

EXPRESSION OF AN ACTIVATED FORM OF INTEGRIN β_2 CHAIN CD18 IN CARDIAC SURGICAL OPERATIONS

Manuela Kudlová¹, Martina Koláčková¹, Pavel Kuneš^{2,1}, Vladimír Lonský², Jiří Mandák², Ctirad Andryš¹, Jan Krejsek¹

Charles University in Prague, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové, Czech Republic: Department of Clinical Immunology and Allergology¹, Department of Cardiac Surgery²

Summary: Background: Myeloid cells are extensively activated in patients undergoing cardiopulmonary bypass (CPB) („on-pump“) in comparison with patients operated without CPB („off-pump“). **Aims:** To evaluate changes in the expression of a novel activation marker expressed on myeloid cells recognized by MEM-148 antibody. **Patients and Methods:** The expression of MEM-148 positive myeloid cells was evaluated by flow cytometry in 40 patients who underwent coronary artery bypass surgery (CABG) randomly assigned to „on-pump“ or „off-pump“ technique. **Results:** The relative and absolute number of MEM-148 positive myeloid cells is significantly diminished during „on-pump“ surgery. A significant increase in their number in postoperative period in both „on-pump“ and „off-pump“ patients was found. There were no significant differences between „on-pump“ and „off-pump“ patients. **Conclusions:** The very trauma of surgery seems to be more relevant in starting on activation of myeloid cells than CPB itself.

Key words: Cardiac surgery; Cardiopulmonary bypass; Myeloid cells; Activation MEM-148

Abbreviations: CABG – Coronary Artery Bypass Grafting; CD – Cluster Designation; CPB – Cardiopulmonary Bypass; ICAM-1 – Intercellular Adhesion Molecule-1.

Introduction

Cardiopulmonary bypass (CPB) in combination with other factors (surgical trauma, anesthesia, medication, hypothermia, ischemia-reperfusion injury) induces inflammatory response in cardiopulmonary patients. This response includes activation of humoral systems (complement, coagulation-fibrinolysis) and cellular components. The crucial role in latter one is devoted to the mutual interplay between activated immune cells in blood vessels and activated endothelial cells. The ultimate goal of this process is extravasation of activated leukocytes into tissues via diapedesis (9). Adhesion of leukocytes to endothelial cells lining which is a multistep process is prerequisite to the diapedesis of leukocytes. The firm adhesion of leukocytes is mediated by the interactions between leukocyte β_2 integrins subfamily and their counterparts ICAM-1, 2, 3 belonging into immunoglobulin superfamily. Members of family of β_2 integrins are heterodimers defined by a CD18 or β_2 subunit that is common to all members of the β_2 subfamily, paired with a unique α chain (CD11 a, b, c,) (16), respectively.

Several avenues of research have converged to reveal the central role of β_2 integrins in the inflammatory response. CD18 chain is essential for the function of β_2 integrins as is evidenced from rare patients suffering from leukocyte adhesion deficiency I. As a result of mutations in the CD18 chain these patients fail to express normal levels of β_2 integrins. As a consequence, neutrophils activation and trafficking is severely impaired.

There are some reports regarding β_2 integrins expression in cardiac surgical patients. It is generally assumed that cardiac surgery using CPB („on-pump“) has more profound impact on leukocytes in comparison with surgery on the beating heart („off-pump“) (11). Indeed, Asimakopoulos et al (1) found that β_2 integrins CD11b/CD18 expression on neutrophils was significantly increased as early as 15 min after onset of CPB. On the contrary, it was reported by Gesler et al (7) that CD11b/CD18 expression was reduced after weaning from CPB.

Recently, a novel activation marker abundantly expressed on the surface of activated monocytes and neutrophils – a 65- to 70-kDa proteolytic fragment of the common β chain of leukocyte (β_2) integrins, CD18, as detected by

a monoclonal antibody produced by MEM-148 hybridoma recognizing unique CD18 epitope was described (5).

The aim of this study was to follow the expression of a novel activation form of CD18 β_2 chain on immune cells of cardiac surgical patients during surgery and an early postoperative period. To the best of our knowledge, no data are available so far concerning the expression of this form of CD18 β_2 integrin chain recognized by MEM-148 monoclonal antibody in cardiac surgical patients. The expression of this marker was compared between „on-pump“ and „off-pump“ patients to ascertain the impact of CPB on the activation of immune cells.

