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Local mRNA Expression of Vascular Cell Adhesion Molecule-1 (VCAM-1), Macrophage Colony-Stimulating Factor (MCSF-1), and its Receptor, *c-fms*, on Rabbit Heart Valves in the Early Phase after Atrioventricular Valve Surgery and *Staphylococcus aureus* Bacteremia

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Contents

Abbreviations

AMV	avian myeloblastosis virus
AV	aortic valve
CAM	cellular adhesion molecule
EC	endothelial cells
ICAM-1	intercellular adhesion molecule-1
IE	infective endocarditis
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MV	mitral valve
MCSF-1 or M-CSF	macrophage colony-stimulating factor-1
PV	pulmonary valve
PA	protein A
PMN	polymorphonuclear leukocyte
RT-PCR	reverse transcription polymerase chain Reaction
S. aureus	*Staphylococcus aureus*
TNF	tumor necrosis factor
TV	tricuspid valve
VCAM-1	vascular cell adhesion molecule-1
VLT-4	very late antigen-4

1. Introduction

1.1 Overview of the roles of VCAM-1, MCSF-1, and *c-fms* in the pathogenesis of inflammation

The vascular endothelium is a regulatory organ ubiquitously distributed throughout the body. Its structural and functional integrity is fundamental to the maintenance of the antithrombotic properties at the interface between blood and the endothelial surface. The endothelium is extremely sensitive to various pathogenic stimuli such as proinflammatory cytokines (e.g. IL-1, TNF-α), bacteria or bacterial endotoxin such as LPS (lipopolysaccharides), hemodynamic shear stress, physical injury, oxidized lipids, hypoxia and nicotine [Verrier, E.D. et al. (1997) and Martin, T.R. (2000)]. In response to these stimuli, endothelial cells undergo profound changes that allow them to participate actively in the inflammatory response.

In the pathogenesis of inflammation in general, one of the initial events is the adherence of mononuclear leukocytes to the endothelial cell surface and the interaction between blood leukocytes and endothelial cells. The endothelium plays a key regulatory role during inflammatory responses, controlling leukocyte adhesion and migration through selective expression of cytokines, chemokines, and adhesion molecules. Adhesion between endothelial cells and circulating leukocytes is mediated at least in part by adhesion molecules in

targeting leukocytes to the sites of inflammation [Ruoslahti, E. (1991)]. Cellular adhesion molecules (CAM) mediate these cellular interactions as well as the cross-talk between endothelial cells and components of the extracellular matrix. Generally, CAMs can be classified in 4 main groups: (a) integrins, (b) cadherins, (c) members of the immunoglobulin superfamily of CAMs (IgCAMs), and (d) selectins [Juliano, R.L. et al. (2002)]. Leukocyte recruitment to the endothelium requires the coordinated expression of cellular adhesion molecules on the endothelium, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin.

A single layer of endothelial cells (ECs) lines the entire cardiovascular system including the cardiac chambers and heart valves [Leask, R.L. et al. (2003)]. CAMs initiate the EC's inflammatory response through the active recruitment of the immune effector cells and may play a crucial role in the development or the resistance to bacterial valvular and endocardial vegetations, as well as to degenerative valvular diseases [Müller, A.M. et al. (2000)].

The vascular cell adhesion molecule-1 (VCAM-1) belongs to the immunoglobulin superfamily, which contains Ig-like extracellular domains that are responsible for cell adhesion. It is present on the membranes of endothelial cells that have been stimulated by proinflammatory substances.

It binds the integrin protein very late antigen-4 (VLA4; $\alpha_4\beta_1$), present on lymphocytic and monocytic cells. It is involved in recruiting these cells from the bloodstream to sites of infection and/or inflammation in the tissues. Previous studies suggest that the expression of VCAM-1 on the endothelium is a hallmark of inflammation and heralds cellular infiltration [Springer, T.A. (1994)]. VCAM-1 expression appears to precede macrophage accumulation in VCAM-1 positive endothelium and VCAM-1 and macrophage levels correlate well in lesion-prone regions [Truskey, G.A. et al. (1999)]. VCAM-1 interacts with VLA-4 on activated lymphocytes and leads to the extravasation of activated lymphocytes into valve tissue in rheumatic carditis [Roberts, S. et al. (2001)] (see Fig.1).

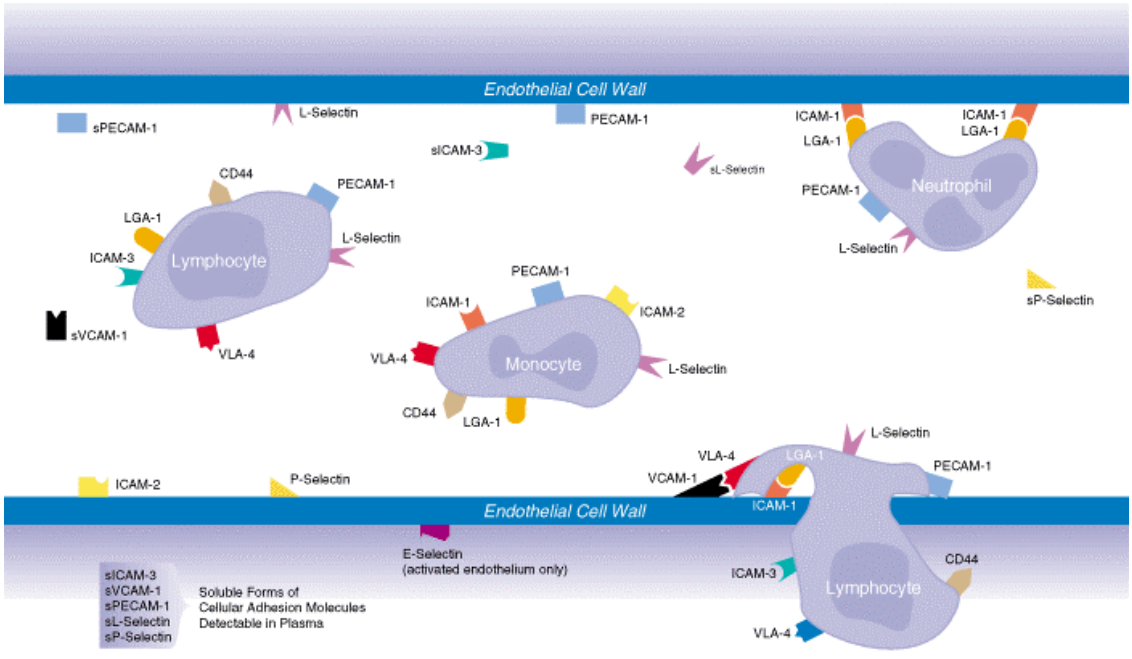


Figure 1: Schematic diagram illustrating leukocyte and endothelial cell interactions mediated by cellular adhesion molecules

Monocytes/macrophages are key players in mechanisms of wound repair, tissue remodeling, and inflammation. The macrophage colony-stimulating factor (M-CSF, also called CSF-1) is a key regulator of monocytic cell lineage development. In bone marrow, M-CSF stimulates proliferation and differentiation of committed progenitors, leading to the production of blood monocytes and tissue macrophages [Stanley, E.R. et al. (1997)]. M-CSF-1 is also a potent proinflammatory cytokine [Zheng, G. et al. (2000)]. All biological effects of M-CSF are mediated by a single receptor, encoded by the proto-oncogene *c-fms*, which is expressed on the surface of cells undergoing monocytic development [Woolford, J. et al. (1985) and Sherr, C.J. et al. (1985)]. *c-fms* is a member of class III of the receptor tyrosine kinase family, which includes Kit, Flt3, and the α- and β-platelet-derived growth factor receptor and interacts with multiple signal transduction proteins including *src* kinase, STAT1, Grb2, and phosphoinositide 3-kinase [Mitrasinovic, O.M. (2001) and Bourette, R.P. et al. (2000) and Rothwell, V.M. et al. (1987)]. Previous studies have shown that recruited monocytes play an important role via the M-CSF/*c-fms* pathway at the site of inflammation [Lan, H.Y. et al. (1997) and Yang, N. et al. (1998) and Kerr, P.G. et al. (1994) and Goto, M. et al. (1993) and Bischoh, R.J. et al . (2000) and Le Meur, Y. et al (2002)].

1.2 Background and objectives of the present study

The pathogenesis of infective endocarditis is poorly understood. For example, the unique propensity of *Staphylococcus aureus* affecting the right heart in intravenous drug-abusers, but mostly the left heart in non-drug-abusing clinical patients is unsolved [Sande, M.A. et al. (1992) and Levine, D.P. et al. (1986)]. Similarly, the phenomenon of spontaneous resolution of right-sided endocarditis in rabbits infected through catheter is as well unclear [Donabedian, H. et al. (1985) and Freeman, L.R. et al. (1979)]. These observations suggest that there are different host responses in the left and the right heart. There are also few studies on the role of VCAM-1, MCSF-1 and *c-fms* in the development of bacterial endocardial vegetation and its effects in valve tissue during bacterial colonization and growth of the vegetation. To our knowledge, there are no studies on mRNA expression of VCAM-1, MCSF-1 and *c-fms* in heart valves, although their roles may be crucial in the pathogenesis of infective endocarditis. In our new experimental model of infective endocarditis in rabbits [Eichinger, W.B. et al. (2002)], we examined the valvular mRNA expression of VCAM-1, MCSF-1 and *c-fms* for the first time. The study was designed to find out whether

(i) early (6 hours after stimulation) gene expression changes are induced by *Staphylococcus aureus*

(ii) early gene expression changes are induced by the presence of an artificial material on the atrioventricular valves, by surgical trauma

and/or by disturbed flow patterns caused through mitral or tricuspid regurgitation which was due to valve insufficiency

The study should help to obtain a better insight into the molecular pathology of infective endocarditis.

2. Materials and Methods

2.1 Establishment of infective endocarditis (IE)

Most patients with IE have pre-existing valve abnormalities. The normal endothelium is nearly resistant to colonization and infection by circulating bacteria [Durack, D.T. et al. (1972) and Durack, D.T. (1975)]. Therefore, we developed a new experimental model of infective endocarditis. By suturing a Dacron patch onto the mitral or tricuspid valve the trias of (i) endothelial lesion, (ii) disturbed hemodynamics and (iii) the presence of an artificial material causes high susceptibility for bacterial adhesion and subsequent development of an acute bacterial endocarditis. Immediately after patch placement, $5*10^6$ colony-forming units of *S. aureus* were admitted intravenously through a peripheral vein. In a prior investigation, this procedure proved to establish infective endocarditis in 64% (9/14) of the animals on the mitral valve [Eichinger, W.B. et al. (2002)] (Figs. 2, 3) and in none of the tricuspid valves (unpublished observation).

Figure 2: Adjacent vegetation in the mitral valve and in the left ventricle 48 hours after Dacron patch implantation

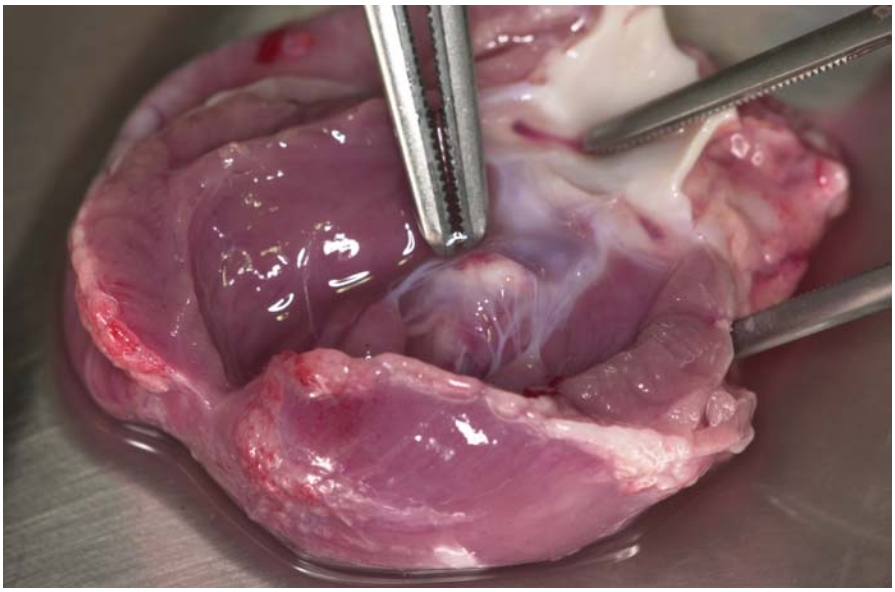


Figure 3: Aspect of the left ventricle with a Dacron patch on the mitral valve 48 hours after the patch implantation. The patch material is covered by a vegetative formation.

2.2 Bacterial Strain and Growth Conditions

We used *Staphylococcus aureus* (ATCC strain 29213) as infective agent, because staphylococci are the pathogens most frequently encountered in infective endocarditis (IE) on prosthetic materials [Durack, D.T. et al. (1994) and Benn, M. et al. (1997) and Hogevik, H. et al. (1995) and Chastre, J. et al. (1995) and Lowy, F.D. (1998) and Moreillon, P.A. et al. (2002)] and because its use is well established in experimental models [Tufano, M.A. et al. (1991) and Strindhall, J. et al. (2002) and Veltrop, M.H. et al. (2000)].

