TLR2 AND TLR4 EXPRESSION ON BLOOD MONOCYTES AND GRANULOCYTES OF CARDIAC SURGICAL PATIENTS IS NOT AFFECTED BY THE USE OF CARDIOPULMONARY BYPASS

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Summary: Cardiac surgery is inseparably linked to the activation of innate immunity cells recognizing danger signals of both endogenous and exogenous origin via pattern recognition receptors such as TLR receptors. Therefore, we followed by flow cytometry TLR2 and TLR4 expression on blood monocytes and granulocytes of patients who underwent coronary artery bypass grafting using beating heart surgery (off-pump, n = 34), with use of standard cardiopulmonary bypass (CPB), (on-pump, n = 30), and miniinvasive CPB (mini on-pump, n = 25), respectively, before, during surgery, and up to 7th postoperative day. TLR2 and TLR4 expression both on monocytes and granulocytes was significantly diminished already at the end of CPB being highly significantly decreased at the end of surgery in all patients' groups. TLR2 and TLR4 expression reached preoperative value at the 1st postoperative day being significantly higher at the 3rd postoperative day. Using intracellular staining we found the peak of TLR2 and TLR4 expression inside of monocytes and granulocytes at the first postoperative day in a subgroup of on-pump patients. In conclusion, TLR2 and TLR4 expression is significantly modulated in patients undergoing coronary artery bypass grafting as a part of adaptive homeostatic mechanisms induced by major surgery. The very surgical trauma is responsible for TLR2 and TLR4 modulation. Surprisingly, cardiopulmonary bypass itself was little contributing to the modulation of TLR2 and TLR4 expression.

Key words: *Cardiac surgery; Inflammation; TLR2 and TLR4 expression; Flow cytometry; Dynamics*

Introduction

Numerous potentially harmful events that may result in onset of an overt inflammatory response are inevitably induced in the course of cardiac surgery. Nowadays it is well established that the protective potential of the inflammatory response is inseparably linked with its destructive counterpart, which might inflict unwanted damage of any body structure.

This response may lead to postoperative complications, including the systemic inflammatory response syndrome, multiple organ dysfunction, and failure, and in a small proportion of patients, even to the death (1, 2). Cardiopulmonary bypass (CPB) is considered to be the trigger of immune cells activation induced by their contact with artificial surfaces of tubing and oxygenators, and also by no turbulent blood flow. In addition, hypoperfusion of critical body organs such as heart and gut is linked to the pronounced ischemia reperfusion damage of these vital organs (3). The effort to remove the harmful effect of the conventional CPB has led to a development of modified (mini) CPB. Mini CPB that has recently been introduced and successfully used, is designed to reduce blood cell activation (4). It provides decreased area of extracorporeal circuit, reduces priming, and also lessens air-blood interface. Another favourable feature of mini CPB is a biocompatible coating that induces higher tolerance of blood cells (5). In spite the fact that the general consensus has not been reached yet, it seems that beating heart surgery is the most physiological approach to surgical myocardial revascularization, coronary artery bypass surgery (CABG). The beating heart surgery is at least avoiding the massive proinflammatory stimuli immanently associated with the use of CPB. Furthermore, pulsate blood flow is maintained with better perfusion of vital body organs thus ameliorating ischemia/reperfusion injury (6).

The inflammatory response in cardiac surgical patients is induced by the exposition of body components, both humoral and cellular to the danger patterns, either exogenous or endogenous origin. Microbial pathogen – associated molecular patterns (PAMPs) and endogenous molecules created upon tissue injury, since called damage-associated molecular patterns (DAMPs), signal the threat of either infection or injury to the host, independently of their non-self or self identity (7). Danger patterns, regardless their origin, are identified by the set of evolutionary highly conserved receptors named pattern recognition receptors (PRR) with subsequent activation of several intracellular activation pathways, culminating predominantly in NFκB transcrip-

tion factor activation. NFκB translocates to the nucleus and mediates an increase on inflammatory cytokine gene expression, leading to pro-inflammatory response. PRR receptors are present within the tissues as membrane glycoproteins, which are expressed on either the outer or inner surfaces of the cellular membrane systems. The broadest expression of all PRR receptors has been proved on innate immunity cells. This heterogeneous cell population comprising dendritic cells, macrophages, and monocytes, is strategically scattered throughout all tissues, including blood and other body fluids, where it serves as sentinel cells that reliably identify, via their PRR receptors, any adverse, potentially noxious, stimuli (8).

The family of Toll-like receptors (TLRs) is prominent. TLRs, of which there are currently 10 described in humans, binding to a range of PAMPs. TLR2 functions a heterodimer with either TLR1 or TLR6 and senses lipopeptides from bacteria. TLR4 binds to lipopolysaccharide of gram-negative bacteria complexed to LBP plasma protein with essential participation of another membrane protein CD14. In addition to the recognition of PAMPs, TLR2 and TLR4 have also been shown to recognise endogenous ligands DAMPs. TLR2 and TLR4 are membrane expressed and have a wide range of putative endogenous ligands which comprise HSP stress proteins, high mobility group box 1 (HMGB1) nucleoprotein and breakdown products of fibronectin, heparansulfate, and hyaluronic acid. All these danger signals are inseparable linked to trauma including surgical injury (9). The broad expression profile of TLRs and their ability to recognise many ligands that are released predominantly as a consequence of tissue injury positions TLR dependent signalling as a rapid response mechanism to tissue damage (10).

Major surgery, including cardiac surgical operations, is characterized by massive generation of DAMPs and the different exposure to PAMPs reflecting the type of surgery being more pronounced in abdominal surgery. Surprisingly, the modulation of TLR sensing system by surgery is, in comparison other clinical situation, still poorly investigated. This is true especially in cardiac surgery. We described the dynamics or TLR2 and TLR4 expression on blood monocytes and granulocytes of cardiac surgical patients undergoing CABG either with or without CPB in our previous work (11). Therefore, this observational study is aimed to substantiate our previous findings. Another aim of this study is to reconcile which surgical approach, either beating heart surgery, or cardiac surgery with CPB or modified CPB, respectively, reveals more pronounced effect on TLR expression.

Patients and Methods

Patients

Eighty-nine patients referred to the first-time coronary artery bypass grafting (CABG) surgery were enrolled in this study. All patients were well informed about purpose of this study and they confirmed their unconstrained participation by a written consent. The study project was approved by the Ethics Committee of the University Hospital in Hradec Kralove, Czech Republic. They underwent either conventional myocardial revascularization with CPB and cardioplegic arrest of the heart (on-pump), or with minimally invasive CPB (mini on-pump) or beating heart surgery without CPB (off-pump). Exclusion criteria consisted of acute inflammation, urgent operation, reoperation, combined operations, operative risk more than 5% (according to logistic Euroscore), preoperative level of serum creatinine above 130 μmol/L, hepatic disease, and malignancies. The demographic and preoperative data of our patients are shown in Table 1.

Methods

Coronary Artery Bypass Grafting with Conventional Cardiopulmonary Bypass (CPB), on-pump technique

All operations were performed via median sternotomy. Heparin was administered intravenously (4 mg/kg of body weight) to maintain an activated clotting time (ACT) above 480 s during the CPB run. The ascending aorta was cannulated by standard aortic cannula and a two-stage venous cannula was placed via the right atrium into the inferior vena cava. The CPB circuit was established using non-coated tubing, a hard shell venous reservoir (Dideco SrL, Mirandola, Italy), a cardiotomy suction device, a hollow fiber membrane oxygenator (Dideco SrL, Mirandola, Italy), a roller pump (Stöckert Instrumente GmBH, München, Germany) and 40.0 μm arterial line filter (Dideco SrL, Mirandola, Italy). The extracorporeal circuit was primed with 500 ml of Ringer's lactate, 500 ml of Rheodextran (Rheomacrodex), 5,000 IU Heparin, 80 ml of Natrium Bicarbonate ($NaHCO_3$ 8.4%), 20 ml of 10% Magnesium Sulphate, and Mannitol (at 1 g/kg of body weight) and calculated to reach hematocrit level above 0.22. The non-pulsatile pump flow was maintained at targeted rate of 2.4 l/min/m^2. At the initiation of CPB, cardiac arrest was achieved by administration of cold blood cardioplegic solution (St. Thomas solution, Ardeapharma, Sevetin, Czech Republic; ratio 4 : 1) in antegrade fashion (10–15 ml/kg of body weight). The heart was cooled topically by cold water and cardioplegia administered repeatedly every 25 minutes. The patient's temperature was allowed to drift to 34–35 °C. Distal anastomoses were performed on the arrested heart in standard manner (every patient received left internal mammary artery graft and different number of saphenous vein grafts). Proximal anastomoses were accomplished while a partial occlusion aortic clamp was applied on the already beating heart. After the termination of CPB, heparinization was reversed with protamine sulphate at a ratio 1 : 1 with Heparin.

Tab. 1: Preoperative characteristics of patients

	MINI	ON	OFF	p
No. of patients	25	30	34	
Gender (no. of females/males)	2/23	5/25	9/25	0.193
Age (years; median, quartiles)	69 (63–73)	67 (61–71)	68 (61–74)	0.807
BMI (median, quartiles)	27 (25–32)	28 (25–32)	29 (27–31)	0.748
Ejection fraction (%; median, quartiles)	60 (50–70)	61 (51–70)	60 (50–70)	0.985
Diabetes mellitus (no.)	7	10	8	0.977
COPD (no.)	4	5	9	0.93
MI (no.)	9	13	15	0.994

Legend: BMI – Body Mass Index; COPD – Chronic Obstructive Pulmonary Disease; MI – Myocardial Infarction

Coronary Artery Bypass Grafting with Minimally Invasive Cardiopulmonary Bypass, mini on-pump technique

The sternotomy, heparinization and arterial cannulation were performed in standard fashion mentioned previously. For venous drainage a 22F dual-stage venous cannula was placed via right atrium into the inferior vena cava and kinetic assisted drainage generated by centrifugal pump (KAVD) was applied. The minimally invasive CPB circuit (Minisystem Synergy, Sorin Group SrL, Mirandola, Italy) consisted of a very short internal face coated tubing (Phosphorylcholine) creating a closed loop, a venous bubble trap, a centrifugal pump, a membrane oxygenator, and 40.0 μm arterial line filter. Cardiotomy suction device was not used. The priming of CPB circuit, pump flow, temperature management and surgical technique were similar to that described in conventional CPB. Retrograde autologous blood prime was used allowing displacement and removing of crystalloid prime by draining blood volume from patient into the circuit just before beginning CPB. Protection of the myocardium during surgery (blood cardioplegia and topical cooling) was the same as in the group of on-pump technique.

Coronary Artery Bypass Grafting without Cardiopulmonary Bypass, off-pump technique

All operations were performed via median sternotomy. Heparin was administered intravenously (3 mg/kg of body weight) to maintain an activated clotting time (ACT) above 300 s during procedure. Patient was kept in normothermia. Exposure to the coronary arteries was performed with the use of suction tissue stabilizer OCTOPUS (Medtronic Inc., Minneapolis, MN, USA) and occasionally a retraction device STAR FISH (Medtronic Inc., Minneapolis, MN, USA). Target coronary arteries were snared proximally and distally using a silastic air-cushioned vascular loop RETRACT-O-TAPE (Quest Medical, Inc., Allen, TX, USA). The operating field was kept free of blood with a humidified CO_2 blower (Medtronic Inc., Minneapolis, MN, USA). Distal anastomoses were performed on the beating heart in a standard manner (every patient received left internal mammary artery graft and different number of saphenous vein grafts). A variety of commercially available intracoronary shunts were used at discretion of the operator. Proximal anastomoses were accomplished while a partial occlusion aortic clamp was applied. Heparinization was then reversed with protamine sulphate at a ratio 0.5:1 with Heparin.

Anesthesiological management has been described previously (12).

Flow Cytometry

Expression of TLR2 and TLR4 was measured on monocytes and granulocytes in venous blood samples. To distinguish between monocytes and granulocytes, staining of cell-surface TLR was performed with the combination of monoclonal antibodies as follows: TLR4 PE/CD14 PerCP/CD45 APC and TLR2 PE/CD14 PerCP/CD45 APC. TLR4 PE, clone HTA125, and TLR2 PE, clone TLR2.3 was purchased from Serotec, while CD14 PerCP, clone MΦP9 was from BD. CD45 APC, clone MEM-28 was purchased from Exbio. All samples were processed immediately upon the blood collection and the expression was measured within 2 h after the collection. A detailed method is described elsewhere (13).

Expression of the receptors was characterized with median fluorescence intensity (MFI), which was set on the basis of background fluorescence of the unstained control sample and the sample stained with isotype control.

Statistics

Statistics of flow cytometric parameters was computed to assess differences both within each group of patients and between all three groups.

Data distribution and homogeneity of variances was tested with Shapiro-Wilk test and Levene's test, respectively. To accept or reject possibility that there are no changes in any given flow cytometric parameters within a group,

values measured during and after surgery were compared to values before surgery using Friedman ANOVA along with Wilcoxon pair test or the sign test. Differences between the groups were determined by Kruskal-Wallis ANOVA or median test. Since the sampling points during surgery matched only between mini on-pump and on-pump group, additionally these groups were compared by Mann-Whitney U test or Kolmogorov-Smirnov test. To assess relationship between values measured at different time points, Spearman's rank coefficient was calculated.

Clinical parameters, which describe each group of patients, were compared using analysis of variance or Kruskal-Wallis ANOVA, and Pearson's X^2 test or Freeman-Halton extension of Fisher's exact test. Differences in duration of cardiopulmonary bypass as well as in duration of aortic clamping between mini on-pump and on-pump group were assesed with Kolmogorov-Smirnov test.

Differences were considered significant if probability (p) was lower than 0.05. Bonferroni correction was applied in case of multiple comparisons. We used Statistica 10 software (StatSoft) to perform the test and plot the results.

In tables, analyzed parameters are characterized by the median value with inter-quartile range in brackets for every single time point. Comparisons within groups are included in tables, and they are described by obtained p value.

Results

TLR4 granulocytes

The expression of TLR4 is relatively weak on granulocytes, but this feature arises from the function of TLR4 on neutrophils that outnumber other immune cells.

Even though the expression was weak, we observed not only the significant increase but also the significant decrease of the expression of TLR4 at the time points we followed in our patients. The course in expression of TLR4 was similar on granulocytes after surgery in all three groups of patients. There was a significant decrease of expression at the end of surgery in every patient. In mini on-pump and on pump patients, we could also see the gradual decrease of expression during surgery, while after the end of surgery, the expression was increasing. The highest values of expression were measured on the 3rd day after surgery. Off pump patients reached higher values on the 1st, 3rd and 7th day after surgery when compared with other groups. This difference was not statistically significant, but still surprising considering the fact that off-pump surgery supposedly activates immune cells to lesser extent than other techniques. Looking at the preoperative data, we can also exclude possibility that granulocytes in off-pump patients were activated before surgery (Tab. 3).

TLR4 monocytes

Changes in expression of TLR4 on monocytes were similar to the changes on granulocytes at the observation times. However, the difference between both populations of cells was the stronger expression of TLR4 on monocytes.

The expression was significantly decreased at the end of surgery, while on the 3rd and 7th day, the expression was enhanced with the maximum on the 3rd day after surgery in all groups of patients. This increase was significant only on the 3rd postoperative day in on-pump and off-pump patients.

Again, there was a significant decrease of expression during surgery in on-pump and mini on-pump group that might be attributed to the shedding of the TLR4 molecule from the cell surface.

Similarly to the expression of TLR4 on granulocytes, off-pump patients also reached the highest expression of TLR4 on monocytes on the 3rd day after surgery when compared with other groups of patients. However, this difference was not significant and there were no differences in any other matching time points between all three groups (Tab. 4).

TLR2 granulocytes

TLR2 as well as TLR4 agonists are potent stimulators of granulocyte activation. In connection with this effect, they also suppress granulocyte apoptosis. Likewise the ex-

Tab. 2: Intraoperative and postoperative characteristics of patients

	MINI	ON	OFF	p
No. of patients	25	30	34	
Duration of surgery (min; median, quartiles)	165 (154–205)	218 (181–255)	165 (130–190)	0.0003
Duration of bypass (min; median, quartiles)	62 (55–88)	85 (59–119)	–	< 0.05
Duration of aortic clamping (min; median, quartiles)	36 (29–41)	40 (31–57)	–	> 0.05
Anastomoses (no. median, quartiles)	2 (2–3)	3 (2–3)	2 (1–2)	< 0.0001
Sepsis (no.)	1	1	1	1
Renal insufficiency (no.)	2	2	1	0.623
Acute myocardial infarction (no.)	1	1	2	1
Multiorgan failure (no.)	1	0	0	0.281

Tab. 3: Expression of TLR4 on granulocytes

	Before surg.	Start of CPB	End of CPB	End of surg.	1st day	3rd day	7th day
Mini on-pump	11.5 (10.4–12.3)	10.7 (9.8–12.3)	9.6 (8.9–10.3)	9.5 (8.7–10)	11.6 (11.1–13.2)	14.6 (12–18.8)	12.6 (10.6–14.9)
			$p < 0.001$	$p < 0.001$		$p < 0.05$	
On-pump	13.5 (11.2–16.1)	11.4 (10.2–12.7)	10 (9.3–11.2)	10.9 (9.2–11.2)	12.6 (10.7–14.3)	15.9 (12.7–21.5)	13.3 (10.7–15.2)
		$p < 0.05$	$p < 0.001$	$p < 0.001$			
Off-pump	12.7 (11.1–14.7)	–	–	10.1 (9.1–11.6)	15.5 (13.3–17.4)	18.0 (14.2–21.7)	14.2 (12–17.3)
				$p < 0.001$		$p < 0.001$	

Tab. 4: Expression of TLR4 on monocytes

	Before surg.	Start of CPB	End of CPB	End of surg.	1st day	3rd day	7th day
Mini on-pump	20.3 (16–24.2)	15.7 (14–18.9)	13.4 (12.7–16.4)	14.0 (12.4–16.5)	20.5 (17–26.2)	25.3 (20.1–30.3)	23.9 (17.8–30)
		$p < 0.001$	$p < 0.001$	$p < 0.001$			
On-pump	22.1 (18.3–31.6)	17.7 (15.4–23)	16.5 (14.4–19.6)	15.6 (14.5–17.7)	20.1 (17.9–31.4)	26.8 (21.3–37.4)	23.3 (18–30.1)
		$p < 0.001$	$p < 0.001$	$p < 0.001$		$p < 0.05$	
Off-pump	25.4 (20.6–32.1)	–	–	18 (15.4–31.8)	25.1 (19.8–36)	35.6 (25.9–43)	30.5 (25.2–39.5)
				$p < 0.001$		$p < 0.05$	

Tab. 5: Expression of TLR2 on granulocytes

	Before surg.	Start of CPB	End of CPB	End of surg.	1st day	3rd day	7th day
Mini on-pump	12.3 (11.1–13)	10.9 (9.9–11.7)	9.4 (8.9–10.6)	9.5 (8.4–10.1)	12.1 (10.8–13.3)	15.7 (12.8–19)	12.8 (11.5–15.2)
			$p < 0.001$	$p < 0.001$		$p < 0.001$	
On-pump	14.1 (12.5–16.7)	12.2 (10.6–13.6)	9.9 (8.8–11)	9.7 (8.4–11)	12.7 (10.3–14.3)	19.8 (16.4–23.3)	12.8 (11.2–15.7)
		$p < 0.05$	$p < 0.01$	$p < 0.01$		$p < 0.05$	
Off-pump	13.3 (11.4–16.2)	–	–	9.6 (8.1–11.6)	14.9 (11.5–15.8)	18.7 (16.3–21.1)	14.2 (12.5–17.3)
				$p < 0.001$		$p < 0.001$	

pression of TLR4, the expression of TLR2 in granulocyte population is less pronounced than in the population of monocytes.

At the end of surgery, the expression of TLR4 was significantly decreased in all groups. The decrease in expression was also possible to observe during surgery in mini on-pump and on-pump patients. In sharp contrast to the profound decrease by the end of surgery, TLR4 was greatly upregulated on the 3rd postoperative day. On the 1st and 7th day after surgery, the expression of TLR was around preoperative values.

In spite of median value of the expression being lower in mini on-pump patients than in other two groups on the 3rd day after surgery, there was not statistically significant difference between all three groups (Tab. 5).

TLR2 monocytes

TLR2 is expressed on monocytes in high density. In post-operative period however, we did not see a great enhancement in the expression of TLR2 on monocytes. The course of expression of TLR2 resembled of the expression

Tab. 6: Expression of TLR2 on monocytes

	Before surg.	Start of CPB	End of CPB	End of surg.	1st day	3rd day	7th day
Mini on-pump	34.9 (25.8–48.3)	24.8 (17.9–30.1)	19.5 (15.2–24.3)	17.5 (14–25)	44.2 (37.2–54.8)	39.7 (29.7–58.5)	35.4 (26.6–49.5)
		$p < 0.001$	$p < 0.001$	$p < 0.001$			
On-pump	42.1 (32.7–49.5)	30.3 (25–37.2)	25.6 (16–33.2)	16.6 (14.3–22.6)	49.6 (34.4–62.7)	52.8 (39.9–60.2)	36.6 (27.4–48.8)
		$p < 0.001$	$p < 0.001$	$p < 0.001$			
Off-pump	44.4 (35.3–53)	–	–	21 (18.2–32.7)	52.3 (47.7–64.1)	58.3 (45.6–88.2)	47.2 (36–56.2)
				$p < 0.001$		$p < 0.05$	

of TLR4 on monocytes. There was a significant decrease in the expression during surgery and at the end of surgery in mini on-pump and on-pump group. Also in off-pump patients, the expression at the end of surgery was low compared to preoperative values. On postoperative days, TLR2 on monocytes was not up-regulated with exception of off-pump group that reached significantly higher expression on the 3rd day after surgery in comparison to preoperative values ($p < 0.05$) as well as in comparison with other two groups of patients ($p < 0.01$) (Tab. 6).