Patients

Forty patients (31 male, mean age 67.9 ± 9 years and 9 female, mean age 66.4 ± 6.4 years, collective mean age 67.6 ± 8.5 years) referred to first-time coronary artery bypass grafting (CABG) were enrolled in this study. Patients underwent either conventional myocardial revascularization with cardiopulmonary bypass and cardioplegic arrest of the heart (‘‘on-pump’’, $n=20$, 16 male, 4 females, mean age 69.4 ± 7 years) or beating heart surgery (‘‘off-pump’’, $n=20$, 15 males, 5 females, mean age 65.9 ± 9.7 years). The patients were randomly assigned either to ‘‘on-pump’’ or to ‘‘off-pump’’ surgery by a member of the cardiac surgical staff outside the research team who was blinded to all variables pertinent to the study design.

Patients in both groups were comparable in age, preoperative left ventricular ejection fraction (median 0.65 in ‘‘on-pump’’, 0.65 in ‘‘off-pump’’ patients, respectively) and the number of performed coronary anastomoses (median 2.0 in ‘‘on-pump’’, 2.0 in ‘‘off-pump’’, respectively). All patients had been taking aspirin 100 mg in one daily dose, which was stopped for five days preceding the operation. Patients treated with anti-inflammatory agents, either steroids or NSAID, were excluded from the study, as were patients with serum creatinine $\geq 130 \mu\text{mol/l}$ or with hepatic disorders. No patients were known to suffer from concomitant malignancies. Patients with active infectious diseases are not admitted to elective CABG in our department. The Ethics Committee of the University Hospital in Hradec Králové approved the study protocol. All participants were informed in detail about the purpose of the study both orally and in writing. They were free to ask any questions. One person refused to participate for reasons he would not specify. All active subjects have given written informed consent.

EUROSCORE is not routinely assessed in our patients.

Cardiopulmonary bypass

CPB was established using a two-stage venous drainage and ascending aortic return. A roller pump (S3 Stöckert®, Stöckert Instrumente GmbH, München, Germany), a membrane oxygenator (Dideco Avant 903®, Dideco Mirandola,

Italy) in a closed modification with collapsible reservoir, a cardiotomy suction device and a 40 μm arterial line filter (Dideco Micro 40®, Mirandola, Italy) were integrated into the extracorporeal circuit. The system surface was not treated with any hemocompatible substance. The priming solution consisted of:

- 500 ml Ringer’s lactate, 500 ml Rheodextran (Rheomacrodex), 5000 IU heparin, 500 000 IU aprotinin, 80 ml natrium bicarbonate (NaHCO_3 8,4 %), 20 ml magnesium sulphate 10 %, 500 mg dexamethasone, manitol 1 g/kg body weight.

The priming volume was calculated to achieve haematocrit levels above 0.22.

Intravenously, heparin was administered at 300 IU/kg body weight to maintain an activated clotting time (ACT) above 480 s during bypass. No patient received either aprotinin or corticosteroid intravenously. Pump flow rates averaged 2.4 l/min/m² body surface areas with pressure maintained at 50–60 mmHg. The patients were kept normothermic. Cardioplegic arrest was induced with 800–1000 ml of a St.Thomas cold crystalloid solution, administered antegradely into the aortic root with added doses of 200–300 ml every 30 minutes whenever needed. All patients received an internal artery mammary graft to the left anterior descending coronary artery (LAD). The central aorto-venous anastomoses were performed during the reperfusion phase of CPB with the heart beating. After termination of bypass, heparin anticoagulation was antagonized by protamine sulphate at a 1:1 dosage.

Cross clamping of the aorta in this group took in average 49 minutes; the duration of cardiopulmonary bypass amounted in average to 84 minutes.

‘‘Off-pump’’ technique

All operations were performed via a median sternotomy incision. Two to three traction sutures in the postero-lateral pericardium were placed. Regional myocardial stabilization was achieved with commercially available suction stabilizers. No preconditioning was performed. The target coronary vessels were snared with a silicone vascular loop proximal to the anastomotic site. An intracoronary shunt was used during construction of the anastomoses. The left internal mammary artery to LAD was the first anastomosis in all patients. The central aorto-venous anastomoses were established with partial occlusion of the ascending aorta. In OPCAB patients, heparin was given at a dosage of 200 IU/kg to achieve an ACT over 300 s. After completion of the final anastomosis, heparin was antagonized with protamine sulphate at a 1:1 dosage to return the ACT to preoperative levels.