Bacteria were routinely grown overnight at 37ºC on Columbia sheep blood agar. This medium was also used to verify the identity of isolates recovered from infected rabbits. Each dilution was confirmed by quantitative culture on blood agar plates.

The cultures were adjusted to an optical density of 0.2 at 620 nm to achieve a concentration of $2*10^8$ CFU/ml dilution. From this starting concentration the cultures were further diluted in 0.9% buffered saline solution to obtain suspensions containing $2*10^6$ CFU/ml.

2.3 Operation Technique

The animal experiments were authorized by the local animal protection

committees. 42 female Chinchilla Bastard rabbits each weighing 3-4 kg were anaesthetized with a combination of 5 mg/kg S-ketamine hydrochloride and 0.15 mg/kg medetomidine hydrochloride injected intravenously, given as a 1.5 ml bolus injection; anesthesia was maintained with further injection at a rate of 0.5 ml/10 minutes. The animals were intubated and mechanically ventilated with 100% oxygen. The ventilator frequency was set at 30 breaths/minute. Continuous surveillance of heart rhythm, heart rate, temperature and mean arterial blood pressure were performed as a standard precaution for any surgical interventions. Perioperative issues involving fluid imbalance (hematocrit, erythrocyte count), electrolyte disturbances, oxygen saturation (pO_2, pCO_2 and pH) and serum bicarbonate levels were controlled every 15 minutes during the operation. All surgical procedures were performed under strict sterile conditions.

The rabbits were shaved around the operation area and rendered aseptic with povidone-iodine (Betadine). Surgical exposure was accomplished via thoracotomy entering the 3rd intercostal space. The lung was retracted carefully to reveal the heart. The pericardium was opened and suspended. Supplying the atrium with a purse-string suture and two Allis clamps around it, a stitch incision in the center of the suture was performed (Fig. 4a). The incision was immediately closed by moving the clamps together,

while the purse-string suture was still left open to permit the subsequent insertion of the Dacron patch. The previously prepared suture (Prolene 5-0) with the Dacron patch was fed through the atrial incision in a single move to the ventricle, perforating the anterior leaflet of the mitral or tricuspid valve, leaving the heart, ending up at the outside of the ventricular myocardium (Figs. 4b - d). The positions of the coronary veins and arteries were identified to prevent injuries during the maneuver. By drawing the ventricular end of the suture, the threaded patch followed the route of the suture through the atrium, and became caught in the valve tissue such that it created valve insufficiency. The mitral insufficiency, which was documented by augmentation of the ventricular wave in the atrial pressure curve, was measured by introducing a tip catheter through the atrial hole and advancing it towards the atrial side of the valve. One measurement was taken before pulling the thread, and another after tying it at the outside of the heart to the ventricular wall (Fig. 5).The atrial end of the Prolene suture was cut and left free to float in the inside of the atrium. Subsequent to the closure of the purse-string suture and the removal of the Allis clamps, pericardial sutures were cut and chest closure was performed after short PEEP ventilation to expand the collapsed lung.

Postoperative analgesia was maintained by intravenous administration of 0.0375mg/kg Piritramide every hour. The animals were monitored

carefully until the euthanasia was performed.

Straight after the operation appropriate dilutions of $5*10^6$ CFU of *S. aureus* were administered intravenously (Groups II, V, VI).

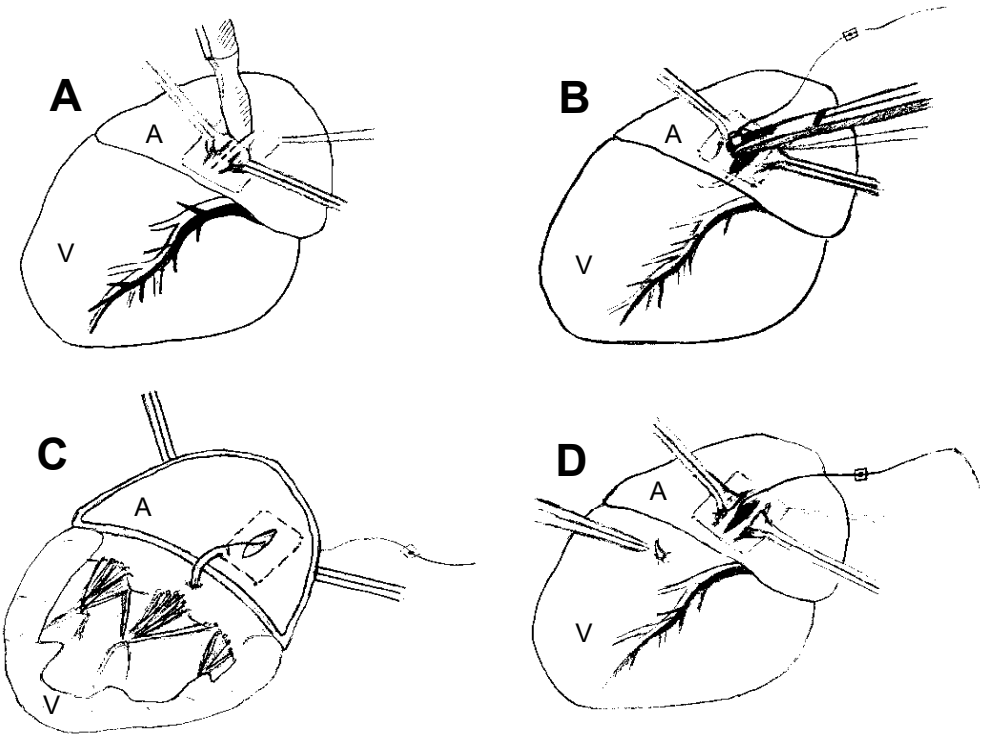


Fig. 4: Surgical technique of patch placement on the mitral valve (from [25])

A - Atrial incision

B - Insertion of the patch through the atrium

C - interior aspect of heart showing perforation of the anterior valve leaflet

D - the final stitch through the ventricle

(A: left atrium; V: left ventricle)

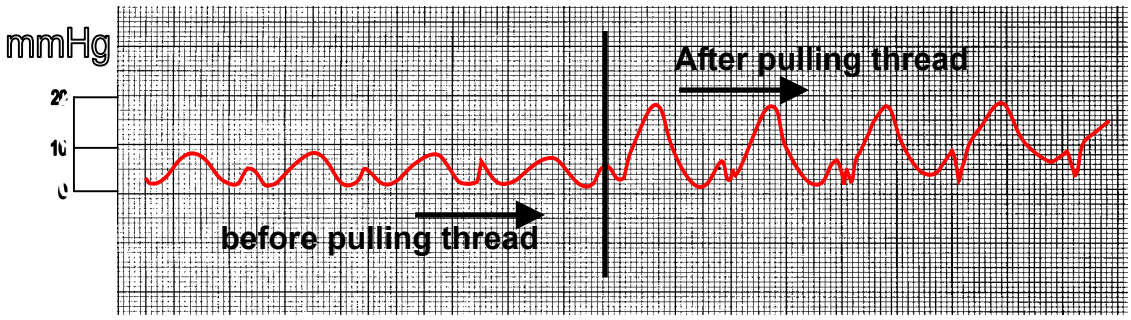


Figure 5: Documentation of mitral insufficiency by left atrial pressure measurement

The rabbits were sacrificed 6 hours after placement of the patch and/or the inoculation of *S. aureus* by rapidly administering 100 mg ketamine hydrochloride intravenously. The time point of 6 hours was chosen because acute modulations in the expression of the genes of our interest is expected to occur within this period as a consequence of bacterial and/or surgical stimulation [Grammer, J.B. et al. (2002)]. The heart was explanted, perfused with sterile phosphate-buffered saline and dissected. Attached fatty tissue was cut off and blood was removed by washing the specimens in 0.9% cold NaCl to obtain clean valvular tissue for RNA isolation. The samples were shock frozen in liquid nitrogen and stored at -75°C.

The operative procedure is shown in the following Figures 6 - 12:

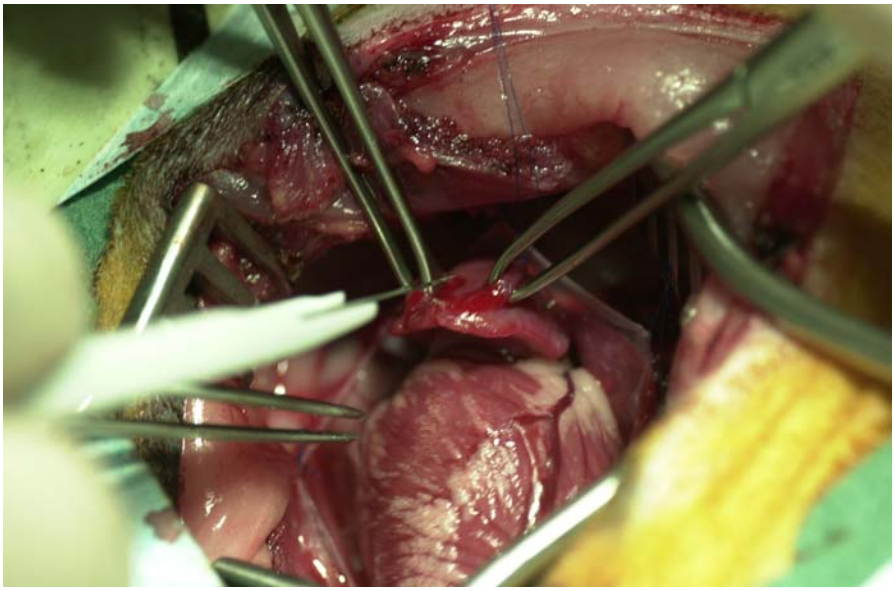


Figure 6: Atrial incision (step 1)

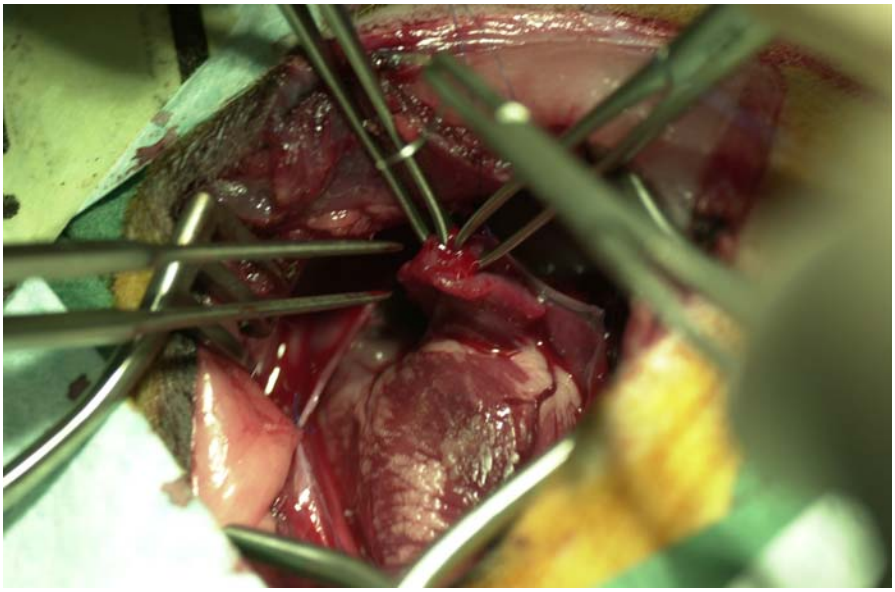


Figure 7: Two Allis clamps around the atrial incision (step 2)

