EFFECT OF MAJOR BURNS ON EARLY AND LATE ACTIVATING MARKERS OF PERIPHERAL BLOOD T LYMPHOCYTES

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SUMMARY. It is known that lymphocytes immunophenotype is a reflection of the functional level of the immune system. The immunosuppressive effect of major burns is also known for many years. T lymphocytes of 50 major burn patients were analyzed in base line (BL) samples at 24 hours and at 1 week and 2 weeks after burn, using monoclonal antibodies of CD3, CD4, CD8, CD25 (IL2R) and HLA-DR by flow cytometry and β2-microglobulin (β2-m) by ELISA. Recorded values were compared with those of 50 healthy donors. There was statistically significant reduction in absolute number of CD3 positive cells (CD3+) (p<0.000) and CD4/CD8 ratio (p=0.01) in the first 24 hours in comparison with controls. CD25 (IL-2R) shows insignificant upregulation on T lymphocytes after burn with significant upregulation of HLA-DR. The absolute number of CD3+ cells began to increase after 2 weeks (p=0.03) but remained less than controls (p=0.08). CD4/CD8 ratio was more or less same as healthy controls after 2 weeks. Upregulation of CD25 was insignificantly increased and that of HLA-DR was markedly increased after 2 weeks (p=0.001). Significant negative correlations were detected between mean values of β2-m and both absolute numbers of CD3 and CD4 positive cells in BL and one week samples. In addition there was significant correlation between mean values of β2-m and values of CD25 expression in the BL samples. The obtained data is suggestive of persistent activation of T lymphocytes two weeks after major burns whereas early shedding of β2-m is related to activation of lymphocytes increasing their susceptibility to apoptosis, both indicative of altered immune response. Burn intensivists and surgeons should be keen to support the patients’ immune system in the first hours following major burns. This support will ensure free-bacteremic blood with a consequent better prognosis.

Keywords: activating markers, peripheral blood, T lymphocytes, major burns

Introduction

In burn injury, infection is still an important cause of morbidity and mortality despite the development of broad spectrum antibiotics and technical advances in life support therapy. Increased susceptibility to infection has been related to impaired immune response. It seems that normal immune defense mechanisms start to become suppressed in burn injuries at around 25% of total body surface area (TBSA).1 The immunosuppressive effect of a major burn has been known for many years; however, a complete understanding of the effects of a burn on the immune system remains elusive.2

In-vitro studies indicate that sHLA-I (β2-microglobulin) antigens may modulate function of lymphocytes in at least two ways. The sHLA-I molecules may bind their physiologic ligands and inhibit T cell function by receptor blockade and/or by induction of apoptosis.3 Moreover, sHLA-I antigens can be phagocytosed by antigen-presenting cells, degraded to peptides, and presented to CD4 positive (CD4+) T cells in the context of membrane HLA class II antigens in a process known as indirect presentation which may lead to either immune tolerance or activation.4

There are many activation antigens which play a significant role and are the markers of immune system activation.5 The activation markers in this study were selected according to their appearance after stimulation including the most well established markers: the early CD25 and late activation marker HLA-DR. CD25 (IL2Rα) is one of the markers of recent activation and impending or recently completed division as shown by Hodge et al.6 study with up-regulation occurring within 4 hours following its stimulation in vitro. Cotner et al.7 study reported its persistence for only a few days. On the other hand, HLA-DR antigens were expressed several days after stimulation when
DNA synthesis is initiated and cell proliferation occurs and remain expressed for several weeks.\textsuperscript{9,10}

HLA-DR as a major histocompatibility complex (MHC class II) is a cell surface receptor encoded by the human leukocyte antigen complex. HLA-DR molecules are up-regulated in response to signaling. In the instance of an infection, the peptide is bound into a DR molecule and presented to T-cell receptors found on T-helper cells. These cells then bind to antigens on the surface of B-cells stimulating B-cell proliferation.\textsuperscript{11} It is known that one of the outcomes of lymphocyte activation may be apoptosis, a phenomenon termed activation-induced cell death (AICD). Moreover, Teodorczyk-Injeyan et al.,\textsuperscript{12} supposed that post burn immunodeficiency may be caused by destruction of immune competent cells by the mechanism of AICD. HLA antigens may modulate function of lymphocytes by inhibition through their receptor blockade and/or by induction of apoptosis.\textsuperscript{5}

As lymphocytes immunophenotype is a reflection of the functional level of immune system, we aimed in this work to generally obtain more insight into blood T-lymphocytes immunophenotyping after major burn, and to particularly determine the activation state of T cells after major burn by detection of CD25, as an early activating marker of T-lymphocytes and HLA-DR expression as a late one and their relation to β2-microglobulin (β2-m). This insight should be actively correlated with timing of this activation to answer the main questions of this work: When should the burn surgeons start supporting the immune system of their patients? How to keep patients free of wound infection, bacteremia, or septicemia?

