu hücrelerde HLA-ABC ve HLA-DR ifade eden hücrelerin oranı ve HLA-ABC ve HLA-DR ifadelerinin şiddeti değerlendirilmiştir. Gruplar arasında ortalama CD4 + hücre sayısı açısından anlamlı fark yoktu. Bununla birlikte, kontrol grubunda daha yüksek CD4 + hücrelerine doğru bir eğilim vardı. IL-4 ve IFN gama, gruplar arasında istatistiksel olarak anlamlı bulunmadı. Kontrol grubu daha düşük IL-4 ve IFN gama ifade değerlerine sahipti. IL-4 ve IFN gama ifade etmeyen ortalama CD4 + hücre sayısı kontrol grubunda diğer gruplara göre istatistiksel olarak daha yüksekti. Kontrol grubunda ise % aktivasyon diğer gruplara göre azdı. Tüm hasta gruplarında CD8 + hücre oranı istatistiksel olarak daha düşük bulundu (p = 0,001). HLA-ABC ve HLA-DR'nin CD8 + hücrelerinde ekspresyonu gruplar arasında benzerdi. İmatinibin sitokin sentezi üzerine herhangi bir etkisi gözlenmemiştir. İmatinib tedavisi altındaki hastalarda T lenfosit fonksiyonlarının etkilendiğine dair klinik belirgin gözlemler olmamakla birlikte, subklinik etki varlığı araştırma konusudur. Yapılan in vitro çalışmaların in-vivo korele olup olmadığının aydınlatılabilmesi için daha ileri çalışmalara gereksinim vardır.

Anahtar Kelimeler: kronik miyelositer lösemi, akım sitometri, imatinib,T hücre

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1. Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease characterized by a reciprocal chromosomal translocation, (t (9:22)(q34;q11) which creates the Philadelphia chromosome (1). This translocation results in the expression of a leukemia-specific oncoprotein, BCR-ABL (2). This oncoprotein has a strong tyrosine kinase protein activity, which directly plays a major role in leukemic transformation (3). Imatinib mesylate, an inhibitor of bcr-abl tyrosine kinase, is currently the most chosen agent in the first-line treatment of CML patients. This drug is well tolerated with only few adverse events and leading to a significant prolongation of hematologic and cytogenetic remission in chronic-phase CML patients (4-6).

There is little information available about the effects of imatinib on the immune regulation after allogeneic stem cell transplantation and effect of graft versus leukemia (GVL). T lymphocytes are primarily acting cells on GVL effect and these are not a part of the leukemic clone in CML (7). Effects of imatinib on T cell functions have not been well defined. However, there are published articles reported that it might have suppressive impact on in vitro T cell functions (8, 9). On the other, there is only one published data existing in the literature studying the in vivo effects of imatinib on T cell functions (10).

The aim of our study was *in vivo* analysis of T lymphocyte functions by flow cytometry and to evaluate the possible functional changes that might occur under imatinib therapy in CML patients.

2. Materials and Methods

Twenty-nine patients (17 men and 12 women) with CML were included in this study. After obtaining informed consent, peripheral blood was collected from CML patients, and healthy subjects (n=9), closely matched for age and sex. Patients were required to have CML based on characteristic clinical and laboratory features, and cytogenetic or molecular evidence for the bcr/abl gene rearrangement. All CML patients were in chronic phase. Eligibility criteria included: patient age>18 years old, none have additional systemic disease and none have received any previous treatment except imatinib or hydroxyurea. CML patients were divided into three groups as newly diagnosed patients having no treatment (group 1), patients who have been receiving imatinib for 1 year (group 2) and patients who have been receiving imatinib more than 1 year (group 3), respectively. Healthy control subjects were regarded as group 4. Local medical ethics committee approved the study. The tenets of declaration of Helsinki were followed.

In this study, CD3, CD4, CD8, CD69, HLA ABC, and HLA DR expression, which were T cell and /or activation markers, were detected by flow cytometry analysis. We examined the percentage of activated CD3+ cells by evaluating CD69 expression on these cells (% activity value). IL-4 and IFN gamma expression on CD4+ T cells were evaluated. The ratio and grade of expression (MFI: mean fluorescent intensity) of HLA ABC and HLA DR on those cells were also examined.

Blood Samples: Seven milliliters of peripheral blood samples were collected into Na-heparinized tubes for every patients and control subjects. Blood samples were immediately delivered to the laboratory for the analyses.