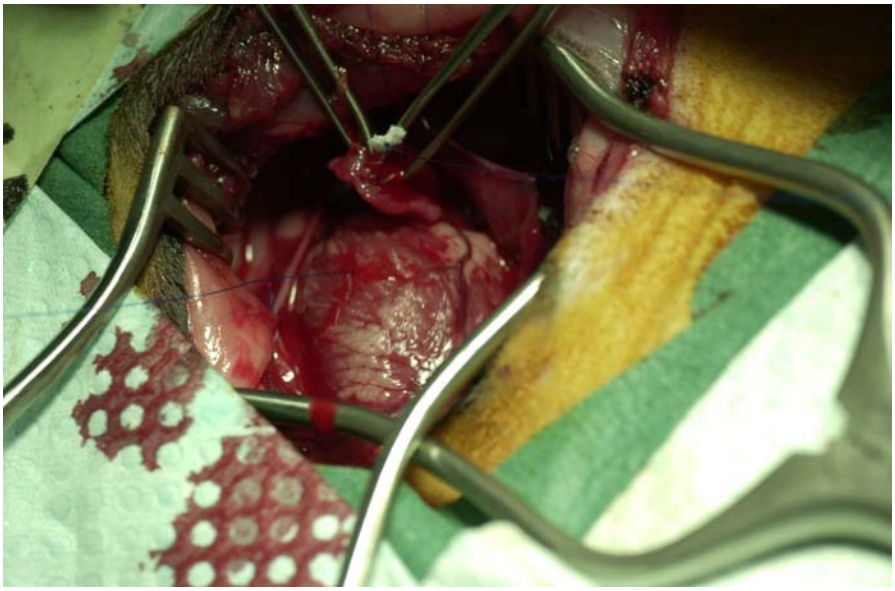


Figure 8: Dacron patch right before pulling through the atrium

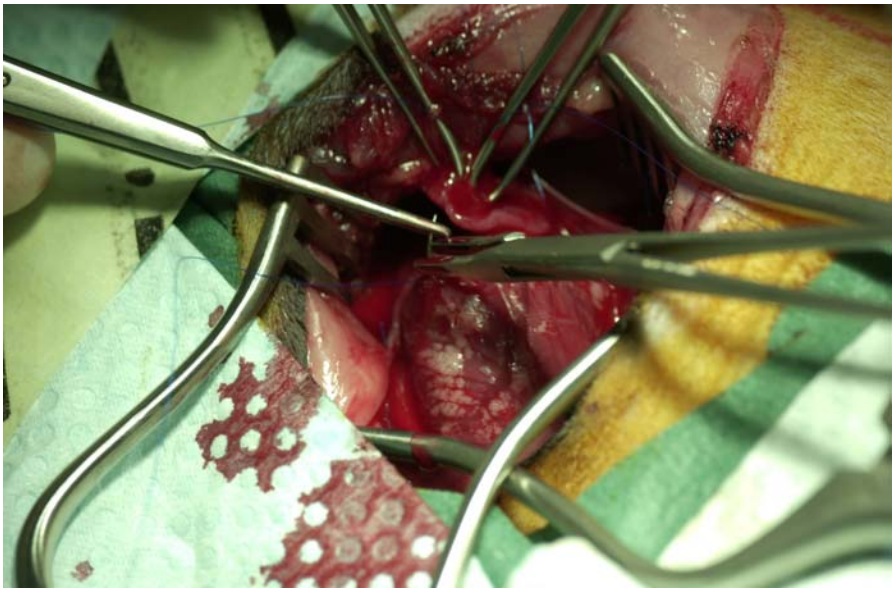


Figure 9: Insertion of the Dacron patch through the atrium (step 3)

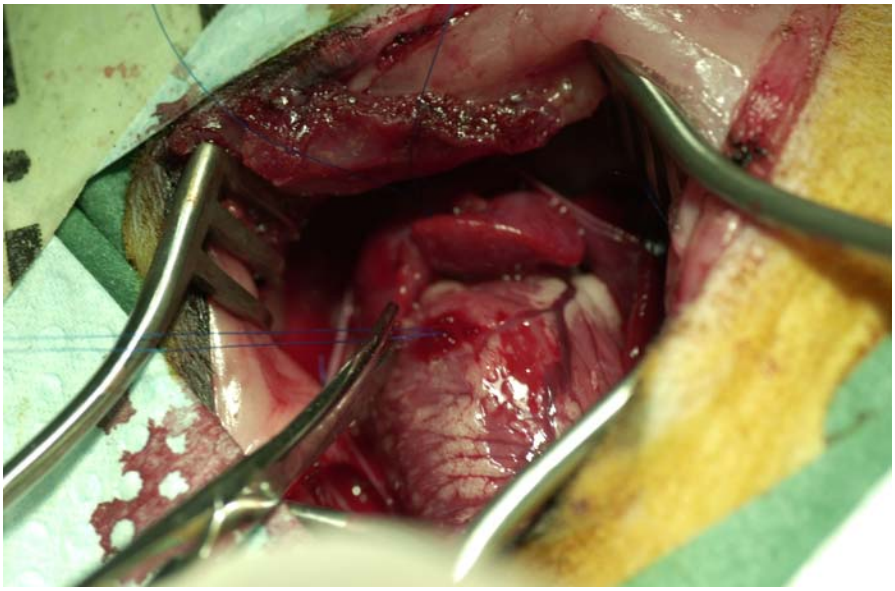


Figure 10: Pulling the thread to measure the atrial pressure (step 4)

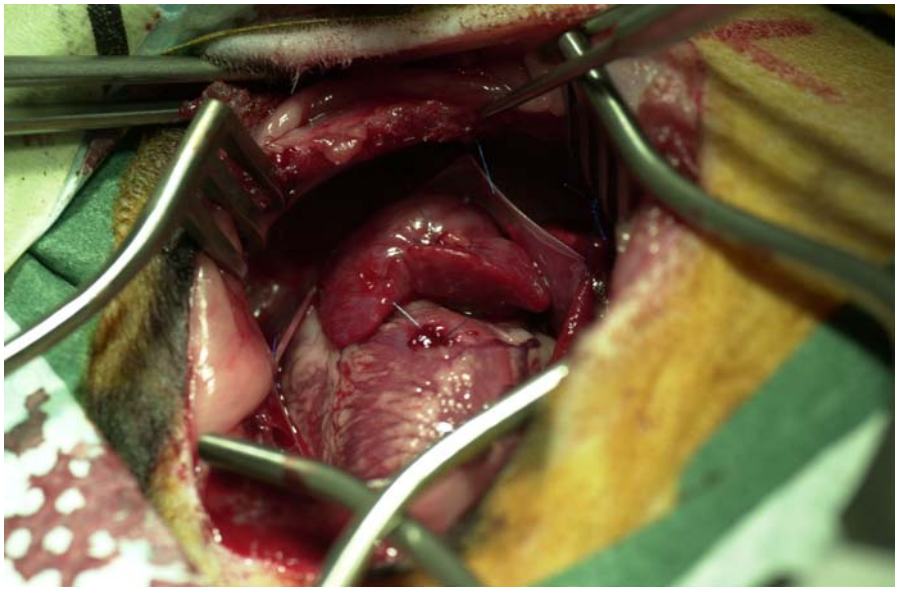


Figure 11: Operative situs after patch placement

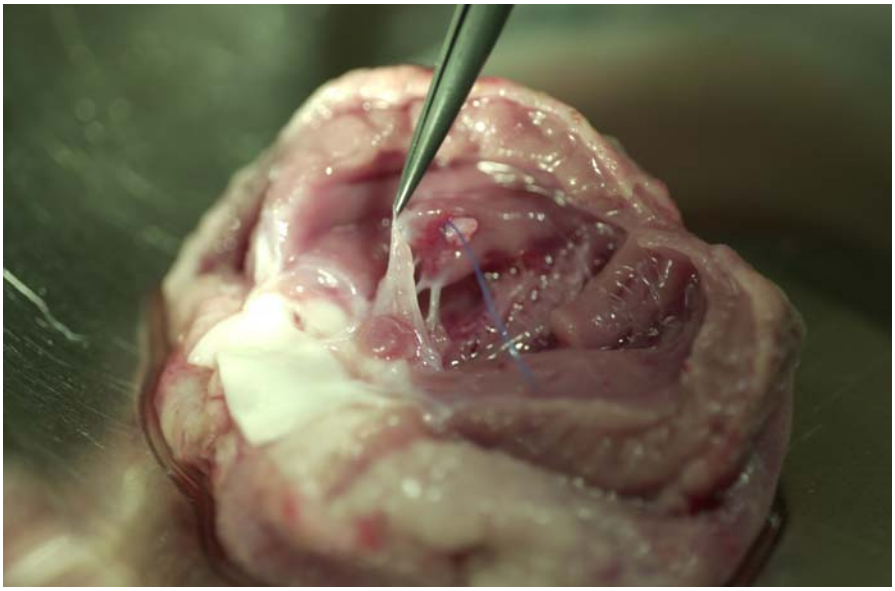


Figure 12: Explantation of the heart 6 hours after patch implantation

2.4 mRNA expression studies

In order to assess up- or down-regulation of mRNAs coding for VCAM-1, MCSF-1 and *c-fms*, the animals were divided into six groups to determine the influence of every stimulus on the expression of the above mentioned mRNAs. The groups were as follows:

Group I: (control, no intervention) this group consisted of 7 untreated rabbits. The explanted valvular tissues were collected, shock frozen in liquid nitrogen and stored at -75°C. This group was used to evaluate interindividual variation in transcriptional regulation without any intervention and to determine the baseline levels of the mRNAs.

Group II: (isolated *S. aureus* bacteremia) 7 rabbits received $5*10^6$ CFU of *Staphylococcus aureus* (ATCC strain 29213) intravenously, but did not undergo valve surgery.

Group III: (isolated tricuspid valve surgery) 7 rabbits were operated. The patch was sewed onto the anterior leaflet of the tricuspid valve via a right-sided thoracotomy. No bacteria were given.

Group IV: (isolated mitral valve surgery) 7 rabbits were operated. The patch was sewed onto the anterior leaflet of the mitral valve via a left-sided thoracotomy. No bacteria were given.

Group V: (tricuspid valve surgery and bacteremia) 7 rabbits were operated.

The patch was sewed onto the anterior leaflet of the tricuspid valve via a right-sided thoracotomy. Immediately after the operation $5*10^6$ CFU of *S. aureus* were injected intravenously.

Group VI: (mitral valve surgery and bacteremia) 7 rabbits were operated. The patch was sewed onto the anterior leaflet of the mitral valve via a left-sided thoracotomy. Immediately after the operation $5*10^6$ CFU of *S. aureus* were injected intravenously.

2.4.1 RNA isolation

Total RNA was isolated from the valve tissues using a modified guanidinium isothiocyanate procedure (RNeasy Maxi, Qiagen, Hilden, Germany), according to the protocol supplied by the manufacturers. RNA concentration and purity were determined spectrophotometrically and by electrophoresis on a 1.2% formaldehyde-agarose gel. In all samples, the 28S and 18S RNA bands were clearly visible without any signs of degradation. The $OD_{260/280}$ ratios were >1.8, indicating high purity.

Since the aortic and pulmonary valve of a rabbit are very small, it was necessary to create aortic and pulmonary valve “pools” from all 7 animals in each group, i.e. 7 aortic (or pulmonary) valves were put into one tube just before tissue homogenization and subsequent RNA isolation. This procedure allowed extraction of enough RNA for the gene expression

analysis experiments. Immediately after isolation, the RNA was stored at -75°C.

2.4.2 Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) amplification from 100ng of total RNA were performed using avian myeloblastosis virus (AMV) reverse transcriptase, *Taq* DNA polymerase, and *Pwo* DNA polymerase (Titan™ One Tube RT-PCR System, Roche, Mannheim, Germany). The specific oligonucleotide primer pairs used for amplification of the cDNAs of the rabbit VCAM-1, MCSF-1 and *c-fms* were designed on the basis of published sequences in GenBank by using the Primer software and corresponded to the following nucleotides:

(i) VCAM-1 826-850 and 1336-1315 (GenBank Accession No.X53051)

(ii) MCSF-1 198-221 and 591-570 (GenBank Accession No.E19817)

(iii) *c-fms* 105-126 and 237-216 (GenBank Accession No. R86522)

Primer pairs to amplify an 18S RNA fragment as internal control were from Ambion (QuantumRNA, Austin, TX, USA).

In the first set of experiments, the linear range of the RT-PCR was determined for all three genes in order to find out those PCR cycle numbers that ensured amplification product yields that were still in the

increasing linear range and did not yet reach the plateau phase (Figs. 13 a-c). Because 18S RNA is present in vast excess in total RNA samples, it is necessary to adjust the amplification efficiency of the 18S signal to the level of the gene of interest [Grammer, J.B. et al. (2000)]. This is achieved using 18S competimers (QuantumRNA), which are specific 18S primers that bind to 18S RNA but prohibit elongation in the PCR. The optimal ratios and detailed RT-PCR conditions for VCAM-1, MCSF-1, and *c-fms* are shown in Table 1. For semiquantitative RT-PCR, coamplifying 18S cDNA as internal control gene, the thermocycler was programmed to give an initial cycle consisting of 50°C reverse transcription for 1 hour and 94°C denaturation for 2 minutes, followed by 10 cycles of 94°C denaturation for 30 seconds, annealing at 57°C (VCAM-1, MCSF-1) or 55°C (*c-fms*) for 30 seconds and extension at 68°C for 45 seconds. In the following cycles, the extension time was prolonged for 5 seconds in each cycle. The final cycle was run with an extension period of 3 minutes at 68°C. PCR products were applied to a 2% agarose gel containing 0.1ug/ml ethidium bromide as DNA labeling substance. Electrophoresis was performed at 100 V for 45 minutes, and densitometry of the bands was carried out using the ImageMaster® VDS System and the ImageMaster®1D Elite software (Amersham-Pharmacia, Freiburg, Germany). To verify the identities of the PCR products, fragments were extracted from the gels and sequenced. Sequencing revealed the desired

511 bp fragment of the rabbit VCAM-1 cDNA, 394 bp fragment of the rabbit MCSF-1 cDNA and 133 bp fragment of rabbit *c-fms* cDNA.