Tab. 7: Correlation of expression of TLR2 or TLR4 between preoperative values and values during and after surgery

TLR4 on granulocytes	Start of CPB	End of CPB	End of surg.	1st post day
Mini on-pump	0.50	0.50	0.58	
On-pump	0.78	0.68	0.70	
Off-pump	–	–	0.61	
TLR4 on monocytes				
Mini on-pump	0.80	0.80	0.80	0.52
On-pump	0.79	0.72	0.69	0.68
Off-pump	–	–	0.76	0.64
TLR2 on granulocytes				
Mini on-pump			0.59	
On-pump			0.61	
Off-pump	–	–	0.50	
TLR4 on monocytes				
Mini on-pump			0.61	
On-pump			0.57	
Off-pump	–	–	0.59	

Legend: The correlation is expressed using the Spearman coefficient.

Expression of TLR4 on granulocytes and monocytes displayed relationship between preoperative values and values at the end of surgery ($r_s = 0.59$ to 0.8) in all groups of patients. Moreover, there was also correlation between preoperative TLR4 values on monocytes and the values on the 1st day after surgery in all groups. In on-pump and mini on-pump patients, we could also observe relationship between preoperative values of TLR4 and the values during surgery ($r_s = 0.5$ to 0.8), even though it was weaker on granulocytes in mini on-pump group ($r_s = 0.5$) (Tab. 7).

Preoperative values of TLR2 were correlated only with values at the end of surgery ($r_s = 0.5$ to 0.61) both in granulocytes and monocytes. Although all groups showed the correlation, for off-pump patients it was the weakest ($r_s = 0.5$) (Tab. 7).

Remarkably, we also found association between expression of TLR4 and TLR2 for both populations of myeloid cells. While the correlation between TLR4 and TLR2 was significant at the end of CPB, end of surgery, and on the 1st and 3rd postoperative day in on-pump and mini on-pump patients, in off pump patients, such relationship existed only on the 1st and 3rd day in monocytes, and on the 3rd day in granulocytes (Tab. 8).

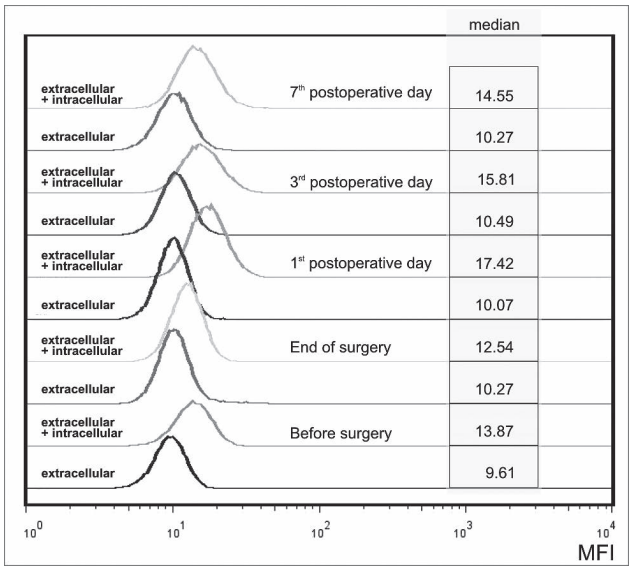
In a subgroup of 5 on-pump patients, we performed the combination of cell surface staining with intracellular stain-

Tab. 8: Correlation between TLR4 and TLR2

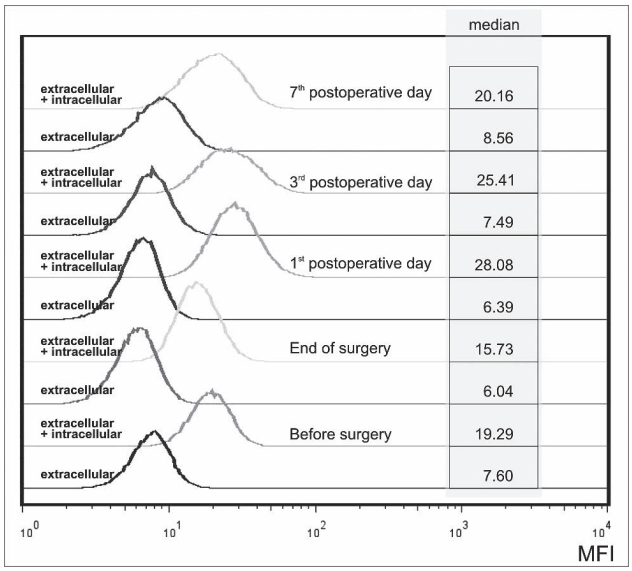
Granulocytes	End of CPB	End of surg.	1st post day	3rd post day
Mini on-pump	0.51	0.59	0.61	0.78
On-pump	0.57	0.51	0.72	0.69
Off-pump	–			0.56
Monocytes				
Mini on-pump	0.57	0.50	0.68	0.71
On-pump	0.73	0.73	0.74	0.77
Off-pump	–		0.62	0.76

Legend: The correlation between TLR4 and TLR2 is expressed using the Spearman coefficient.

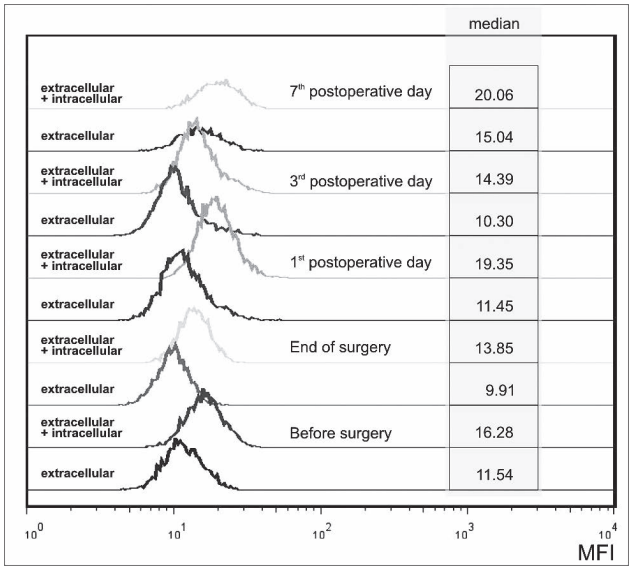
ing and compared the results with the cell surface staining only. We observed cytoplasmatic expression of TLR4 and TLR2 in both granulocytes and monocytes (Fig. 1A–D). The highest cytoplasmatic expression of TLR4 and TLR2 was measured on the 1st day after surgery in both populations of cells, even though very high expression of intracellularly localized receptor was detected in the case of granulocyte TLR2 at all sampling points (Fig. 1B). Since the cell surface expression of TLRs increased on the 3rd day after surgery, we could assume that intracellularly localized TLR was at least partially displaced to the outside surface of plasma membrane. Since TLR4 and TLR2 are also expressed on membranes inside the cells, we combined cell surface staining with cytoplasmatic staining. Following the staining of cell surface antigens, we used 1% paraformaldehyde for 15 min to fix the surface proteins. Then the treatment with 0.5% saponin enabled us to perform the intracellular staining with monoclonal antibodies (light lines). The control samples were incubated with monoclonal antibodies prior to the paraformaldehyde and saponin treatment but the intracellular staining was not performed. These samples showed the expression of TLR in outside



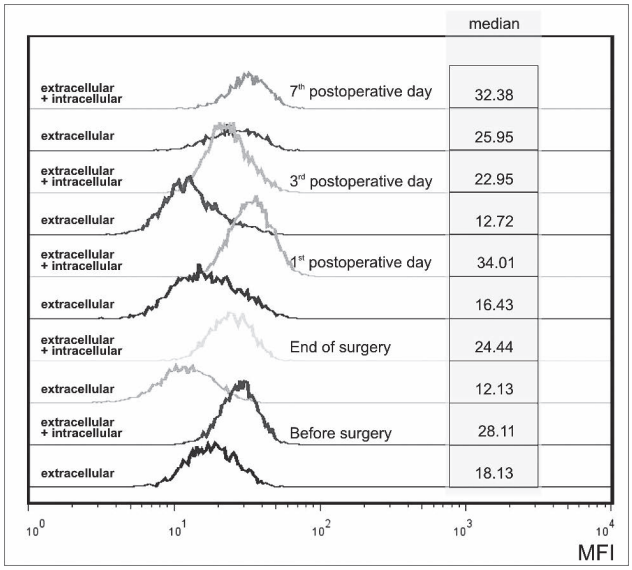
A) TLR4 in population of granulocytes



B) TLR2 in population of granulocytes



C) TLR4 in population of monocytes



D) TLR2 in population of monocytes

Fig. 1: The example of a patient whose blood samples were stained with the combination of cell surface and intracellular staining

plasma membrane (dark lines). Non-specific binding was prevented using the phosphate-buffered saline with 2.5 mM EDTA, 0.2% BSA and 5% FBS. Unstained and IgG control samples were used to reveal background fluorescence and the binding to Fcγ receptors, respectively (data not shown).

Discussion

Acute inflammation directed by the innate immune response has evolved to efficiently combat infection and is critical to host defense. However, such innate mechanisms may also be activated as a result of tissue injury resulting from pathophysiological or exogenous sources. These events lead to what is termed a sterile inflammatory response (14). Patients who underwent cardiac surgery are exposed to both infectious and noninfectious danger stimuli the later being more pronounced in majority of cases.

There is a growing body of evidence linking TLRs, particularly TLR2 and TLR4, to the deleterious inflammatory effects seen in ischemia reperfusion injury associated with myocardial infection, and trauma, including cardiac surgery. This issue is recently reviewed by Arslan et al. (10). The modulation of TLR2 and TLR4 expression on the peripheral blood monocytes and granulocytes of cardiac surgical patients with CABG was the matter of our interest of our previous research which results have already been published (11). We found that the intensity of both TLR2 and TLR4 is diminished during and after surgery. In this study we try to substantiate our previous result as only a few further studies aiming the dynamics of TLR receptors in CABG surgery have been published since the original observation of Dybhal et al. (15). In addition to our already published data on TLR4 and TLR2 dynamics in off-pump and on-pump patients, patients with modified CPB, "mini on-pump" patients were enrolled to this study.

Two different methods of CPB were compared in this study- standard CPB versus miniaturized CPB. Standard, conventional CPB, which is daily used at our department, has no special chemical or biological coat on an internal tubing system surface. On the other hand the tubes of miniaturized CPB were coated by phosphorylcholine. We wanted to analyze different systems of CPB with all standard components in this study. That is why we did not use any coated tubing system for conventional CPB.

The expression of TLR receptors was determined by highly reproducible flow cytometry separately for blood granulocytes and monocytes. Both TLR2 and TLR4 on granulocytes was highly significantly diminished in all three groups of patients at the end of surgery regardless the use of CPB. It seems from this that CPB itself is not contributing substantially to the modulation of granulocyte TLR4 and TLR2 expression. It is a very surprising observation because there is the firm consensus that CPB is a strong inducer of granulocytes priming and activation (16). Thus, the very trauma associated with CABG surgery is responsible for TLR2 and TLR4 modulation on granulocytes. The decrease in TLR2 and TLR4 expression was already significant at the end of CPB, it means, approximately 1 hour before the end of surgery suggesting that TLR2 and TLR4 down modulation is relatively early event in the course of cardiac surgery. The significantly decreased TLR2 and TLR4 expression on granulocytes at the end of surgery in patients operated with the use of CPB was reported by Hadley et al. (17). However, their group comprised six patients only who were followed up to the first postoperative day only.

The evaluation of TLR2 and TLR4 expression on granulocytes is scarce. Neutrophils are considered to be predominantly effector cells in inflammatory response. Much interest is paid to monocyte/macrophages cells. This cells population is together with dendritic cells strategically distributed in blood and tissues serving as sentinel cells sensing both exogenous and endogenous harm stimuli by PRR receptors. Blood monocytes express all TLR receptors with higher density compared to granulocytes (18). Monocyte macrophages are the rich source of proinflammatory cytokines, such as IL-1β, TNFα, which are released early during inflammation. These cells are activated in tissue by surgical injury, local oxidative stress and by microbial PAMPs, such as bacterial lipopolysaccharides penetrating from gut microflora of cardiac surgical patients with transiently impaired gut mucosa immunity. The expression of TLR2 and TLR4 on monocytes was significantly diminished as early as at the start of CPB. In addition, there was no such apparent increase in TLR2 and TLR4 expression at the 3rd postoperative day on monocytes, compared to granulocytes. Thus monocytes seem to be the first leukocyte population which is stimulated by cardiac surgery. Decrease in TLR membrane receptors is mediated by either receptor internalization or shedding. Downmodulation of membrane receptors is relevant to the cells physiology. It is very likely that in cardiac surgical patients the exposition to proinflammatory stimuli is so extensive that various antiinflammatory homeostatic mechanisms are switch on to prevent the development of overwhelming inflammatory response. Indeed, several such protective mechanisms have already been described in cardiac surgery. The increase in IL-10 level which is recognized as antiinflammatory homeostatic cytokine after cardiac surgery is consistently reported (12, 19). Monocyte/macrophage population is functionally heterogeneous. The number monocytes expressing scavenger receptor for hemoglobin CD163 is increased early after cardiac surgery (20, 21). Macrophages expressing CD163 are designated as M2c subset. They are considered to dampen inflammatory response among other by the production of IL-10 and TGF beta as well as glucocorticoid signaling. The increased TLR2 and TLR4 expression on effector granulocyte population at the 3rd postoperative day is probably induced by elevated level of proinflammatory cytokines, e.g. TNFα, which level is peaked early after cardiac surgery. Dybdahl et al. (15) in their early work found significantly decreased TLR2 expression on monocytes at

the end of surgery with significant increase at the 2nd day after surgery. TLR4 expression was only non significantly decreased early after surgery with significant increase up to 2nd day after surgery when the observation was finished. Versteeg et al. (22) followed rather heterogeneous group of patients who underwent surgery on the heart arteries. These patients were examined during surgery up to the 1st postoperative day. In concordance with our results both TLR2 and 4 expression on monocytes was significantly diminished after surgery. In contrast to our results their expression was significantly increased already at the 1st postoperative day. In our study this increase was postponed to the 3rd postoperative day. It is not possible to reconcile in this regards because in the third available work by Hadley (17) the observational period was finished again only 20 hrs after surgery. In addition, the value of this work is limited by the fact that 6 patients only were enrolled. Various surgical traumas seem differently modulate TLR expression. Hei et al. (23) reported significantly increased TLR2 and TLR4 expression on blood mononuclear cells of patients with liver transplantation already at the first postoperative day. In contrast to these results, TLR2 and TLR4 expression was significantly diminished at that time in our cardiac surgical patients.

It is very difficult to interpret our observation that the significant relationship of TLR2 and TLR4 expression both on monocytes and granulocytes especially between preoperative values and values at the end of surgery was found. Remarkably, we also found association between TLR2 and TLR4 expression for both monocytes and granulocytes. These associations seem to be more pronounced in on-pump patients in comparison with off-pump patients probably reflecting the impact of CPB itself. Relevant data already published which address these issues are not available.

Original in essence seems our determination of both extracellular and intracellular TLR2 and TLR4 expression in the populations of monocytes and granulocytes in a subgroup of on-pump patients. TLR2 and TLR4 are considered to be membrane expressed. However, we found very high expression of intracellularly localized TLR2 in granulocytes at all observational points. The highest intracellularly localized expression of TLR2 and TLR4 both in monocytes and granulocytes was reached at the 1st postoperative day when membrane expression of these receptors was similar to preoperative values being significantly diminished at the end of surgery. From this dynamics it seems unlikely that increased intracellular expression of TLR2 and TLR4 receptors is caused by receptors internalization. This increase is more likely the early physiological response reflecting body demand since the cell surface expression of TLR receptors is significantly increased at the 3rd day after surgery. We could assume that intracellularly localized TLRs are displaced to the outside surface of plasma membrane. However, the possibility that intracellularly localized TLR2 and TLR4 receptors are also serving as PRR receptors of danger signals remains to be elucidated.

Recently, gene regulation network connecting ischemia/reperfusion with systemic inflammation in cardiac surgery with CPB identified by whole blood transcriptomics was reported by Liangos et al. (24). They found the upregulation of key sensors of ischemia/reperfusion which activation initiated a concerted inflammatory response via upregulation of TLR4, 5, IL6, IL1beta/IL18, and pentraxin 3. The former components of immune response are recognized in cardiac surgery as prominent proinflammatory response elements. Pentraxin3 exerts antiinflammatory homeostatic activity (25) and is less pronounced than C-reactive protein increase after surgical procedures (26). In present study no significant differences in TLR2 and TLR4 expression between either on pump, mini on pump or off pump patients were found. Emerging data suggest that the inflammatory response is activated regardless the use of CPB (27).

In conclusion, the current concept of contact activation of leukocytes by CPB might be oversimplified. The proinflammatory response evoked by cardiac surgery is balanced by antiinflammatory and pro-survival response. The modulation of TLR2 and TLR4 expression on innate immunity cells of cardiac surgical patients described in this work is very likely the part of body effort to maintain homeostasis.

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ORIGINAL ARTICLE

THE EFFECT OF CARDIAC SURGERY ON PERIPHERAL BLOOD LYMPHOCYTE POPULATIONS

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***Summary*: Background:** Cardiac surgery using cardiopulmonary bypass (CPB) is associated with some adverse postoperative complications caused by an altered immune response. An alternative approach to cardiac surgery, operating without the use of CPB (i.e. off-pump surgery), seems to display less adverse impacts on the immune response. **Patients and Methods:** Peripheral blood lymphocytes in 40 patients undergoing cardiac surgery either with CPB ("on-pump") or without CPB ("off-pump") were followed using flow cytometry. The samples of peripheral blood were taken at five intervals: preoperatively, after termination of the surgery, on the first, on the third and on the seventh postoperative day, respectively. **Results:** The most substantial changes appeared on the first postoperative day in both subgroups of patients. While the percentage of both total T cells and $CD4^+$ T cells were decreased, the percentage of HLA-DR^+ activated lymphocytes was increased. These changes were more profound in the "on-pump" subgroup compared to the "off-pump" subgroup. **Conclusion:** Our results may suggest that the "off-pump" surgical approach reveals less adverse impact on adaptive immune responses.

Key words: *Cardiac surgery; CPB (cardiopulmonary bypass); Beating heart surgery; Lymphocytes; Flow cytometry*

Introduction

Cardiac surgical operations are followed by numerous changes in the immune reactivity (4, 18). Massive activation of innate immunity very early during cardiac surgery is elicited by the extensive exposure of this branch of immunity to "danger" signals which emanate from body integrity destruction, tissue and organ hypoperfusion, followed by an exaggerated reactive oxygen species generation and decreased barrier functions of gut mucosa to emphasize only some of many. Moreover, in "on pump" patients overwhelming contact activation of both humoral and cellular components of blood has to be added to the top of the complex list of adverse effects raised during cardiac surgery. Adaptive immunity, meaning T and B cell systems, is affected with some delay in the course of surgery, peaking in the early postoperative period. The dynamics of immune response is thus following the very nature of immune responses (17). Both activation and inhibition processes could be identified during cardiac surgery and in the course of postsurgical recovery. The optimal balance between these processes is the ultimate goal, leading to full recovery.

Two principally different attempts either using cardiopulmonary bypass (CPB) ("on-pump") surgery or without such arteficial support, meaning beating heart surgery ("off- pump") are now being used (11). It is claimed that an extensive and sometimes overwhelming systemic inflammatory response (SIRS) followed by profound immune depression leading to severe infectious complications are due to CPB (22). Based on results of some studies, it is suggested that the beating heart surgery is superior to "on pump" surgery (5, 6). However, data which are gathered in this field are contradictory. Further work is necessary to reconcile these doubts.

At least transient lymphopenia and suppression of specific immunity is induced by anesthesia and any surgical operations (13, 14). Global immunosuppression elicited by "on-pump" cardiac surgery seems to be more profound compared to other types of surgery (18). Lymphopenia, which is typically seen during an early postoperative period in "off-pump" patiens, is especially caused by the reduction of T cell populations (10, 17). Whereas the number of $CD4^+$ helper inducer T cells is significantly decreased, the number of $CD8^+$ suppressor cytotoxic T cells is not affected or is even increased in the response to the cardiac surgery. As a result, the CD4/CD8 T cell ratio is substantially decreased (13, 17, 21). There is a shift from Th1 subset activity to Th2 subset regulations mirrored by the changes in the spectrum of cytokines produced (8, 13, 15, 16, 17). Regarding natural cytotoxicity mediated by NK cells, re-

sults are contradictory. Both diminished NK cell numbers and activity or unaffected or even enhanced NK cell activity are mentioned by many authors (1, 13, 18, 19, 21).

There is the consensus that "on-pump" surgery is associated with more extensive lymphocyte activation. The expression of numerous activation markers, such as an early activation molecule (AIM) CD69 and the late activation molecules HLA-DR and α subunit of IL-2 receptor (CD25), is increased in lymphocytes of cardiac surgical patients (2, 9, 14, 22, 23, 24).

In the context of our previous work dealing with different patterns of cytokine production in "on-pump" and "off-pump" cardiac surgical patients (12), the aim of this study is focused on the changes of adaptive immunity cell substrate and the expression of activation markers on these cells in the course of cardiac surgery and during early the postoperative period.

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 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 surgery 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).

The Ethics Committee of the University Hospital in Hradec Kralove approved the study protocol. Written informed consent was given by each participant.

Blood sampling

Peripheral venous blood from an antebrachial vein was withdrawn into heparinized testing tubes manufactured by Saarstedt (Germany) at the following time points: introduction to anaesthesia (sample 1), after termination of the operation (sample 2), the first postoperative day (sample 3), the third postoperative day (sample 4), and the seventh postoperative day (sample 5).

Methods

Direct double immunofluorescence whole blood lysing method was used. Lymphocytes were stained by monoclonal antibodies purchased from Immunotech (France): the following combinations of monoclonal antibodies CD19 FITC/CD5PE, CD3FITC/CD4PE, CD8FITC/CD56PE, CD3FITC/CD69PE, CD3FITC/HLADR-PE. To identify lymphoid cells precisely, the combination of CD45 FITC and CD14 PE monoclonal antibodies were used. Samples were analyzed by FACS Calibur flow cytometer (B.D., USA) using CELLQuest software.

