IMMUNOLOGICAL INVESTIGATION OF THE CELLULAR AND MOLECULAR COMPOSITION OF PERICARDIAL FLUID IN PATIENTS WITH OPEN HEART SURGERY

PH.D. DISSERTATION

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1 Abbreviations

- **AB**: antibody
- **ACS**: acute coronary syndrome
- **AMI**: acute myocardial infarction
- **APC**: allophycocyanin
- **AR**: aortic regurgitation
- **AS**: aortic stenosis
- **AVR**: aortic valve replacement
- **B**: Bursa
- **BASO**: basophil monocytes
- **BB**: *Borrelia burgdorferi*
- **BMI**: body mass index
- **BSA**: bovine serum albumin
- **CABG**: coronary artery bypass graft
- **CAD**: coronary artery disease
- **CAS**: calcific aortic stenosis
- **CCL2**: C-C motif chemokine ligand 2
- **CD**: cluster of differentiation
- **CS**: citrate synthase
- **CP**: *Chlamydia pneumoniae*
- **CRP**: C-reactive protein
- **CTLs**: cytotoxic (or cytolytic) T lymphocytes
- **C3**: Complement C3
- **C4**: Complement C4
- **CH50**: 50% hemolytic complement
- **DM**: diabetes mellitus
- **EF**: ejection fraction
- **ELISA**: Enzyme Linked Immunosorbent Assay
- **E-N-T**: ear, nose and throat
- **EO**: eosinophil monocytes
- **ESC**: European Society of Cardiology

- **ESR**: erythrocyte sedimentation rate
- **FACS**: fluorescence-activated cell sorting
- **FITC**: fluorescein isothiocyanate
- **FL**: fluorescence
- **FSC** light: forward scattered light
- **γ/δ T**: gamma delta T, gd T
- **h**: human
- **HLA**: human leukocyte antigen
- **HIV**: human immunodeficiency virus
- **HP**: *Helicobacter pylori*
- **HRP**: horseradish peroxidase
- **H_2O_2**: hydrogen peroxide
- **H_2SO_4**: Sulfuric acid
- **H_3PO_4**: Phosphoric acid
- **IF**: immunofluorescent staining
- **Ig**: immunoglobulin
- **IL6**: Interleukin-6,
- **IL8**: Interleukin-8
- **iNKT**: invariant natural killer T
- **IQR**: interquartile range
- **kD/kDa**: kilodaltons
- **LAD**: left anterior descending coronary artery
- **LDH**: Lactate dehydrogenase
- **LVEF**: left ventricular ejection fraction
- **LY**: lymphocyte
- **M**: molarity
- **MCP-1**: monocyte chemoattractant protein-1
- **MI**: myocardial infarction
- **MONO**: monocytes
- **MP**: *Mycoplasma pneumoniae*

- **N**: number of participants/samples
- **NEU**: neutrophil
- **ng/ml**: nanogram/milliliter
- **NK**: natural killer
- **NKT**: natural killer T
- **Nm**: nanometer
- **OD**: optical density
- **OPD**: o-phenylenediamine
- **P-value**: probability value
- **PBS**: phosphate buffered saline
- **PCI**: percutaneous coronary intervention
- **PerCP**: peridinin chlorophyll
- **PF**: pericardial fluid
- **R**: correlation coefficient
- **RDB**: Reagent Dilution Buffer
- **RT**: room temperature
- **sCD40L**: soluble CD40 ligand
- **SEM**: standard error of mean,
- **SB**: Setup Beads
- **sP-sel**: soluble P-selectin
- **SSC light**: side scattered light
- **Streptavidin-PE**: Streptavidin-Phycoerythrin
- **T**: thymus
- **Tact**: activated T cells
- **TCRs**: T-cell receptors
- **T_H**: helper T
- **TMB**: tetramethylbenzidine
- **Tmemo**: memory T cells
- **Tnaive**: naive T cells
- **TNF**: tumor necrosis factor
- **tPA**: tissue plasminogen activator

- **Treg**: regulatory T
- **YE**: *Yersinia enterocolitica*
- **WBC**: white blood cell

2 Introduction

2.1 Physiology and composition of pericardial fluid

Pericardial fluid (PF) is a clear, pale yellow fluid located in the pericardial space between the parietal and visceral layers of the pericardium and has a volume of approximately 15-50ml (Burns *et al*., 1992; Barreiro, Stewart and Whitman, 2011; Gaughan and Kobel, 2014). There are two pericardial layers, the serosa and the fibrosa. The monocellular serosa lined by mesothelial cells, directly covering the heart surfaces is the visceral pericardium, also called "the epicardium". The fibrosa together with the reflections of the serosal sac internally attached to it is the parietal pericardium, also referred to simply as "the pericardium" (Lambert, 1998) (Fig.1.). Interestingly, the luminal surface of the mesothelial cells have microvilli with occasional cilia that presumably bear friction and facilitate fluid and ion exchange (Iaizzo, 2009).