Flow cytometric analysis

Reagents: Phorbol myristate acetate (PMA) , ionomycin, brefeldin A (BFA), phosphate buffered saline (PBS), FACS permeabilizing solution 2, paraformaldehyde %1, ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich, St. Louis, MO.

Cell Induction: T cells were induced by PMA and ionomycin, which were non-specific cell inducers. Two different tube series were prepared with either inducer or not (negative control). For the first tube series, one milliliter peripheral blood sample was treated with 20 µL ionomycin (1 µg/) and 25 µL PMA (25 ng/mL) for 2 hours at 37°C. At the end of the

second hour, 1:10 diluted BFA (1 μg/mL) was added in order to keep the cytokines within the cells. These cells were incubated at 37°C for 4 hours. 50 μL EDTA was added into the solution and mixed briefly with vortex. Upon incubation 15 minutes at room temperature, mixed with vortex again for 10 seconds. Five milliliters of FACS lysis solution was added and incubated for 10 minutes. These prepared tubes were stored at -80°C until their use for flow cytometric analysis.

Seven milliliters wash buffer was added onto the cells in the tubes and centrifuged at 500xg for 10 minutes. Supernatant was removed and 0.5 ml wash buffer was added onto the remaining cell pellet. These cells were ready to incubate with specified antibodies.

Marking cells with antibodies

Tubes were prepared as follows

1st tube**:** $IgG1_{FITC}$ / $IgG1_{PE}$ 4th tube: $CD4_{FITC}$ / $IL\text{-}4_{PE}$

2nd tube: $cIgG1_{PE}$ 5th tube: $CD69_{FITC}$ / CD3

3rd tube: $CD4_{FITC}$ / $IFN\gamma_{PE}$ 6th tube**:** HLA ABC_{FITC} /$CD8_{PE}$/HLA DR_{PC5}

100 μL of cell suspension were delivered to each tube and mixed with 0.5 ml 1xFACS permeabilizing solution 2 by vortexing for 10 seconds. Upon incubation 10 minutes at room temperature 2 ml of wash buffer was added and centrifuged at 500xg for 5 minutes. Supernatant was removed, 20 μL of each antibodies were added, and incubated for 30 minutes in dark at room temperature. At the end of this period, 2 ml wash buffer was added and centrifuged at 500xg for 5 minutes again. Supernatant was removed, 200 μL 1% paraformaldehyde was added, and flow cytometric readings were started.

Flow cytometric evaluation: Routine daily calibration of the flow cytometer (FC-500, Beckman Coulter, Florida, USA) was performed before the acquisition of the samples. Two hundred thousand (200.000) cells/tube were counted and data set was analyzed by special software, RXP analysis (Beckman Coulter, Florida, USA). During the analyses, after selection of CD4 + cells using the CD4-SSC (side scatter: light scattered with 90°) plot for the 3rd and 4th tubes, the expression of IL-4 and IFN gamma was determined by setting the markers according to isotypic control signals. On the 5th tube the percentage activated of CD3+ cells were determined by evaluating CD69 expression on these cells (% activity value). The ratio of cells, which expressed CD8+, HLA ABC and HLA DR and the grade of expression of HLA ABC and HLA DR on those cells were analyzed in the 6th tube.

Statistical Analyses

All statistical analyses were performed using statistical software package (SPSS Version 20.0 for Windows, SPSS, Chicago, IL, USA). The significance of the difference between means was determined by the Kruskal-Wallis test. p-values <0.05 were considered statistically significant.

3. Results

Thirty-nine patients (17 males, 12 females) who had a diagnosis of CML and 9 (5 males, 4 females) healthy subjects were enrolled in this study. The mean ± standard errors of mean age were 51.5 ± 2.11 (range: 27–69) and 47.4 ± 3.49 (range: 32-62) for the CML patients and healthy subjects, respectively ($p>0.05$).

CD4+ cells and IL-4 and IFNγ expressions

After selection of CD4 + cells from CD4-SSC graph (Figure 1A), we found that, there was no statistically significant difference between the groups in terms of IL-4 and IFN gamma expressions and total CD4+ cell counts. Although CD4+ cell counts were not significantly different between groups, it showed a tendency towards higher levels in-group 4. There was no significant difference in the cytokine expressions between the groups. However, it is interesting to note that lower values of both cytokines (IL-4 and IFN gamma) were found in the control group. Figure 1B shows the cells that express IL-4 and IFN gamma among the groups. In addition, mean cell counts that express neither

IL-4 nor IFN gamma were analyzed and significant differences were found between the groups (Figure 1C). Group 4 showed significantly higher values when compared to that of group 1, 2, and 3 (p=0.01). The difference between group 2 and group 4 and group 3 and group 4 was strikingly different than the difference between the group 1 and group 4.