Statistical analysis

Changes in the relative numbers of lymphocytes within a group and between both groups ("on-pump", "off-pump") were evaluated. Data were analyzed using ANOVA and post-hoc tests. The dynamics of changes is expressed as medians. A probability (p) value < 0.05 was considered significant. Statistical analysis was performed with Statistica 5.5 software (Statsoft, USA).

Results

Significant differences in the relative number of $CD5^+$ lymphocytes were found. The relative number of $CD5^+$ lymphocytes in "on-pump" patients at the end of surgery was significantly lower compared to "off-pump" patients (61.8 % vs. 77.8 %, respectively; $p<0.01$). The nadir in the relative number of $CD5^+$ lymphocytes was reached at the first postoperative day in both groups (54.8 % in "on-pump"; 64.3 % in „off-pump, respectively). There was a gradual increase in their numbers thereafter (Fig. 1). The same results were obtained by staining with monoclonal antibody against CD3.

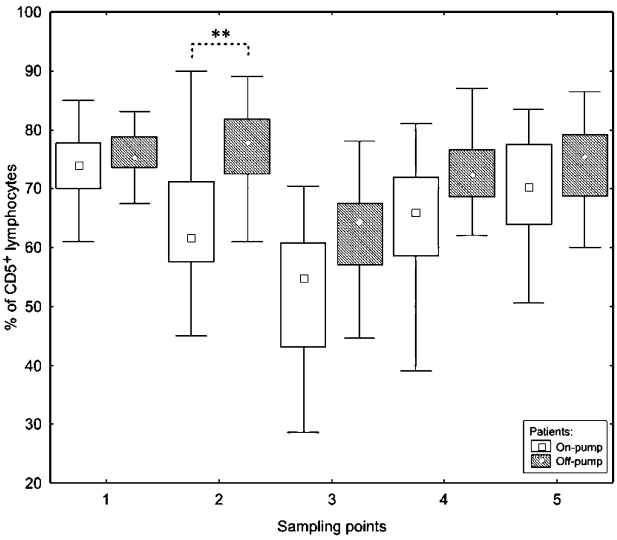

Fig. 1: The relative number of $CD5^+$ lymphocytes in cardiac surgery patients ("on-pump" and "off-pump" group) in the course of surgery and in the early postoperative period (1 – introduction to anaesthesia, 2 – after termination of the operation, 3 – the first postoperative day, 4 – the third postoperative day, 5 – the seventh postoperative day), ** probability level 0.01–0.001).

A similar pattern in the relative number of $CD4^+$ helper inducer T cells both "on-pump" and "off-pump" patients was delineated by us. The relative number of $CD4^+$ helper T cells was significantly decreased in "on-pump" patients

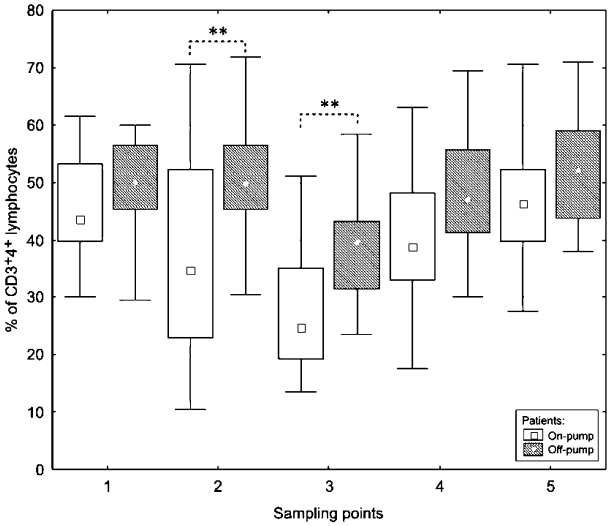

Fig. 2: The relative number of $CD3^+/CD4^+$ lymphocytes in cardiac surgery patients ("on-pump" and "off-pump" group) in the course of surgery and in the early postoperative period (1 – introduction to anaesthesia, 2 – after termination of the operation, 3 – the first postoperative day, 4 – the third postoperative day, 5 – the seventh postoperative day), ** probability level 0.01–0.001).

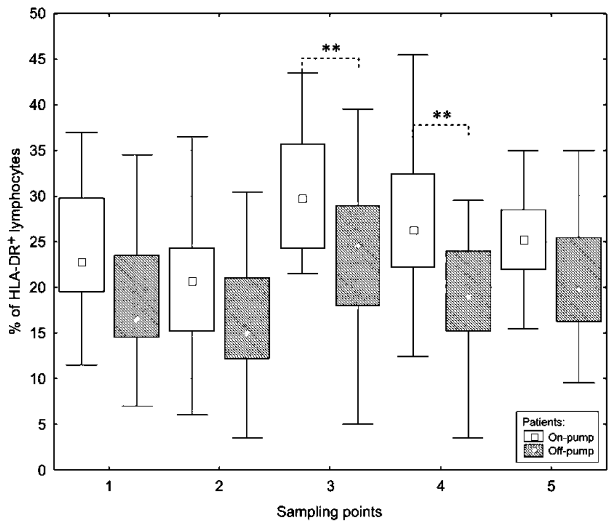

Fig. 3: The relative number of $HLA\text{-}DR^+$ lymphocytes in cardiac surgery patients ("on-pump" and "off-pump" group) in the course of surgery and in the early postoperative period (1 – introduction to anaesthesia, 2 – after termination of the operation, 3 – the first postoperative day, 4 – the third postoperative day, 5 – the seventh postoperative day), ** probability level 0.01–0.001).

(34.8 %) compared to "off-pump" patients (49.8 %; $p<0.01$) at the end of surgery. The relative number of $CD4^+$ T cells was even lower at the first postoperative day, being significantly decreased in "on-pump" patients (24.8 %) compared to 39.5 % in "off-pump"; $p<0.01$. The gradual recovery to the preoperative levels of $CD4^+$ T cells was identified in both patient groups in the late postoperative period (Fig. 2).

There were no significant differences between "on-pump" and "off-pump" patients regarding the relative number of $CD8^+$ cells. Initially, there was a non-significant increase in their numbers during surgery, followed by a decrease in their relative numbers reaching statistical significance at the 7th postoperative day for both groups.

Significant differences between "on-pump" and "off-pump" patients were not found either in the case of $CD56^+$ NK cells. The maximum in the relative number of $CD56^+$ NK cells was reached at the first postoperative day, with a subsequent decrease in both groups.

The expression of an early activation marker CD69 was not influenced by cardiac surgery, being nonsignificantly different between "on-pump" and "off-pump" patients.

In contrast to this, the expression of the late activation marker HLA-DR on lymphocytes was significantly influenced by the different surgical approaches. The dynamics of HLA-DR expression on lymphocytes was similar for both groups, reaching the maximum at the first postoperative day. The number of $HLA\text{-}DR^+$ lymphocytes at the first postoperative day was higher in "on-pump" patients (29.8 %) compared to "off-pump" patients (24.5; $p<0.01$). A similar pattern was found also at the 3rd postoperative day, being 26.3 % for "on-pump" and 19.0 % for "off-pump", respectively; $p<0.01$. The maximum of $HLA\text{-}DR^+$ lymphocytes was reached on the 1st postoperative day in both the "on-pump" and "off-pump" patients (Fig. 3).

Discussion

In agreement with others (17, 22), we found significantly lower numbers of T cells in peripheral blood of cardiac surgical patients in the postoperative period. The nadir in the relative number of T cells was reached on the 1st postoperative day in both "on-pump" and "off-pump" patients. The same pattern was found also in the case of $CD4^+$ helper inducer T cells in accordance with previous studies (4, 13, 17). The population of $CD8^+$ cells is covers predominantly cytotoxic suppressor T cells but small populations of natural cytotoxic NK cells expressing CD8 are also included. There was a gradual decrease in both $CD8^+$ cells and NK cell populations in the entire postoperative period, reaching a minimum on the 7th postoperative day, being nonsignificantly different between "on-pump" and "off-pump" patients. This pattern is in contrast to the very rapid decrease in the relative number of $CD4^+$ helper T cells which reached their minimum already on the 1st postoperative day. Based on the previous fact, there is a decrease in the value of the

immunoregulatory index as shown by others (13, 18, 21, 23). These changes are not caused by the haemodilution during surgery as proven by others (8, 21). It is suggested that both redistribution between peripheral blood and bone marrow pools, together with tissue sequestration of activated lymphocytes subsets, are reasons for this (10, 13).

We also followed the activation of lymphocytes after cardiac surgical operation. We did not found any significant changes in the expression of the C-lectin type early activation molecule CD69 in either "on-pump" or "off-pump" surgery. In contrast, there was a significant increase in the expression of a late HLA-DR activation marker in the postoperative period in both groups of patients, reaching a maximum on the first postoperative day. An increased number of activated lymphocytes in "on-pump" patients has been published by others, but such studies in "off-pump" patients are very sparse (8, 21, 22, 23). It was proven that lymphocyte activation is functionaly linked to anergy and apoptosis of T cells, especially Th1 subset helper – inducer T cells and cytotoxic $CD8^+$ T cells (4, 8, 14, 20). As a consequence of this, there is a substantial shift towards Th2 – driven immune response in "on-pump" patients as seen from cytokine patterns (3, 13, 15, 16, 17). This shift from Th1 – driven cytotoxic reactivity with many potentially adverse effects on body structures toward Th2 – driven response, culminating in much more mild humoral response and production of antiinflammatory mediators such as IL-10, has to be recognized as a principal regulatory and homeostatic mechanism to maintain body homeostasis.

One of the principal aims of our study was to discover if there are different variables, inducing lymphocyte activation, raised by different surgical approaches. Data from this are discordant. It was claimed by Abbas (1) and Gasz (9) that "on-pump" surgery is associated with more profound changes compared to "off-pump" surgery. In contrary, Diegeler (6), Blacher (2), and Franke (7) are in aggreement that activation of immune response is comparable regardless of "on-pump" or "off-pump" surgical approaches. It seems from their results that the very surgical trauma itself is the most important variable.

It could be summarized from our results that there are substantial changes in lymphocyte populations in both "on-pump" and "off-pump" patiens, being more profound in former group. The majority of these changes was found on the first postoperative day on which significantly lower level of $CD4^+$ helper T cells and a significantly higher number of HLA-DR expressing lymphocytes in "on-pump" patients were identified.

Predominantly antiinflammatory and immunosuppressive mechanisms which are typical for an early postoperative period in cardiac surgical patients are associated with enhanced risk of infection complications (4, 18, 19, 22). To overcome such transiently impared immune response in cardiac surgical patients some immunomodulatory interventions have been recently discussed (16). Our work adds some data to favour the opinion that some alternative surgical approaches could attenuate adverse effects of cardiac surgery on lymphocyte populations.

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ORIGINAL ARTICLE

RANK/RANKL EXPRESSION IS INDUCED BY CARDIAC SURGICAL OPERATION

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Summary: Background: Cardiac surgery provokes a systemic inflammatory response in any patient. This complex body reaction involves also RANK/RANKL molecules which have been recently identified as principal regulators of bone metabolism. Aims: To follow the changes in the expression of RANK/RANKL molecules on innate immune cells of cardiac surgical patients. Patients and Methods: Twenty-six patients undergoing cardiac surgical were assigned to undergo coronary artery bypass grafting using either cardiopulmonary bypass ("on-pump") or modified "miniinvasive on-pump". The expression of RANK/RANKL was performed by flow cytometry. Results: Significantly increased expression of RANK on monocytes of "miniinvasive on-pump" patients was found at the 1st, the 3nd, and 7th postoperative days. The similar pattern was found also for monocyte RANKL expression. In addition, RANKL expression was significantly increased at the 3rd postoperative day in "on-pump" patient. No significant differences between "miniinvasive on-pump" and "on-pump" cardiac surgical patients were found. Conclusion: The expression of both RANK and RANKL molecules is significantly enhanced on monocytes of "miniinvasive on-pump" cardiac surgical patients.

Key words: *RANK; RANKL; Monocytes; Cardiac surgery; Cardiopulmonary bypass*

Introduction

Cardiac surgical operation provokes a systemic inflammatory response in any patient. This inflammatory response is a result of very complex interplays based on both inherited individual predispositions and many variables including extent of body trauma, impact of cardiopulmonary bypass, and ischemia-reperfusion injury to list the most important (4). Such adverse conditions are accompanied by the development of danger patterns which are identified by a limited number of so called pattern recognition receptors (PRR). Both immune cells and cells of non immune origin are activated via PRR receptor with subsequent release of various proinflammatory mediators, including cytokines with proinflammatory activities, such as TNFα, IL-1, and chemokines. Their contribution to the development and maintainance of systemic inflammatory response (SIRS) in cardiac surgical patients is well established now (2, 14, 16). The modulation of both innate (9, 11) and specific immune response (7) were followed in cardiac surgical patients also by our group.

The impact of cardiac surgery is really pluripotent affecting numerous body systems. However, although the participation of inflammatory mediators in the cardiac surgery has become widely recognized, identification and characterization of other actors of inflammation is still awaited. Receptor activator of nuclear factor-κB ligand (RANKL) and its receptor RANK are among such promising molecules. These molecules are members of TNF receptor superfamily. They are either expressed as membrane receptors or are shed in a soluble form into the body fluids. These factors have previously been indentified as essential mediators for paracrine signalling in bone metabolism (12, 17). However, their contribution to the body homeostasis is much complex. It has recently been confirmed that RANK and RANKL are also involved in modulation of the immune response through interaction with both innate and adaptive immunity cells, such as dendritic cells, macrophages, T and B cells, respectively (1, 3).

Furthermore, there is an increasing number of reports regarding RANK/RANKL involvement in some cardiovascular disorders such as acute coronary syndrome (18), acute myocardial infarction and heart failure (6). However, there is an apparent lack of informations to which extent is RANK/RANKL signalling system modulated in patients undergoing cardiac surgery. We found in this our pilot study that there was a significant increase in the expression of both RANK and RANKL on monocytes of cardiac surgical patients operated using „miniinvasive" cardiopulmonary bypass during an early postoperative period.

Patients

Twenty-six patients (4 females and 22 males) were enrolled to this study. They were assigned by the cardiac surgeon outside of research team to undergo coronary artery bypass grafting (CABG) using either cardiopulmonary bypass (CPB), „on-pump" surgery or miniinvasive CPB, „miniinvasive on-pump" surgery. Thirteen patients underwent „on-pump" surgery (3 females, 10 males; mean age 66.2 ± 8.3 years). Thirteen patients underwent „miniinvasive on-pump" surgery (1 female, 12 males; mean age 65.8 ± 8.6 years).

Patients in both groups were comparable in age and preoperative ejection fraction. In „on-pump" patients ejection fraction was 61, range 53.5 to 68.3; median of anastomoses was 2.5. In „miniinvasive on-pump" patients ejection fraction was 60, 49.5 to 70; median of anastomoses was 2. No significant differences were found between groups regarding duration of CPB.

The exclusion criteria were concomitant surgery (valvar or aortic), an emergency procedure, patients with local or systemic infection or inflammation, severe left ventricular dysfunction (ejection fraction < 30 %), renal failure (serum creatinine >180 $\mu mol.l^{-1}$ or active renal replacement therapy). The potential enrollee needed to meet the criteria for „on-pump", and „miniinvasive" CPB procedures.

Elective patients discontinued antiplatelet agents, aspirin 100 mg in one daily dose, at least five days prior to surgery. Each subject passed a sreening examination including medical history, physical examination, blood and urine test, ECG, X-ray of the chest and echocardiography.

Anaesthetic management

Food and fluid intake was discontinued at midnight on the day preceding surgery.

Anaesthesia was induced with intravenous thiopenthal or midazolam and sufentanyl, muscle relaxation with cisatracurium and was maintaned by infusion of cisatracurium, sufentanyl and propofol. Isoflurane was added in oxygen.

All patients were monitored according to general protocol used worldwide during open heart procedures. Median sternotomy was a routine surgical approach in all cases. The left internal mammary artery and great saphenous vein were harvested.

„On-pump" surgery

After median sternotomy and pericardotomy cardiopulmonary bypass was established by standard aortic cannulation and two-stage venous cannulation of the right atrium. Target ACT time was over 480 seconds. Cardiac arrest was instituted by antegrade infusion of cold crystalloid cardioplegia (St. Thomas solution, Ardeapharma, Sevetin, Czech Republic) or cold blood cardioplegia (blood to St. Thomas solution in ratio 4:1), repeated every 20 minutes, and topical cooling for myocardial protection were employed.

The extracorporeal circuit consisted of membrane oxygenator (Polystan Safe Maxi, Maquet Cardiopulmonary AG, Hirrlingen, Germany) and roller pump with non-pulsatile flow (Stőckert S3, Sorin Group, Műnchen, Germany). Oxygenator and tubing were primed with a mixture of Hartmann's solution, 10% Rheodextran solution (molecular weight 40,000), 10% Mannitol solution, 8.4 % Sodium bicarbonate, Magnesium sulphure, 5,000 IU of heparin. Normothermic perfusion with calculated blood flow 2.4 $ml.l^{-1}.m^{-2}$ was used.

Once completing all distal anastomoses, the aortic cross-clamp was removed and the proximal anastomosis were performed with tangential aortic clamp.

„Miniinvasive on-pump" surgery

„Miniinvasive on-pump" surgery was established using a small 22F two-stage venous drainage and ascending aortic return. Minisystem Synergy Sorin® (Dideco S.p.A., Mirandola, Italy) was used.

Oxygenator and tubing were primed with a mixture of 500 ml Ringer's lactate, 5000 IU heparin, 80 ml natrium bicarbonate ($NaHCO_3$ 8,4 %), 20 ml magnesium sulphate 10 %, manitol 1 g/kg body weight.

Normothermic perfusion with target ACT above 480 s and Calafiore cardioplegic arrest was used. 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.

Ethics Commitee approval

The study protocol was approved by the Ethics Committee of the University Hospital in Hradec Králové. All participants were informed in detail about the purpose of the study both orally and in writing. They were free to ask any questions. All active subjects have given written informed consent.

Blood sampling

Peripheral venous blood from an antebrachial vein was withdrawn in the operating room and in the intensive care unit. Samples were collected into tubes Vacutainter treated with lithium heparin, manufactured by Becton Dickinson, UK.

In all „on-pump", and „miniinvasive on-pump" groups of 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
2) the termination of operation
3) the first postoperative day
4) the third postoperative day
5) the seventh postoperative day

Methods

Double immunofluorescence standard whole blood staining method was used. Briefly, 25 µl of heparinized venous was incubated with given pair of monoclonal antibodies (2x3 µl) for 20 minutes at room temperature. After subsequent lysis (10 min) of red blood cell (Optilyse C, Immunotech, France) samples were washed by buffered saline solution (PBS) and resuspended in PBS with azide. Following combination of monoclonal antibodies labeled either with fluoresceine isothiocyanate (FIC) or phycoerythrine (PE) were used: CD45-FITC/CD14-PE, isotypic control IgG1-fITC/IgG2a-PE (Immunotech, France). Monoclonal antibodies reacting with RANK (CD265, Receptor Activator of NFκB) was IgG2a rat monoclonal antibody, clone R12–31 PE purchased from eBioscience, USA. Monoclonal antibodies reacting with RANKL (CD265, Receptor Activator of NFκB Ligand) was IgG2b mouse monoclonal antibody, clone MIH24 PE purchased from eBioscience, USA. Measurements were performed using FACSCalibur flow cytometer and data acquired by CellQuest software (BD Bioscience, NY, USA). Lymphocytes, monocytes and granulocytes were identified on the basis of different CD45 v. CD14 expression (leukogate). Results of flow cytometric analysis were expressed as a median fluorescence intensity (MFI) for a given population.

Statistical analysis

Within group differences were evaluated by comparison of RANK/RANKL expression separately for monocytes and granulocytes expressed as MFI using Friedman ANOVA and Wilcoxon pair test. Differences between the groups of patients were tested using Kruskal-Wallis ANOVA. Bonferroni correction was applied when Wilcoxon test and Kruskal-Wallis ANOVA were used for multiple comparisons. Clinical data were analyzed by Fisher exact test, Mann-Whitney U test and t-test.

Differences were considered significant at $p<0.05$.

Data are expressed as medians and interquartile ranges in plots. Plots also display the range of non-outliers values.

Results

Dynamics in the expression of RANK (CD265) separately for monocytes and granulocytes were followed in „on-pump“ and „mini on-pump“ cardiac surgical patients after surgery and during an early postoperative period. Results are expressed as percentage of changes of RANK MFI and compared to the preoperative value which is considered as baseline. The expression of RANK on both monocytes and granulocytes was decreased after operation being significant in former one followed by significant increase during an early postoperative period in „mini on-pump“ patients only, being without any change in „on-pump“ patients. These changes were more prominent in monocytes compared to granulocytes. There was no significant differences between „on-pump“ and „mini on-pump“ patients. Results are show in Fig. 1, 2.

Dynamics in the expression of RANKL (CD254) are shown in Figs. 3 and 4. There were no significant changes in the expression of RANKL on granulocytes of cardiac surgical patients. In contrast, monocyte RANKL expression was significantly increased after surgery and during postoperative period in „mini on-pump“ patients, being increased at the 3rd postoperative day in „on-pump“ patients as well. There were no significant differences between patients groups suggesting that the very surgical trauma is responsible for the induction of RANK/RANKL on leukocytes of cardiac surgical patients being minimally influenced by CPB.

Discussion

Current view of an inflammatory response induced by cardiac surgery is more and more complex. New markers are followed not only to understand better to the pathophysiology of this reaction but their putative clinical value is also tested. RANK/RANKL molecules and the decoy receptor osteoprotegrin represent a novel triad with pleiotropic effects on bone metabolism, immune system, and inflammatory response (12, 17). Recently, it has been found that RANK/RANKL regulatory axis have an important role in the immunopathogenesis of vascular diseases.

Sandberg et al (18) reported increased expression of RANKL on monocytes of unstable angina patients. Furthermore, RANKL enhanced the release of monocyte chemoattractant protein in mononuclear cells from unstable angina, and promoted matrix metaloproteinase activity. Persistent inflammation appears to play a role in the development of heart failure. In addition to TNFα, several other members of TNFα or TNFαR superfamilies such as RANK/RANKL also could be involved in the development of myocardial failure. Indeed, Ueland et al (20) found increased systemic expression of both RANKL on T cells and sRANKL in serum of patients with myocardial failure.