Patients and methods

A prospective study included 50 patients (27 females and 23 males), their ages ranged from 18 to 40 year old with a mean age of ± 27.6 years. The patients suffered from acute second and third degree major burns ranging from 25 to 40% of TBSA and were admitted to burn units of Assiut University and El-Mataria teaching hospitals in Upper Egypt and Lower Egypt territories respectively. Three samples were collected from each patient; the first sample was in the first 24 hours as a baseline (BL) of acute burn condition, while the second and third samples were collected after one and two weeks from the beginning of burn injury, respectively. A control group composed of 50 healthy individuals was selected during the same period from blood donors. Their ages ranged from 20 – 42 years with a mean age of ± 29.5. The research framework of this study was examined and approved by the research ethical committee of Assiut University. All patients and controls were subjected to the following:

- Complete blood cell counts performed on EDTA-treated blood, using an automated hematology analyzer.
- Immunophenotyping: Three colors staining of monoclonal antibodies were carried out using a standard protocol. The panel of monoclonal antibodies used was: Isotypic control, CD4/CD8/CD3, and CD25/HLA-DR/CD3.
- Analysis was performed at the first 24 hours after burn (BL), after 1 week and after 2 weeks on a FACS Caliber flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA, USA) at the flow cytometry laboratory, South Egypt Cancer Institute, Assiut University. Dead cells were excluded by gating on FSC/SSC and lymphocytes gate was selected. Marker expression was recorded as percentage and absolute count of positive cells (Fig. 1).
- Detection of β2-m was done using enzyme immunoassay (EIA) kit (QuantiKine IVD. R&D Systems, Inc. USA)

![Fig. 1 - A: Lymphocytes gate (R1) is determined by low forward and side angle light scatter (FSC & SSC respectively). B: Percentage of positive cells for the 2 subsets CD4 and CD8. C: Cells are re-analyzed on a scatter diagram combining CD3 with CD25. D: A scatter diagram combining CD3 with HLA-DR.](image-url)
The quantitative variables SPSS (Statistical Package for Social Sciences) version 15.0 was used for data analysis expressed as mean ± standard deviation. Comparison studies were done using t-test for 2 independent groups and one way ANOVA (Kruskal Wallis test) for more than two independent groups. Correlations between quantitative variables were performed by using Pearson correlation. P-value is significant at 0.05 levels.

**Results**

There were statistically significant reduction in absolute number of CD3+ cells (p<0.000) and CD4/CD8 ratio (p=0.01) in the first 24 hours (BL) in comparison with controls. CD25 (IL2R) shows insignificant up-regulation on T lymphocytes after burn with significant up-regulation of HLA-DR after one week (*Table I*).

The absolute number of CD3+ cells began to increase after 2 weeks (p=0.03) but still remained less than controls (p=0.08). CD4/CD8 ratio was more or less the same as healthy controls after 2 weeks. Up-regulation of CD25 was insignificant contrary to HLA-DR which was markedly increased after 2 weeks (p=0.001). The mean values of β2-m during the whole duration of the study were significantly higher in burn patients than that of the controls but with no significant differences between the 24 hrs, 1 week or 2 weeks measurements (*Table I*).

Significant negative correlations were detected between BL and in one week samples of mean values of β2-m and both values of absolute number of CD3 and CD4 positive cells. In addition there was significant positive correlation between mean values of β2-m in BL samples and HLA-DR expression in one week samples and both values of CD25 and HLA-DR positive cells as well as between mean values of β2-m after two weeks and all tested values (*Table II*).

**Discussion**

Increased levels of β2-m indicate that there is a problem, but they are not diagnostic for a disease or a condition. They do reflect, however, disease activity and tumor burden. The increase of β2-m in blood of patients with burn injury can be a consequence of cell activation with subsequent shedding of sHLA-I from cells participating in compensatory mechanisms after burn trauma. Patenaude and his co-workers suposed that post-burn immunodeficiency

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**Statistical analysis**

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may be caused by destruction of immune competent cells by the mechanism of AICD which explains the significant reduction in the absolute number of CD3 after one week of burn injury we have observed. This was also confirmed by data obtained by Lebedev et al. who reported also a decrease of Fas/CD95 expression. This decrease in FAS receptor (FasR) also known as apoptosis antigen 1 (APO-1 or APT) expression is the mechanism of peripheral blood lymphocytes protection from AICD. Burn injury causes change of apoptosis threshold induced through Fas-FasL system. In turn, sHLA-I can indirectly influence the FasL interaction. Apoptosis of Fas+ cells may be induced via soluble FasL which is released following binding of sHLA-I antigen to CD8 molecule. Also Madihally et al. explained the down regulation of different markers in peripheral blood lymphocytes in burned patients in the first week as a result of shedding of some molecules as a consequence of early post burn lymphocyte activation. Extensive destruction of tissues by the mechanisms of necrosis may be another reason for increase of β2m serum level and significant reduction in lymphocytes.