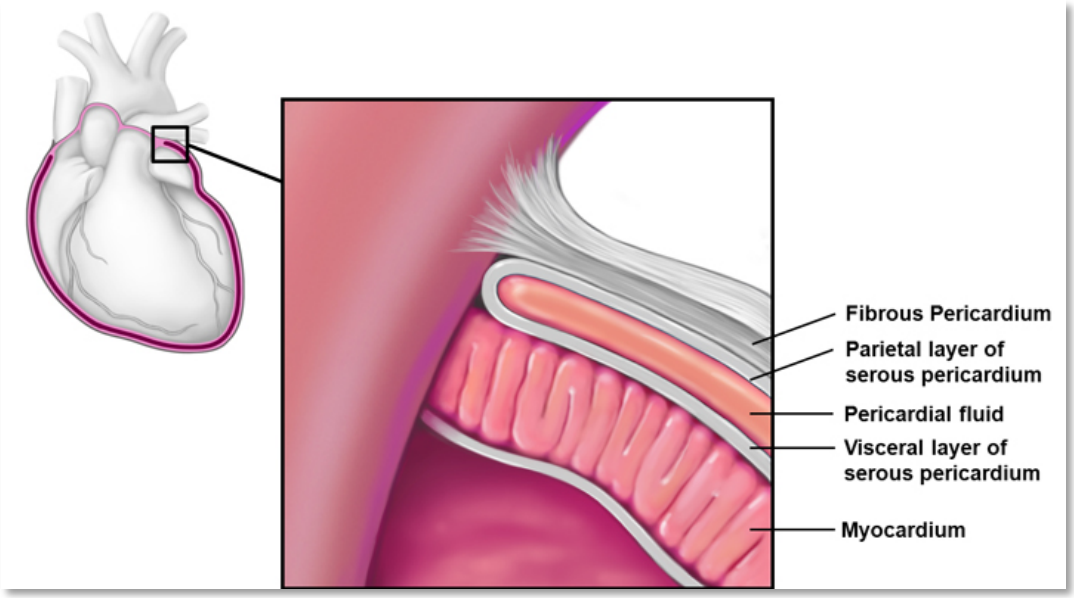

Figure 1. Anatomy of the pericardium Source: http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/cardiology/pericardial-disease/

Similar to other serosal fluids, the small amount of physiologic PF is believed to be a transudate generated by the net result of the hydrostatic pressure

and the osmotic gradient between plasma and PF (Ben-Horin *et al.*, 2005, 2007) through the epicardial capillaries (and probably the parietal pericardium), as well as a small amount of interstitial fluid from the underlying myocardium (Elie *et al.*, 2018), during the cardiac cycle (Stewart *et al.*, 1997). Thus, PF is mainly an ultrafiltrate of plasma that includes some overflow of myocardial interstitial fluid as well as myocardial lymph drainage. Therefore, PF reflects the composition of cardiac interstitium (Mebazaa, 1998). The fluid drainage is mainly accomplished through the parietal pericardium lymphatic capillary bed (in sheep) (Yuan *et al.*, 2000). The whole procedure is not fully understood because of the difficulty to study pericardial fluid dynamics under normal conditions (Vogiatzidis *et al.*, 2015).

PF is remarkable for high LDH and protein content and for the predominance of lymphocytes, especially memory type T-cells, whereas only scant literature is available on the physiological composition of it (Tan *et al.*, 1974; Gibson and Segal, 1978; Perez-Cárceles *et al.*, 1995; Soós *et al.*, 2002; Bechtloff *et al.*, 2011).

Protein concentration is lower than in plasma, but with a relatively high albumin component, due to albumin's lower molecular weight and ease of transmembrane transport as compared to a complete protein. Proteins with a molecular mass below 40 kDa can diffuse through the epicardium into the pericardial space (Limana *et al.*, 2007). Electrolyte concentrations are as predicted for a plasma ultrafiltrate, yielding a PF osmolarity consistent with such an ultrafiltrate and therefore less than plasma osmolarity (Lambert, 1998; Ege *et al.*, 2003).

Regarding the cell population, studies in human normal PF have shown the presence of a heterogeneous cell population. There are mesothelial cells, lymphocytes (53%), granulocytes (31%), macrophages (12%), eosinophils (1.7%), and basophils (1.2%) (Tbl.1.). This means that the PF "lymphocytosis" should always be under critical consideration and characterized as pathological only when it exceeds 60% of the whole cell population (Gibson and Segal, 1978; Benhaiem-Sigaux *et al*., 1985; Vogiatzidis *et al*., 2015).

