

## Immunophenotype of peripheral blood lymphocytes following thermal injury in patients

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### Abstract

**Background:** Severe immunosuppression occurs after large thermal burn and probably contributes substantially to patient morbidity and mortality. In this study we investigated the range and distribution of T-lymphocyte subsets CD3<sup>+</sup> (T cells), CD4<sup>+</sup> (T helper/inducer cell, th), CD8<sup>+</sup> (T suppressor /Cytotoxic cells, TS/C), CD3<sup>+</sup>CD4<sup>+</sup>/CD3<sup>+</sup>CD8<sup>+</sup> ratio, CD19<sup>+</sup> (B cells) and CD16<sup>+</sup> (NK cells) in patients following thermal injury.

**Methods:** Forty male, aging 18-60 years with major thermal injury were studied. The total body surface area of the burn injury, ranged from 30 to >70%. Whole blood samples were collected at three and seven days postburn. Partec flowcytometry system and triple color flowcytometry reagents (Dako Co), were used to evaluate peripheral blood lymphocytes population of patients admitted at the Motahary Burn Center in Tehran.

**Results:** Compare to healthy controls, patients with burns have shown a significant reduction in relative number of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells at three and seven days postburn. CD4<sup>+</sup>/CD8<sup>+</sup> ratio were below normal range in three days and remained in normal range in seven days following injury. CD19<sup>+</sup> B cell populations were elevated in burn patients at both three and seven days. The number of CD16<sup>+</sup> NK cells were significantly declined in three days and moderately increased on day seven, following injury. Thus, the data showed that thermal burn injury suppressed T-lymphocyte subsets proliferation in various days. In addition, all compartments showed phenotypic changes in the 3th and seventh days after burn, in different groups of age. Thermal burn injury suppressed T cell subsets proliferation on day 3 and 7 postburn, when compared to normal controls. ( $P \leq 0.05$ ) at 3 and 7 days post burn.

**Conclusion:** Significant changes in lymphocytes population could be an important factor in immunosuppression and development of sepsis in thermal burn patients.

**Keywords:** burn, T lymphocytes, B lymphocytes, NK cells, CD4<sup>+</sup>/CD8<sup>+</sup> ratio

### Introduction

Many years ago the basic pathology of the burn wound was the main problem of morbidity,

mortality and long term disability, throughout the world and especially in the economically developing countries [1].

Severe injury induces a temporal shift in im-

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immune reactivity that can lead to serious complications or even death [2,3]. Increased susceptibility of burn patients to infections is due to deficient T helper(Th1) responses [4]. Despite improvements in burn wound, care and treatment, an understanding of postburn physiology and involved immunological factors are mainly unknown. The complex interaction of structure and function within the dermis allows the skin to act as an innate barrier and also as an integral part of the adaptive immune responses mediated by lymphocytes [5,6]. One of the primary characteristic of the skin is to play role as an immuno-competent organ .

Peripheral blood lymphocytes circulate through the skin, performing an immuno- surveillance role. Lymphocyte immunophenotype is a reflection of the functional level of immune system. Changes in lymphocyte cells count may affect the activation level of immune system in burn patients [7, 8] and severe immunosuppression occurs after broad thermal burns [9-11]. The recent literature contains more experimental and clinical studies on the changes of immune status following thermal injury [12-14] and requires more study for complete understanding of the burn effects on the immune system. In Iran burn injuries have caused significant morbidity and mortality, especially among the pediatric population [15,16]. Therefore, the aim of this study was to determine alteration in peripheral blood lymphocyte subsets changes in Iranian thermal burn patients and evaluate its correlation to burn percentage in third and seventh days of post burn injuries, and also compare cell count in different age groups. To the best of our knowledge this is the first study in Iran performed, to evaluate T-lymphocyte subgroups, B cells, CD16 (NK) cells for the Iranian burned patients.

### Methods

Forty male patients with burn injury were examined at the Motahary (Burn center-Tehran) hospital within three hours after their injury.

The age of patients was varied from 18-60 years. The total body surface area burned (TBSAB)" was between 30->70 percent. In most cases, the cause of burn was flame. All patients received standard treatment and classified into three groups: First group (n=20), with TBSAB between 30-50%, second group (n=12) between 50-70% and third group (n=8) more than 70%. Questionnaire was administer to each study subject in order to collect demographic, lifestyle and medical information. Immediately after their arrival at the hospital, whole blood samples (2ml) were collected. Blood samples were also taken at three and seven days post burn by venipuncture into a steriale EDTA (Ethylene Diamine Tetra Acetate). Complete blood counts (C.B.C) were performed by culter counter (Coulter Co, USA) and white blood cell differential counts were conducted on the smear of all patients, except subjects whit abnormal values. The evaluation of lymphocytes population of patients was performed using triple color antibodies by partec flowcytometric immunofluorescence analysis.