Anaesthesiological management

All patients were anaesthetised according to the current protocol of our department. Anaesthesia was induced with

thiopental and midazolam. Muscular relaxation was achieved with cisatracurium. Anaesthesia was maintained with isoflurane and intermittent sufentanyl. Continuous propofol was used as a supplement if needed. Volume-controlled ventilation with FiO_2 of 0.50 was employed. Mean arterial pressure was kept over 50 mmHg with norepinephrine administered whenever required.

Blood sampling

Venous blood (peripheral venous blood from an antebrachial vein) was withdrawn in the operating room and during postoperative period in the intensive care unit. Samples were collected into heparinized tubes manufactured by Greiner, Germany.

In "on-pump" patients, blood was withdrawn at following time points:

- 1) introduction to anaesthesia, which in both groups represented the baseline or reference value for all parameters measured thereafter
 - 1a) before cross-clamping of the aorta
 - 1b) after aortic cross-clamp release
 - 1c) after termination of CPB
- 2) after termination of the operation
- 3) the first postoperative day
- 4) the third postoperative day
- 5) the seventh postoperative day.

In "off-pump" patients, blood was withdrawn at:

- 1) introduction to anaesthesia
- 2) after termination of the operation
- 3) the first postoperative day
- 4) the third postoperative day
- 5) the seventh postoperative day.

Methods

The expression of activated immune cells expressing novel activation marker recognized by MEM-148 monoclonal antibody was determined by phycoerythrin labeled monoclonal antibody purchased by Serotec, UK, Cat.No. MCA2086RPE in combination with anti CD45 panleukocyte marker (Immunotech, France). Direct double immunofluorescence whole blood lysing method was used.

To identify lymphoid and myeloid cells precisely, the combination of CD45 FITC and CD14 PE monoclonal antibodies were purchased from Immunotech, France, respectively.

Relative and absolute number of MEM-148 positive cells, separately for lymphoid and myeloid cells, were determined. Shift in the intensity MEM-148 expression on myeloid cells during surgery and in the postoperative period was expressed as MFI value (mean fluorescence intensity).

Results were measured by FACS Calibur flow cytometer (B.D., USA) using CELLQuest software. Irrelevant $\text{IgG}_1\text{FITC}/\text{IgG}_1\text{PE}$ monoclonal antibodies serve as negative isotopic control.

Statistical analysis

We analyzed changes in the relative and absolute numbers of immune cells expressing activated form of CD18 molecule recognized by MEM-148 monoclonal antibody in both group of patients ("on-pump", "off-pump"). Samples taken at the introduction to anaesthesia were considered as reference. Differences between "off- and on-pump" patients were also evaluated.

Data were analyzed using two-way ANOVA for repeated measures with Fisher test for multiple comparisons. To exclude confounding effect of different age and sex in both groups, unpaired t-test and chi-square test were performed. Correlations were assessed using Pearson's correlation coefficient.

A probability (p) value < 0.05 was considered significant.

Statistical analysis was performed with Statistica 5.5 software (Statsoft, USA).

Results

1. Expression of CD18 β_2 integrin recognized by MEM-148 on lymphocytes

The expression of a truncated form of CD18 β_2 integrin chains recognized by MEM-148 monoclonal antibody on lymphocytes was without any change during cardiac surgical operations either operated with or without CPB. The percentage of MEM-148 positive lymphocytes was in range from 3 % to 6 %. There was an insignificant decrease after declamping aorta in „on-pump“ patients. Slight, insignificant increase in both groups was found after surgery. There are were no differences between „on-pump“ and „off-pump“ patients ($p=0.28$, data are not shown).

2. Expression of CD18 β_2 integrin recognized by MEM-148 on myeloid cells

2.1. Changes in the relative number of MEM-148 positive myeloid cells

The relative number of MEM-148 positive myeloid cells was significantly decreased in „on-pump“ patients in the whole entire period of surgery from cross-clamping aorta to the end of surgery in comparison with preoperative level ($p<0.001$). In spite of a slight increase on the 1st postoperative day this relative number remained significantly diminished ($p<0.001$). This number was normalized on the 3rd postoperative day followed by a significant decrease on the 7th postoperative day in „on-pump“ patients (Fig. 1).