Another study by Mabrouk et al. determined IL-6, CD3 and its subsets CD4 and CD8 in patients with burn injury. On the fourth day after burn injury, they recorded significant decrease in absolute numbers of CD3 and CD4/CD8 ratio compared with controls. They explained the decline in these markers in the post-burn injury period by immunosuppressive factors. One of these factors is a burn toxin that was characterized as a polymerized complex of cell membrane lipid proteins (lipid protein complex). This has been shown to inhibit the proliferation of normal T lymphocytes in response to stimulation. The T lymphocyte decrease in post-burn period has profound implications for patient susceptibility to infection and as the decline in CD4/CD8 ratio is limited to the first week, it could provide valuable information as to the establishment of early treatment in order to avoid likely future complications.

Buchanan et al. examined the effect of burn injury on CD4 and CD8 T cell haemostatic proliferation after irradiation. They observed that CD8 T cells are more powerful than CD4 T cell in their proliferative response after injury. Another study showed that the burn injury induces a change in T cell homeostasis and affects mainly CD4 T after 10 days of thermal injury showing significant reduction in their absolute number. They interpreted this reduction in CD4 lymphocytes, by the presence of high percentage of naive and effector/memory CD4 T-activated lymphocytes ten days after burn injury, and added that these cells are effectively primed, as previously presented in an earlier study. In another study, these primed cells could be more susceptible to apoptosis thus multi-factorial modulation of T lymphocytes function following burn injury leads to disruption of the homeostatic controls which may explain the increase in occurrence of sepsis or infection. The significant negative correlations detected in our study between mean values of β2-m in BL and after one week samples and both values of absolute number of CD3 and CD4 +ve cells confirm that the increased susceptibility to apoptosis related to β2-m increase is mainly affecting CD4 +ve cells which may be the cause of disturbed CD4/CD8 ratio in burns.

A reliable method of detection of subclinical infection may be the demonstration of activated lymphocytes, which can be conducted rapidly and before the isolation of the infective organism. Hodge et al. had previously shown that detection of upregulation of CD45RO, an activated/memory isofrom of CD45 present on T cells, is a sensitive and reliable marker of neonatal infection. Another early marker of activation on T cells was CD25 (IL-2Ra) and a late marker was HLA-DR. In the present study, CD25 (IL2R) showed insignificant upregulation on T lymphocytes after burn with significant upregulation of HLA-DR. In addition, the obtained data suggests persistent activation of T lymphocytes 2 weeks post major burns and that upregulation of HLA-DR expression was a more sensitive indicator and can reflect post burn lymphocyte activation and proliferation.

In our study, there was a significant correlation between mean values of β2-m in the BL samples and values of CD25 expression and there was not any correlation between mean values of β2-m in BL samples and HLA-DR expression as well as between mean values of β2-m in one week and two weeks samples and both values of CD25 and HLA-DR positive cells, which suggests that the shedding of β2-m is limited to the first days. As this shedding increases the susceptibility to apoptosis in CD4+ cells, it seems that boosting the immune system is much more useful in the first hours following a major burn.

**Conclusion**

In our study, the upregulation of HLA-DR appeared greater than that of CD25, suggesting that HLA-DR expression is a more sensitive indicator and can reflect post burn lymphocyte activation and proliferation. The findings presented in this project are suggesting that the shedding of β2-m is limited to the first days and increasing the susceptibility of apoptosis related to this shedding is more in CD4+ cells. This implies that supporting the immune system is much more useful in first hours after major burn injury. Patients with major burns should have their immunological support coincident with other primary measures of early burn management including empirical antibiotic coverage before the end of the first week, as the second week samples indicated no correlations between mean values of β2-m in BL samples and HLA-DR expression as well as between mean values of β2-m in one week and two weeks samples and both values of CD25.
and HLA-Dr positive cells, which suggests that the shedding of β2-m is limited to the first days. The supported immunological condition of those patients along with wound control, specific antimicrobial therapy, sufficient nutritional support, and the hemodynamic and electrolyte balance will keep them free of bacteremia and/or septicemia with expected faster wound healing and/or successful skin grafting. In view of the results of this study, current guidelines restricting empirical prophylactic antibiotic usage in patients with severe burn injuries need to be revised. However, benefits of early prophylactic antibiotic administration need to be balanced with the major disadvantage of emergence of resistant bacterial strains. Another study to answer this dilemma is recommended.

**BIBLIOGRAPHY**