To underline a broad physiological role of RANK, its expression is ubiquitous comprising skeletal muscle, liver, gut, thymus, adrenal gland. It is also expressed on a majority of immune cells, such as dendritic cells, monocytes, B cells, NK cells, and granulocytes (3). RANKL is expressed on dendritic cells, T cells, monocyte/macrophage cells. Expression of both RANK and RANKL is inducible in the presence of proinflammatory cytokines, such as TNFα, IL-1β, GM-CSF, and chemokines. RANKL expression is further induced by glucocorticoids (19).

Numerous proinflammatory stimuli are raised by cardiac surgery as a result of local trauma, cardiopulmonary bypass as well as pulmonary and myocardial reperfusion (4). In an attempt to reduce the inflammatory response which is inseparable linked to any cardiac surgery, procedures either avoiding cardiopulmonary bypass or mini-

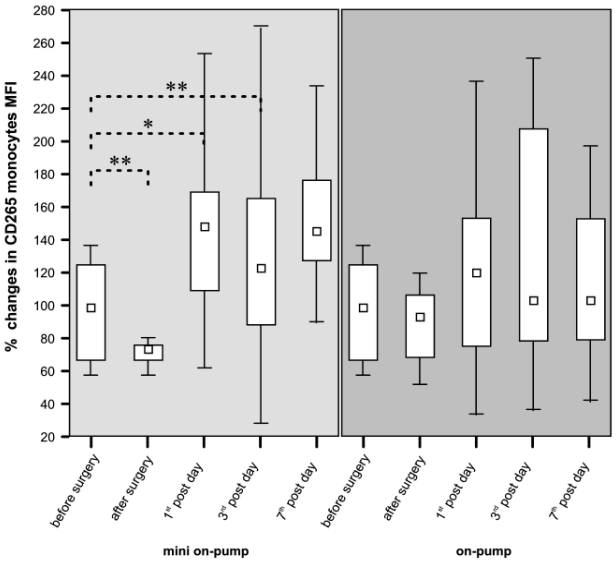

Fig. 1: Comparison of % changes in CD265 monocyte MFI between mini on-pump patients and on-pump patients.

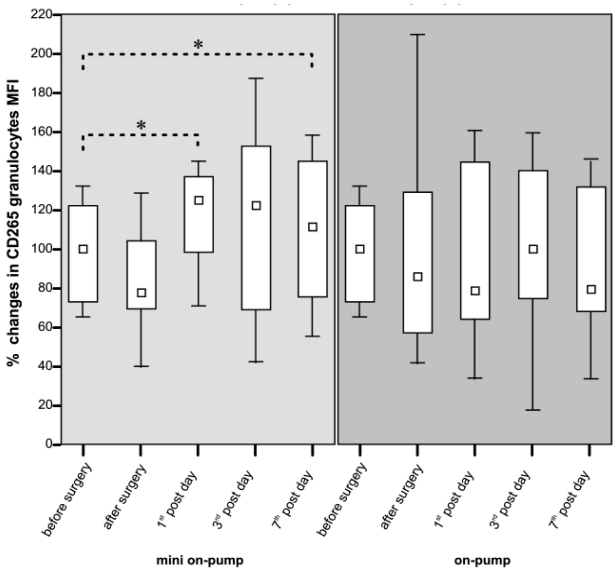

Fig. 2: Comparison of % changes in CD265 granulocyte MFI between mini on-pump patients and on-pump patients.

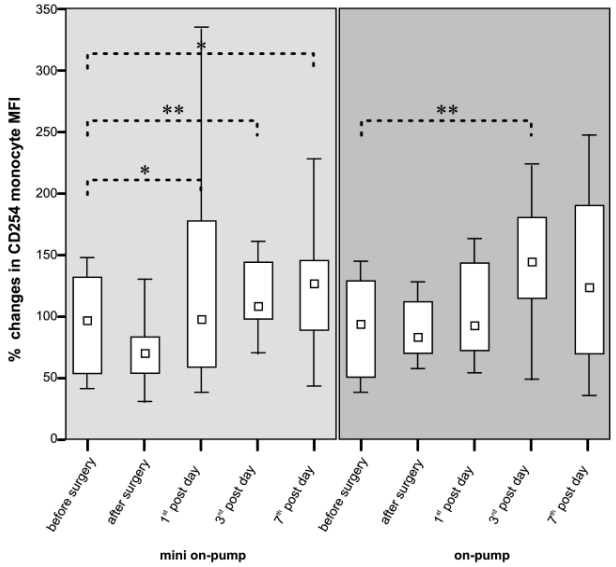

Fig. 3: Comparison of % changes in CD254 monocyte MFI between mini on-pump patients and on-pump patients.

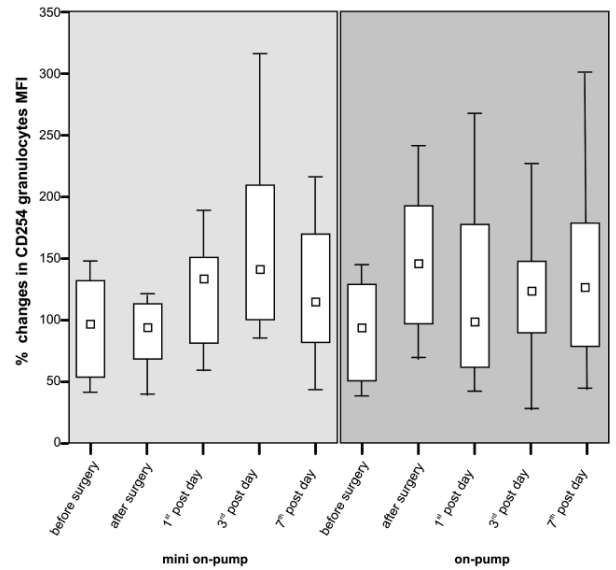

Fig. 4: Comparison of % changes in CD254 granulocyte MFI between mini on-pump patients and on-pump patients.

mizing its adverse effects are now extensively studied (5). Our study was addressed two aims. The first one was to follow the changes in the expression of RANK and RANKL on monocytes and granulocytes of patients undergoing cardiac surgery. The second aim was to test wheather there are differences in the RANK/RANKL expression in patients undergoing CABG using standard CPB or modified „miniinvasive“ CPB. Suprisingly, there was more significant dynamics in the expression of both RANK, RANKL in „miniinvasive“ CPB patients compared to „standard“ CPB patients in whom only nonsignificant changes were found.

Cells of monocyte/macrophage origin are considered as sentinel cells sensing danger signals by their surveillance receptors, such as TLR receptors, in the onset of inflammatory reaction. In opposite, granulocytes are the principal effector inflammatory cells. Based on this paradigm the expression of both RANK and RANKL is significantly increased on monocytes of cardiac surgical patients after surgery and during postoperative period. This phenomena is linked to overall monocytes activation as other surface molecules with both proinflammatory potential such as TLR receptors (10) and antiinflammatory capacity such as sca-

venger receptor for hemoglobin display the similar pattern (8).

As data regarding changes in the expression of RANK/RANKL during surgery and in an early postoperative period are entirely absent it is difficult to disscus them in the context of other works. Increased RANK/RANKL expression could contribute to inflammation, leukocyte recruitment and matrix degradation as described in acute coronary syndrome by Sandberg et al (18). This was clearly proved by an excellent clinical and experimental study by Poubelle et al. (15) focused on neutrophils RANK/RANKL expression in rheumatoid arthritis. They found that neutrophils influenced by proinflammatory substances are expressing both RANK/RANKL. RANK/RANKL expression is induced by TNFα, GM-CSF, and IL-4 *in vitro*.

It might be hard to draw a firm conclusion from this pilot study. It is apparent from our results that RANK/RANKL expression, especially on monocytes, is modulated by cardiac surgical operation. Wheather it is the regulatory attempt to modulate inflammatory response as shown e.g. in animal model of CD40L – deficient mice (13) or it is the only epiphenomena remains to be established.

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ORIGINAL ARTICLE

THE EXPRESSION OF CD38 ADP-RIBOSYL CYCLASE ECTOENZYME IN IMMUNE CELLS OF CARDIAC SURGICAL PATIENTS

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***Summary*: Background:** This study was aimed at following the changes in the expression of CD38 ADP-ribosyl cyclase ectoenzyme on peripheral blood immune cells of patients undergoing cardiac surgical operations. **Patients and Methods:** The expression of CD38 on lymphoid and myeloid cells was determined by immunofluorescence and flow cytometry in forty cardiac surgical patients assigned to surgery either using ("on-pump", n=20) or without the use ("off-pump", n=20) of cardiopulmonary bypass. **Results:** There was a very rapid upregulation of CD38 expression in "on-pump" patients, becoming significant at declamping of aorta ($p<0.01$) for myeloid cells and at the weaning from CPB ($p<0.001$) for lymphocytes. The increased expression of CD38 on lymphocytes in "off-pump" patients was prolonged for the entire observation period. However, significant differences in the expression of CD38 between "on-pump" and "off-pump" patients were not found either in lymphoid or myeloid cells. **Conclusion:** CD38 expression in immune cells of cardiac surgical patients is upregulated early during surgery, providing additional activation stimuli to the cell substrate of the inflammatory response induced by cardiac surgery.

Key words: *Cardiac surgery; Immune cells; Activation; CD38; Expression*

Introduction

Numerous events, which may results in the onset of an overt systemic inflammatory response, are inevitable induced in the course of cardiac surgery (12). Among these potentially harmful events, many stand out as the most clinically relevant to the very nature of surgical injury and the inevitable mechanical manipulation of the heart muscle. In addition, the contact of both humoral and cellular components of blood with artificial surfaces together with transient endotoxemia results in most cases from splanchnic hypoperfusion, and last but not least, from ischemia. Reperfusion injury of the heart and the lungs skew bodily regulations towards inflammation (1, 4, 18). Massive, sometimes exaggerated, immune cell activation is a characteristic feature of this process. Cellular activation is inseparably linked to the calcium mobilisation in which the CD38 molecule is clearly involved. Indeed, it has now been firmly established that CD38 is a multi-functional enzyme catalyzing the metabolism of two distinct Ca^{2+} messengers, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (14).

CD38 is a 42 kDa type II transmembrane glycoprotein with its enzymatic active site located outside the cell (6). Thus, CD38 is an extracellular enzyme or an ecto-enzyme, similar to some other leukocyte markers, including CD13, CD26, CD39, CD73 and CD157 (5). CD38, originally identified to be expressed in cells of lymphoid lineage and displaying stage – related variations, has much broader expression, regarding nowaday knowledge.

It is ubiquitously expressed in virtually all tissues. It is present not only on cell surfaces but also in various intracellular organelles, including the nucleus (3). It now appears that CD38 plays multiple roles in regulating the survival, activation, differentiation, migration and death of immunocytes (20). Indeed, CD38 expression profiles, as defined by staining with anti CD38 antibodies, are still widely used as diagnostic and prognostic markers for human diseases such as AIDS and chronic B-cells leukemias (17).

Our aims were to follow the dynamics of the expression of the CD38 molecule on lymphoid and myeloid cells in the peripheral blood of patients undergoing cardiac surgery either with ("on-pump") or without ("off-pump") the use of cardiopulmonary bypass. Another goal of our study was to discover if there are significant differences in the expression of CD38 between "on-pump" and "off-pump" patients.

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 for first-time coronary artery bypass grafting 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 surgery 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 100 mg of aspirin in one daily dose, which was stopped five days before the operation. Patients treated with anti-inflammatory agents, either steroids or NSAID, were excluded from the study, as were patients with serum creatinine ≥ 130 μmol/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 study protocol was approved by the Ethics Committee of the University Hospital in Hradec Králové. All active subjects had given informed written consent.

Cardiac surgical procedure

Cardiopulmonary bypass, "off-pump" technique and anesthesiological management have been recently described in detail elsewhere (13).

Blood sampling

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

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

Double immunofluorescence standard whole blood staining method was used. Briefly, 25 μl of heparinized venous blood was incubated with the given pair of monoclonal antibodies (2x3μl) for 20 minutes at room temperature. After subsequent lysis (10 min) of red blood cell (Optilyse C, IMMUNOTECH, Marseille, France) samples, they were washed with buffered saline solution (PBS) and resuspended in PBS with azide. The following combinations of monoclonal antibodies were used: CD45-FITC/CD14-PE (LeukoGate) to distinguish between myeloid cells and lymphoid cells, isotypic control IgG1-FITC/IgG1-PE, CD45-FITC/CD38-PE monoclonal antibodies which were purchased from IMMUNOTECH, Marseille, France. Measurements were performed using FACSCalibur flow cytometer and data acquired by CellQuest software (BD Bioscience, NY, USA). Lymphocytes and myeloid cells were identified on the basis of different CD45 v. CD14 expression (leukogate). No attempt was made to distinguish between granulocytes and monocytes. Results of flow cytometric analysis were expressed as a percentage of positive cells in the population of lymphocytes and in the population of myeloid cells.

Statistical analysis

We analyzed changes in the relative numbers of immune cells expressing CD38 in both groups of patients ("on-pump", "off-pump"). Samples taken at the introduction to anesthesia were considered as a reference. Differences between "off- and on-pump" patients were also evaluated.

Data were analyzed using two-way ANOVA for repeated measures with Fisher LSD test and Dunnett test for multiple comparisons. To exclude the confounding effect of different ages and sexes in both groups, unpaired t-test and Fisher's exact test were performed.

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

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

Results

Expression of the CD38 molecule on lymphoid cells

In "on-pump" patients, the change in the expression of CD38 was already very rapid reaching significant increase ($p<0.001$) at the weaning from CPB compared to preoperative levels. This increase continued through the finishing

of surgery and the 1st postoperative day, followed by a decline to the preoperative levels at the 3rd and 7th postoperative days (Fig. 1).

In "off-pump" patients, the expression of CD38 was significantly increased ($p<0.01$) at the 1st postoperative day and remained significantly increased for the rest of the observation period (Fig. 1).

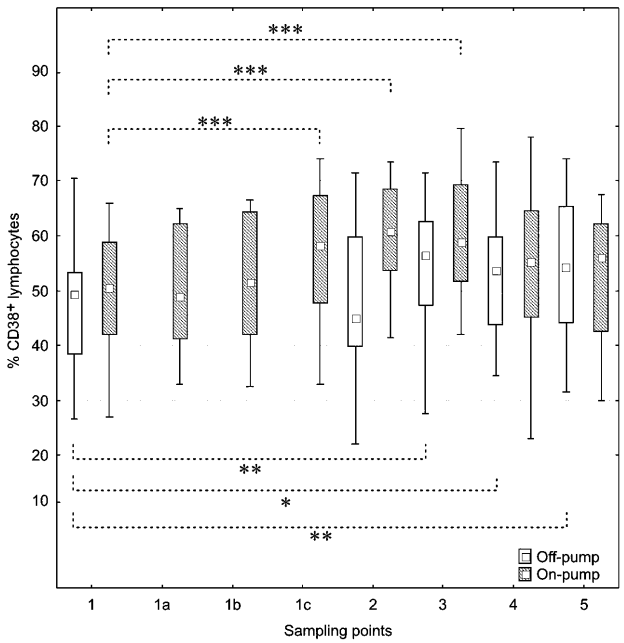

Fig. 1: Relative percentage of CD38 positive lymphocytes in cardiac surgical patients.

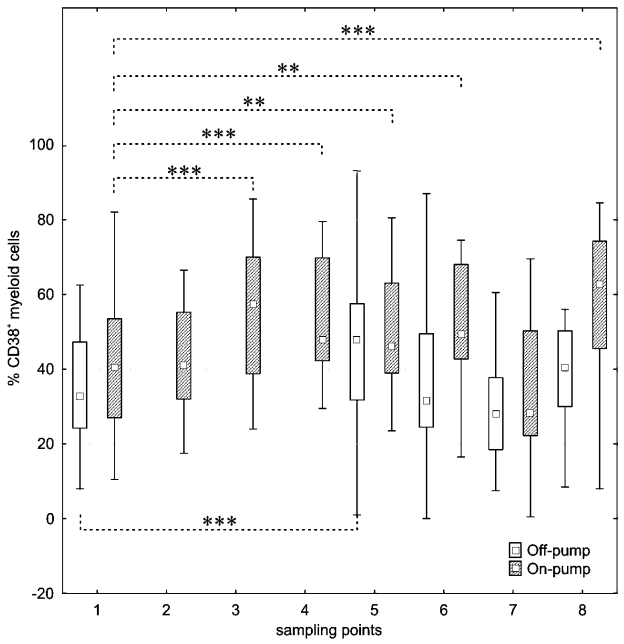

Fig. 2: Relative percentage of CD38 positive myeloids cells in cardiac surgical patients.

Comparing the dynamics of CD38 molecule expression between "on-pump" and "off-pump", a significant difference was not found. There was only a trend towards higher expression of the CD38 molecule in "on-pump" patients.

Expression of the CD38 molecule on myeloid cells

In "on-pump" patients the changes in the expression of CD38 in myeloid cells arrived even quicker, at declamping of the aorta, compared to lymphoid cells ($p<0.01$). This increase persisted almost through the whole observation period except the 3rd postoperative day (Fig. 2).

In "off-pump" patients, the dynamics of CD38 expression is rather monotonous compared to "on-pump" patients, being significantly higher only at the finishing of surgery ($p<0.001$). The percentage of CD38 expressing myeloid cells during the postoperative period was similar to the preoperative values (Fig. 2).

Comparing the dynamics of CD38 molecule expression between "on-pump" and "off-pump", a significant difference was not found. There was only a trend towards higher expression of CD38 molecule in myeloid cells in "on-pump" patients.

Discussion

Broadly expressed multifunctional ectoenzyme CD38 is physically associated with cell surface receptors that mediate signalling cascades (14). It is seen early during differentiation of immune cells on various types of progenitor cells. Subsequently, its expression is heavily upregulated in immune cells after their appropriate stimulation. CD38 principally participates in metabolism of two distinct Ca^{2+} messengers, cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). The former is a cyclic nucleotide that modulates the ryanodine receptor and mobilizes the endoplasmic Ca^{2+} stores at neutral or alkaline pH. NAADP is structurally distinct from cADPR and targets separate Ca^{2+} stores, for example, acidic organelles like lysosomes (14).

CD38 regulates inflammatory responses by modulating the activity of both leukocytes and nonhaemopoietic cells in the inflammed tissue. Enzymatic generation of cADPR by CD38 regulates intracellular calcium release and calcium influx during neutrophil activation, including their adhesion and chemotaxis (2). This is very relevant to our cardiac patients, as their blood cells granulocytes are heavily stimulated during surgery both by the contact with artificial surfaces in "on-pump" patients or by numerous proinflammatory stimuli that are raised in all cardiac surgical patients irrespective of the surgical strategy used.

Many parameters of granulocytes describing their activation status have already been identified and published. Upregulation in the expression of an activated form of leukocyte β_2 chain integrin CD18 recognized by the MEM-148 monoclonal antibody was found in our previous study (11). Another activation marker of phagocytic cells, high affinity

receptor for Fc part of IgG immunoglobulins (FcγRI, CD64) determined by a novel quantitative approach utilizing Leuko64^{TM} kit, is also induced by cardiac surgery, as we have shown (9).

We believe, in accordance with the suggestion of Partida-Sanchez et al (20) who utilized infection in the modelling of CD38 activity, that inflammatory cells such as neutrophils constitutively express low levels of CD38 on their surfaces. However, under normal conditions in vivo, neutrophils produce only very small quantities of cADPR because the substrate (NAD^+) is limiting (7). If neutrophils encounter free NAD^+ in their extracellular environment, as might be expected if cell damage has occurred, then the neutrophils will be able to produce cADPR in much larger quantities. Increased cADPR will then allow the neutrophils to respond to various danger signals, including chemoattractive compounds, by activating plasma membrane ion channels. This sustained calcium signal will allow the neutrophils to activate and migrate. When injury or infection is resolved, extracellular NAD^+ levels would return to baseline, cADPR production by the neutrophil would be reduced and proinflammatory signalling throught the various surface receptors would be attenuated.

The physiological functions of CD38 are clearly widespread. Not only innate immunity in its inductory and regulatory arms but also adaptive immune response is regulated by the CD38 molecule (15, 19). Regarding its role in the differentiation and activation in B cells lineage antigen, the driven immune response of mature B cells is influenced by the activity of CD38. Such cells express CD38 and undergo a few rounds of replication before being recruited to germinal centres within secondary follicles. These cells then undergo the process of isotypic switching and somatic hypermutation in this microenvironment. CD38 expression is now appreciated as a prognostic marker in some B lymphoproliferation disorders (16).

CD38 functions in T cells are mediated by cell surface association with TcR CD3 complex receptors and other membrane molecules. Little expression of CD38 is seen in the resting T cells. However, CD38 is rapidly upregulated upon lymphocytes activation, as also evident from our results. CD38 ligation induces activation of transcription factors involved in the development of inflammatory responses, such as NFκB. Activation of cells via CD38 leads to increased production of cytokines, especially INFγ by T cells (22). Cardiac surgery also leads to T cell activation (8). In our previous work, the percentage of HLA DR expressing lymphocytes in "on-pump" patients was significantly higher compared to "off-pump" patients on the 1st and 2nd postoperative days (8). It was a surprise for us to see that the number of CD38 positive lymphocytes in "off-pump" patients was significantly increased through the entire observation period in comparison to "on-pump" patients in whom the number of CD38 positive cells was already returning back to the preoperative value on the 3rd postoperative day.

In conclusion, we are in harmony with the opinion of others utilizing infection as a model of inflammation (20). We propose that CD38 in cardiac surgical patients works in a fashion ressembling surveillance receptors such as Toll-like receptor activity (10). When cell damage occurs, ecto-enzyme CD38 is activated by increased substrate availability and produces significant quantities of calcium – mobilizing metabolites that can modulate proinflammatory activities of a complex cell substrate of inflammation raised in cardiac surgical patients by the presence of danger signals (23). When the cellular damage has been controlled, metabolites produced by CD38 molecule activity regulating calcium mobilization in cells are reduced and proinflammatory activities of cells are attenuated. CD38 does not recognize danger signals directly as regular pattern recognition receptors do. Instead, CD38 acts essentially as a sensor for endogenous cell damage and necrosis.