Changes in the relative number of activated MEM-148 expressing myeloid cells in „off-pump“ patients were resembling the pattern found in „on-pump“ patients. The significant decrease on the 1st postoperative day ($p<0.05$) was followed by the return back to the preoperative value on the 3rd postoperative day with subsequent decrease on the 7th postoperative day ($p<0.001$) (Fig. 2).

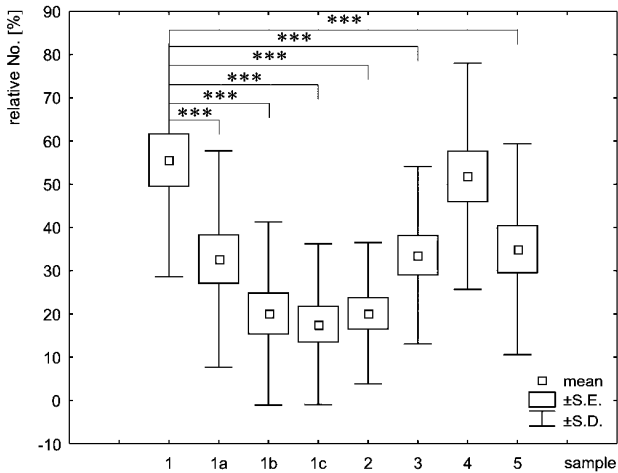


Fig. 1: Changes in the relative number of MEM-148 positive activated myeloid cells in “on-pump” patients.

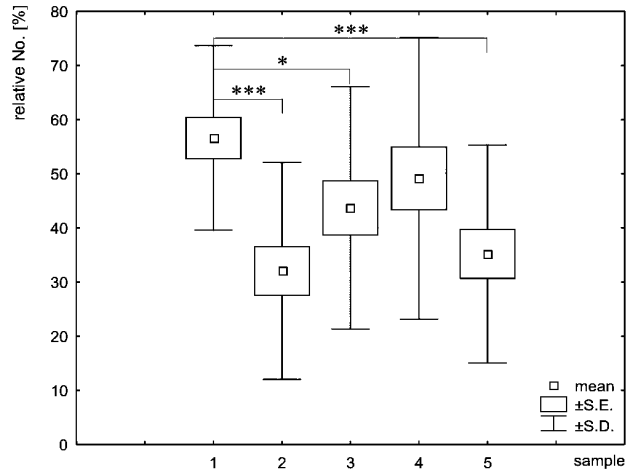


Fig. 2: Changes in the relative number of MEM-148 positive activated myeloid cells in “off-pump” patients.

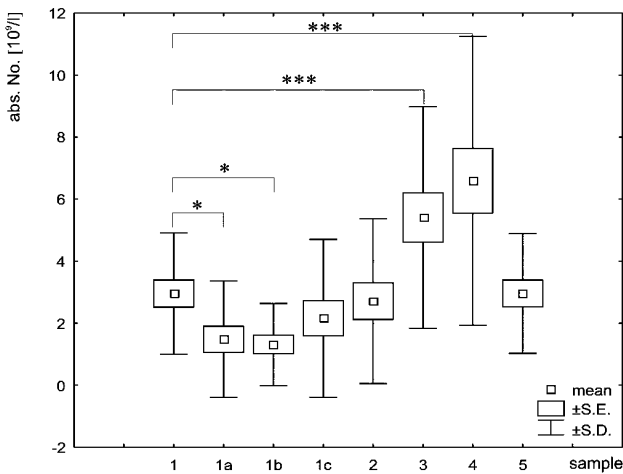


Fig. 3: Changes in the absolute number of MEM-148 positive activated myeloid cells in “on-pump” patients.

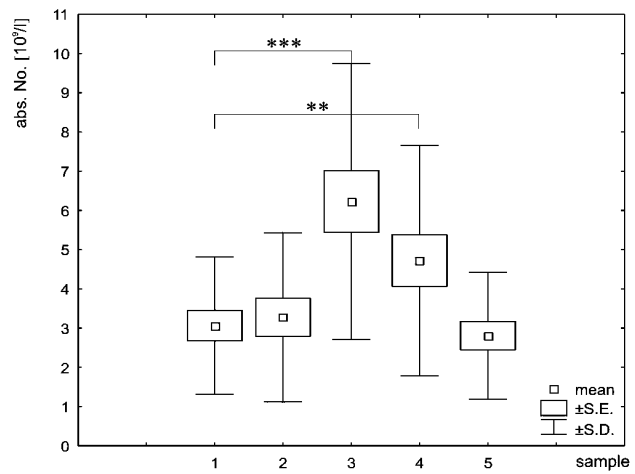


Fig. 4: Changes in the absolute number of MEM-148 positive activated myeloid cells in “off-pump” patients.