All reagents were obtained from DAKO (Denmark CO). Triple colour Antibody reagents are included; Anti-human CD4<sup>+</sup>/F.I.T.C (Fluorescein isothiocyanate)+Anti-humanCD8<sup>+</sup>/RPE (Phycoerythrin)+Anti-human CD3<sup>+</sup>/RPE+ cy5, Anti human CD16<sup>+</sup>/FITC +Anti human CD19<sup>+</sup>/RPE + Anti human CD3<sup>+</sup>/RPE - cy5, negative control mouse IgG1/FITC+ Mouse IgG1/RPE + mouse IgG1/RPE-cy5, Uti-lyse, erythrocyte-lysing reagent.

Using these different reagents the cells with surface antigens CD3<sup>+</sup>,CD4<sup>+</sup>,CD8<sup>+</sup>, CD19<sup>+</sup> and CD16<sup>+</sup> were identified. Then one of monoclonal antibody reagents were added to one-hundred microliter patient whole blood , the fluoro-ochrome - labelled antibodies bind specifically on the surface of lymphocytes. Then stained samples were treated with lysing solution. Finally, all samples were washed and analyzed by flowcytometry technique.

Table 1. Statistical results of peripheral blood lymphocytes immunophenotype in burn patients at three and seven days post burn ( $P < 0.05$ ).

Cell Type	Percent mean $\pm$ SD In third day	Percent mean $\pm$ SD In seventh day	Significance level
CD3 <sup>+</sup> T cell	44.9 $\pm$ 14 $\pm$ 36	32.25 $\pm$ 19.75	0.001
CD3 <sup>+</sup> CD4 <sup>+</sup> T cell	23.22 $\pm$ 8.22	16.22 $\pm$ 10.68	0.003
CD3 <sup>+</sup> CD8 <sup>+</sup> T cell	20. 51 $\pm$ 11.08	14.92 $\pm$ 11.76	0.009
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	1.37 $\pm$ 0.67	1.56 $\pm$ 1.08	0.149
CD19 <sup>+</sup> B cell	19.56 $\pm$ 7.76	18.98 $\pm$ 9.6	0.112
CD16 <sup>+</sup> NK cell	12.13 $\pm$ 8.6	17.19 $\pm$ 15.79	0.05

**Statistics:** All variables were expressed as mean standard deviation (SDM). The significance differences were evaluated by the student's t-test, differences at  $P \leq 0.05$  regarded to be significant, and analyzed by using the Microsoft flow max program.

### Results

The phenotypic analysis of T-lymphocyte subsets (CD3<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, CD3<sup>+</sup> CD8<sup>+</sup>), CD4<sup>+</sup>/CD8<sup>+</sup> ratio, B-lymphocyte (CD19<sup>+</sup>) and NK cells (CD16) demonstrated that patients suffering from thermal burns, would express

these antigens, in different days of postburn injury. The TBSAB was between 30- >70% (Table 1).

The mean percentage of CD3<sup>+</sup> T cells population was decreased in third and seventh day postburn when compared to the normal level. Statistical analysis showed there was significant differences between third and seventh day postburn ( $P < 0.001$ ). As shown in Table 1, the number of CD3<sup>+</sup>CD4<sup>+</sup> T cells appeared to be higher in the third when compared to the seventh day postburn, with significant differences ( $P < 0.003$ ).

Table 2. Mononuclear cell subsets at three and seven days post burn according to different age groupS ( $P < 0.05$ ).