CD3+ and CD69+ cells and % activity values

The analysis of CD+3 and CD69+cells are shown in Figure 2A. No statistically significant difference was found in terms of CD3+ cell counts (p=0.893) and CD69+ expression (p=0.118). However, activity % values were found to be significantly different (Figure 2B). The activity % in-group 4 was lower than the other groups. There was no difference between group 1, 2, and 3.

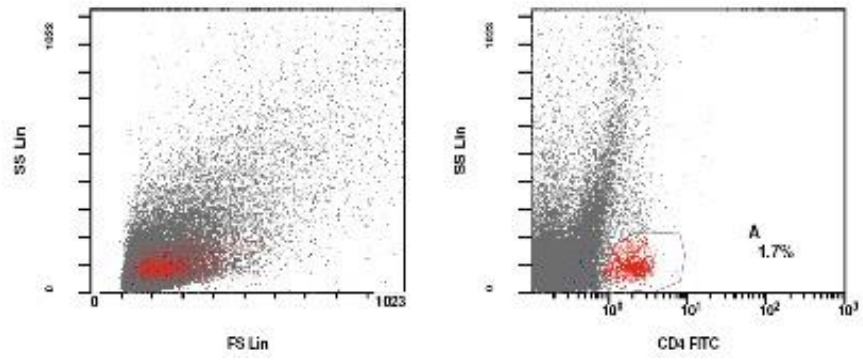

Figure 1A. Flow cytometric analyses which show CD4+ cells in total leukocyte pool.

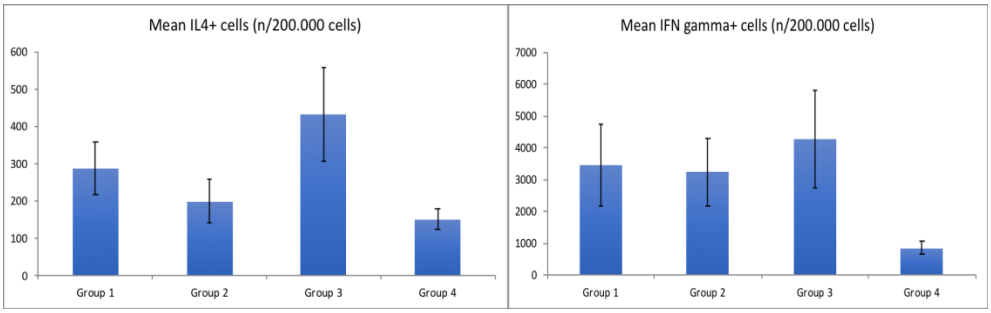

Figure 1B. Mean cell counts of IL4 and IFN gamma expressing CD4+ cells.

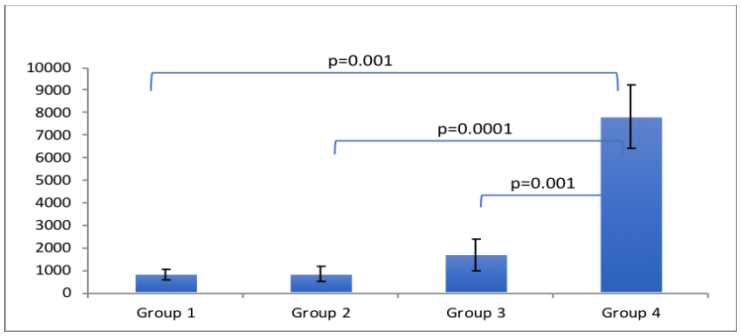

Figure 1C. Distribution of CD4+ cells, which expressed neither IFN nor IL4.