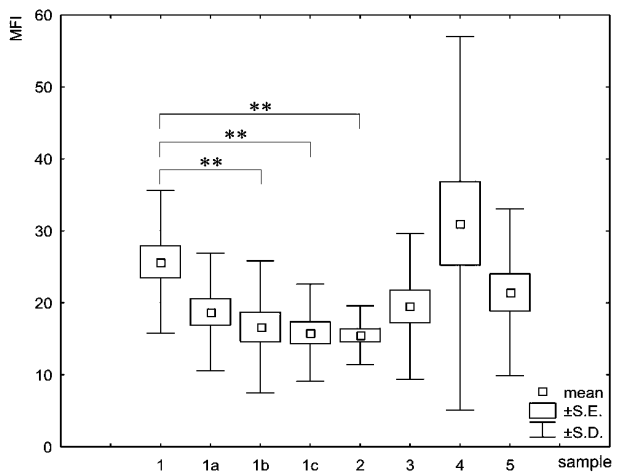


Fig. 5: Changes in the density of expression of MEM-148 activation marker on myeloid cells in “on-pump” patients.

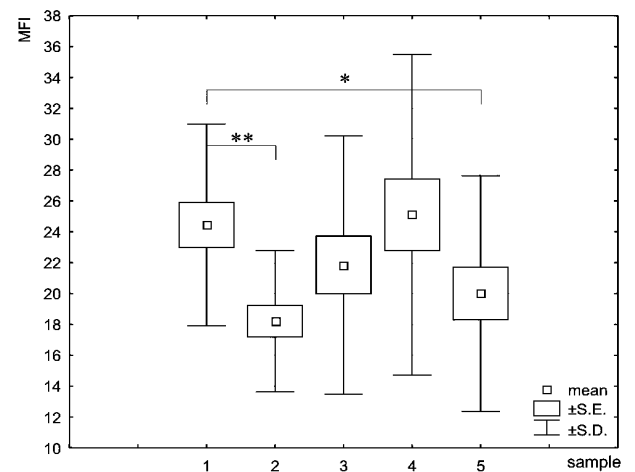


Fig. 6: Changes in the density of expression of MEM-148 activation marker on myeloid cells in “off-pump” patients.

There were no significant differences between „on-pump“ and „off-pump“ patients regarding relative number of MEM-148 positive myeloid cells ($p=0.44$, data are not shown).

2.2. Changes in the absolute number of MEM-148 positive myeloid cells

Changes in the absolute number of myeloid cells in „on-pump“ patients resembled relative count pattern. The significantly decreased number was found at the cross clamping ($p<0.05$) and declamping aorta ($p<0.05$), respectively.

This transient decrease was followed by the return to the preoperative baseline value at the weaning from CPB and at the finishing of surgery. The absolute count of activated myeloid cell was subsequently highly significantly increased ($p<0.001$) on the 1st and 3rd postoperative days, returned to the baseline value on the 7th postoperative day (Fig. 3).

The changes of the absolute number of activated myeloid cells in „off-pump“ patients were very similar compared to „on-pump“ patients. The slight, insignificant increases at the finishing surgery were followed by the statistically highly significant increase on the 1st postoperative day ($p<0.001$) and the 3rd postoperative day ($p<0.01$), respectively, with subsequent normalization on the 7th postoperative day (Fig. 4).

There were no significant difference between „on-pump“ and „off-pump“ patients regarding absolute number of MEM-148 positive activated myeloid cells ($p=0.86$, data are not shown).

3. Changes in the intensity of expression of activated epitope CD18 recognized by MEM-148 monoclonal antibody

The density of the expression of particular surface molecule is reflecting the physiology of cells. Flow cytometry enables to estimate mean fluorescence intensity (MFI) as a surrogate parameter of surface molecules density.