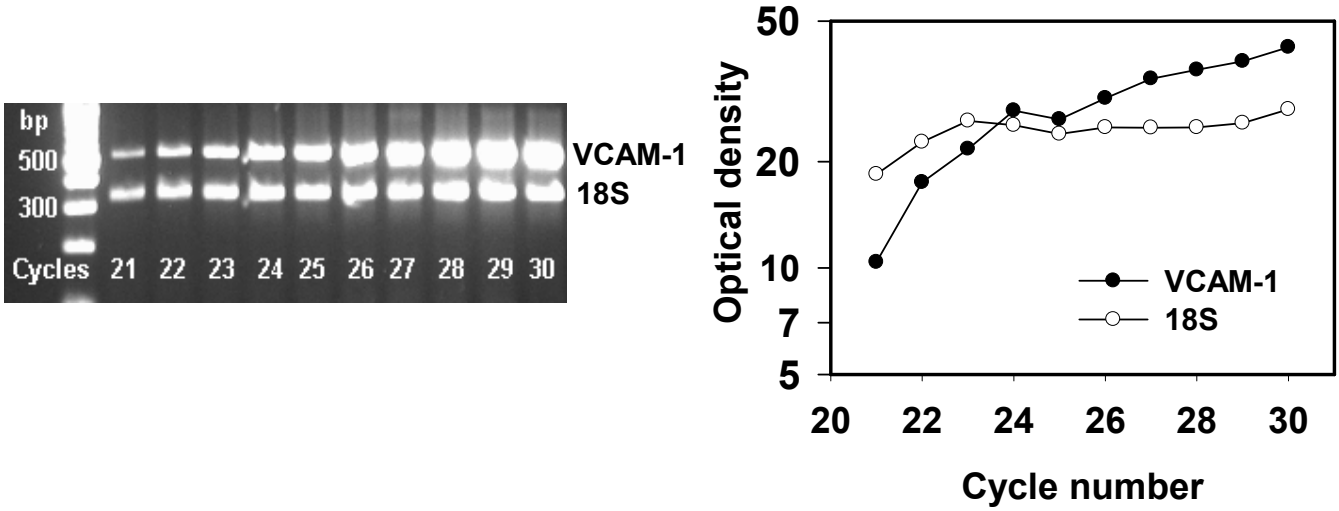


Figure 13a: Duplex-RT-PCR amplifying a 511 bp fragment and a 325 bp fragment of the rabbit VCAM-1 cDNA and the 18S cDNA, respectively, dependent on the cycle number and using 100ng pooled total RNA from the mitral valve of animals in group IV. 21-30 cycles with an annealing temperature of 57°C were run. Primer concentrations were 0.4 µM, the 18S primer/competimer (Quantum RNA, classic II) ratio was 2.5:7.5. The optimal cycle number was found to be 23.

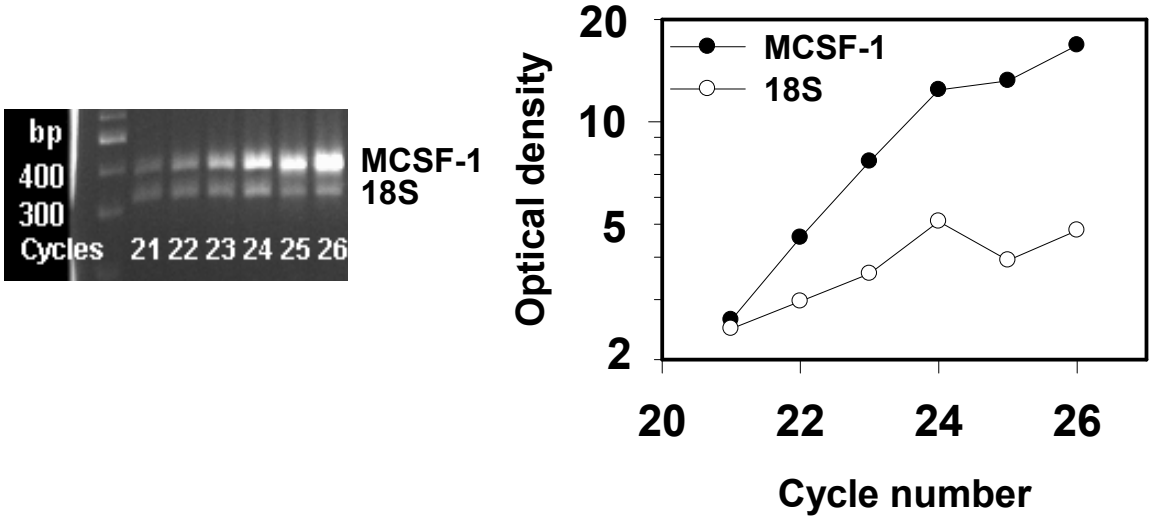


Figure 13b: Duplex-RT-PCR amplifying a 394 bp fragment and a 325 bp fragment of the rabbit MCSF-1 cDNA and the 18S cDNA, respectively, dependent on the cycle number and using 100ng pooled total RNA from the tricuspid valve of animals in group IV. 21-26 cycles with an annealing temperature of 57°C were run. Primer concentrations were 0.4 µM, the 18S primer/ competimer (Quantum RNA, classic II) ratio was 2:8. The optimal cycle number was found to be 24.

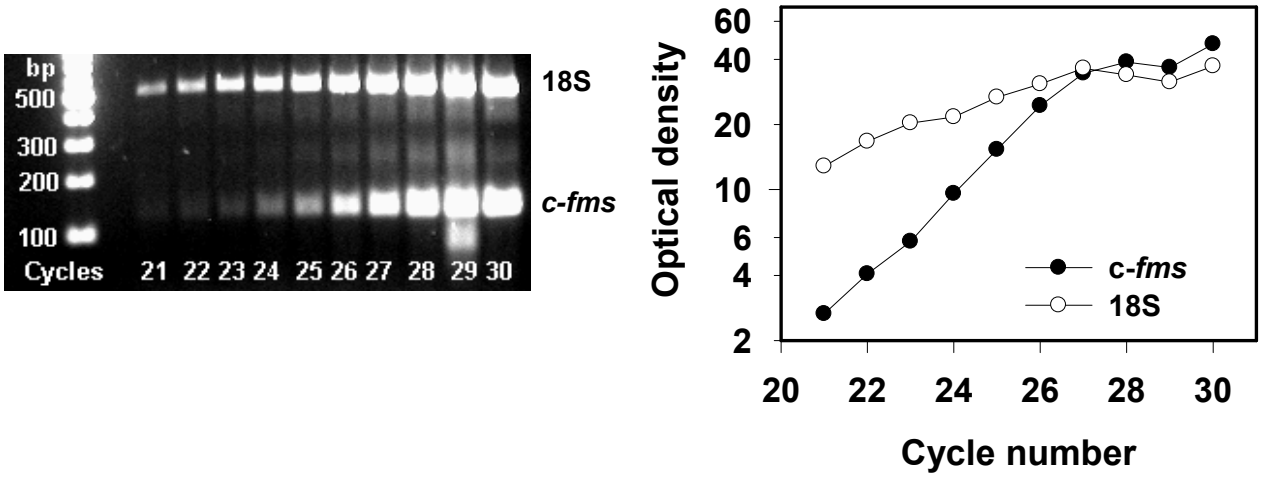


Figure 13c: Duplex-RT-PCR amplifying a 133 bp fragment and a 487 bp fragment of the rabbit *c-fms* cDNA and the 18S cDNA, respectively, dependent on the cycle number and using 100ng pooled total RNA from the mitral valve of animals in group IV. 21-30 cycles with an annealing temperature of 55°C were run. Primer concentrations were 0.4 µM, the 18S primer/ competimer (Quantum RNA, classic I) ratio was 3:7. The optimal cycle number was found to be 25.

Gene of interest	Cycle number	18S primer/ competimer ratio	Annealing temperature (°C)	RT-PCR product length (bp)
VCAM-1	23	2.5/7.5(CL.II)	57	511
MCSF-1	24	2:8 (CL.II)	57	394
c-fms	25	3:7 (CL.I)	55	133

Table 1: Experimental conditions found to be optimal in duplex-RT-PCRs for the genes of interest together with 18S RNA as internal control. The 18S products had either 487 (Classic I) or 325 (Classic II) base pairs. The 18S primer sets which interfered least with the primers for the gene of interest were chosen. The ideal primer/competimer ratios were determined in separate experiments (data not shown). The annealing temperatures were calculated based on the primer properties.

2.5 Statistical Analysis

Values are expressed as mean ± standard deviation. Statistical analysis of gene expression data was performed using the student's *t*-test, $p<0.05$ was considered statistically significant.

3. Results

In order to simplify and clarify the data presentation and interpretation, we defined two series of experimental groups:

- right-sided surgery series, consisting of groups III and V
- left-sided surgery series, consisting of groups IV and VI

Both series were compared with groups I and II, serving as non-surgery control groups. Because there are two different stimulations provoked by mitral or tricuspid surgery, leading to different hemodynamic conditions and micro-environment in the left and right heart chambers, we emphasize gene transcription in the tricuspid and pulmonary valves from the right-sided surgery series and the transcription in the mitral and aortic valves from the left-sided surgery series.

3.1 mRNA expression of VCAM-1

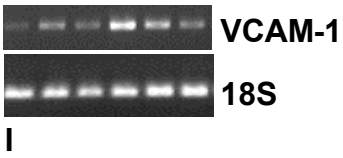
3.1.1 mRNA expression of VCAM-1 after isolated bacteremia

Isolated *S. aureus* bacteremia did not significantly influence the expression levels of VCAM-1 mRNA in any of the four heart valves ($p>0.05$ compared to control).

3.1.2 mRNA expression of VCAM-1 in mitral and aortic valves in the left-sided surgery series (see Fig. 14)

There were no significant mRNA expression changes of VCAM-1 in mitral valves 6 hours after isolated *S. aureus* bacteremia, mitral surgery alone and mitral surgery combined with *S. aureus* bacteremia as shown in Fig. 13 ($p>0.05$ compared to control).

However, there were strong changes in the aortic valves (Fig. 13). When the rabbits were stimulated by isolated *S. aureus* bacteremia and mitral surgery separately, the VCAM-1 mRNA expression was about 3- and 7-fold increased, respectively, compared to the control group, whereas mitral surgery combined with bacteremia resulted in an only about 2-fold upregulation of the VCAM-1 mRNA.



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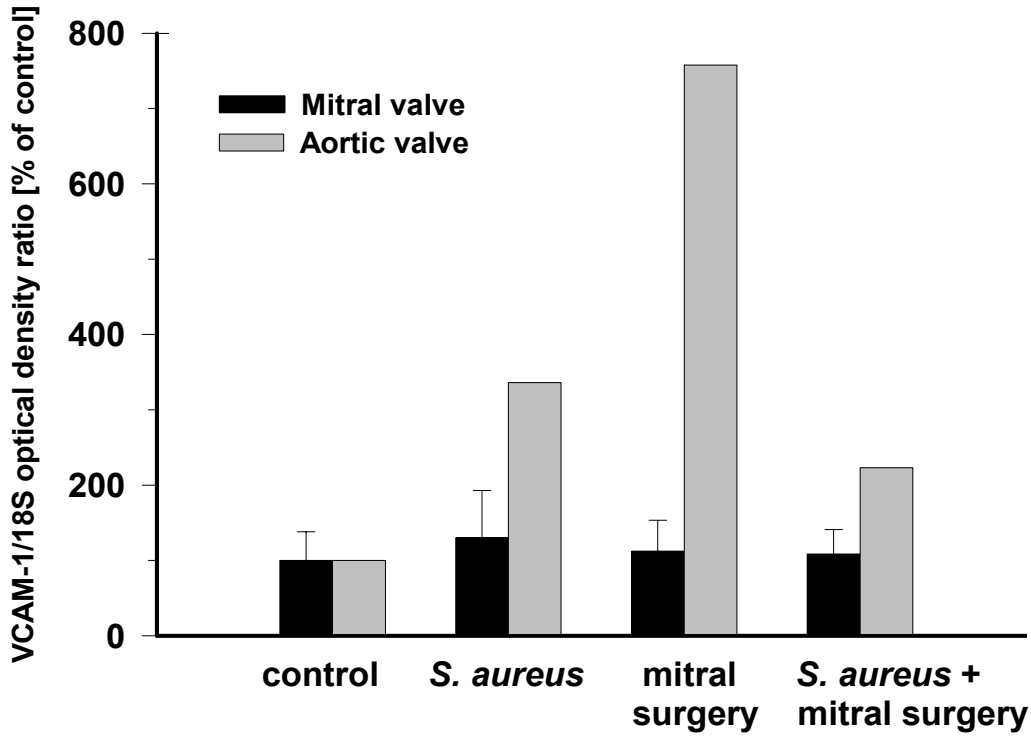


Figure 14: Expression of VCAM-1 mRNA in mitral and aortic valves after stimulation by bacteria and mitral surgery. Due to the use of "pooled" aortic valves, there are no standard deviations (see 2.4.1).