CD38 is now the focus of interest as a promising target to modulate inflammatory response (21). Several compounds with the potential to modulate CD38 activity are now being tested exploiting *in vitro* and animal models. Based on the substantial progress in this field, it is plausible that CD38 agonists could enrich therapeutical armamentarium of all physicians coping with undesired inflammatory response in their patients, including cardiac surgeons.

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Clinical Study

The Effect of Conventional and Mini-Invasive Cardiopulmonary Bypass on Neutrophil Activation in Patients Undergoing Coronary Artery Bypass Grafting

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Interleukin-10 (IL-10) is considered to be a cytokine with potent anti-inflammatory properties, which have been previously linked to increased incidence of sepsis. The level of IL-10 is elevated by cardiac surgery when cardiopulmonary bypass (CPB) and methylprednisolone are used. In our study, we compare the level of IL-10, IL-10 Receptor (IL-10R), and percentage of neutrophils between two groups of cardiac surgical patients undergoing Coronary Artery Bypass Grafting, both of which were not given methylprednisolone. The first group was operated with conventional CPB, while the second group was operated with minimally invasive CPB (mini-CPB). We detected enhanced level of IL-10 during surgery and at the end of surgery in both groups of patients. While no correlation between IL-10 and IL10R was found, IL-10 was positively correlated with increased percentage of neutrophils at the time points when the level of IL-10 peaked.

1. Introduction

Cardiac surgery is connected to profound inflammatory response, characterized by increased level of both proinflammatory and anti-inflammatory mediators. Cardiopulmonary bypass (CPB) is considered to be a potential trigger of cytokine release, therefore, the impact of cardiac surgery on morbidity and mortality is often correlated with the use of CPB [1, 2]. Surgery conducted with the use of CPB is accompanied by ischemia-reperfusion injury, and above that, CPB also represents the environment where blood cells become activated by contact with artificial surfaces, direct air-contact, and also nonturbulent flow. The effort to remove the harmful effect of conventional CPB has led to a development of new surgical devices and techniques. Mini-CPB, that has recently been introduced and successfully used, is designed to reduce blood cell activation: it provides decreased area of extracorporeal circuit, reduces priming, and also lessens air-blood interface. Another favorable feature of mini-CPB is a biocompatible coating that induces higher tolerance by blood cells. The impact of mini-CPB on suppressing inflammatory response is already described and published [3, 4].

As the blood reaches all the body compartments, inflammatory response to surgical injury exceeds local reaction and becomes systemic. Although the increased production of cytokines is essential for protection against infection and also healing the wounds, unregulated inflammatory response is likely to have a harmful effect. Regulating mechanisms consist of anti-inflammatory molecules such as cytokines, cell surface enzymes, receptor antagonists, and soluble receptors [5]. The net impact of the produced cytokines, whether with proinflammatory or anti-inflammatory properties, is determined by the ability of the immune system to balance the inflammatory response properly [6].

IL-10 is a regulatory and immunosuppressive cytokine that is produced by a variety of cells [7]. Upon binding to its receptor (IL-10R), IL-10RB subunit transmits a signal into the cell which activates STAT3- and STAT3-resposive

Table 1: Demographic and preoperative data.

	Conventional CPB		Mini-CPB		*P* value
Patients (no.)	22		22		
Women/men (no.)	4/18		2/20		0.66
Age (years)	68	(63–71)	69	(66–74)	0.57
Body mass index	28.5	(25.3–32)	26.5	(25–31.3)	0.38
Acetylsalicylic acid (no.)	21		21		
Beta blockers (no.)	21		20		
ACE inhibitors (no.)	16		18		0.72
Statins (no.)	21		20		
Diabetes mellitus (no.)	4		6		0.72
COPD (no.)	3		4		
Prior myocardial infarction (no.)	12		9		0.37
Leukocytes (cells $\times 10^9$/L)	7.5	(6.8–8.5)	7.1	(6.3–8.8)	0.8
Ejection fraction (%)	64.5	(51.3–69.8)	60	(48.5–69.5)	0.35

Parameters marked as "no." display the number of positive cases in a group of patients or number of patients treated with a given medication. Other parameters are characterized by median value and interquartile range in brackets. If both groups contain the same number of cases or if they unequal by a case, then *P*-value is above 0.99 and is not displayed. ACE: angiotensin-converting enzyme; COPD: chronic obstructive pulmonary disease.

genes [8]. As a result, the production of proinflammatory cytokines, such as IL-1 and TNF-α, is inhibited [9].

The changes in production of IL-10, IL-1, and TNF-α have already been extensively evaluated in patients undergoing CABG surgery with conventional CPB [10]. However, the information about kinetics of IL-10 in mini-CPB patients is sparse. We compared the changes of IL-10 serum level, the expression of IL-10 membrane receptor, and the percentage of neutrophils in conventional and mini-CPB patients, as well as between these groups of patients. We also examined the incidence of sepsis and acute renal dysfunction in both groups.

2. Material and Methods

2.1. Patients. Forty-four patients, undergoing elective coronary artery bypass grafting (CABG) surgery on an arrested heart using CPB, were enrolled into our study. All patients were well informed about the purpose of the study and they confirmed their unconstrained participation by a written consent. The study project was approved by the Ethics Committee of the University Hospital in Hradec Kralove, Czech Republic. Patients, included in the study group in the period from December 2006 to December 2007, were randomly assigned to surgery either with the use of conventional CPB ($n = 22$) or mini-CPB ($n = 22$). Exclusion criteria consisted of acute inflammation, urgent operation, reoperation, combined operations, operative risk more than 5% (according to logistic Euroscore), preoperative level of serum creatinine above 130 μmol/L, hepatic disease, and malignancies. The demographic and preoperative data of our patients are shown in Table 1.

2.2. Conventional Cardiopulmonary Bypass (CPB). CPB was established using a two-stage venous drainage and ascending aortic return. A roller pump (Stöckert Instrumente GmBH, München, Germany), a membrane oxygenator (Dideco SrL, Mirandola, Italy) in a closed modification with a collapsible reservoir, a cardiotomy suction device, and a 40 μm arterial line filter (Dideco SrL, 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 of Ringer's lactate, 500 mL of Rheodextran (Rheomacrodex), 5,000 IU heparin, 80 mL of Natrium Bicarbonate ($NaHCO_3$ 8,4%), 20 mL of 10% Magnesium Sulphate, and Mannitol (at 1 g/kg body weight). The priming volume was calculated so that hematocrit level reached above 0.22. Heparin was administered intravenously at 300 IU/kg body weight to maintain an activated clotting time (ACT) above 480 s during bypass procedure. Patients received neither aprotinin nor corticosteroids intravenously. Pump flow rates averaged 2.4 L/min/m^2 of body surface area with pressure maintained at 50–60 mmHg. The patients were kept normothermic. Cardioplegic arrest was induced with a cold blood cardioplegic solution, which consisted of blood mixed with St. Thomas solution (Ardeapharma, Sevetin, Czech Republic) in 4 : 1 ratio. It was administered antegradely into the aortic root with doses added every 20 min or as needed. All patients received an internal artery mammary graft to the left anterior descending coronary artery. The central aortovenous anastomoses were performed during the reperfusion phase of cardiopulmonary bypass with the heart beating. After the termination of CPB, heparin anticoagulation was antagonized using Protamine Sulphate at a dose of 1 : 1.

2.3. Minimally Invasive Cardiopulmonary Bypass (Mini-CPB). Mini-CPB was established using a small 22F two-stage venous drainage and ascending aortic return. Minisystem Synergy (Sorin Group SrL, Mirandola, Italy) consisted of a centrifugal pump, membrane oxygenator, 40 μm arterial line filter, and a venous bubble trap. Cardiotomy suction was not used. The whole system, consisting of a closed loop with the surface treated with PH.I.S.I.O phosphorylcholine

coating (Sorin Group SrL, Mirandola, Italy) and very short tubing, was placed close to the operating field. The priming solution, heparinization, pump flow, temperature, and surgery technique were identical with the conventional CPB procedure described above. Cardioplegic arrest, induced according to the Calafiore warm blood-cardioplegia protocol, was administered antegradely into the aortic root. At the beginning of CPB, crystalloid priming solution was flushed retrogradely together with the blood coming from the arterial line to minimize the hemodilution of the patient.

2.4. Anesthesiological Management. All patients were anesthetized according to the current protocol of our department. Anesthesia was induced using Thiopental and Midazolam. Muscular relaxation was achieved with Cisatracurium. Anesthesia was maintained with Isoflurane and intermittent use of Sufentanyl. Continuous infusion of Propofol was used as a supplement if needed. Volume-controlled ventilation with FiO_2 0.5 was employed. Mean arterial pressure was kept above 50 mmHg, with norepinephrine administered as required.

2.5. Sample Collection and Data Acquisition. Blood samples were withdrawn from subclavian vein before and during surgery and from antebrachial vein in postsurgery period. The samples were collected into anticoagulant-untreated Vacutainer tubes as well as heparinized Vacutainer tubes (Becton Dickinson, UK) at the following time points: before surgery (introduction of anesthesia), at the beginning of CPB, at termination of CPB, at the end of surgery, and on the 1st, 3rd, and 7th postoperative day.

Serum was separated from the blood cells by centrifugation at 1000 g for 15 min. Samples were stored at -80°C prior to analysis. IL-10 concentration was determined by enzyme-linked immunosorbent assay (ELISA), with a sensitivity of 1 pg/mL. ELISA kit was purchased from Bender MedSystems (Austria). IL-10 concentrations were measured using spectrophotometer (Labsystems Multiskan RC, USA) with Genesis software.

Heparinized blood samples were processed immediately after collection. 50 μL of blood was incubated with titrated monoclonal antibodies anti-CD3 FITC/IL-10R PE/CD14 PerCP/CD16 APC. Antibodies, except for anti-IL-10R, were purchased from BD Biosciences (USA), while anti-IL-10R was purchased from BioLegend, USA. Following the 20 min incubation, red blood cells were lysed by hypotonic lysis and then the samples were washed. The data acquisition was performed with FACS Calibur flow cytometer using CellQuest software (BD Biosciences, USA), and the analysis was done using FlowJo software (Tree Star, USA). Expression of IL-10R on cells was displayed as median fluorescence intensity (MFI). Expression of IL-10R during surgery was not assessed.

2.6. Statistical Analysis. Values of IL-10, IL-10R, and percentage of neutrophils during and after surgery were compared to preoperative values. Normal distribution of the data was determined by Shapiro-Wilk test. Changes within a group were evaluated using ANOVA for repeated measures and Dunnett's test or Friedman ANOVA and Wilcoxon paired test. To determine the differences between both groups of patients, values of IL-10, IL-10R, and percentage of neutrophils were compared at matching time points using two-way ANOVA for repeated measures and Fisher's LSD test or Mann-Whitney U test. The relationship between IL-10 and percentage of neutrophils was assessed by Spearman correlation coefficient. Demographic and clinical data were analyzed by Fisher exact test, Mann-Whitney U test, and Student's t-test. All tests were performed at a significance level of 0.05. Bonferroni correction was used in case of multiple comparisons. Results of statistical analysis were expressed as medians unless stated otherwise.

3. Results

3.1. Differences in IL-10 in Serum within the Groups. Changes in IL-10 serum level were similar in both groups of patients, with respect to the preoperative serum level of 1 pg/mL in conventional CPB group and 1.45 pg/mL in mini-CPB group. In the conventional CPB group, serum level of IL-10 significantly increased at the termination of CPB (52.7 pg/mL, $P < 0.001$), at the end of surgery (50.5 pg/mL, $P < 0.001$), and on the 1st postoperative day (7.3 pg/mL, $P < 0.01$). Similarly, the mini-CPB group experienced an increase in serum level at the termination of CPB (from 1.5 pg/mL to 10.6 pg/mL, $P < 0.01$), at the end of surgery (21.2 pg/mL, $P < 0.001$), and on the 1st postoperative day (5.8 pg/mL, $P < 0.05$). Postsurgery monitoring revealed that the level of IL-10 was gradually decreasing in both groups and did not significantly differ from preoperative levels (Figure 1).

3.2. Differences in IL-10 in Serum between the Groups. There was significant serum-level difference ($P < 0.01$) between both groups at the termination of CPB: the conventional CPB group topped the mini-CPB group by reaching the value of 52.7 pg/mL. At this time point, IL-10 in mini-CPB group was enhanced, but only reached 10.6 pg/mL. In mini-CPB group, the level of IL-10 was highest at the end of surgery (21.2 pg/mL). In postsurgery period, the level of IL-10 decreased and did not differ between both groups (Figure 1).

3.3. Percentage of Neutrophils. Percentage of neutrophils was significantly increased ($P < 0.001$) in both groups of patients at the end of CPB, at the end of surgery, and also on the 1st and 3rd postoperative day. The baseline was 55% of neutrophils in conventional CPB group and 53% in mini-CPB group. The highest percentage of neutrophils was measured on the 1st postoperative day in both groups of patients (80% in conventional CPB group and 81% in mini-CPB group). There was statistically significant difference in percentage of neutrophils between both groups at the end of surgery ($P < 0.05$) when conventional CPB group reached 79% and mini-CPB group reached 75% of neutrophils (Figure 2).

The percentage of neutrophils correlated with the maximum level of IL-10 at the termination of CPB ($r_s = 0.73$, $P < 0.001$), at the end of surgery in conventional CPB group

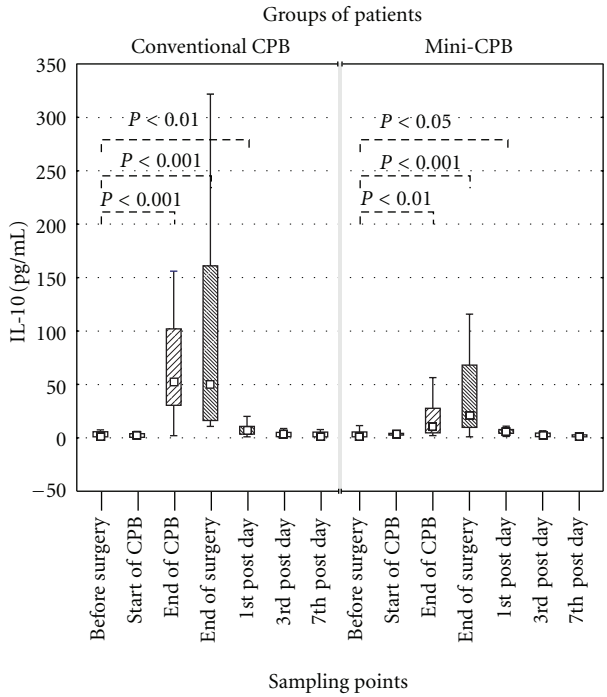


Figure 1: IL-10 level in serum of patients operated either with conventional CPB or mini-CPB. The IL-10 level was detected by ELISA. The level of IL-10 differed between conventional CPB and mini-CPB group after termination of CPB ($P < 0.01$). Even though the difference at the end of surgery was not statistically significant, IL-10 greatly varied when comparing both groups. Squares display median, boxes are quartiles, and whiskers display the range of nonoutlier values.

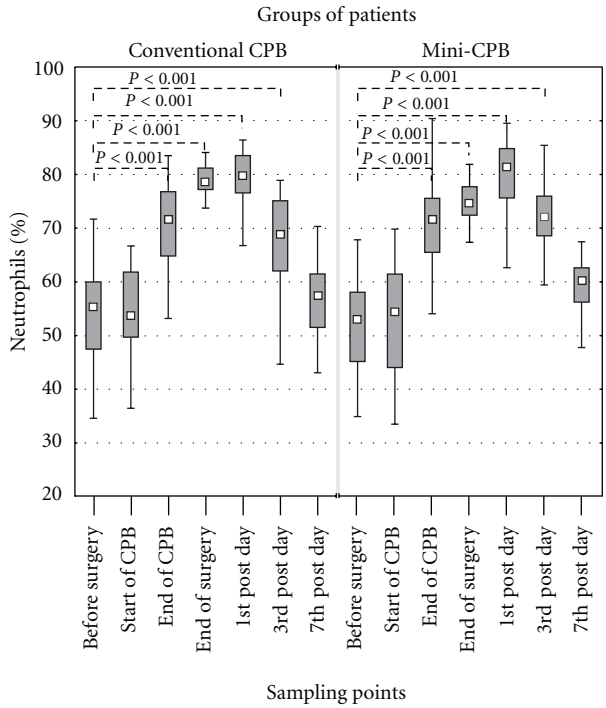


Figure 2: Percentage of neutrophils in peripheral blood samples of patients with conventional CPB or mini-CPB. The percentage of neutrophils was evaluated by flow cytometry. The percentage of neutrophils significantly differed between both groups of patients at the end of surgery (79% of neutrophils in conventional CPB group versus 75% of neutrophils in mini-CPB group, $P < 0.05$). Squares display, median, boxes are quartiles, and whiskers display the range of nonoutlier values.

($r_s = 0.54$, $P < 0.01$), and at the end of surgery in mini-CPB group ($r_s = 0.54$, $P < 0.01$) (Figures 3(a)–3(c)).

3.4. Expression of IL-10 Receptor (IL-10R). Since IL-10R is expressed on T lymphocytes, neutrophils, and monocytes, the expression was statistically evaluated in all of these populations. In lymphocytes and neutrophils, no differences were found at any time point when comparing MFI values within a group of patients or between both groups of patients.

The expression of IL-10R on monocytes significantly decreased at the end of surgery in both groups: the preoperative expression of IL-10R dropped from MFI of 10.2 to 7.6 ($P < 0.01$) in conventional CPB group, as well as in mini-CPB, where the preoperative level dropped from MFI of 9.8 to 7.6 ($P < 0.05$). On the 3rd postoperative day, the expression of IL-10R on monocytes was significantly enhanced (Figure 4), reaching MFI of 10.6 in conventional CPB ($P < 0.05$) and 11.3 in mini-CPB ($P < 0.05$). After that, the IL-10R expression decreased again and there was no significant difference between the preoperative value and the value on the 7th day after surgery in both groups (Figure 5).

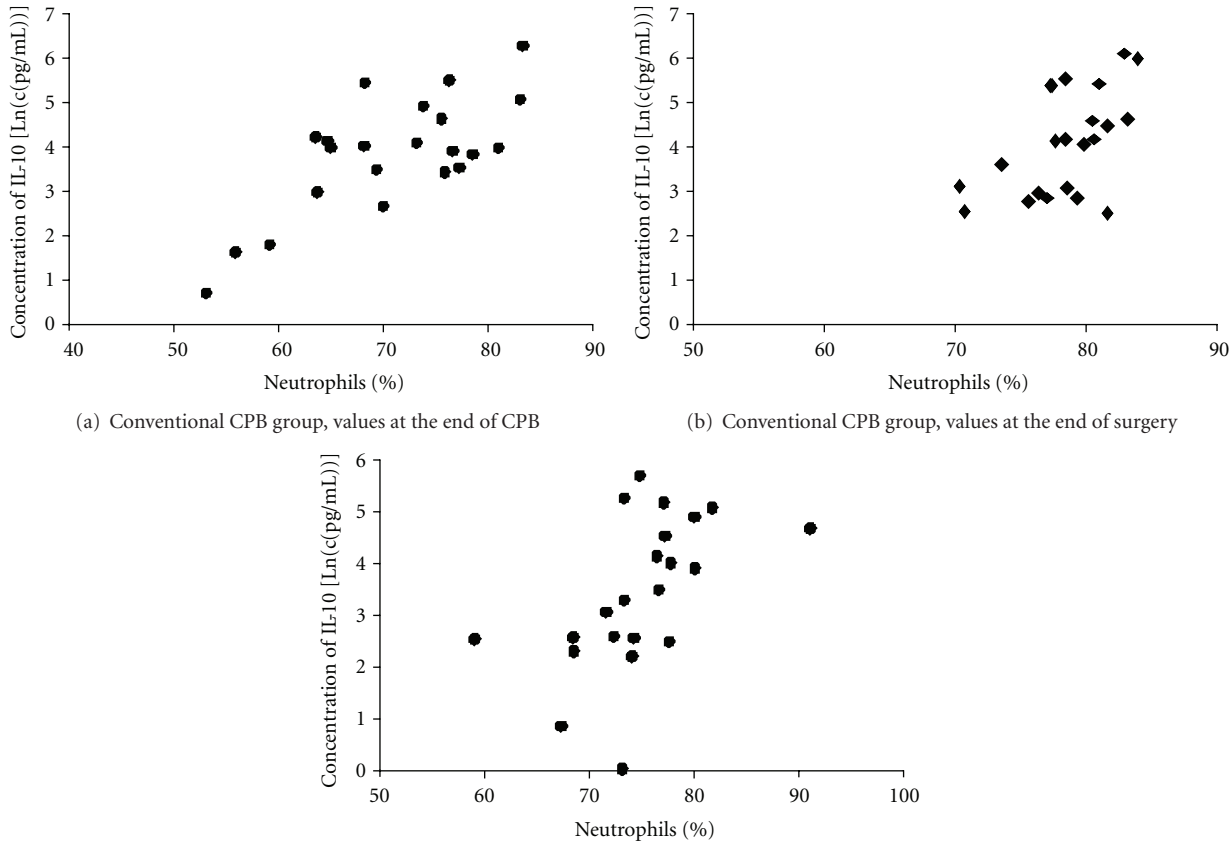
There was no correlation between serum level of IL-10 and expression of IL-10R on monocytes.

No difference in IL-10R expression on monocytes was observed when comparing both groups of patients (Figure 5).

We observed single- as well as multiple-organ dysfunction or failure in both groups of patients. IL-10 level in each group is listed in Table 2. Only two patients were considered suffering from sepsis. Intraoperative and postoperative data of patients are displayed in Table 3.