We followed the density of a truncated form of β_2 integrin chain CD18 on myeloid cells of cardiac surgical patients. The density of this marker expressed as MFI was significantly diminished at de-clamping aorta, weaning from CPB and at the end of surgery, respectively ($p<0.01$). This parameter was insignificantly changed in the whole postoperative period compared to baseline preoperative value in „on-pump“ patients (Fig. 5).

There was significantly diminished density of expression of truncated form of CD18 molecule on myeloid cells at the finishing of surgery compared to the preoperative level in „off-pump“ patients ($p<0.01$). This decrease was followed by the normalisation on the 1st and 3rd postoperative days. In accord with a relative number of MEM-148 positive myeloid cells, the density of this marker was significantly diminished on the 7th postoperative day ($p<0.05$) (Fig. 6).

There were no significant differences in the density of the truncated CD18 molecule between „on-pump“ and „off-pump“ patients ($p=0.798$, data are not shown).

Discussion

The inflammatory reaction in cardiac surgical patients is the result of a complex interplay between numerous humoral factors and cell substrate of inflammation. Amongst cells involved in this process special role is devoted to innate immunity monocyte-macrophages and granulocytes. Whereas monocyte-macrophage cells are the richest source of pluripotent proinflammatory cytokines upon activation, activated granulocytes are recruited into tissues by stepwise interaction between adhesion molecules on the surface of leukocytes and their corresponding receptors expressed on the luminal surface of inflamed endothelium (16). There is a substantial long lasting effort to identify activated neutrophils in blood of patients with systemic inflammatory response induced by various stimuli either to identify patients at the risk of development of overwhelming inflammatory response potentially ultimating into multiple organ failure syndrome (MOFS) or to implicate the causative agent of such inflammatory response e.g. bacterial infection (2).

Such promising marker is Fc γ -receptor I (CD64), a high affinity receptor for IgG₁ and IgG₃ subclasses of immunoglobulins. Numerous substances of both exogenous and endogenous origin are rapidly upregulating Fc γ RI expression on the surface of neutrophils (13). In contrast to Fc γ RI, informations regarding the expression of a novel activation marker, CD18 chain recognized by MEM-148 monoclonal antibody; are very sparse and in the case of cardiac surgery are entirely lacking.

Activation of myeloid cells by various physiological and experimental stimuli is accompanied by multiple surface changes associated predominantly with degranulation, it means externalization and thus enhanced surface expression of several membrane proteins stored in cytoplasmic granules, simultaneously with proteolytic shedding, and internalization of distinct sets of molecules. Thus, activated blood myeloid cells typically upregulate surface expression of chemotactic receptors, complement receptor type 3 (CR3; CD11b/CD18), and down-modulate surface density of lipopolysaccharide receptor CD14, adhesion receptors CD44 and CD62L, or antiadhesion sialoglycoprotein CD43 (6).

Recently, it has been reported that CD18 β_2 chain is proteolytically cleaved on the surface of activated myeloid cells. The resulting free 65 to 70-kDa fragment of CD18 is expressed apparently as a free molecule unassociated with CD11 α chains or other molecules and represents a novel abundant activation marker of myeloid cells (5). This fragment is not likely produced by proteases released from secretory granules of the activated cells or by activated membrane-associated proteases and comes predominantly

from integrin molecules stored intracellularly in resting cells. Transmembrane fragments of CD18 produced by the activation-induced proteolytic cleavage obviously lose their association with CD11 chains and expose the epitope recognized by monoclonal antibody MEM-148, which is the sterically hidden in the intact β_2 integrin heterodimer (4).

The expression of activated CD18 β_2 chain (MEM-148 positive) on lymphocytes in cardiac surgical patients is without any significant changes either during surgery or in the postoperative period regardless "on-pump" or "off-pump" patients. These indicate a low impact of cardiac surgery on this population in an early period. This finding is reflecting the fact, that specific immunity is induced later during inflammatory response.

On contrary, the CD18 expressing (MEM-148 positive) myeloid cells displayed substantial changes both in the relative and absolute counts. The relative and absolute number of MEM-148 positive myeloid cells was significantly diminished during surgery in "on-pump" patients. This drop could be explained by the selective entrapment of activated myeloid cells on the surfaces of CPB circuits. Indeed, such selective decrease, particularly population of CD16/CD14⁺ monocytes during "on-pump" surgery, was reported by Wehlin et al. (19).