Although PF has a grossly "obvious" lubricant function; a variety of surfactant phospholipids within it are capable of greatly reducing friction between otherwise hydrophilic surfaces (Lambert, 1998; Pfaller *et al*., 2019). Accumulating evidence shows that PF plays more than one important role in the physiology and pathophysiology of the heart and its surrounding tissues. (Selmeci *et al*., 2000; Soós *et al*., 2002; Nagy *et al*., 2009) The pericardial cavity stocks many bioactive substances, such as cardiac hormones, growth factors, prostaglandins, and cytokines, whose implications in heart function are still not entirely clear, but may include paracrine regulation of heart contractility, vasodilation and cardiac sympathetic stimulation. These functions are supported by the predominance of biological processes related to the response to stress, stimulus and to wounding, according to the gene ontology enrichment analysis of the pericardial fluid proteins (Trindade *et al*., 2019). Prostacyclins, small amounts of complement (C3, C4, CH50), other immune factors, myocardial cellular enzymes, and related compounds are continually released into the PF by the mesotheliumans (Lambert, 1998).

2.2 Pericardial fluid under pathologic conditions

In everyday cardiac surgery practice PF plays a role in the case of pericardial effusion.

Pericardial effusion is identified as any excessive pericardial contents due to inflammatory exudation, systemic fluid retention, bleeding, gas, or combinations of these (Cohn, 2007).

The more common causes of pericardial effusions include infections (viral, bacterial, especially tuberculosis), cancer, connective tissue diseases, pericardial injury syndromes (post-myocardial infarction effusions, post-pericardiotomy syndromes, post-traumatic pericarditis), metabolic causes (especially hypothyroidism), myopericardial diseases (especially pericarditis, but also myocarditis, and heart failure), and aortic diseases (Imazio *et al.*, 2010), although the etiology remains unclear in almost 30% of the cases (Levy *et al.*, 2006).

The onset of symptoms, ranging from insidious to rapid to sudden, is related to the tempo of physiologic impairment. As little as 150 mL of rapidly added pericardial contents (usually blood) can be lethal in minutes. Primarily inflammatory or irritative bloody pericardial effusions' critical cardiac compression may first appear well over a liter. (Lambert, 1998)

Tamponade results from cardiac compression due to increased intrapericardial pressure with low cardiac output and elevated systemic venous pressure due to compromised cardiac filling (Cohn, 2007). This is a clinical diagnosis suggested by pulsus paradoxus and Beck's triad (jugular venous distension, distant heart sounds, and hypotension) (Gluer *et al.*, 2014). Pericardiocentesis is a life-saving procedure in decompensated cardiac tamponade and is indicated for effusions of 20 mm in diastole visualized by echocardiography. Pericardiocentesis can be guided by fluoroscopy, echocardiography, or a surgical approach (Levy *et al.*, 2006). The subxiphoid approach is most commonly used: introduce the needle between the xiphisternum and left costal margin at an angle of 30-45 directed towards the left shoulder (Gluer *et al.*, 2014). However, pericardiocentesis is mandatory, not only for cardiac tamponade, but also for moderate to large effusions when bacterial or neoplastic etiologies are suspected (Imazio *et al.*, 2010).

2.3 Indications of coronary artery bypass graft (CABG) – and aortic valve repair/replacement surgery

Atherosclerotic coronary artery disease (CAD) is one of the leading causes of death (Gaughan and Kobel, 2014; Sipahi, 2015) and nonrheumatic, calcificating aortic valve stenosis (CAS) is the most common cause of aortic valve operations in developed countries (Lindroos *et al.*, 1993).

2.3.1. CABG

CABG surgery is the surgical revascularization of the heart, bypassing the occluded or stenotic coronary arteries with autologous or artificial vessel(s). CAD remains the most common pathology with which cardiologists and cardiac surgeons are faced. Given projections that the number of Americans older than 65 years of age will double from 30 million currently to 62 million over the next 25 years, and the anticipated increase in the prevalence of important risk factors for CAD such as diabetes mellitus (DM) and obesity, the need for coronary revascularization in the next two decades can be expected to increase (Cohn, 2007). Symptoms of CAD can vary from asymptomatic or mild angina pectoris to acute coronary syndrome (ACS) and myocardial infarction (MI).