Cell type	Days/Post burn	Age (yr)			
		18-30	31-40	41-50	51-60
CD3 <sup>+</sup> T cell	Third day	40.65 $\pm$ 14	43.97 $\pm$ 14.91	47.69 $\pm$ 12.19	52.96 $\pm$ 20.82
	Seventh day	32.56 $\pm$ 21.09	25.52 $\pm$ 21-57	29.56 $\pm$ 20.56	48.82 $\pm$ 12.89
CD3 <sup>+</sup> CD4 <sup>+</sup> T cell	Third day	21.06 $\pm$ 8.8	22.88 $\pm$ 8.53	25.79 $\pm$ 11.38	16.04 $\pm$ 6.36
	Seventh day	16.3 $\pm$ 11 .44	13.31 $\pm$ 10.1	17.39 $\pm$ 13.89	15.49 $\pm$ 9.34
CD3 <sup>+</sup> CD8 <sup>+</sup> T cell	Third day	18.2 $\pm$ 9.01	19.37 $\pm$ 12.57	21.62 $\pm$ 8.83	35.21 $\pm$ 12.29
	Seventh day	14.96 $\pm$ 11.4	11.31 $\pm$ 13.14	11.79 $\pm$ 8.68	32.84 $\pm$ 13.08
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	Third day	1.34 $\pm$ 0.62	1.47 $\pm$ 0.62	1.48 $\pm$ 0.9	0.45 $\pm$ 0.02
	Seventh day	1.52 $\pm$ 1.07	1.7 $\pm$ 1.19	1.69 $\pm$ 0.96	0.6 $\pm$ 0.6
CD19 <sup>+</sup> B cell	Third day	22.62 $\pm$ 10.73	19.34 $\pm$ 6.03	21.25 $\pm$ 5.21	28.73 $\pm$ 16.14
	Seventh day	18.19 $\pm$ 9.22	15.6 $\pm$ 8.64	24.84 $\pm$ 10.69	15.67 $\pm$ 5.32
CD16 <sup>+</sup> NK cell	Third day	11.42 $\pm$ 8.66	9.06 $\pm$ 4.32	11.57 $\pm$ 8.5	16.25 $\pm$ 9.23
	Seventh day	13.53 $\pm$ 14.4	9.17 $\pm$ 9.81	14.62 $\pm$ 13.57	28.6 $\pm$ 10.71

The mean percentages of CD3<sup>+</sup>CD8<sup>+</sup> level was higher in third day postburn than seventh day in post burn ( $P<0.009$ ). The mean CD4<sup>+</sup>/CD8<sup>+</sup> ratio in Third and seventh day post-burn remained in nearly normal range.

The mean number of CD19<sup>+</sup> B lymphocytes appeared to be higher in the third days when compared to the seventh day postburn and was statistically insignificant ( $P>0.05$ ). The mean percentage of CD16<sup>+</sup> NK cells on the third day was lower than seven days. The difference between the two days was statistically significant ( $P<0.05$ ). Peripheral white blood cells counts, (CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte subset values, CD4<sup>+</sup>/CD8<sup>+</sup> ratio, CD16<sup>+</sup> and CD19<sup>+</sup>) from burned patients in different groups of age at third and seven days post burn, were analysed to determine differences in age and its relation with changes in the proportion of mononuclear cell subsets and, results are shown in Table 2. The proportion of CD3<sup>+</sup> lymphocytes at third days post burn were modified by a few percentages for every decade of life. CD3<sup>+</sup> T cells at seventh day post burn were reduced in all groups of age when compared to day three post burn, however, CD3<sup>+</sup> T cells at day seven were significantly less in patients aged 18-30 years compared to aged 51-60 years ( $P<0.05$ ). CD3<sup>+</sup> T cell counts in all groups of age in third and seventh day were diminished when compared to the normal level in each group of age. Mean percentages of CD4<sup>+</sup> T cells as well as CD8<sup>+</sup> T cells in seventh day post burn, in all groups of age were decreased when compared to third days but it was insignificant ( $P>0.05$ ). The results obtained showed a significant increase in the percentages of CD8<sup>+</sup> T cells in the above 50 age group in third and seventh days post burn when compared to controls. In addition, no significant change was observed in the mean percentages of CD4<sup>+</sup>/CD8<sup>+</sup> ratio in third and seventh days post burn in different groups of age, and ratio levels were close to normal ranges except patients aged 51-60 years that had less than normal values. Our data indicated a rise in per-

centages of CD19<sup>+</sup> B lymphocytes in three days post burn of all age groups. CD19<sup>+</sup> counts at day seven remained near in normal ranges for all groups of age except aged 41-50 years ( $P<0.05$ ), compared to 18-30 years. Finally, in this study a significant difference existed in the number of CD16<sup>+</sup> NK cells with the 51-60 years between day three and seven post burn ( $P<0.05$ ). Results of present study were compared to our previously published results as a reference ranges [17].