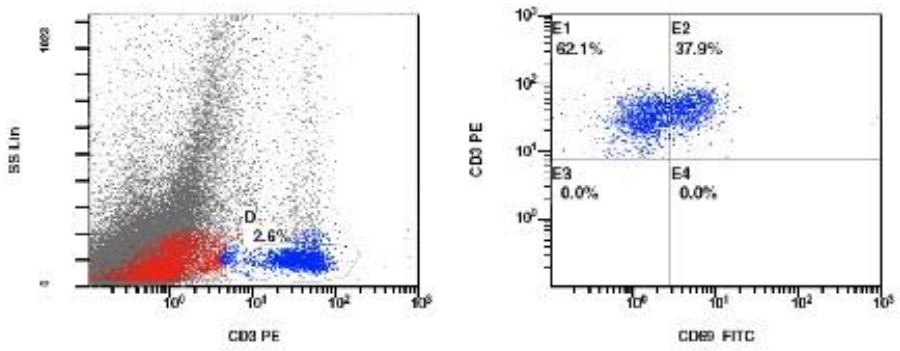

Figure 2A. Flow cytometric analysis of CD3+ cells and CD69+ expressing CD3+ cells.

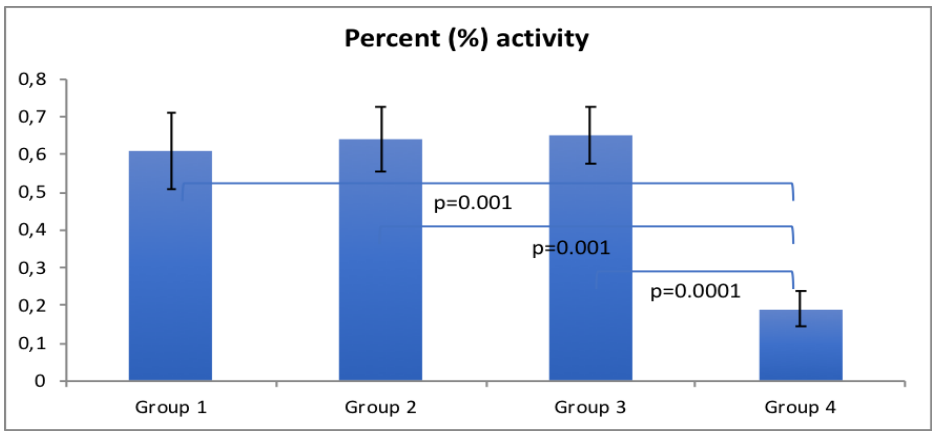

Figure 2B. The percentage of activated T cell for each group

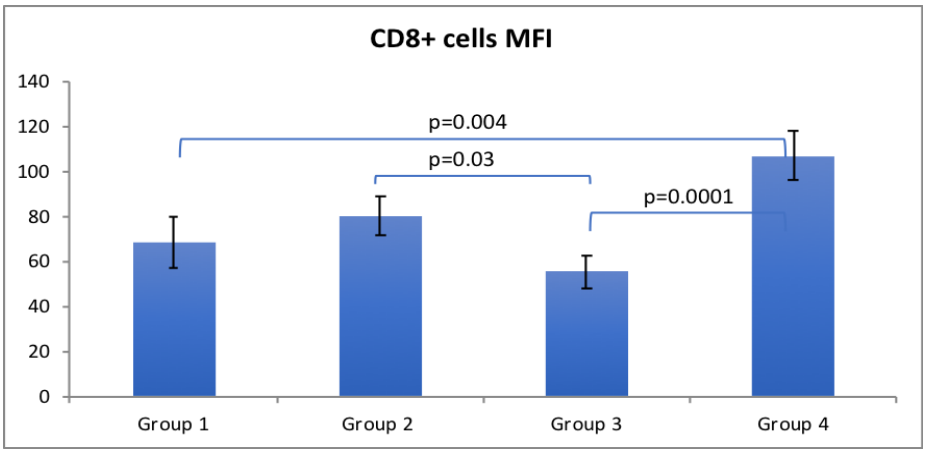

Figure 3. CD8+ cells between the groups.

CD8+ cells and HLA ABC and HLA-DR expressions:

The mean CD8+cell count and HLA ABC and HLA DR expression on these cells were analyzed. There were significant differences in terms of CD8+ cell counts between groups 2-3, 1-4, and 3-4 (Figure 3). There was no significant difference between HLA ABC and HLA DR expression on CD8+ cells among the groups (p=0.964 and 0.873, respectively). In addition, the grade of expression of HLA ABC and HLA DR on CD+8 cells were compared and no differences were found among four groups.

4. Discussion

CML is a clonal malign disease of hematopoietic stem cell which is characterized by translocation BCR and ABL genes between chromosomes 9 and 22 (2). The BCR-ABL fusion protein has a tyrosine kinase activity that is responsible for the leukemic transformation (11). Since BCR-ABL protein is new to the immune system and specific to the leukemic cells, it is a potential target for leukemic specific T cell response (12, 13). It gains more importance because T-cells are not a part of leukemic clone in CML (14). Imatinib is currently the most chosen agent in the treatment of CML patients. There are published articles reporting that imatinib has suppressive effects on T cells. In this current study, we analyzed that in vivo T cell functions in CML and effects of imatinib on those cells.