3.1.3 mRNA expression of VCAM-1 in tricuspid and pulmonary valves in the right-sided surgery series (see Fig. 15)

There was a significant upregulation of VCAM-1 mRNA in tricuspid valves induced by isolated tricuspid surgery compared to the control group ($p<0.005$). Additional bacteremia did not result in a further upregulation compared to the isolated tricuspid surgery group ($p>0.05$).

In pulmonary valves, there was the same trend as in tricuspid valves: surgical trauma and/or local perturbed flow in the right ventricle resulted in 5-fold higher VCAM-1 mRNA amounts and obviously played a major trigger role in the upregulation of VCAM-1 mRNA after tricuspid surgery and *S. aureus* bacteremia.

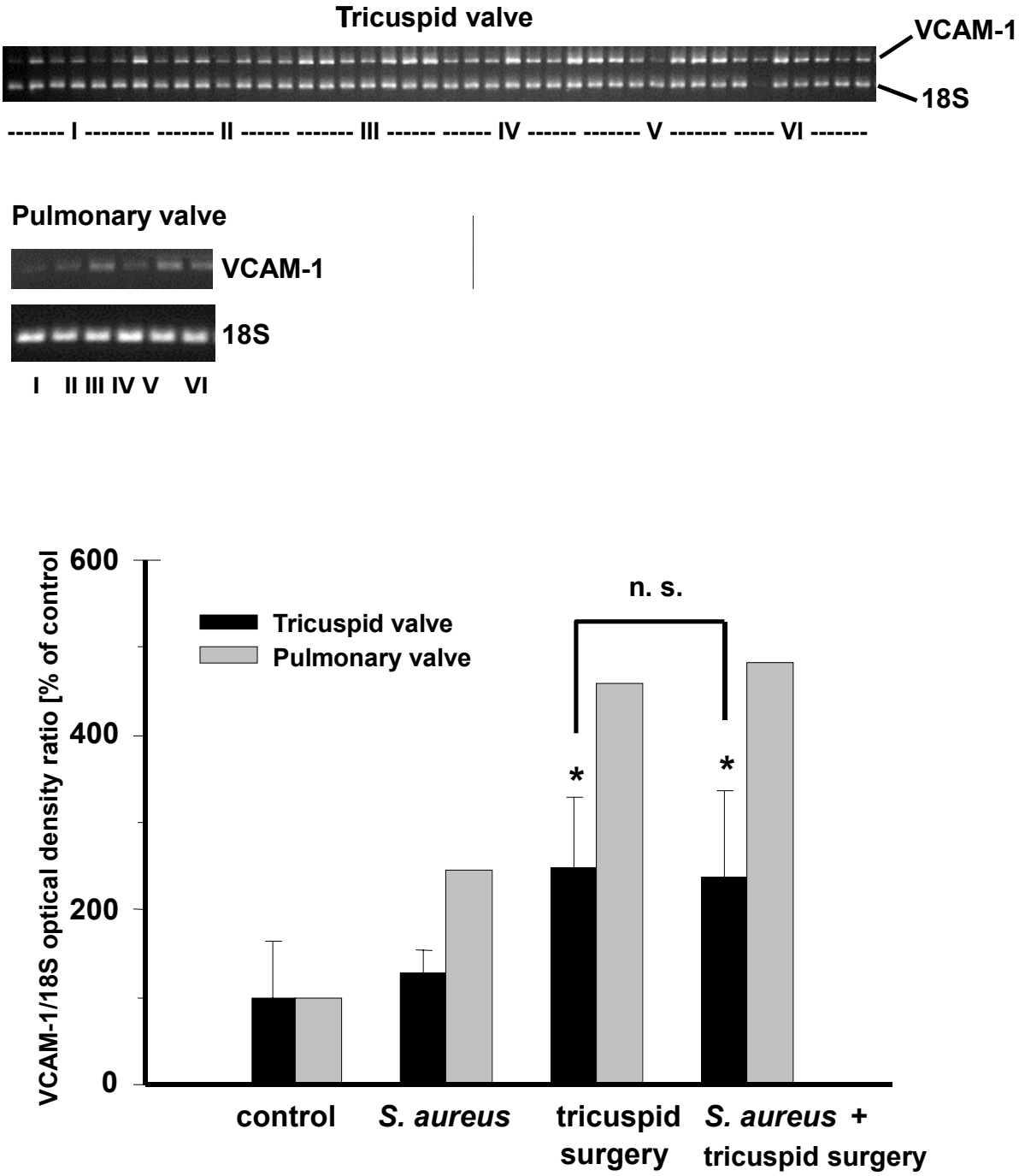


Figure 15: Expression of VCAM-1 mRNA in tricuspid and pulmonary valves after stimulation by bacteria and tricuspid surgery. Due to the use of "pooled" pulmonary valves, there are no standard deviations (see 2.4.1).

3.2 mRNA expression of MSCF-1

3.2.1 mRNA expression of MCSF-1 after isolated bacteremia

Intravenously injected *S. aureus* alone induced a weak upregulation of MCSF-1 mRNA in all four heart valves, but this was not statistically significant when compared to the control group ($p>0.05$).

3.2.2 mRNA expression of MCSF-1 in mitral and aortic valves in the left-sided surgery series (see Fig. 16)

After isolated mitral surgery, the MCSF-1 mRNA in the mitral valve was significantly upregulated compared to the control group ($p<0.001$).

Furthermore, the amount of MCSF-1 mRNA significantly increased after combination of mitral surgery with *S. aureus* bacteremia ($p<0.0001$ compared to the control group, and $p<0.01$ compared to the isolated mitral surgery group).

In the left-sided surgery series, the MCSF-1 mRNA expression in the aortic valve was highest after the combination of mitral surgery and bacterial administration. Here, surgery and bacteremia seemed to evoke a synergistic effect on MCSF-1 mRNA expression.

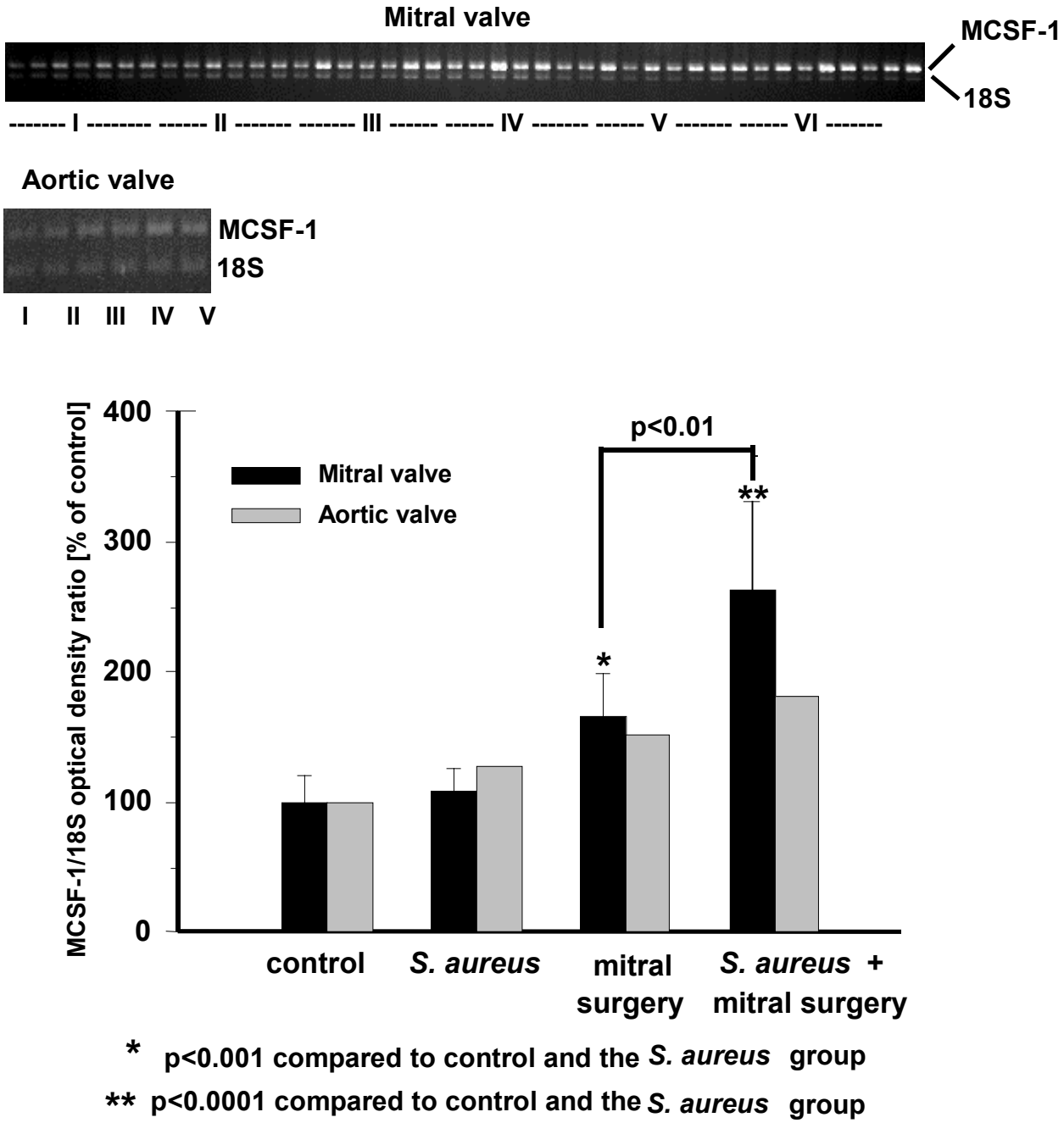


Figure 16: Expression of MCSF-1 mRNA in mitral and aortic valves after stimulation by bacteria and mitral surgery. Due to the use of "pooled" aortic valves, there are no standard deviations (see 2.4.1).

3.2.3 mRNA expression of MCSF-1 in tricuspid and pulmonary valves in the right-sided surgery series (see Fig. 17)

The mRNA expression of MCSF-1 in the tricuspid valve was significantly upregulated after isolated tricuspid surgery compared to the control group ($p<0.005$).

However, no further upregulation was observed after the combination of tricuspid surgery and bacterial stimulation ($p>0.05$ compared to the isolated tricuspid surgery group and $p<0.005$ compared to the control group).

Regarding the MCSF-1 mRNA expression in the pulmonary valve after tricuspid surgery, there was a similar synergistic upregulation after combination of surgery with bacterial administration as observed in the aortic valve (compare Figs. 16 and 17).

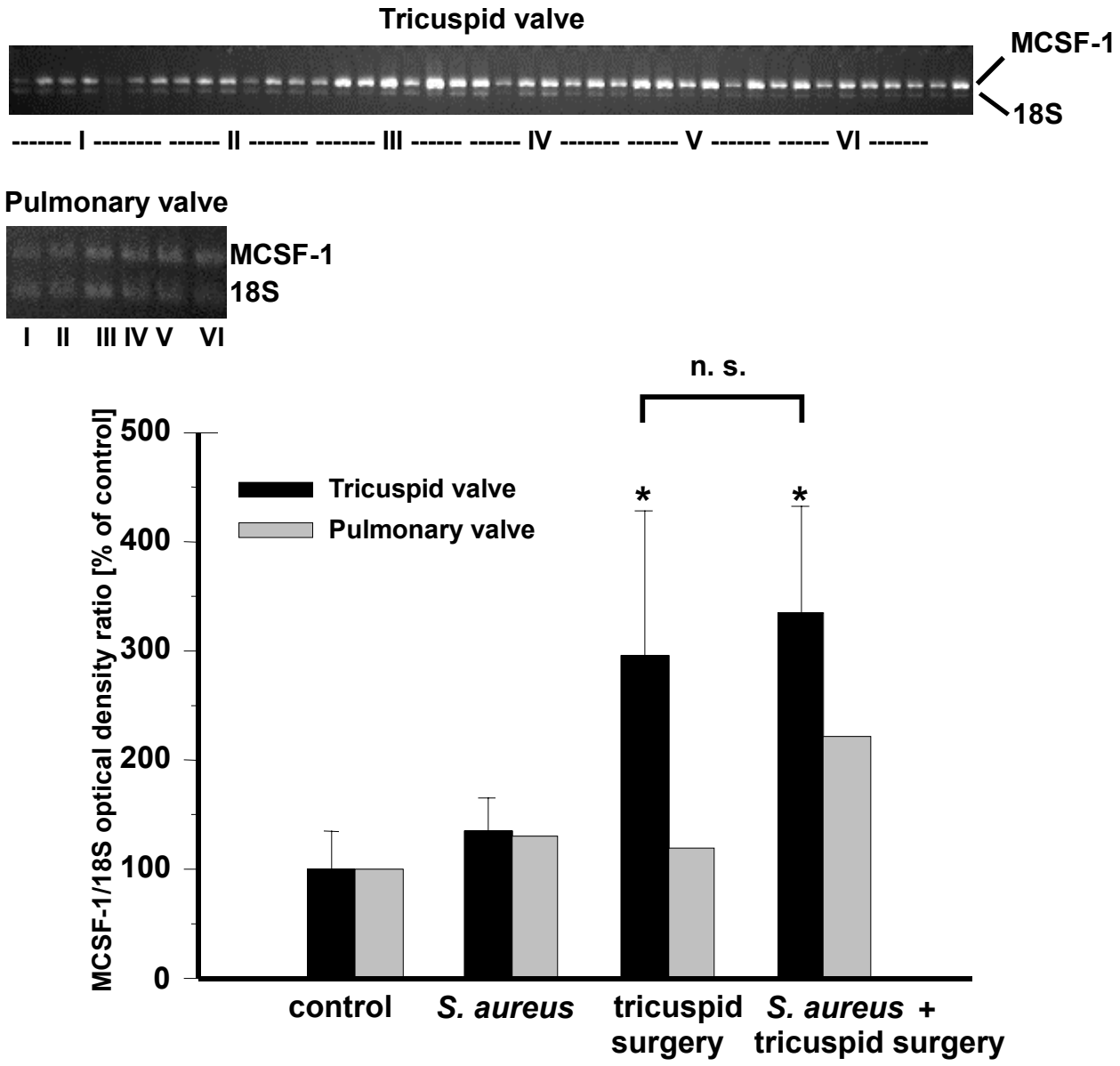


Figure 17: Expression of MCSF-1 mRNA in tricuspid and pulmonary valves after stimulation by bacteria and tricuspid surgery. Due to the use of "pooled" pulmonary valves, there are no standard deviations (see 2.4.1).