4. Discussion

IL-10 is an important cytokine, maintaining the proinflammatory response balanced. Although IL-10 can keep cells unresponsive, receptors on the cells affected by IL-10 are still capable of binding and internalizing proinflammatory cytokines. As a result, the cytokines are removed from blood circulation without subsequent activation of the cells [11, 12]. This kind of decoy activity is thought to be of favorable contribution of IL-10 in ischemic heart after myocardial reperfusion, and it is likely to prevent against heart failure [13]. However, high level of IL-10 can also have an adverse effect by increasing the likelihood of sepsis. Nonetheless, a significant association between sepsis and higher level of IL-10 determined by—1082 base pair single polymorphism in promoter region of IL-10 gene is equivocal [14, 15]. It has been



(a) Conventional CPB group, values at the end of CPB

(b) Conventional CPB group, values at the end of surgery

(c) Mini-CPB group, values at the end of surgery

Figure 3: Relationship between percentage of neutrophils and serum level of IL-10. (a) Correlation, $r_s = 0.73$, in conventional CPB group at the termination of CPB ($P < 0.001$). (b) Correlation, $r_s = 0.54$, in conventional CPB group at the end of surgery ($P < 0.01$). (c) Correlation, $r_s = 0.54$, in mini-CPB group at the end of surgery ($P < 0.01$)

Table 2: IL-10 level in groups of patients without and with organ dysfunction or failure.

	No organ affected ($n = 15$)	One and more organs affected ($n = 7$)	One organ affected ($n = 5$)	More organs affected ($n = 2$)
Conventional CPB ($n = 22$)	89 (41–451)	203 (148–731)	181 (148–385)	467 (203–731)
	No organ affected ($n = 11$)	One and more organs affected ($n = 11$)	One organ affected ($n = 6$)	More organs affected ($n = 5$)
MINI-CPB ($n = 22$)	35 (13–219)	75 (23–192)	105 (23–183)	55 (49–192)

Groups of patients were divided by number of organs suffering from dysfunction or failure. Upper value displays median of IL-10 (pg/mL), values in the brackets describe the range from minimum to maximum characterizing a group.

reported that CPB induces the release of IL-10, which can be further enhanced by the use of corticosteroids in patients undergoing CABG [16]. In our study, corticosteroids were not applied, and yet we observed that CPB induced a profound release of IL-10. We found significant difference in IL-10 production between the two different types of CPB used. Mini-CPB, which is considered to be a less harmful approach of cardiac surgery, elicited a lower release of IL-10. In spite of this fact, the increase of IL-10 does not seem to impact clinical outcome. Although the level of IL-10 was lower in mini-CPB group, the number of patients suffering from organ dysfunction or failure was nonsignificantly higher in mini-CPB group than in conventional CPB group ($P < 0.22$) (Table 2). While we would be able to predict organ dysfunction or failure according to IL-10 level in conventional CPB group, the similar relation was unclear in mini-CPB group. We also looked at the IL-10 value in patients with sepsis and patients without sepsis. Microbiologically confirmed, there were only two septic patients, one in each group, both reaching values of IL-10 in serum that fell within quartiles of IL-10 calculated for each sampling time point in any given group. Therefore, in our study groups, even though the IL-10 level was very high in some patients, IL-10 could not be used as a predictive marker related to sepsis. Increased level of IL-10 along with the medical treatment (Ampicillin-sulbactam) of our patients might have prevented the onset of

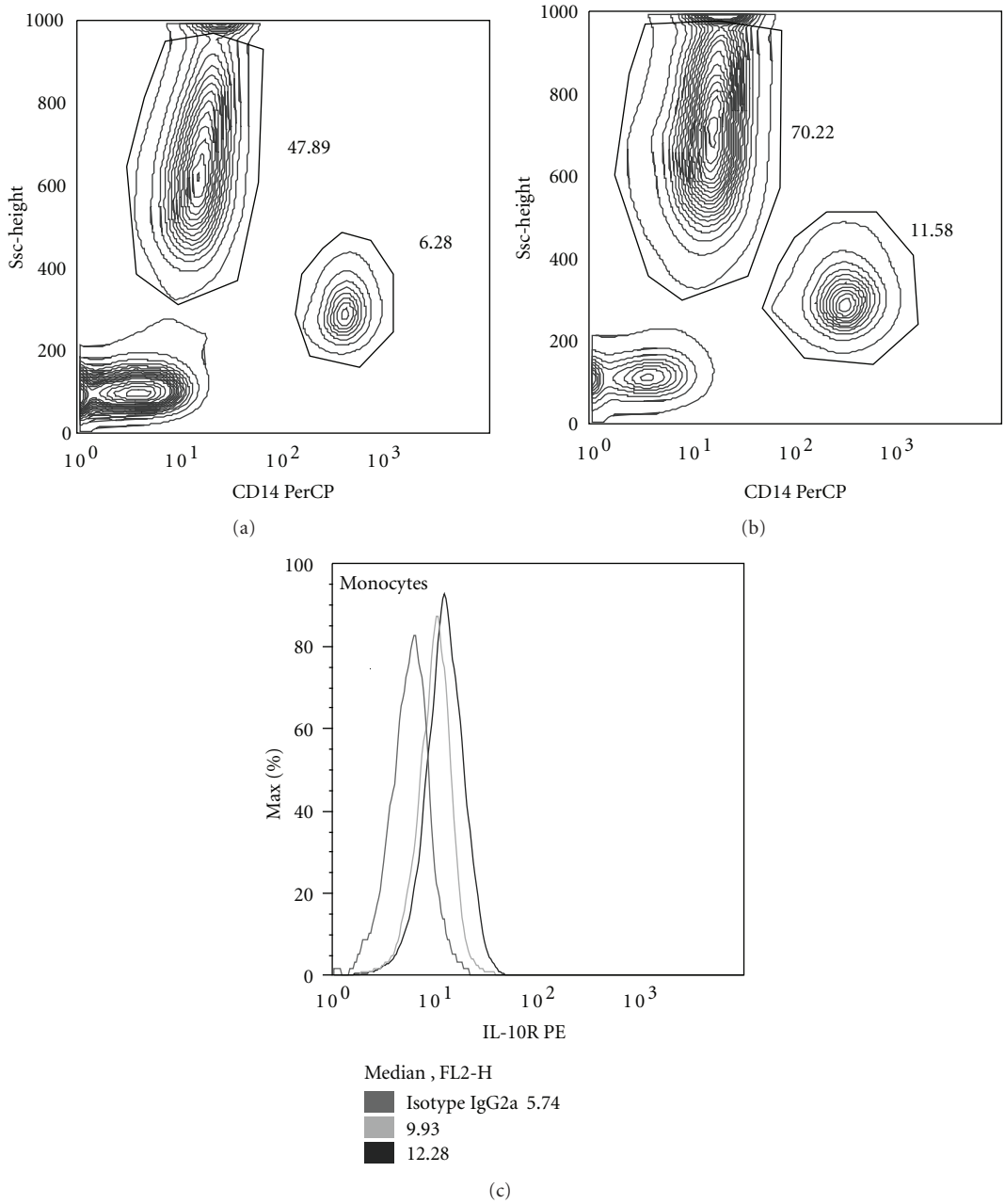


Figure 4: Illustration of changes in the expression of IL-10R on monocytes of conventional CPB patient. Monocytes are discriminated from other populations of cells by high expression of CD14 (x-axis). (a) Leukocytes before surgery. (b) Leukocytes on the 3rd postoperative day. (c) Overlaid histograms of monocytes from (a) and (b). Although the expression of IL-10R on monocytes enhances on the 3rd day after surgery (black line), it is relatively weak considering the isotype control (dark grey line). Intracellular staining did not reveal higher expression of IL-10R in cells (data is not shown).

sepsis. Such a beneficial role of IL-10 is in concordance with experimental studies [17, 18].

In previous works, IL-10 level was also found to be significantly increased in patients who suffered from postoperative renal dysfunction [19], which was characterized by creatinine level raised above 176 μmol/L [20]. The mini-CPB group had two patients that were suffering from acute renal dysfunction, while there was only one patient in conventional CPB group. The patient from conventional CPB group reached 522.5 pg/mL at the termination of CPB. This patient had the highest value of IL-10 in serum out of both groups. However, the other two patients did not exceed quartiles of IL-10 calculated for each sampling time point. According to this result, there is no simple relation between enhanced level of IL-10 and increased probability of acute renal dysfunction.

We compared the percentage of neutrophils to IL-10 level when IL-10 in serum was at its highest level. We observed significant correlation between IL-10 and percentage of

Table 3: Intraoperative and postoperative data of patients.

	Conventional CPB		Mini-CPB		P value
Duration of surgery (min)	210	(161–250)	165	(155–203)	0.15
Duration of CPB (min)	70	(56–111)	62	(55–76)	>0.1
Priming solution (mL)	1600	(1425–1800)	1100	(1000–1300)	<0.001
Anastomoses (no.)	2	(2-3)	2	(2-3)	0.7
Intraoperative blood loss (mL)	1000	(725–1000)	700	(500–950)	0.14
Postoperative blood loss in 24 h (mL)	650	(450–1075)	600	(500–950)	>0.1
Acute renal dysfunction and failure (no.)	1		2		
Respiratory dysfunction and failure (no.)	4		9		0.19
Postsurgery myocardial dysfunction and AMI (no.)	5		6		
Sepsis (no.)	1		1		

Parameters marked as "no." display the number of positive cases in a group of patients with the exception for number of anastomoses which denotes median value. All other parameters are characterized by median value and interquartile range in brackets. If both groups contain the same number of cases or if they unequal by a case, then P-value is above 0.99 and is not displayed. AMI: acute myocardial infarction.

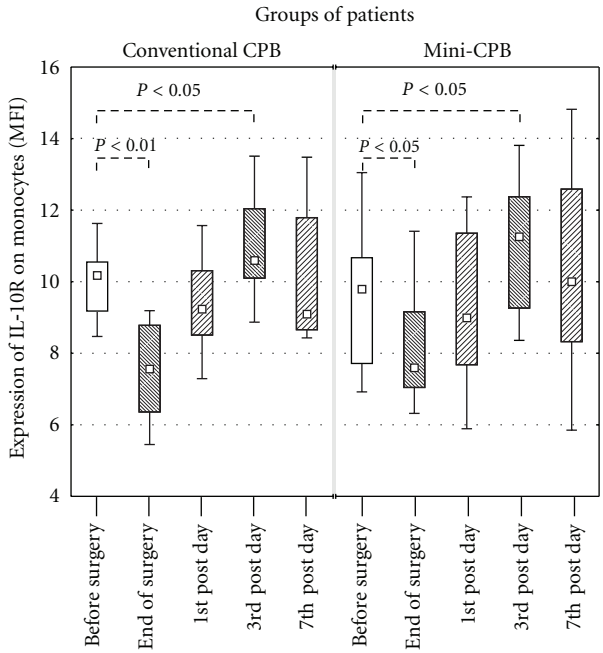


Figure 5: Expression of IL-10R on monocytes in peripheral blood samples of conventional CPB or mini-CPB group as a result of flow cytometry data analysis. The expression of IL-10R did not significantly differ between both groups of patients. Squares display median, boxes are quartiles, and whiskers display the range of nonoutlier values.

neutrophils at the termination of CPB in conventional CPB group ($r_s = 0.73$, $P < 0.001$) and at the end of surgery in mini-CPB group ($r_s = 0.54$, $P < 0.01$). We also found lower—but still significant—correlation ($r_s = 0.54$, $P < 0.01$) between IL-10 and percentage of neutrophils at the second highest level of IL-10 (at the end of surgery) in conventional CPB group. Our data suggests that neutrophils were the main producers of IL-10 in most of our cardiac surgical patients. However, the lower correlation coefficient at the end of surgery indicates other cells might also have participated in IL-10 production. The observation is in agreement with recently published findings that show that neutrophils are an important source of IL-10 [21] and that mini-CPB attenuates neutrophil activation and cytokine release after coronary bypass surgery [3].

IL-10 exerts its function through the binding to IL-10R [22]. It seems that higher level of IL-10 in conventional CPB group is exclusively linked to the surgery technique and devices used. Since we found no significant difference in expression of IL-10R between both groups of patients, we can hypothesize that the expression of IL-10R on hematopoietic cells exceeds the maximum level of its ligand, IL-10. It has been discovered that other cells, such as fibroblasts and epithelial cells [23, 24], also express IL-10RA after induction; thus IL-10 may have a much broader effect on tissues and organs, an effect which cannot be explained by the possible correlation of serum IL-10 and the expression of IL-10R on monocytes.

5. Conclusion

IL-10 level and percentage of neutrophils are significantly affected by the type of cardiac surgery employed. Although IL-10 level may have statistical relation to sepsis or renal dysfunction, the generally accepted critical level that would enable to unambiguously distinguish between patients with worse or good prognosis does not exist. However, in certain conditions, like using conventional CPB, IL-10 may represent a supporting tool, that, along with other parameters, would help rank the patients in likelihood of organ dysfunction or failure. The observed correlation between increased level of IL-10 and higher percentage of neutrophils in both groups of patients suggests functional relationship between both parameters.

Acknowledgments

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Clinical Study

Early Expression of FcγRI (CD64) on Monocytes of Cardiac Surgical Patients and Higher Density of Monocyte Anti-Inflammatory Scavenger CD163 Receptor in "On-Pump" Patients

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Objective. Activation of innate immunity cells is inseparably linked to cardiac surgical operation. The aim of this study was to assess the kinetics in the expression of receptor for Fc part of IgG, FcγRI (CD64), and scavenger receptor CD163 on peripheral blood cells of cardiac surgical patients and to examine the effect of cardiac bypass as a separable influence on the systemic acute inflammatory response. *Methods*. Forty patients, twenty in each group, were randomly assigned to CABG surgery performed either with "on-pump" or without "off-pump" cardiopulmonary bypass. Standardized quantitative flow cytometry method was used to determine the expression of surface markers. *Results*. The density of CD64 molecule on monocytes reached maximum on the 1st postoperative day ($P < .001$) whereas the peak for CD64 molecule expression on granulocytes was postponed to the 3rd postoperative day ($P < .001$). The expression of CD163 scavenger molecule on monocytes reached maximum on the 1st postoperative day ($P < .001$). The density of CD163 molecule on monocytes on the 1st postoperative day is significantly higher in "on-pump" patients in comparison with "off-pump" patients ($P < .001$). *Conclusion*. In cardiac surgical patients the expression of activation marker FcγR1 (CD64) on monocytes is increased earlier in comparison with granulocytes in both "on-pump" and "off-pump" patients. The expression of scavenger molecule CD163 on monocytes is significantly higher in "on-pump" patients.

1. INTRODUCTION

Numerous events, potentially generating an inflammatory response, are induced during cardiac surgery. Amongst them, the combination of surgical injury, mechanical manipulation with the heart, the contact of blood components with the artificial surfaces of the cardiopulmonary bypass circuit, transient endotoxemia and ischemia-reperfusion injury of the heart and lungs are relevant [1]. The inflammatory reaction 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 lumenal surface of inflamed endothelium [2]. There is a substantial long lasting effort to identify activated monocytes and 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, for example, bacterial infection [3].

Activation of myeloid cells by various physiological and experimental stimuli is accompanied by multiple surface changes associated predominantly with degranulation. Thus, activated blood myeloid cells typically upregulate surface expression of chemotactic receptors, complement receptor type 3 (CD11b/CD18), and downmodulate surface density of lipopolysacharide (LPS) receptor CD14, the low affinity FcγRIII or CD16 receptor, adhesion receptors CD44 and CD62L [4].

The Fcγ-receptor I, FcγRI, (CD64), is a high affinity receptor for IgG1 and IgG3 subclasses of immunoglobulins. FcγRI is constitutively expressed with high density on monocytes and macrophages, less so on eosinophils, but only to a very low extent on resting neutrophils.

Numerous substances both exogenous and endogenous origins rapidly upregulate FcγRI expression on the surface of neutrophils [5]. Microbial cell wall components such as LPS, endogenous complement split products, and cytokines, such as IFNγ and TNFα, are some of the activators. The expression of FcγRI is determined by immunofluorescence and flow cytometry. The introduction of a new diagnostic kit Leuko64 enables standardized and quantitative approach to the determination of FcγRI expression on immune cells.

Hemoglobin scavenger receptor CD163 is a group B cysteine-rich scavenger receptor expressed exclusively by cells of monocyte-macrophage lineage. This glycoprotein is characterized as a scavenger receptor for hemoglobin, mediating endocytosis of hemoglobin-haptoglobin complexes. Previous studies have indirectly linked CD163 scavenger receptor to anti-inflammatory phenomena [6]. High CD163 expression correlates with the Mo2 anti-inflammatory properties of monocytes and macrophages [7].

The aim of this study was to follow the changes in the expression of granulocyte activation markers FcγRI and anti-inflammatory CD163 scavenger receptor in patients undergoing cardiac surgical operation either with the use of cardiopulmonary bypass ("on-pump") or operated on the beating heart during "off-pump" operation and in the postoperative period using quantitative flow cytometric approach.

2. PATIENTS

Forty patients (31 males, mean age 67.9 ± 9 and 9 females, mean age 66.4 ± 6.4, 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 males, 4 females, mean age 69.4 ± 7) or beating heart surgery ("off-pump," $n = 20$, 15 males, 5 females, mean age 65.9 ± 9.7).

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, resp.) and the number of performed coronary anastomoses (median 2.0 in "on-pump," 2.0 in "off-pump," resp.). The study protocol was approved by the Ethics Committee of the University Hospital in Hradec Králové. 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 consents.

Cardiopulmonary bypass, "off-pump" technique, and anesthesiological management have been recently described in detail elsewhere [8].

3. BLOOD SAMPLING

Peripheral venous blood from an antebrachial vein was withdrawn in the operating room and in the intensive care unit. Samples were collected into heparinized tubes Vacutainer, Cat. no. 36884 manufactured by Becton Dickinson.

In both "on-pump" and "off-pump" patients, blood was withdrawn at the following time points:

(1) introduction to anaesthesia, which in both groups represented the baseline or reference value for all parameters measured thereafter,
(2) after termination of the operation,
(3) the first postoperative day,
(4) the third postoperative day,
(5) the seventh postoperative day.

Additional samples were taken from "on-pump" patients:

(1a) before cross-clamping of the aorta,
(1b) after aortic cross-clamp release,
(1c) after termination of CPB.

4. MATERIALS AND METHODS

Leuko64 kit manufactured by Trillium Diagnostics, LLC, (www.trilliumdx.com, Brewer, Me, USA) was used to determine the expressions of CD64 and CD163 on leukocytes of blood samples. Leuko64 kit is composed of a reagent cocktail of two monoclonal antibodies with specifities to CD64 (clones 22 and 32.2, both FITC conjugated) and monoclonal antibody to CD163 (clone Mac2-148, phycoerythrin conjugated) and a fluorescence beads suspension used for instrument calibration and standardization of leukocyte CD64 and CD163 expressions in human blood. The assay was run according to the instruction for use provided by manufacturer. Briefly, 50 microliters of blood and the Leuko64 monoclonal cocktail reagent are incubated for 10 minutes, red cell lysis buffer is added and incubated for additional 15 minutes, and 5 microliters of bead suspension are added prior to flow cytometric analysis. Results were measured by an FACSCalibur flow cytometer (BD Biosciences, San Jose, Calif, USA) using CELLQuest software. The listmode data were analyzed using Leuko64 software (Trillium Diag.). Results are expressed as indexes of positivity for CD64 and CD163 expressions on granulocyte and monocyte populations as provided by the Leuko64 software.

5. STATISTICAL ANALYSIS

We compared changes in the intensity of expressions of CD64 and CD163 in both groups of patients ("on-pump," "off-pump") separately. Samples taken at the introduction to

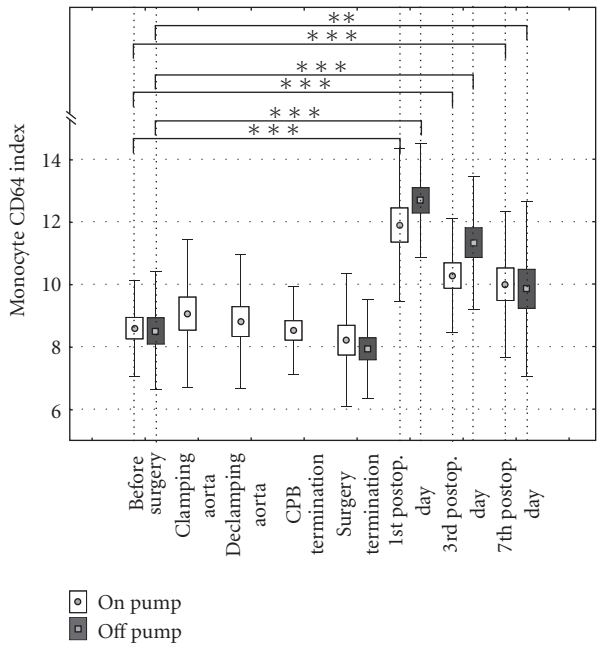

Figure 1: CD64 molecule expression on monocytes in "on-pump" and "off-pump" patients.

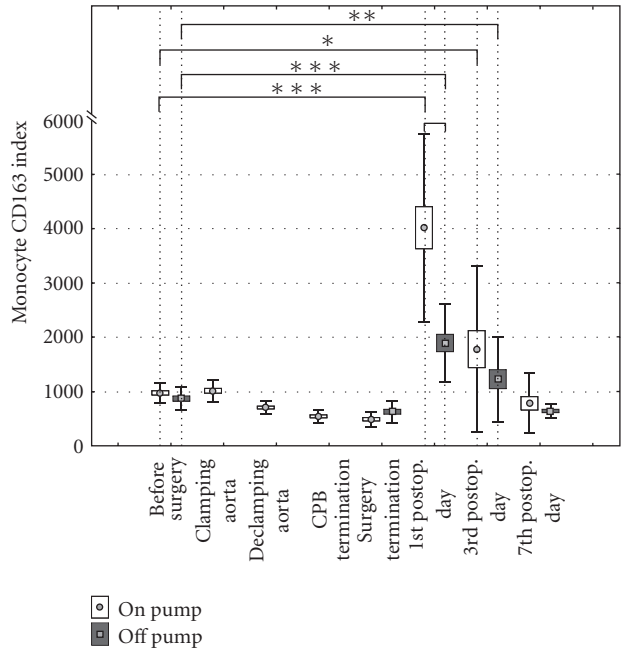

Figure 2: CD163 molecule expression on monocytes in "on-pump" and "off-pump" patients.

anesthesia were considered as reference or baseline expressions of CD64 and CD163. 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 presentation in both groups, unpaired t-test and chi-square were performed. A probability (P) value < .05 was considered significant. Statistical analysis was performed with Statistica 5.5 software (Statsoft, Okla, USA).