In our study, we found that there was no significant difference in terms of mean CD4+ cell counts among the groups. Moreover, the expression of IL4 and IFN gamma was not found to be different between the groups. In a similar study, Gao et al showed that T cells which produces TH1 cytokines were not different in CML patients receiving imatinib when compared to that of healthy controls (15).. They hypothesized that T cells that were induced with PMA uses protein kinase C pathway and this pathway does not show any homology with Src kinase family. Our study further supports this theory and in addition we thought that because PMA mainly induces IFN, it is more convenient to use anti-CD3/CD28 which uses T cell receptor (TCR) pathway.

CD4+ cells which did not express IL4 and IFN gamma were also analyzed in our study. It was found significantly higher values in control group (group 4) when compared to the other groups. Contrary to our study, Tsuda et al found that IL4 and IFN gamma negative cells increased in CML patients who did not receive imatinib therapy when compared with healthy controls (16). On the other hand, Leder et al found the similar results like in our study (17). Given the effect of imatinib on CD4+ T cell functions are unknown and possible phenotypic changes on dendritic cells, it can be hypothesized that the same effect might occur on CD4+ T cells. Also, there was a slightly bias to IL4 and IFN gamma negative cells in total CD4+ T cells. This bias can be interpreted as T cells might have not been induced sufficiently.

In the current study, we did not find any differences in terms of mean CD3+ T cells and CD69 expressions on these cells. However, there was significant difference in percent activity values which define percentage of CD69+ cells in all CD3+ T cell population. Cwynarski et al studied in vitro effects of imatinib on T cells (8). They showed that imatinib suppressed CD25, CD69 and HLA DR expressions. In a similar study by Seggewiss et al found that imatinib inhibited TCR mediated T cell proliferation and activation in a dose dependent manner (9). In contrast, Dietz et al reported that imatinib did not inhibit CD69 and CD25 expression that were activation markers for T cells (18). We thought that this inconsistency in the published literature might be explained by using various T cell stimulatory agents in those studies. Cwynarski et al and Seggewiss et al used CD3/CD28 as a stimulatory agent (8,9). On the other hand, Dietz et al used PMA, like in our study (18). To the best of our knowledge, we did not find any reports regarding high percent activity values in CML patients receiving imatinib therapy. In our study, we did not find any significant difference between the groups for mean CD69+ cells within CD3+ T cell pool. While

CD69+ cells within CD3+ T cells were not different among the groups, the significant differences between percent activity values might be attributed to individual differences in terms of mean T cell counts. This finding also supports the theory that PMA might not induce T cells adequately.

CD8+ cells were significantly lower in CML patients either receiving imatinib or not when compared with healthy controls, like in published literature (9, 18-21). Additionally, this difference was much more prominent between the control group and patients who had been receiving imatinib more than 1 year (group 3). At this point we can hypotheses that longer exposure to imatinib might result with more dominant decrease in CD8+ cells. Seggewiss et al reported that imatinib inhibited CD8+ T cells in a dose dependent manner in vitro (9). Dose dependent changes in CD8+ T cells in this in vitro study might be considered as similar with time dependency in our in vivo study. On the other hand, Mumprecht et alshowed that imatinib did not affect cell cycle and activity of memory CD8+ T cells in vivo but there was a selective inhibition of memory cytotoxic T cells which encountered with specific antigen (22).

HLA DR expression on CD8+ T cell is an indicator of T cell activation. In our study, we analyzed these expressions and did not find any significant differences between the groups. Cwynarski et al(8) reported that HLA DR expression was suppressed by imatinib in vitro but in this study HLA DR expression was detected on CD3+ cells contrarily to our study.

There are pitfalls of our study that must be addressed. First, limited number of patients and heterogeneity of groups. Second, in this study we used PMA and ionomycine for T cell induction. It has been known that these agents are non-specific indicators. We thought that for a stronger and non-selective induction, more suitable agents might be used such as CD3/CD28.

In conclusion**,** we could not show any inhibitory effect of imatinib on T cell functions that could support clinical observations. It will be a research area how this interaction will be in new and more potent 2nd and 3rd generation tyrosine kinase inhibitors.

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