### Discussion

Significant thermal injuries induce a state of immunosuppression that predisposes burn patients to infectious complication. Thermal injury is among the most severe forms of trauma and could affect the body both locally and systemically. Immunological factors have been suggested as playing a central role in the disruption of normal processes of wound healing, tissue remodeling, suppression in the number of the total lymphocytes occurs in burned patients [7,18,19]. Studies of immunity in burned patients are more difficult because many of the drugs administered to the patients, including antibiotics, and also blood transfusions, surgical procedures, anesthesia and nutritional deficiencies could alter the outcomes [20-22]. Immuno alteration observed following thermal injury may be due, in part, to a redistribution of cellular elements responsible for generation of the immune response [13,23]. In the last decades various studies have been done on the effects of lymphocyte subsets on burn patients at different day post burned [7,8, 14, 24]. In the present study comparison of T cell subsets, B cells and NK cell values (according to different day and age) was made with those published earlier [14,21,23].

Our study showed a reduction in the number of CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes level as well as CD4<sup>+</sup>/CD8<sup>+</sup> ratio, three and seven days after the initiation of the thermal injury. This injury-related dysregulation of the cellular im-

mune response appears to be associated with an altered T-lymphocyte capacity for activation. This and other studies have shown that there is pronounced immunosuppression following thermal injury [3]. The number of CD19<sup>+</sup> B cells on the 3th and 7th day was higher than normal controls. Mechanisms of changes of antibody formation after burn injury are still unclear. The defect appeared because of immunological factors affecting B cell function in two days. (Third and seventh post burn) [7,18,19]. The increase in number of CD16<sup>+</sup> NK cells in seven days post burn may activate immune system, and is inevitable after thermal burn. Our finding was compatible with some part of earlier reports [6, 14, 22].

We found special correlation between a CBC with differential (which was scheduled every days or as necessary) and lymphocytes number, in these patients. We also observed, reduction in the number of lymphocytes population as well as white blood count, which may participate in impairment of general mechanisms for immunoregulation during burn shock and transition of blood to the level of self-regulation. That is the sign of immunosuppression development.

However, these studies give more insight into the role of immune system in general in burn or wound healing and may provide tools to interfere in cases of disturbed wound healing [5,7,23-25]. Burn injury induces a systemic hypermetabolic response, resulting in inflammation, immune compromise [26,27,28]. Changes in lymphocytes population were identified on the third and seventh days of different age groups in the burn patients. Insignificant changes were observed in the level of CD19<sup>+</sup> B lymphocytes among different age groups at three and seven days postburn. No significant increase in the CD16<sup>+</sup> NK cell population with increasing age was observed except aged 51-60 years. The increase in number of CD16<sup>+</sup> NK cells activation in this period may induce apoptosis a phenomenon now termed "activation-

induced cell death (AICD)". Moreover postburn immunodeficiency may be caused by destruction of immune competent cells by the mechanism of activation-induced apoptosis. A part from age differences, these discrepancies could be attributed to factors such as smoking habits, diet, climate and /or hygienic conditions [14].

Therefore, the results obtained in this study correlated with others report [7,30-32]. And this study may confirm that determination the mean percentages of cellular immune system in thermal burn patients in different groups of age had positive effects on survival. Cell mediated immunity and antibody production may be affected by nutritional deficiencies. Infection and malnutrition may setup a various cycle, resulting in decrease immunity in the burn patients [24, 28]. With early treatment and nutritional support may prevent decline in immunity and death [14,27,28,33]. There is a direct but inverse relationship between age and survival of any burn size. In patients with TBSAB from 30-70% or more, the number of the activated cells was varied. The duration of post burn lymphocyte activation and its outcome were dependent on burn severity, in patients with TBSAB mostly >70% [8,34]. However the immunological response to thermal injury is a depression in both the first and second lines of defense [9,19]. Therefore, defects in cellular immunity in the burned patient may play an important role in sepsis and death, these defect and changes are not the unique cause of sepsis, factors are various [10,28,35-37]. Among the several factors perhaps one of the most important in determining the degree of injury (a part of TBSA) is the peripheral blood lymphocyte counts in burn patients. No report was found in the current literature investigating the cellular immune system in Iranian thermal burn patients. Hence, this study was performed.

### Conclusion

The potential importance of this finding is

highlighted by the differences at three and seven days postburn associated with the different lymphocyte populations, and the level of these cells are an important markers of immune system activation. The number of lymphocytes and their subsets may be influenced by both genetic and environmental factors, and that needs to be investigated in future study.

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