6. RESULTS

We found substantial dynamics in the expressions of both CD64 and CD163 molecules on immune cells in our cardiac surgical patients. These changes are expressed as changes in CD64 and CD163 indexes separately for monocytes and granulocytes. Preoperative levels were taken as reference points. There were no significant changes in the monocyte CD64 expression during cardiac operation in "on-pump" patients (data not shown). The monocytes CD64 index was significantly increased in both "on-pump" and "off-pump" patients in postoperative period from the first to the seventh day ($P < .01$). Comparing monocyte CD64 index between "on-pump" and "off-pump" patients, there were no significant differences ($P < .587$) during operation and in the postoperative period (Figure 1). The similar patterns were found for monocyte CD163 index in our cardiac surgical patients with following exceptions. Monocyte CD163 index, in contrast to the monocyte CD64 index showed a significant difference as a function of the pump status. There was a statistically significant increase in monocyte CD163 index in "on-pump" patients compared to "off-pump" patients at the first postoperative day ($P < .001$) (Figure 2). The significant increase in monocyte CD163 index in both "on-pump" and "off-pump" patients compared to the baseline expression was found only at the 1st and 3rd postoperative days ($P < .01$).

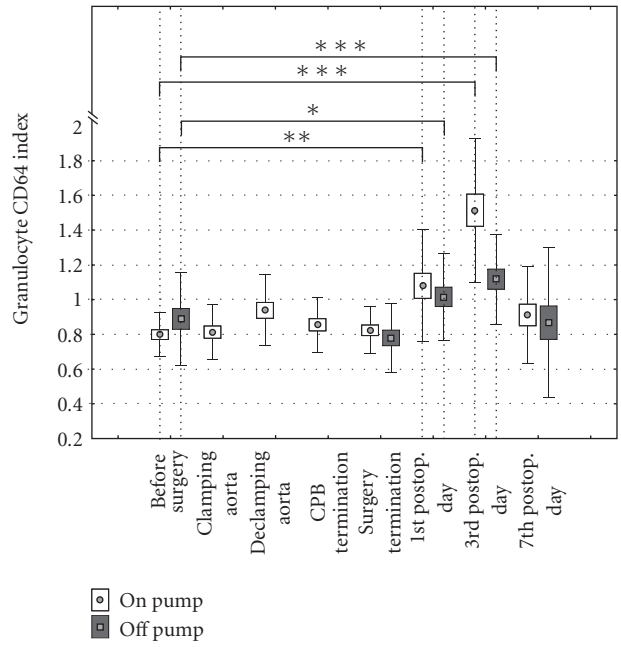

Figure 3: CD64 molecule expression on granulocytes in "on-pump" and "off-pump" patients.

The granulocyte CD64 index was significantly increased both in "on-pump" and "off-pump" patients at the 1st and

3rd postoperative days. Statistically significant differences of granulocyte CD64 indexes between "on-pump" and "off-pump" were not reached ($P < .195$) (Figure 3). The original and pathophysiologically very important observation in our results is that the maximum of CD64 expression on monocytes (1st postoperative day) precedes the maximum of CD64 expression on granulocytes (3rd postoperative day). The granulocyte CD64 expression returned to baseline or normal levels by the 7th postoperative day.

7. DISCUSSION

A long-term sustained effort is devoted to the better understanding of inflammatory reaction which is inseparably linked to every cardiac surgical operation. Numerous humoral and cell-mediated parameters of immune system have been studied to identify markers describing the development of such inflammatory reaction. The ultimate goal of such studies is to identify those patients who are at substantial risk for development of overwhelming systemic inflammation (SIRS), a condition of complex physiology that might severely compromise the outcome of surgery or even lead to death. Many studies conducted in the last few years have shown reduced inflammation in patients operated on by the "off-pump" technique compared to "on-pump" surgery [9]. 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 has not been reported. The very trauma of surgery seems to be more relevant in initiating SIRS rather than cardiopulmonary bypass itself, the latter adding a CPB specific fraction on top of unfavorable events [10].

The objective of this study was to compare the degree of activation of innate immunity cells between "on-pump" and "off-pump" patients. The novel diagnostic Leuko64 kit was used by us to dissect such differences. This kit enables quantitative determination of CD64/CD163 molecule expression on immune cells by flow cytometry.

FcγR1 receptor (CD64) is constitutively expressed on macrophages, monocytes, and eosinophils, but its expression is negligible on resting neutrophils. Neutrophil CD64 molecule expression is one of many activation-related surface receptors changes manifested during the normal innate immunity response. Microbial cell wall components such as LPS, complement split products, and cytokines (IFNγ, TNFα) are some of the activators [3]. In contrast to numerous other neutrophilic activation surface antigens, such as CD11b/CD18, CD14, and CD16 with large storage intracellular pools, CD64 has limited intracellular storage, but de novo synthesis can be induced in the presence of proinflammatory conditions. In comparison with former neutrophilic activation markers, CD64 expression on neutrophils is thus much less affected by stimuli of degranulation to which these cells are extensively exposed both during cardiac surgical operation and during blood sample processing [5]. Thus CD64 molecule appears uniquely suited as a surrogate marker of neutrophil activation or systemic acute inflammatory response as its expression starts from less than 2000 sites per cell and becomes upregulated in a graded fashion depending upon intensity of stimulation by cytokines [4].

Clinical usefulness of CD64 determination has been proven in differential diagnosis of sepsis of bacterial origin. Changes in the expression of CD64 on circulating leukocytes in patients undergoing cardiothoracic surgery were also reported [11]. Some of these previous works are suffering from the lack of precise quantitation since CD64 positivity was for example expressed as mean fluorescence intensity [11, 12]. This means of quantitation is valid on a single platform for day to day comparison, but lacks standardization necessary for a routine clinical laboratory test, whereas the Leuko64 kit approach does allow for interlaboratory comparisons. Application of a new standardized diagnostic kit in our study overcomes above mentioned limitations. Using calibration fluorescence beads and specialized software, CD64/CD163 density is expressed as index positivity separately for granulocytes and monocytes. As expected, CD64 positivity on monocytes is one log higher in comparison with granulocytes. A significant increase in both monocytes and granulocytes CD64 expressions has been found on the 1st and 3rd postoperative days. No significant differences between "on-pump" and "off-pump" patients have been recognized. Whereas CD64 expression on monocytes is nearly identical for both "on-pump" and "off-pump" patients, there is a tendency for higher granulocyte CD64 index on the 3rd postoperative day in "on-pump" patients. This likely reflects the greater sensitivity to cytokine effects of granulocytes compared to monocytes. Our results are in contrast to the work of Stefanou et al. [13] who did not report any induction of CD64 expression on monocytes of cardiac surgical patients post CPB. However, only 10 patients were enrolled to their study with some differences in CPB compared to our patients. Furthermore, our attempt using Leuko64 kit ensures better standardisation of method in comparison with previous work, where positivity was expressed as a simple MFI.

The neutrophils CD64 index is designed so that normal inactivated cells yield value of <1.00 and blood samples from individuals with documented sepsis or SIRS typically show values of >1.50. In our patients, the perioperative and postoperative period was uneventfull with the only one exception, the patient, who will be discussed below. It is our original observation that the maximum of CD64 expression on monocytes of our cardiac surgical patients has already been reached on the 1st postoperative day in contrast to granulocytes in which the maximum of CD64 expression has been postponed to the 3rd postoperative day. This observation implies that FcγRI expression on monocytes is upregulated by a different cellular mechanism very early during operation by the exposition of monocytes to various danger patterns which are raised during operation. The upregulation of FcγRI on granulocytes is a secondary event mediated by their exposition to proinflammatory cytokines and mediators formed by monocytes-macrophages.

The FcγRI receptor (CD64) on white blood cells integrate responses involving both the innate and acquired immune systems and are very important for effective phagocytosis of bacteria and immune complexes. A new, intrinsic role for this receptor has recently been proposed by Devaraj

et al. who have described the participation of FcγR on internalization of C-reactive protein by endothelial cells, with subsequent release of chemoattractive proinflammatory IL-8, decrease of eNOS, and increased ICAM-1 and VCAM-1 adhesion molecules [14]. In conclusion, FcγRI reveals proinflammatory activities in general.

Proinflammatory pathways have to be tightly counterbalanced by numerous anti-inflammatory processes, that is production of anti-inflammatory cytokines such as IL-10 [15], to maintain protective physiological level of inflammatory response in cardiac surgical patients. Another approach to calm down inflammatory reaction is removing of pleiotropic proinflammatory species by the action of so-called scavenger receptors which are differentially expressed on cells of both immune and nonimmune origins [6]. Unique characteristics amongst these scavenger receptors reveal hemoglobin scavenger receptor (CD163). CD163 scavenger receptors represent a highly efficient system to remove potentially toxic and proinflammatory hemoglobin from the circulation and local sites of inflammation [16]. Cardiac surgical patient are extensively exposed to large amounts of free heme/hemeproteins due to intravascular haemolysis and tissue damage. There is accumulating evidence that an excess of free heme can cause cell damage and tissue injury. Heme catalyzes the formation of reactive oxygen species (ROS), resulting in oxidative stress. Because the low-molecular weight iron chelated heme is lipophilic, it can easily intercalate in the membrane and impair lipid bilayers and organelles, such as mitochondria and nuclei, and destabilize cytoskeleton [17]. Several defense mechanisms against free heme-mediated oxidative stress and inflammation exist. They consist of intra- (e.g., heme oxygenase-2 and heme oxygenase-3) and extracellular (e.g., hemopexin, albumin) scavengers, antioxidative enzymes, and heme oxygenase-1 during hemolysis.

Intravascular free hemoglobin is captured by plasma scavenger protein haptoglobin. It is generally accepted that stable hemoglobin-haptoglobin complexes are subsequently delivered to the reticuloendothelial system by CD163 receptor-mediated endocytosis [16]. Any free vascular heme is bound to the plasma protein hemopexin or albumin, which transport it to the liver for degradation in reticuloendothelial system. However, when large amounts of free heme proteins or heme (locally) accumulate, like in a blood clot or after vascular deposition, the scavengers get overwhelmed or are unable to reach them. This enables heme to exert its damaging effects. Therefore, the amount of free heme must be tightly controlled to maintain homeostasis and avoid pathological conditions.

The monocyte CD163 index has been found to be between 5 000 and even 40 000 in our study. There was a sharp increase of monocyte CD163 in the postoperative period reaching statistically highly significant maximum ($P < .001$) on the 1st postoperative day for both "on-pump" and "off-pump" patients followed by decrease to the baseline preoperative levels. Furthermore, the patients' monocyte CD163 index is significantly higher ($P < .001$) in "on-pump" patient group on the 1st postoperative day. Reports regarding monocyte CD163 expression in cardiac surgical patients are very sparse. In a study by Goldstein et al. [18], a significant increase in the monocyte CD163 expression on the 1st postoperative day was found. This is in accord with results of our study. But there were several limitations in a previous study. CD163 molecule was expressed as mean fluorescence index (MFI) in comparison with our study in which standardized quantitative approach using calibration fluorescence beads was exploited yielded much more relevant data. The changes in the entire perioperative period and during postoperative period up to the 7th postoperative day have been followed by us in comparison with a previous study.

The link of CD163 hemoglobin scavenger receptor to anti-inflammatory phenomena in cardiac surgical patients has been proven by Philippidis et al. [19]. In their study, elevated expression of CD163 on circulating monocytes during the resolution phase of the systemic inflammatory response to cardiopulmonary bypass surgery was reported. Furthermore, binding of hemoglobin-haptoglobin complexes to CD163 expressing monocytes elicited potent interleukin-10 secretion. It was reported by Goldstein et al. [18] that an increase in the monocyte CD163 expression was 14 times higher in their "on-pump" patients who were treated by bolus administration of metylprednisolone perioperatively in comparison with untreated patients. Such therapeutical intervention was not performed in any our patients. It seems unlikely that higher monocyte CD163 expression in our "on-pump" patients was elicited by metylprednisolone which is a regular component of CPB fluids used at our department. More pronounced proinflammatory stimuli raised during "on-pump" surgery are more relevant. There will be the unique chance to dissect between the influence of corticosteroids present in CPB fluid and other variables involved in "on-pump" surgery. Currently, CPB protocol used at our department has been changed and methylprednisolone has been ommited. CD163 is rapidly shed from the surface of monocytes when activated by LPS or phorbol esters. In addition, also cross-linking of FcγR triggers shedding of CD163 [20]. This phenomenon is very likely counterbalanced by the fact that such shedding is followed by upregulation of this hemoglobin scavenger receptor, as was shown for metaloproteinases-mediated CD163 shedding [21]. This is probably true for our cardiac surgical patients. Very recently, it was proven by Weaver et al. [22] that CD163 shedding is also induced via stimulation with TLR-4, TLR-2, and TLR-5.

Initially included in this study was a single patient, male, 73 years old who was excluded from our study due to his death on the 2nd postoperative day. This patient underwent uncomplicated cardiac surgery using cardiopulmonary bypass. He developed acute diaphragmatic myocardial infarction two hours after finishing surgery. Patient was reoperated and reanastomosis was performed. In spite of this effort, cardiogenic and subsequent hemorrhagic shock was developed ultimating to death next day in the morning. Body temperature was below 36°C so that infection is unlikely.

The expression of both CD64 and CD163 molecules before surgery and up to the end of surgery was comparable with other patients. Granulocyte CD64 index reached 1.72 value which is significant for the development of SIRS [23]. However, such values were found in additional four patients

without any impact on their postoperative course. Remarkable is the fact that monocyte CD163 index in this patient was the highest between our patients and was approximately two times higher in comparison with all our patients investigated. It could be concluded from this our anecdotal observation that the increase in CD163 monocyte positivity could be the marker with predictive value of worse outcome in cardiac surgical patients.

In conclusion, using standardized quantitative methods we revealed substantial dynamics in the expression of the activation marker FcγRI and scavenger receptor CD163 in both "on-pump" and "off-pump" cardiac surgical patients during operation and in the postoperative period. The maximum in the expression of CD64 on monocytes in postoperative period precedes the maximum in the expression of this molecule on granulocytes by two days.

ACKNOWLEDGMENT

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Clinical Study

Lipopolysaccharide Binding Protein and sCD14 are Not Produced as Acute Phase Proteins in Cardiac Surgery

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Objectives. The changes in the serum levels of lipopolysaccharide binding protein (LBP) and sCD14 during cardiac surgery were followed in this study. *Design*. Thirty-four patients, 17 in each group, were randomly assigned to coronary artery bypass grafting surgery performed either with ("on-pump") or without ("off-pump") cardiopulmonary bypass. LBP and sCD14 were evaluated by ELISA. *Results*. The serum levels of LBP were gradually increased from the 1st postoperative day and reached their maximum on the 3rd postoperative day in both "on-pump" and "off-pump" patients (30.33 ± 9.96 μg/mL; 37.99 ± 16.58 μg/mL), respectively. There were no significant differences between "on-pump" and "off-pump" patients regarding LBP. The significantly increased levels of sCD14 from the 1st up to the 7th postoperative day in both "on-pump" and "off-pump" patients were found with no significant differences between these groups. No correlations between LBP and sCD14 and IL-6, CRP and long pentraxin PTX3 levels were found. *Conclusions*. The levels of LBP and sCD14 are elevated in cardiac surgical patients being similar in both groups. These molecules are not produced as acute phase proteins in these patients.

1. INTRODUCTION

Numerous events, potentialy generating inflammatory response, are induced during cardiac surgical operation on the open heart. Amongst them, the combination of surgical injury, mechanical manipulation with the heart, the contact of blood components with artificial surfaces of the cardiopulmonary bypass circuit, transient endotoxemia, and ischemia-reperfusion injury of the heart and lungs are relevant [1, 2].

The systemic inflammatory response syndrome (SIRS) viewed as basically useful has been conserved during evolution in order to provide support for the host to survive in an unfriendly environment, such as strenuous exercise of the "fight" or "flight" nature, multiple injuries or burns, infections or, more recently, major surgery. Whichever the underlying case, tight control of every step of the inflammatory reactions must be executed both on local and on systemic level where activities of the neuroimmune, endocrine, and circulatory systems overlap. If this control fails, morbidity and mortality increase dramatically [3].

The presence of bacterial lipopolysaccharide (LPS) or endotoxin in systemic circulation is sensed as a very strong danger pathogen associated molecular pattern (PAMP) by innate immunity. This identification could be followed by an exagerated and sometimes overwhelming systemic inflammatory reaction [4]. In cardiac surgical patients, transient endotoxemia is a manifestation of insufficient blood supply to the splanchnic vascular bed after a substantial amount of blood volume has been diverted from the patient's own vasculature into the tubing circuit of the heart-lung machine. Gut wall ischemia results in an increase of villous capillary permeability with ensuing translocation of lipopolysaccharide or even of the patient's own enteral flora into the systemic circulation.

The presence of LPS is identified by numerous phylogenetically highly conserved receptors of innate immunity which are called pattern recognition receptors (PRRs). PRRs are either humoral or membrane molecules which are sometimes shed into the body fluids. The crucial role in the identification of LPS is devoted to the humoral lipopolysaccharide binding protein (LBP) and two membrane receptors

CD14 and toll-like receptor-4 (TLR-4, CD284), respectively. All these molecules are working in concert providing stimulatory signals to innate immunity cells such as monocyte-macrophages and granulocytes [5]. However, the former two molecules display a dual role in an inflammatory reaction. While present in plasma in low concentrations, CD14 being in the soluble form, early during inflammatory response, LBP and sCD14 are serving as a part of an early alarm system aimed at recognizing and binding of LPS and other danger signals, and thus enhancing the activation of the immune system. In the late phase, both sCD14 and LBP could play a role by preventing the lethal side effects of overwhelming inflammatory reaction induces by the presence of "danger."

This study was aimed to follow the serum levels of LBP and sCD14 in cardiac surgical patients undergoing coronary artery bypass grafting (CABG) either with the use of cardiopulmonary bypass (on-pump) or without the use of cardiopulmonary bypass (off-pump). The levels of these markers immediately after surgery and up to seventh postoperative days were compared to preoperative level. Whereas LBP is recognized as a typical acute phase protein principally synthesized by hepatocytes, sCD14 molecules are either produced de novo as acute phase protein or are released into body fluids by shedding from cell surfaces. To ascertain the sources of sCD14, its level was correlated to the level of IL-6 which is the most potent stimulus for liver synthesis of acute phase proteins and to the levels of two pentraxins; C-reactive protein (CRP) and long pentraxin (PTX3), respectively.

2. PATIENTS AND METHODS

Forty patients (31 male, mean age 67.9±9 and 9 female, mean age 66.4±6.4, 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, $n = 2$, 16 male, 4 females, mean age 69.4 ± 7) or beating heart surgery (off-pump, $n = 2$, 15 males, 5 females, mean age 65.9 ± 9.7).

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, resp.) and the number of performed coronary anastomoses (median 2.0 in on-pump, 2.0 in off-pump, resp.). 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 μmol/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 study protocol was approved by the Ethics Committee of the University Hospital in Hradec Králové. 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.

Cardiopulmonary bypass, off-pump technique and anesthesiological management have been recently described in detail elsewhere [6].

3. BLOOD SAMPLING

Venous blood (central venous blood from arteria pulmonalis, peripheral venous blood from an antebrachial vein) was withdrawn in the operating room and on the first postoperative day in the ICU. Afterwards, only peripheral venous blood was taken due to the removal from the patients' vascular bed of all superfluous indwelling cannulas. Since there were practically no differences in results representative of blood samples originating from the respective sampling sites, for the sake of clarity only values obtained from the peripheral venous blood are indicated as results representative of the entire period of investigation. Samples were collected into tubes manufactured by Greiner, Germany.

In both on-pump and off-pump patients, blood was withdrawn at the following time points:

(i) introduction to anaesthesia, which in both groups represented the baseline or reference value for all parameters measured thereafter;
(ii) after termination of the operation;
(iii) the first postoperative day;
(iv) the third postoperative day;
(v) the seventh postoperative day.

3.1. Blood sample analysis

Untreated blood samples were allowed to clot at room temperature. Serum samples were obtained after centrifugation (2000 g per 8 minutes), aliquoted and immediately freezed. Samples were thawed only once. Serum level of LBP was determined by ELISA kit, cat.number HK315, HyCult biotechnology b.v., The Netherlands. Serum level of sCD14 was evaluated by sCD14 EASIA kit, cat.number KAS0231, BioSource Europe S.A., Belgium. IL-6 was quantitatively measured by commercially available ELISA kit (BenderMed Systems) according manufacturer's instructions. Results were evaluated by spectrophotometry at 450 nm (Multiscan photometer) using Genesis software. CRP was assessed by immunonephelometry on IMAGE 800 (Beckman). PTX3 was detected using detection set (Alexis Biochemicals, Switzerland) cat.no. ALX-850-299-KI01 for sandwich ELISA application that provided capture monoclonal antibody to PTX3 (700 ng/mL), detection polyclonal antibody to PTX3 (25 ng/mL), and recombinant PTX3 (standard). Plates (96 wells, NuncmaxiSorb 446612) were read at 405 nm by an automatic reader (Multiscan photometer) and evaluated by Genesis software.

3.2. Statistics

Serum level changes within a time and differences between both groups of patients were compared by two-way analysis of variance for repeated measures and Fisher's post hoc test. Results are expressed as medians and quartiles. Relationships

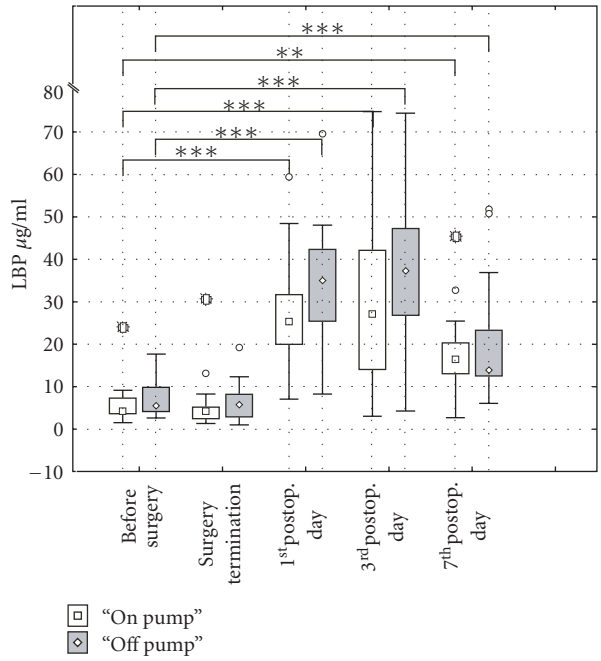

Figure 1: Comparison between serum levels of LBP in "*on-*" and "*off-pump*" patients F (1.32) = 1 .96; P < .l75.