The return back to the preoperative values of a relative count of MEM-148 positive myeloid cells which was found on the 3rd postoperative days in both "on-pump" and "off-pump" patients, was probably caused by the massive release of granulocytes at this period. Unmature, physiologically not fully competent granulocytes (bands) are exported to the periphery at that time. These cells are probably unable to be properly activated and are not expressing activated form of CD18 molecule. Subsequently, the relative number of these cells was again significantly diminished on the 7th postoperative day. At that time normal one replaces not fully immunocompetent granulocytes. Due to still persistent proinflammatory conditions in cardiac surgical patients, highly activated myeloid cells are sequestered in the injured tissue lowering the relative number of activated myeloid cells in blood.

The similar explanation could be used to explain significantly diminished density of expression of activated CD18 (MEM-148 positive) molecules on myeloid cells during "on-pump" surgery up to the 1st postoperative day. This decrease expressed as a change in MFI value, was found on the 1st postoperative day in "off-pump" patients as well. In general, priming and activation of immune cells is followed by a transient down-modulation of membrane molecules density. This phenomenon is supposed to relieve from potentially harmful overactivation of immune cells (6). Our results are in a concordance with the work of Tarnok et al. (17) who found decreased density of common β_2 (CD18) chain during surgery up to the 2nd postoperative day in "on-pump" patients.

The absolute number of activated (MEM-148 positive) myeloid cells is significantly increased on the 1st and the 3rd

postoperative days in both "on-pump" and "off-pump" patients. This increase, in spite of diminished MFI and relative count of these cells, is simply caused by substantial neutrophilia, which is typical for this period.

Many studies conducted in the last few years, which investigated various inflammatory markers, have shown reduced inflammation in patients operated on by the "off-pump" technique compared to "on-pump" surgery (3). On the other hand, any definitive proof in favor of "off-pump" surgery in terms of reduced long-term mortality compared to its "on-pump" counterpart is still missing. Moreover, some studies dealing with the inflammatory response elicited in "on-pump" versus "off-pump" CABG patients arrived at the conclusion that most of the differences observed between the two procedures were mostly of quantitative rather than qualitative nature (10, 18, 20). These controversies might be reconciled by taking into account the basic fact that any major surgery, no matter whether cardiac or non-cardiac, elicits disruption of the whole-body integrity due to skin and tissue incisions, bleeding, heart rate or blood pressure instability, and other possible derangements to a smooth perioperative course. All of these inconveniences can be prevented only to a certain degree, even if utmost care is expended. Thus, the very trauma of surgery seems to be more relevant in starting on SIRS rather than cardiopulmonary bypass itself, the latter adding a CPB-specific fraction on top of other unfavorable events (12, 14, 15).

Our results are supporting this idea. We did not find any significant differences between "on-pump" and "off-pump" patients neither in the relative or absolute number of MEM-148 positive myeloid cell nor in its density of expression.

Another opened issue in cardiac surgery is whether depletion of activated circulating leukocytes by arterial line leukocyte filters might alleviate inflammatory response. Rationale, obtained from the results of previous studies was supporting this idea. Very recently these opinions have been rejected by Ilmakunnas et al (8) who have found that passing of leukocytes through arterial filters was enhancing activation of neutrophils expressed as changes in CD11b/CD18 β_2 integrins expression.

This study was aimed to follow the expression of free 65 to 70-kDa fragment of CD18 in cardiac surgical patients. This fragment is expressed apparently as a free molecule unassociated with CD11 chains and represents a novel abundant activation marker of myeloid cells (5). It is possible to speculate that the proteolytic cleavage and concomitant dissociation of the major CD18 fragment may uncover ligand-binding sites in the α chain (CD11) thus formed unconventional form of high-affinity conformation of the β_2 integrin molecule. On the other hand, the observed cleavage of CD18 may simply be a first step of a degradative process down-regulating the amounts of functional cell surface β_2 integrins in the later phases of the adhesion process (5).

Regardless above speculations, this study clearly showed that there are no significant differences in the expression of

activated CD18 β_2 integrins recognized by MEM-148 antibody between "on-pump" and "off-pump" cardiac surgical patients.

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Corresponding author:

Jan Krejsek, Prof., University Hospital Hradec Králové, Department of Clinical Immunology and Allergy, Sokolská 581, 500 05 Hradec Králové, Czech Republic, e-mail: krejsek@fnhk.cz