3.3 mRNA expression of *c-fms*

3.3.1 mRNA expression of *c-fms* after isolated bacteremia

After bacterial stimulation, the mRNA expression of *c-fms* was only slightly upregulated in the pulmonary valve but downregulated in the other heart valves. The changes were not significantly different from the control group ($p>0.05$).

3.3.2 mRNA expression of *c-fms* in mitral and aortic valves in the left-sided surgery series (see Fig. 18)

The mRNA expression of *c-fms* in mitral valve was significantly downregulated after isolated mitral surgery ($p<0.05$, compared to the control group). There was a tendency of reduced *c-fms* mRNA downregulation in the mitral and in the aortic valve after combination of surgery with bacterial application, however, these changes were not significantly different from the control group and from the group that underwent only surgery ($p>0.05$).

Neither bacteria or surgery alone nor the combination of both led to significantly different changes in the *c-fms* mRNA expression in the aortic valves.

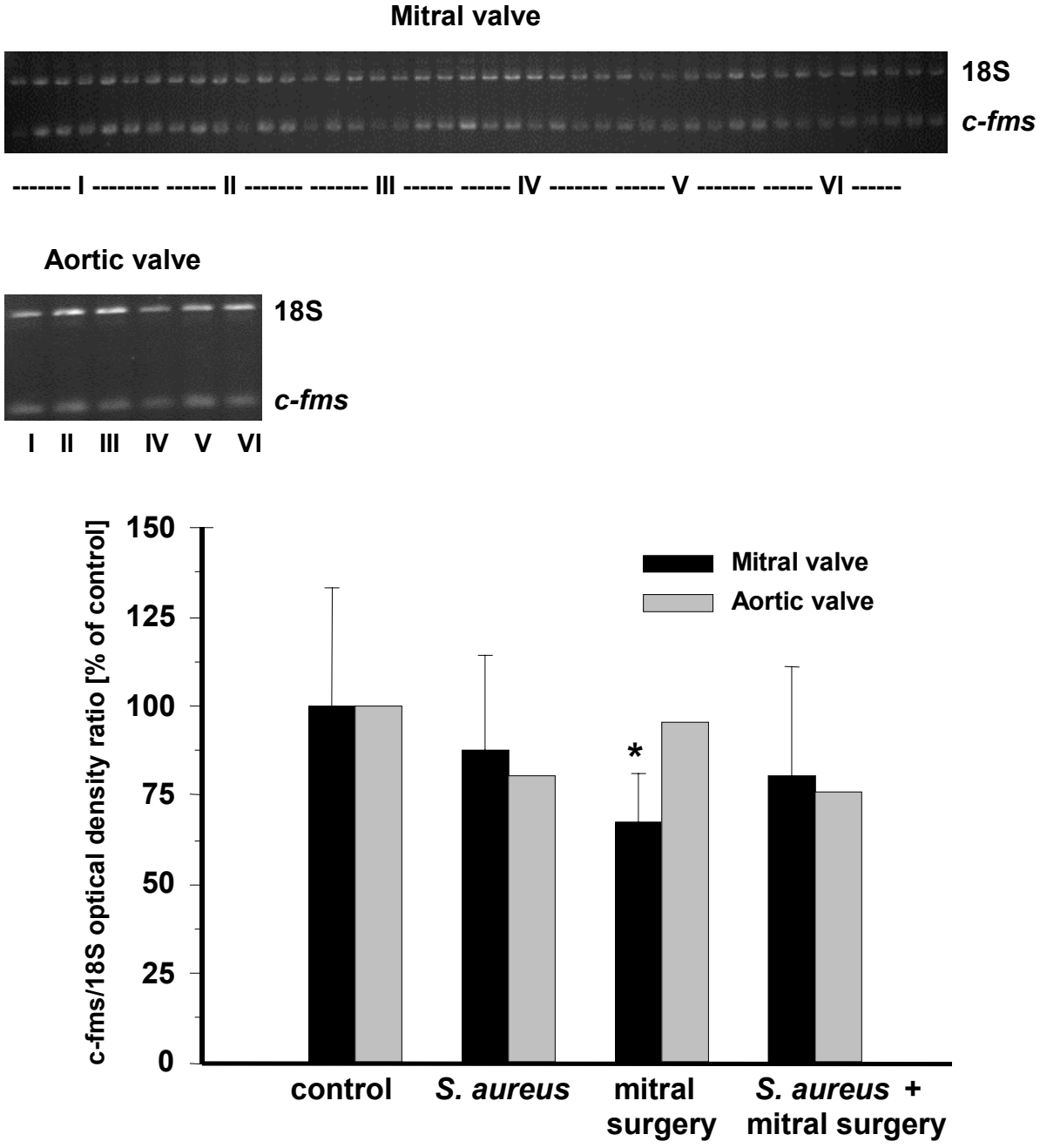


Figure 18: Expression of *c-fms* mRNA in mitral and aortic valves after stimulation by bacteria and mitral surgery. Due to the use of "pooled" aortic valves, there are no standard deviations (see 2.4.1).

3.3.3 mRNA expression of *c-fms* in tricuspid and pulmonary valves in the right-sided surgery series (see Fig. 19)

The mRNA expression of *c-fms* in the tricuspid valve was significantly decreased after isolated tricuspid surgery ($p<0.05$, compared to the control group). The downregulation in the tricuspid valve after surgery followed by bacterial stimulation was not significantly different compared to the control group and to the animals which only underwent tricuspid surgery group ($p>0.05$).

Neither bacteria or surgery alone nor the combination of both led to significantly different changes in the *c-fms* mRNA expression in the pulmonary valves.

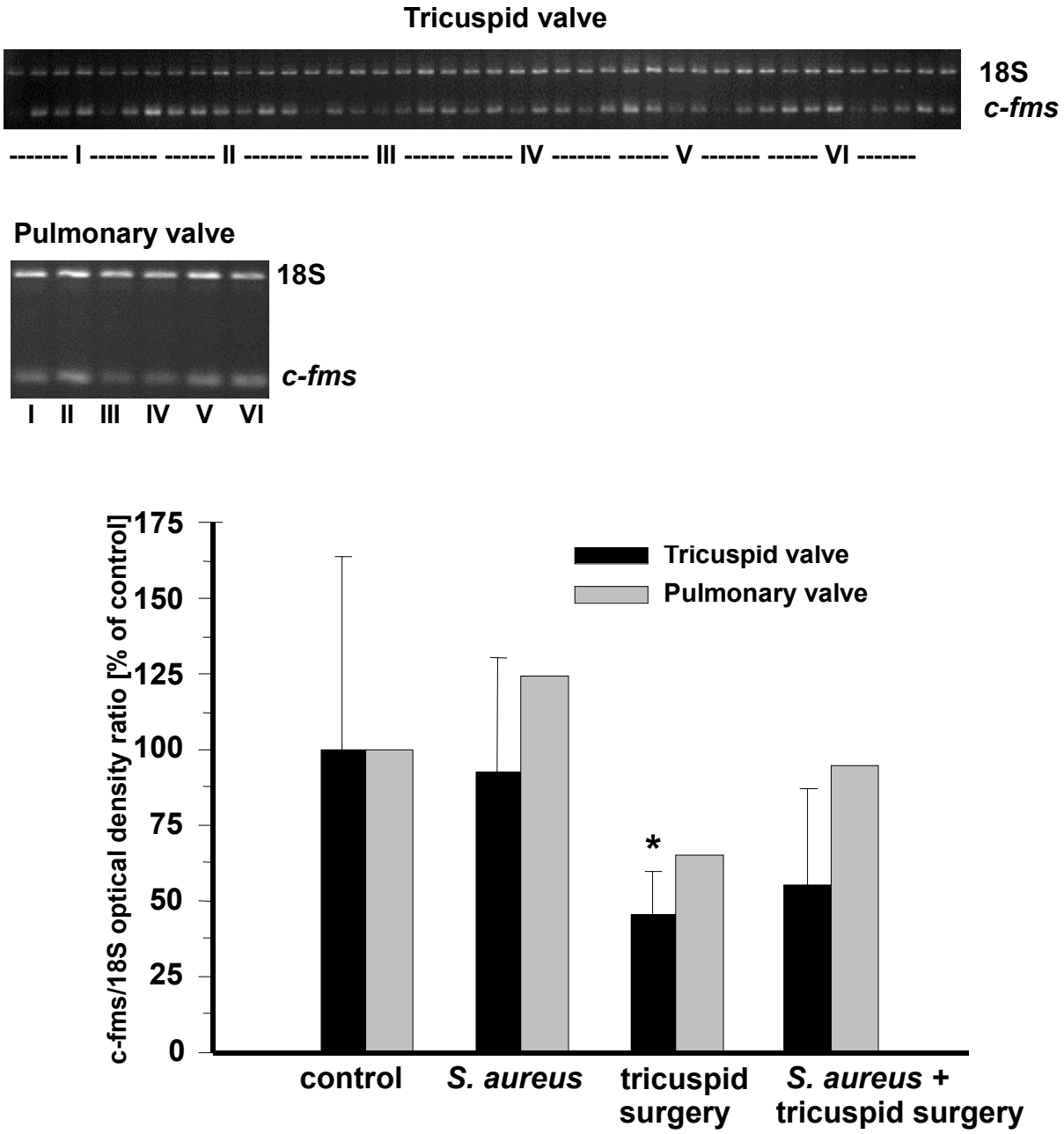
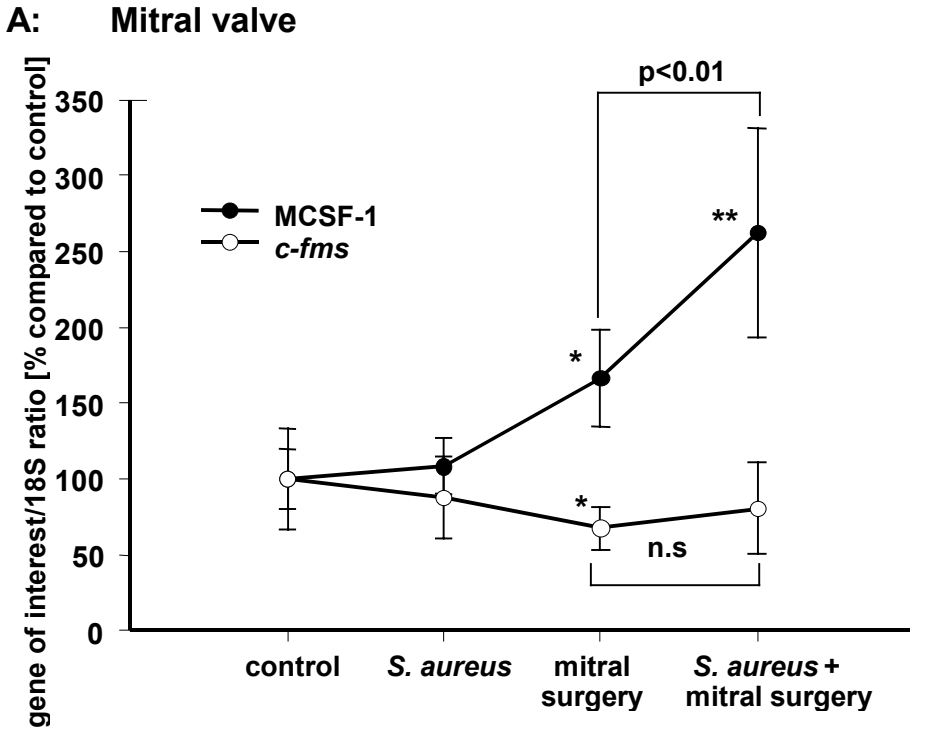