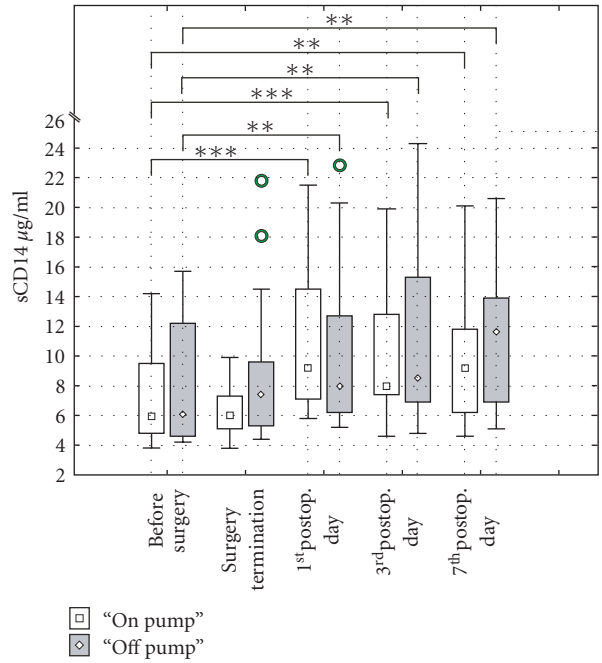

Figure 2: Comparison between serum levels of sCDI4 in "*on-*" and "*off-pump*" patients F (1.32) = 0.86; P < .4I7.

between concentrations of different cytokines were assessed using Pearson's correlation. Probability values of <.05 were considered statistically significant. Statistical analyses were performed with Statistica 6 software (StatSoft, USA).

4. RESULTS

4.1. Changes in the serum level of LBP

The baseline preoperative levels of LBP to which LBP concentrations were statistically compared were nearly identical in both on-pump and off-pump patients (6.25 ± 5.12 μg/mL; 7.61 ± 4.79 μg/mL, resp.). The same situation was found after finishing surgery indicating that LBP levels are not influenced neither by surgery itself nor by cardiopulmonary bypass. The sharp statistically significant increase of LBP concentrations was found on the 1st postoperative day in both groups of patients. The maximal level of LBP was reached on the 3rd postoperative day in both on-pump and off-pump patients (30.33 ± 9.96 μg/mL; 37.99 ± 16.58 μg/mL, resp.). The serum levels of LBP declined therafter being still significantly higher on the 7th postoperative day in both on-pump and off-pump patients (18.11 ± 9.96 μg/mL; 20.15 ± 13.79 μg/mL, resp.). Suprisingly, the concentrations of LBP were slightly higher in off-pump patients comparing with on-pump patients. However, the statistical significance was not reached comparing on-pump and off-pump patients (P < .1715). Results are shown in Figure 1.

To test the hypothesis that LBP is serving as one of acute phase proteins, the correlation between serum levels of LBP and IL-6 which is the principal cytokine regulating acute phase proteins by hepatocytes, and the two members of prototypic pentraxin family acute phase proteins, C-reactive proteins, and long pentraxin 3, respectively, were tested. No correlations were found thus rejecting our hypothesis (data are not shown).

4.2. Changes in the serum level of sCD14

The baseline preoperative levels of sCD14 to which sCD14 concentrations were statistically compared were very similar in both on-pump and off-pump patients (7.29 ± 3.32 μg/mL; 8.30 ± 4.26 μg/mL, resp.). There was a slight, nonsignificant decrease of sCD14 level in on-pump patients after surgery (P = .170), whereas sCD14 concentration was nonsignificantly elevated in off-pump patients at that time. There were different trends in LBP concentration in on-pump and off-pump patients during postoperative period. The maximum of sCD14 concentration in on-pump patients was reached on the 1st postoperative day (11.30 ± 4.93 μg/mL; P = .0001) followed by a gradual decrease up to the 7th postoperative day (9.41 ± 4.80 μg/mL; P = .009) being still above preoperative baseline level. In contrast, there was a gradual increase of sCD14 serum level from the end of surgery up to the 7th postoperative day when the maximum was reached (11.05 ± 4.57 μg/mL; P = .002). In spite of these differences, no significant differencies between on-pump and off-patients were found (P = .417). Results are shown in Figure 2.

To test the hypothesis that sCD14 is serving as one of acute phase proteins, the correlation between serum levels of sCD14 and IL-6 which is the principal cytokine regulating acute phase proteins by hepatocytes, and two members of prototypic pentraxin family acute phase proteins, C-reactive proteins, and long pentraxin 3, respectively, were tested. No

correlations were found thus rejecting our hypothesis (data are not shown).

5. DISCUSSION

Numerous danger patterns, both endogenous and exogenous origins, are generated in patients undergoing cardiac surgical operation. Sensing of these danger patterns via innate immunity pattern recognition receptors (PRRs) is followed by the development of inflammatory response. Numerous PRRs are now fully characterized. Amongst them LBP, CD14, MD-2, and TLR-4 are implicated as key factors in innate immunity cells activation by bacterial endotoxin [7].

LBP is a 50-kDa polypeptide mainly synthesized in hepatocytes and is released as a 60-kDa glycoprotein into blood stream after glycosylation. Other sources of LBP synthesis have been identified, such as epithelial cells of mucosa as well as the smooth muscle cells of lung arteries, and heart muscle cells. The amino acid sequence of LBP revealed substantial homology to bactericidal permeability increasing (BPI) protein, another LPS-binding protein originated in innate immunity.

LBP binds to the amphipathic lipid A moieties of LPS with high affinity and has been shown to facilite the process of LPS monomerization and subsequent presentation to other cellular and humoral binding sites. It catalyzes the transfer of LPS to a binding site of membrane-bound mCD14, which represents one part of cellular-LPS receptor. Adding LBP to a serum-free cell system enhances the LPS-mediated stimulation of CD14-positive cells 100- to 1000-fold. In addition, LBP transfers LPS to soluble sCD14 molecule [8].

We found significantly increased serum levels of LBP in our cardiac surgical patients from the first postoperative day up to the 7th postoperative day in comparison with preoperative level. The maximum, approximately 30 μg/Ml, was reached on the 3rd postoperative day with subsequent decline in both off-pump and on-pump patients. It was suprizing to recognize that LBP level was even higher in off-pump patients, but no significant differences between on-pump and off-pump patients were found. We have no informations regarding LBP measurement in cardiac surgical patients to compare our results. The only one exception is the work by Fransen et al. [9] who followed LBP concentrations in on-pump patients. Unfortunately, their observation period was only up to 18 hours after declamping aorta. They found significantly increased LBP level at the 8th hour with subsequent increase at the 18th hour after start of reperfusion but maximum was not reached in their observation period.

Several in vivo and in vitro experiments demonstrated that LBP is a secretory class 1 acute phase protein whose gene is transcriptionaly activated by cytokine-inducible nuclear proteins. The transcriptional regulation is induced by IL-1 alone or synergistically by IL-1 and IL-6 leading to a maximum LBP concentration within 24–48 hours after stimulation. This response can be strongly enhanced by TNF-α and dexamethasone [10]. The dynamics of LBP concentrations in our cardiac surgical patients was resembling this kinetics but with prolongation up to the 7th postoperative day. To test the effect of proinflammatory cytokines on LBP synthesis, the level LBP was correlated with the level of IL-6 which is a principal cytokine regulating acute phase proteins synthesis in the liver. No such correlation was found (data are not shown). The possibility that LBP concentration in blood is influenced by exogenous corticosteroid is also unlikely. There was no significant difference between on-pump and off-pump patients despite the fact that former patient's group is exposed to methylprednisolone which is a standard component in CPB fluid used in our setting.

In humans LBP is constitutively in serum at concentrations of 5–15 μg/mL. It is in a good concordance with its baseline level in our cardiac surgical patients. Its level is raised 10- to 50-fold during the acute phase reaction [11]. The similar findings were revealed by us in our patients. In contrast to C-reactive protein which level is peaked at the 3rd day in our patients and then rapidly declined, increased level of LBP is sustained up to the 7th postoperative day. It probably reflects the dual role of LBP in an inflammatory reaction. Whereas serving as a potent pattern recognition receptor at low concentrations early during inflammation to amplify the immune response rendering, for example, TNF-α production in macrophages, high concentrations inhibits danger-pattern-induced host cell activation [11]. It can be partly explained by the ability of LBP to transfer LPS to serum lipoproteins thus neutralizing the bioactivity of LPS. LPS has been shown to be physically associated with apoA- or apoB-containing lipoproteins and to transfer LPS into high- (HDL) and low-density lipoproteins (LDL) resulting in the clearance of LPS from the bloodstream. These capabilities were also reported for very-low-density lipoprotein (VLDL) and chylomicrons [12].

Up to now, few studies have been published evaluating the value of LBP as a diagnostic marker in patients with SIRS of noninfectious versus infectious origin and as potential prognostic marker predicting outcome [13]. These results can not be proven in our cardiac surgical patients because no cultivation-confirmed bacterial infections were found in this group. Information regarding LBP level during cardiac surgery are very sparse. It is reported by Vreugdenhil et al. [12] that plasma level of LBP is gradually increased from the 8th to 18th hour after declamping aorta but the maxim was not reached in their observation period. It is resembling our data but in our patients the maximum in LBP production was reached on the 3rd postoperative day. The role of LBP as acute phase proteins is unlikely in our cardiac surgical patients because no correlations with either IL-6, CRP, or PTX3 were found. It is extremly interesting that no significant differences in the serum level of LBP between on-pump and off-pump patients were found in our study. It is generally assumed that splanchnic hypoperfusion during extracorporeal circulation together with steady laminar blood flow that is generated by the heart-lung machine instead of the pulsative blood flow generated by each cardiac contraction, result in gut wall ischemia, subsequent increase of villous capillary permeability and transient endotoxemia. It is supposed that these changes are not so profound in off-pump patients. Lack of differences in LBP concentration between on-pump and off-pump patients could be interpreted in at least two

ways. First, the intensity of the exposition to bacterial danger pattern is similar in both on-pump and off-pump patients. Second, LBP production is stimulated by another still unknown danger pattern which is identical in both on-pump and off-pump patients.

The second aim of our study was to follow the changes of soluble form of CD14 molecule (sCD14) during cardiac surgical operation. CD14 is a glycosylphosphatidyl-inositol-anchored protein constitutively expressed on the surface of various cells, including monocytes, macrophages, neutrophils, B-cells, dendritic cells, as well as several other cell types of nonhematopoietic origin. Aside of this membrane-bound state, CD14 is also found in a circulating soluble form [14]. CD14 molecule is the part of a receptor system of innate immunity cells to identify danger patterns of both exogenous and endogenous origin. This system is represented by the combined actions of the membrane-bound isoform of CD14 with the central transmembrane signaling unit of TLR-4 and the accessory protein MD-2 [15].

Two opposite functions have been described for sCD14. It can either reduce endotoxin-induced activities by competing with mCD14 for LPS binding or mediates the LPS-induced activation of non-CD14-expressing endothelial, epithelial, and smooth muscle cells. In addition, CD14 may function as a receptor for other microbial products, human heat shock proteins Hsp60, and other endogenous ligands such as ceramides, phospholipids, and modified lipoproteins [16].

We found significantly increased serum level of sCD14 from the 1st up to 7th postoperative days compared to the preoperative values in both on-pump and off-pump patients. Suprisingly, there are no significant differences between on-pump and off-pump patients. Evenmore, while reaching the maximum on the 1st postoperative day in on-pump patients, sCD14 level was gradually increasing in off-pump patients up to the 7th postoperative day (end of observation). It is not easy to discuss our findings. The level of sCD14 is not commonly assessed in cardiac surgical patients. It could be possible to extrapolate from patients undergoing major elective abdominal surgery as was reported by Hiki et al. [17]. They found slight decrease 6 hours after incision of skin, reaching the maximum on the 1st postoperative day thereafter decline to approximately baseline preoperative level on the 10th postoperative day. This pattern is resembling our off-pump patients with the exception that maximal concentration in our patients was slightly higher (11.3 μg/mL versus 9.4 μg/mL).

Several clinical studies have reported elevated serum levels of sCD14 in various inflammatory conditions such as Kawasaki disease [18]. Furthermore, correlation between sCD14 and severity of the trauma in polytraumatized patients have also been published [19]. Besides its function in LPS signaling, sCD14 might therefore play a role in inflammatory processes by controlling the immune system level of response. It has recently been demonstrated that sCD14 is a regulatory factor capable of modulating cellular and humoral immune responses by interacting directly with T and B cells [20]. Moreover, it has been suggested that sCD14 could be an acute phase protein, because apart from proteases-mediated shedding, sCD14 is also produced by hepatocytes, which represent the major source of acute phase proteins. Indeed, Bas et al. [14] in their clinical and experimental studies clearly showed that in patients suffering from rheumatoid arthritis, serum level of sCD14 did not correlate with the number of leukocytes, thus excluding an important source from leukocyte membrane-bound CD14, by protease-mediated shedding. In contrast, serum levels of sCD14 in these patients correlated with those of C-reactive protein, a classical acute phase protein, and IL-6, a cytokine known to regulate the synthesis of APP in the liver. We also sought for such correlations in our cardiac surgical patients. No statistically significant correlations between serum level of sCD14 and either IL-6 concentration or CRP and PTX3 levels were found in our study. It could be concluded from our results that sCD14 is not produced as one of acute phase proteins in cardiac surgery. The source of sCD14 in these patients remains enigmatic. Whether sCD14 in cardiac surgery originates mainly from leukocytes by protese-mediated shedding warrants further investigations.

In conclusion, we found significantly increased levels of both sCD14 and LBP in the early postoperative period in cardiac surgical patients. We found no significant differences between on-pump and off-pump patients in the serum levels of these parameters. Finally, both LBP and sCD14 molecules do not seem to act as acute phase proteins in cardiac surgical patients.

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ABBREVIATIONS

IL: Interleukin
LBP: Lipopolysaccharide binding protein
LPS: Lipopolysaccharide
PAMP: Pathogen associated molecular patterns
PRR: Pattern recognition receptors
PTX3: Long pentraxin 3
sCD14: Soluble form of CD14 molecule
SIRS: Systemic inflammatory response syndrome
TLR: Toll-like receptors

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ORIGINAL ARTICLE

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

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Summary: ***Background:*** Myeloid cells are extensively activated in patients undergoing cardiosurgical operations. It is supposed that this activation is more profound in patients operated with 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 differencies 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 them 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 cardiosurgical 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 prerequisited 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 beloging 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 Gessler 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 cardiosurgical 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 cardiosurgical 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 ≥ 130 μmol/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 (NaHCO3 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/m^2 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 cisatrakurium. 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 lymfoid 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 IgG_1FITC/IgG_1PE monoclonal antibodies serve as negative isotypic 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).

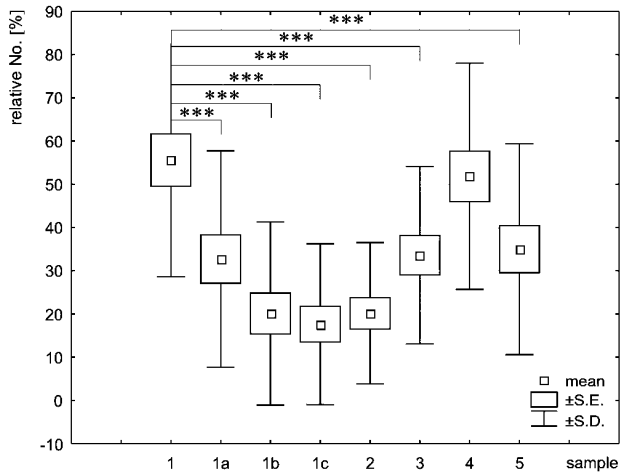

Fig. 1: Changes in the relative number of MEM-148 positive activated myeloid cells in "on-pump" patients.

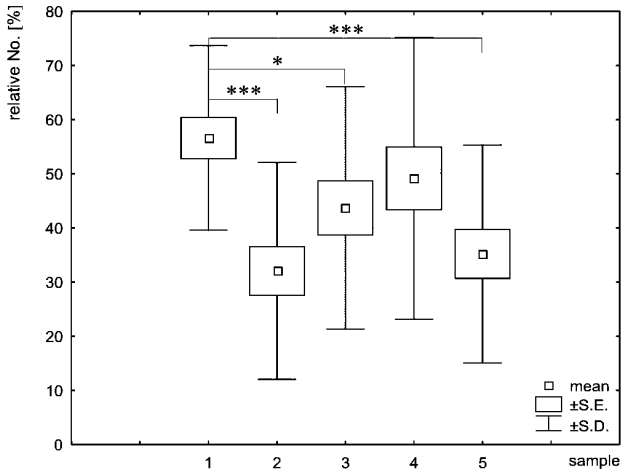

Fig. 2: Changes in the relative number of MEM-148 positive activated myeloid cells in "off-pump" patients.

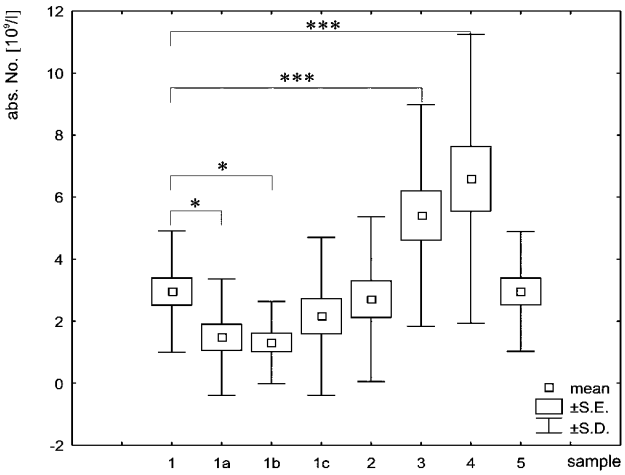

Fig. 3: Changes in the absolute number of MEM-148 positive activated myeloid cells in "on-pump" patients.

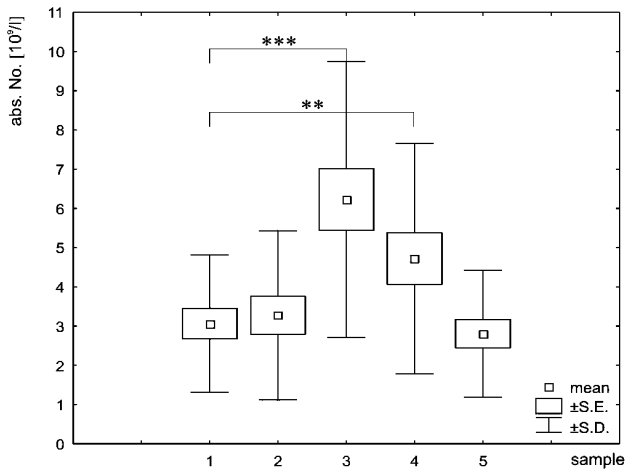

Fig. 4: Changes in the absolute number of MEM-148 positive activated myeloid cells in "off-pump" patients.

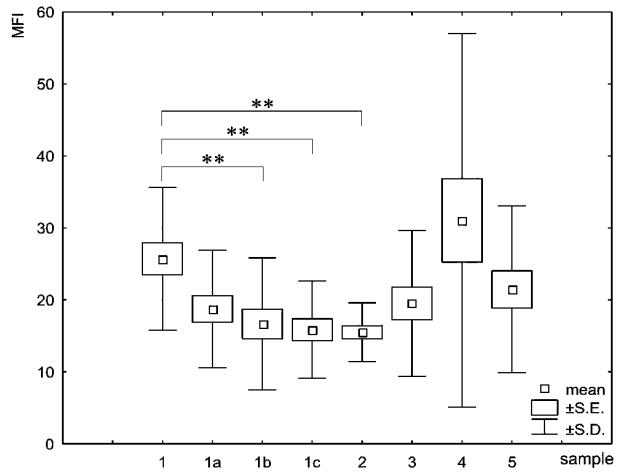

Fig. 5: Changes in the density of expression of MEM-148 activation marker on myeloid cells in "on-pump" patients.

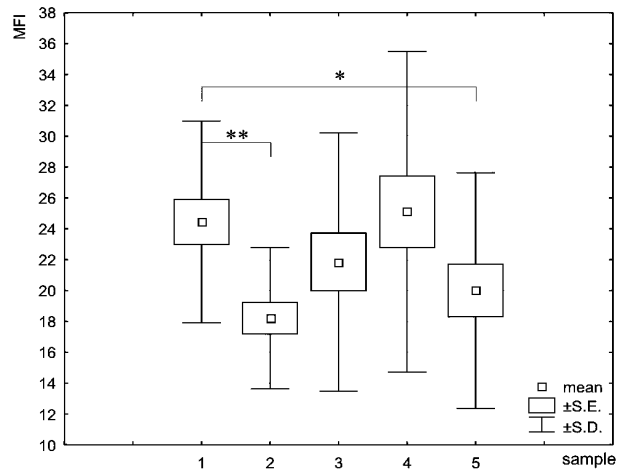

Fig. 6: Changes in the density of expression of MEM-148 activation marker on myeloid cells in "off-pump" patients.

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 lumenal 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 Fcγ-receptor I (CD64), a high affinity receptor for IgG_1 and IgG_3 subclasses of immunoglobulins. Numerous substances of both exogenous and endogenous origin are rapidly upregulating FcγRI expression on the surface of neutrophils (13). In contrast to FcγRI, 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 CD11α 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 intracellulary in resting cells. Transmembrane fragments of CD18 produced by the activation-induced proteolytic cleavage obviously loose 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 latter 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 circuites. 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 persistant 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 suppossed to relieve from potentially harmfull 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 wheather 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 obeserved 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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