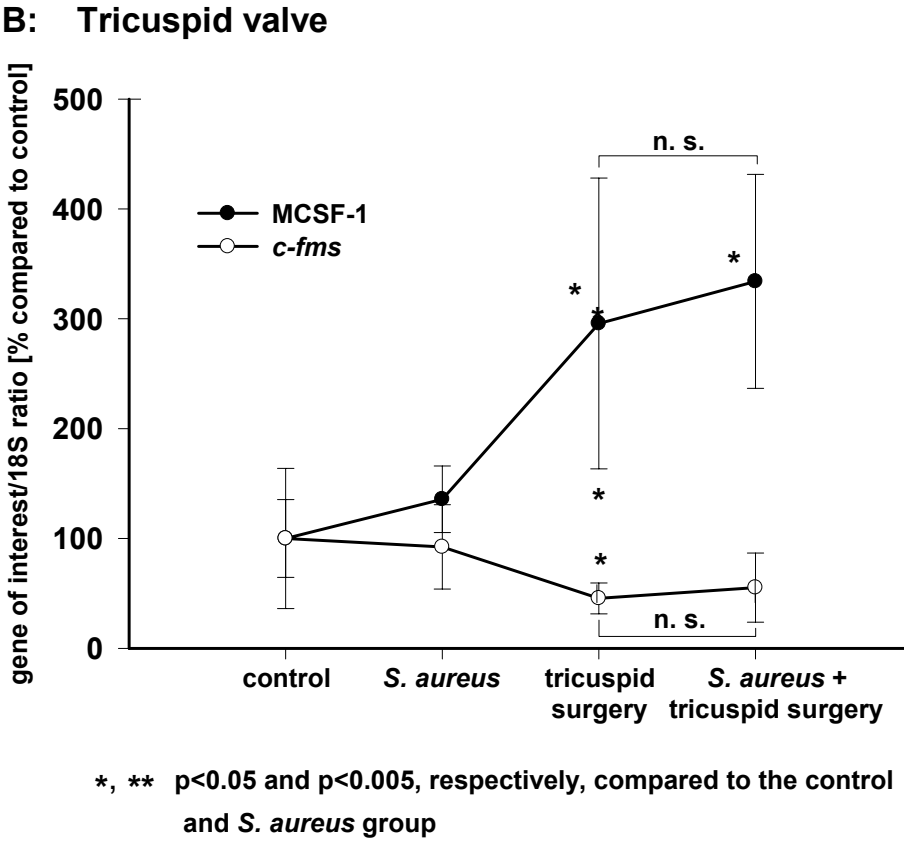
Figure 19: Expression of *c-fms* mRNA in tricuspid and pulmonary valves after stimulation by bacteria and tricupid surgery. Due to the use of "pooled" pulmonary valves, there are no standard deviations (see 2.4.1).

3.4 Comparison between MCSF-1 and *c-fms* mRNA expression

The juxtaposition of the mRNA data for MCSF-1 and *c-fms* in the mitral and the tricuspid valve is displayed in Figs. 20 A and B. Both graphs clearly show that in the operated groups, where the MCSF-1/18S mRNA ratio was significantly higher than in the control group, the *c-fms* mRNA expression did not parallel the MSCF-1 course, instead, there was even a significant decrease in *c-fms* expression in the isolated surgery groups and the *cfms*/18S ratios were still lower (without reaching significance) in the groups where valve surgery had been combined with the administration of bacteria.



*, ** p<0.05 and p<0.001, respectively, compared to the control *S. aureus* group



Figures 20 A, B: Comparison of MCSF-1 and *c-fms* mRNA expression in the atrioventricular valves after bacterial stimulation and mitral/tricuspid surgery

4. Discussion

Endothelial cells play an active role in the process of leukocyte adhesion to the vascular vessel wall and to their subsequent extravasation into underlying inflamed tissue [Cronstein, B.N. et al. (1993) and Osborn, L.

(1990)]. The molecules involved in endothelial cell-leukocyte interaction and expressed on the surface of endothelial cells have been identified and characterized in many studies and recent reviews give an excellent overview about their specific roles in inflammation and vascular disease [Szmitko, P.E. et al. (2003) and Szmitko, P.E. et al. (2003) and Keller, T.T. et al. (2003)]. Stable adhesion of leukocytes to the vascular endothelium is necessary before “rolling” on the vessel endothelial cell surface can occur [Ley, K. et al. (1993) and von Andrian, U.H. et al. (1992) and Dore, M. et al. (1993)]. “Rolling” is a result of the dynamic equilibrium between the tractive forces induced by the blood stream and the resistance of the endothelial cell surface. The complex phenomenon of cell rolling on the endothelium involves strong interaction between adhesion molecules on the endothelial cell surface and counter-receptors on the surface of adhering leukocytes. Such an event implies a continuous process of binding to and detachment of circulating cells from the endothelial surface. Two major classes of endothelial adhesion molecules are involved in this process [Ley, K. (1996)]. The first belongs to the family of selectins [Lawrence, M.B. et al. (1993) and Lawrence, M.B. et al. (1991)], such as E-selectin and P-selectin, both of which are expressed on the endothelial cell surface. In this process, cytokine activation plays a pivotal role [Montgomery, K.F. et al. (1991)]. The second major class consists of members of the immunoglobulin superfamily: the most important

molecules are ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1). These CAMs are expressed on the endothelial surface [Wuthrich, R.P. (1992)] and mediate leukocyte adhesion through specific leukocyte integrins. ICAM-1 mediates firm adhesion of neutrophils, monocytes, and lymphocytes, while VCAM-1 is primarily responsible for the adhesion of lymphocytes and monocytes, but not of neutrophils. Moreover, VCAM-1 is unique in that its expression is largely restricted to lesions and lesion-predisposed regions, whereas ICAM-1 and other CAM expression extends also into uninvolved and lesion-protected regions [Iiyama, K. et al. (1999)], the main reason why we focused our interest among CAMs to VCAM-1. The temporal expression of these CAMs, in response to inflammatory cytokines, and recognition of cell-specific counter-ligands, orchestrates the recruitment and activation of leukocytes into inflamed tissues to fight infections. However, in certain pathological conditions, leukocyte infiltration contributes to tissue damage and organ injury.

In our experimental rabbit model, the surgical procedure involves many factors that can affect endothelial cell activation, e. g. disturbed flow from valvular insufficiencies, artificial material on the atrioventricular valves (AV valves), endothelial injury and systemic inflammation.

4.1 Influence of disturbed flow on VCAM-1 expression in heart valves

Hemodynamic flow conditions have a major influence on the process of leukocyte adhesion to endothelium *in vivo* [Lipowsky, H.H. et al. (1991)]. Specifically, the flow velocity gradient near the vessel wall induces traction forces (shear stress) tangential to the endothelial cell surface that oppose leukocyte adhesion. The effects of flow on leukocyte-endothelial interaction have been mimicked *in vitro* using parallel-plate perfusion chambers and videomicroscopy [Lawrence, M.B. et al. (1990)]. However, leukocyte adhesion *in vivo* and *in vitro* differs greatly because adhesion can take place at much higher shear stress levels *in vivo* (up to 30 dynes/cm^2 = 3N/m^2) than *in vitro* (<5dynes/cm^2 = 0.5N/m^2). The reason for this is unknown. Aside from affecting the interaction between circulating cells and the endothelial surface, mechanical forces induced by the blood flow (i. e. hydraulic pressure and flow velocity) can directly modulate the structure and function of vascular endothelial cells [Walpola, P.L. et al. (1995)]. Because fluid shear stress regulates a genetic program that alters the synthesis and release of endothelial cell-derived molecules, the effects of modified flow conditions on endothelial cell expression of CAMs has been explored repeatedly. Recent results indicate that steady unidirectional laminar flow increases the gene expression of ICAM-1 in human umbilical vein endothelial cells [Chiu, J.J. et al. (1997) and Sampath, R. et al.

(1995)]. It was demonstrated that shear stress induces endothelial VCAM-1 expression and increases monocytic cell adherence via a VCAM-1/$\alpha_4\beta_1$ mechanisms [Gonzales, R.S. et al. (1996)]. ICAM-1 and VCAM-1 are required for stable adhesion and emigration of the cells [Pober, J.S. et al. (1990)].

In the present study, we examined the mRNA expression of VCAM-1 in the four heart valves in an experimental rabbit model 6 hours after stimulation by surgical trauma and/or generation of perturbed blood flow, which is likely to result from mitral or tricuspid insufficiency. This disturbed flow pattern is very complex, involving flow separation, re-circulation, flow reattachment, non-uniform shear stress distribution, pulsatile flow, and oscillatory and fluctuating flow pattern [Ku, D.N. et al. (1985)]. Our results show that the mRNA expression of VCAM-1 in the four heart valves is upregulated to a different level, but the difference in mitral valves compared to the baseline expression did not reach significant values.

On the other hand, VCAM-1 mRNA expression in tricuspid valves was significantly enhanced in the group that underwent tricuspid surgery ($p<0.05$). This could be due to disturbed blood flow resulting from tricuspid insufficiency but also a response to trauma induced by surgery.

However, additional bacteremia did not result in a further upregulation compared to the isolated tricuspid surgery group ($p>0.05$). These data suggest that the left heart valves (mitral and aortic valves) and the right heart valves (tricuspid and pulmonary valves) display different VCAM-1 responses to surgical trauma and/or local disturbed blood flow. The results suggest that (i) the surgical trauma and/or locally disturbed flow resulting from surgery play a prominent role in the mRNA upregulation of VCAM-1 in tricuspid valves and that (ii) *S. aureus* bacteremia does not significantly influence the VCAM-1 mRNA expression in tricuspid valves under these conditions in the early phase.

In the present study, the conditions of disturbed flow at the atrioventricular valves lasted only 6 hours. It is possible that a longer exposure of endothelial cells to such flow disturbances may exert a stronger effect on the adhesive properties on heart valve endothelial cells, including those lining the mitral valve.

The modified hemodynamics and surgical trauma seemed to display different abilities to upregulate mRNA expression of VCAM-1 in different valves in the early phase. The aortic valve showed a stronger response to disturbed flow in the left ventricle (7-fold increase). This indicates that circulating inflammatory cells may readily adhere to the surface of aortic

valves and may lead to inflammation. This is in agreement with the pathogenesis of aortic valve disease, whereas this phenomenon did not take place in mitral valves. The reason for this observation remains largely unknown and may involve differences in gradient pressure, flow velocity, flow direction and shear stress. A preliminary immunohistochemical study on aortic valves from surgical and autopsy cases which suffered from degenerative disease suggested that the increased expression of adhesion molecules (E-selectin, ICAM-1 and VCAM-1) on the valvular endothelium is more likely to be cytokine-induced rather than due to shear forces. This hypothesis was based on their findings of adhesion molecules on autopsy valves that were diseased but not stenotic and therefore not subject to hemodynamic disturbances [Ghaisas, N.K. et al. (2000)]. The data presented here also suggest, that increased VCAM-1 mRNA expression in the aortic valve after mitral surgery is not a consequence of local trauma or disturbed flow properties (because the aortic valve remained untouched and mitral insufficiency does not necessarily affect the hemodynamic situation at the aortic valve), but more likely the effect of a systemic reaction like cytokine-induced mechanisms.

Regardless of the signaling mechanisms, the expression of VCAM-1 in heart valves with preexisting pathological changes, e. g. valvular insufficiency, local disturbed flow, heart valve lesions or the presence of an

artificial material, may be major contributors to the development of heart valve inflammation. Whether the transcriptional regulation of VCAM-1 in our model may serve as a predictor for the risk of a heart valve to suffer from inflammatory reactions remains unsolved and has to be investigated in more detail.

4.2 Influence of bacteremia on VCAM-1 expression in heart valves

Adherence to cell surfaces is the first step in the establishment of bacterial disease. Many bacterial germs have evolved the capacity to adhere to CAMs. They are able to bind to CAMs by mimicking or acting in place of host cell receptors or their ligands [Boyle, E.C. (2003)]. Recruitment of different types of leukocytes elicited by bacteria is dependent on multiple parameters, including intercellular signaling molecules, the types of involved cells, tissues or organs and the nature of the bacterial stimulus [Mizgerd, J.P. (2002)]. *Staphylococcus aureus* has a unique capacity to cause endovascular infections, such as infective endocarditis, thrombophlebitis, and vascular and heart valve prosthetic infections [Moreillon, P. et al. (2004)]. The surface components of *S. aureus*, nameley protein A (PA) and lipoteichoic acid (LTA), are proinflammatory. They stimulate the production of IL-1, IL-4, IL-6, TNF, and IFN, which can modulate CAM expression [Tufano, M.A. et al (1991)]. Recent data

indicate, that PA and LTA are able to directly induce the production of IL-6 and IL-8 and increase ICAM-1 expression at 2 and 4 hours after LTA and PA stimulation in human dermal fibroblasts [Perfetto, B. et al. (2003)]. Furthermore, clinical isolates of *S. aureus* were found to induce the expression of E-selectin and ICAM-1 after 4-6 hours in human endothelial cells and provoke an inflammatory response [Strindhall, J. et al. (2002)]. Increased P-selectin expression was demonstrated on cultured human vein endothelial cells (HUVECs) after incubation with *S. aureus* α-toxin [Krull, M. et al. (1996)]. This toxin represents a prototype of pore-forming exotoxins and is the major cytotoxin of *S. aureus* [Bayer, A.S. et al. (1997) and Valeva, A. et al. (1996) and Bhakdi, S. et al. (1991) and Bhakdi, S. et al. (1996)]. In addition, enhanced P-selectin-mediated adherence of human polymorphonuclear leukocytes (PMN) to rat aortic vascular endothelium was observed when aortic rings were stimulated with α-toxin [Buerke, M. et al. (2002)]. However, anti-P-selectin monoclonal antibodies and fucoidin could not inhibit PMN adhesion completely, suggesting that other mechanisms or additional adhesion molecules might be involved (e. g. ICAM-1, ICAM-2, VCAM-1).

In the present study, *S. aureus* bacteremia did not activate the valvular endothelium since no significant VCAM-1 upregulation was found in the heart valves of the rabbits. This may be due to the chosen time point of 6

hours which might have been too short to see a change in VCAM-1 expression because VCAM-1 seems to be induced more slowly than E-selectin, which is regarded as being expressed by endothelial cells in the early and active states of inflammation, whereas VCAM-1 seems to characterize later phases of inflammation [Cybulsky,M.I. et al. (1991) and Kuzu, I. et al. (1993) and Abe, Y. et al. (1996)]. Our results support the common observation, that intact endothelium is almost resistant to colonization and infection by bacteria [Durack, D.T. et al. (1975)]. However, when the valves were exposed to surgical trauma and/or disturbed flow, *S. aureus* bacteremia led to a significant upregulation of the VCAM-1 mRNA in the tricuspid valve, but not in the mitral valve. The meaning of this differential regulation remains unclear, however, in light of early [Freemann, L.R. (1987)] and own recent observations that left-sided valves are much more susceptible to bacterial infection than right-sided valves, the role of VCAM-1 in this special situation has to be investigated in more detail. In our model, a Dacron patch had been placed onto the tricuspid valve in 12 rabbits, but when explanted after 48 hours, none of the animals showed bacterial vegetations on the valves (unpublished data). In contrast, bacterial vegetations were found in 9 of 14 (64%) rabbits, which received Dacron patches on the mitral valve [Eichinger, W.B. et al. (2002)]. This also mirrors the clinical situation where mainly left-sided endocarditis occurs with the exception of i. v. drug-abusers, in whom

right-sided endocarditis is common.

4.3 The effects of surgery and bacteremia on MCSF-1 and *c-fms* mRNA expression on heart valves

In bacterial endocarditis, the endocardial vegetation on the surface of valves consists of a tight network of fibrin, which contains the infectious microorganisms and a few phagocytic cells [Scheld, W.M. et al. (1990)]. It was suggested that monocytes play a role in the formation of heart valve vegetations and may have a protective effect during the course of *S. aureus* endocarditis [Veltrop, M.H. et al. (2000)]. In the past, macrophage accumulation has been mostly attributed to the recruitment of circulating blood monocytes and to some extent to prolong cell survival as a response to stimuli in many diseases [van Furth, R. (1989)]. This concept was based on the findings that resident tissue macrophages have only little or no proliferative capacity and that macrophage recruitment from blood monocytes is the response to stimuli that require enhanced phagocytic activity. However, it is now well established that recruited monocytes can proliferate locally partly via M-CSF/*c-fms* pathway at the site of inflammation [Lan, H.Y. et al. (1997) and Yang, N. et al. (1998) and Kerr, P.G. et al. (1994) and Goto, M. et al. (1993) and Bischoh, R.J. et al. (2000) and Le Meur, Y. et al. (2002)]. These studies suggest that local macrophage

proliferation may be a more important contributor to macrophage accumulation during inflammation than the recruitment of circulating monocytes.

In a previous study it has been shown that MCSF-1 is capable of inducing its receptor; similarly, the CSF-1 receptor, *c-fms*, can also activate its own growth factor ligand, thus initiating an autocrine loop resulting in cellular transformation, proliferation and tumorigenesis [Keshava, N. et al. (1999)].

In the present study, the mRNA expression of MCSF-1 and *c-fms* in heart valves was investigated (Figs. 20A, B). While in the mitral valve there was a significant upregulation of MCSF-1 mRNA after surgery and an even stronger upregulation after combined surgery and bacterial stimulation, in the tricuspid valve the MCSF-1 mRNA upregulation was only significant in the surgery group (compared to the control group). No further upregulation was observed after additional bacteraemia. The reason for this finding is unclear, at least, it may be hypothesized that the transcriptional response of the mitral and the tricuspid valve to *S. aureus* bacteraemia, regarding the cytokine MCSF-1 and under conditions of valve trauma and/or disturbed flow, is different. This phenomenon may imply that MCSF-1 reflects the “inflammatory status” of a tissue, i.e. the progression of macrophage accumulation and proliferation at the site of inflammation

[Le Meur, Y. et al. (2002)].

In our animal model, *c-fms* mRNA was significantly downregulated on mitral valves, as well as on tricuspid valves after corresponding atrioventricular valve surgery but not after bacterial stimulation alone. Correspondingly, MCSF-1 upregulation was not induced by bacteremia, but was significant in the operated groups. The presented data are in accordance with reports showing that *c-fms* mRNA and protein has been downregulated in murine macrophages stimulated with granulocyte/ macrophage-CSF [Horiguchi, J. et al. (1987)] and in human monocytes treated with MCSF-1 [Sariban, E. et al. (1989)]. However, another *in vitro* study demonstrated that highly concentrated lipopolysaccharide downregulated *c-fms* mRNA in a murine macrophage cell line [Gusella, G.L. et al. (1990)]. Possible explanations for the discrepancy between this and our results might be the use of different stimulative agents (live bacteria vs. purified LPS), applied dose, model (*in-vivo* vs. *in-vitro*), and tissue examined (heart valve vs. pure macrophage cell line). The levels of *c-fms* expression are also dependent on the stages of macrophage differentiation and proliferation as well as on the stimulus applied [Gusella, G.L. et al. (1990)]. The need for downregulation of *c-fms* by proinflammatory stimuli may be dictated by the ability of such a stimulus to induce MCSF-1 production (like in our model by surgical trauma and/or

bacteremia) in cells that constitutively express high levels of *c-fms*. This could be a mechanism by which macrophages avoid a prolonged autocrine stimulation provoked by their own growth factor.

It is known, that recruited monocytes at least in part proliferate locally at the site of inflammation induced by the MCSF/*c-fms* pathway [Roberts, S. et al. (2001) and Lan, H.Y. et al. (1997) and Yang, N. et al. (1998) and Le Meur, Y. et al. (2002)]. Moreover, blockade of the MCSF/*c-fms* signaling pathway with anti-*c-fms* monoclonal antibody can drastically reduce macrophage accumulation during an inflammatory response [Le Meur, Y. et al. (2002)]. At the same time, we know that MCSF-1 can induce the transcription of its receptor, *c-fms*, through an autocrine loop [Keshava, N. et al. (1999)]. Vice versa, these authors also showed that the MCSF-1 receptor, *c-fms*, can induce its own growth factor ligand and that overexpression of both, MCSF-1 and *c-fms,* in normal ovarian granulosa cells leads to cell proliferation and tumorigenesis. They suggested the possibility of a role for these genes in progression of ovarian cancer. Hence, we can hypothesize that the *c-fms* downregulation in the presence of increased ligand concentrations may be a self-protection against excessive cell growth and dedifferentiation. To investigate the expression of the MCSF-1/*c-fms* system on the protein level in heart valves it would be useful to stain heart valve tissue sections for the presence of these proteins

and also to identify recruited macrophages in the valve tissue at various time points and in dose-response experiments.

4.4 Study limitations

There are a few points that have to be mentioned because they represent restrictions and should be kept in mind while interpreting the data. First, the study groups consisted of only 7 rabbits and, second, there was only one time point in the early phase after stimulation when the mRNA expression was analyzed. Hence, statistical safety could be improved with a higher number of animals and a time-dependent analysis of gene expression would give much more information about the time course of tissue remodeling and ongoing of inflammatory reactions. Third, groups II, V, and VI received only 1 dosis of bacteria. It would be interesting to perform a dose-dependent analysis and to use different bacterial strains with different virulence to evaluate the tissue responsiveness under these conditions. Fourth, protein expression in heart valves would greatly enhance the information, however, rabbit heart valves are so tiny, so that simultaneous expression analysis of mRNA and protein in one valve is not applicable. Another set of experiments for protein detection would be necessary. And finally, fifth, RT-PCR experiments allow the measurement of “steady-state” levels of mRNA, which are of course, subject to

degradation and new synthesis. In addition to this information, it would be valuable to perform nuclear run-on experiments in order to assess the rates of mRNA expression, which reflect a dynamic change upon stimulation.

4.5 Conclusions

(1) Each heart valve shows a different VCAM-1 response after mitral or tricuspid surgery in the early phase. Disturbed flow and/or an endothelial lesion resulting from the surgical procedure lead to a significant upregulation in the tricuspid valve, but not in the mitral valve. Therefore, considerable interest exists in the role of VCAM-1 for the development of infective endocarditis.

(2) The MCSF-1 mRNA is significantly upregulated in the mitral and tricuspid valve after surgery. When bacterial endocarditis develops, a further increase of MCSF-1 mRNA expression can be observed only on mitral valves. This obvious difference between the AV-valves in the MCSF-1 response to proinflammatory stimuli might be one of the reasons why we could not induce right-sided endocarditis in our experimental setting. The results also suggest that the MCSF-1 mRNA expression level reflects the “inflammatory status” of the tissue, i.e. number and differentiation of recruited macrophages.

(3) The coincidence of upregulated MCSF-1 and downregulated *c-fms* in macrophages upon traumatic and/or proinflammatory stimuli may be

interpreted as a necessary negative autocrine feed-back loop in which high ligand concentrations, which leads to downregulation of the receptor in order to elude prolonged stimulation by their own growth factors.

5. Summary

Objective: To explore the roles of vascular cell adhesion molecule-1 (VCAM-1), macrophage colony-stimulating factor-1 (MCSF-1), and its receptor, *c-fms*, in the development of bacterial endocardial vegetations and to analyze their effects on valve tissue during bacterial colonization and vegetation growth, we studied their mRNA expression in rabbit heart valves in the early phase after atrioventricular valve surgery and *S. aureus* bacteremia.

Materials and Methods: Seven rabbits received $5*10^6$ colony forming units *Staphylococcus aureus* i.v.; seven rabbits underwent mitral surgery; seven rabbits underwent tricuspid surgery; seven rabbits underwent both mitral surgery and i.v. *S. aureus* administration; and seven rabbits underwent both tricuspid surgery and i.v. *S. aureus* administration. The hearts were explanted 6 hours after the intervention and the valvular tissues were shock frozen in liquid nitrogen. Total RNA was isolated and mRNA

abundance determined by semiquantitative duplex RT-PCR using 18S RNA as internal control. Results were compared to the control group consisting of seven untreated rabbits.

Results: *S.aureus* bacteremia alone did not lead to significantly changed mRNA levels of any studied molecules. In the mitral valve, the VCAM-1 expression was not significantly upregulated after mitral surgery and after additional bacteremia, but it was significantly more abundant in the tricuspid valve after tricuspid surgery ($p<0.005$). Additional *S.aureus* bacteremia did not result in a further upregulation compared to the isolated tricuspid surgery group ($p>0.05$). The mRNA expression of MCSF-1 on atrioventricular valves was significantly upregulated after corresponding isolated AV-valve surgery. Moreover, in the mitral valve but not in the tricuspid valve, a significant further increase in MCSF-1 mRNA was observed after additional *S.aureus* bacteremia ($p<0.01$ compared to isolated surgery). The mRNA expression of *c-fms* in atrioventricular valves was significantly downregulated only after corresponding isolated AV-valve surgery ($p<0.05$).

Conclusions: (i) Each heart valve shows a different VCAM-1 response after surgical procedures and *S.aureus* bacteremia in the early phase. Theremore, considerable interest exists in the role of VCAM-1 in the development of infective endocarditis. (ii) The MCSF-1 mRNA expression level seems to reflect the inflammatory status of the heart valve tissue, i.e.

number and differentiation of recruited macrophages. (iii) A necessary negative autocrine feed-back loop may exist between MCSF-1 and *c-fms* upon traumatic and/or proinflammatory stimuli in heart valves, which leads to downregulation of the receptor in order to elude prolonged stimulation by their own growth factors.

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7. Appendix

7.1 Resume

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