CABG is performed in patients with asymptomatic, or mild, or stable angina, or with unstable angina that have significant left main coronary artery stenosis or left main equivalent: significant (greater than or equal to 70%) stenosis of the proximal left anterior descending coronary artery (LAD) and proximal left circumflex artery. CABG can be beneficial for patients with asymptomatic or mild angina who have proximal LAD stenosis with 1- or 2-vessel disease. (This recommendation becomes a Class I if extensive ischemia is documented by noninvasive study and/or left ventricular ejection fraction (LVEF) is less than 0.50). CABG is recommended for patients with stable angina who have 3-vessel disease. (Survival benefit is greater when LVEF is less than 0.50). CABG is recommended in patients with stable angina who have 2-vessel disease with significant proximal LAD stenosis and either EF less than 0.50 or demonstrable ischemia on noninvasive testing (Eagle *et al.*, 2004).

2.3.2. Aortic valve repair/replacement

Age-related degenerative calcific aortic stenosis (CAS) is currently the most common cause of acquired aortic stenosis (AS) in adults and the most frequent reason for aortic valve replacement (AVR) in patients with AS, while rheumatic AS represents the least common form. Calcified bicuspid aortic valve represents the most common form of congenital AS.

The prevalence of aortic valve abnormalities as detected by population-based echocardiographic study increases with age, with 2% of people 65 years of age or older having isolated CAS, whereas 29% exhibit age-related aortic valve sclerosis without stenosis. CAS is also observed in a number of other conditions, including Paget disease of bone and end-stage renal disease.

AS causes gradual obstruction to left ventricular outflow. Critical obstruction to the left ventricular outflow is reached with (1) an increase in peak systolic pressure gradient of greater than 40 mm Hg in the presence of normal cardiac output, and (2) a decrease in effective aortic orifice area of less than 0.5 cm^2/m^2 of body surface area (usually 0.8 cm^2). The cardinal manifestations of acquired AS are angina pectoris, syncope, and, ultimately, heart failure. Aortic valve replacement is indicated in patients with symptomatic AS.

Aortic regurgitation (AR) is a diastolic reflux of blood from the aorta into the left ventricle owing to failure of coaptation of the valve leaflets during diastole. AR has numerous causes, which can be grouped according to the structural components of the valve affected. Calcific aortic disease, idiopathic degenerative disease, active or chronic aortic valve endocarditis, rheumatic disease, a bicuspid aortic valve, and myxomatous proliferation of aortic valve tissue all prevent the valve cusps from closing properly. Aortic dissection, trauma, chronic systemic hypertension, aortitis from syphilis, viral syndromes, or other systemic arteritis and connective tissue disorders such as Marfan syndrome, Reiter disease, Ehlers-Danlos syndrome, osteogenesis imperfecta, and rheumatoid arthritis all lead to annular dilatation and valvular insufficiency. Most commonly, aortic valvular insufficiency is seen in combination with AS (Cohn, 2007).

2.4 About the immune system

2.4.1. Characteristics of the investigated lymphocyte populations

The innate immune system responds rapidly to infectious agents, whereas the adaptive response requires cell division and differentiation of effector cells. The

role of lymphocytes is mediating mainly adaptive immunity. The main populations of circulating lymphocytes in the peripheral blood originate from the common lymphoid precursor, and are called T-, B, and natural killer (NK) cells based on their role in the immune response and their cell surface structures. In circulation they are present mostly in naive form (mature cells that have never encountered foreign antigen). Naive lymphocytes are activated by antigens to proliferate and differentiate into effector and memory cells. Naive and memory lymphocytes are both called resting lymphocytes because they are not actively dividing, nor are they performing effector functions. Memory cells may survive in a functionally quiescent or slowly cycling state for years, even lifelong, without a need for stimulation by antigen and presumably after the antigen is eliminated. Subsets of B and T lymphocytes exist with distinct phenotypic and functional characteristics. (Fig. 2.)

B lymphocytes are the only cells capable of producing immunoglobulins (antibodies), thus functioning as the mediators of humoral immunity. Conventional or B2 ($CD19^+$) B cells represent 5-15% of circulating lymphocytes in healthy individuals. A special subpopulation of B lymphocytes are the $CD5^+$ B1 cells (5% of all B lymphocytes) which can be found in pleural and peritoneal cavities and are able to produce autoantibodies as well. Memory B lymphocytes may express certain classes of membrane immunoglobulin, such as IgG, IgE, or IgA, whereas naive B cells express only IgM and IgD. In humans, CD27 expression is a marker for memory B cells. (Abul K. Abbas, Andrew H. Lichtman, 2015)

The major lymphocyte group consists of T lymphocytes, which have a restricted specificity for antigens; they recognize peptides derived from foreign proteins that are bound to antigen presenting cells. T lymphocytes do not produce antibody molecules. T cell subsets are $CD4^+$ helper T lymphocytes and $CD8^+$ cytotoxic (or cytolytic) T lymphocytes (CTLs). $CD4^+$ regulatory T cells are a third subset of T cells; their function is to inhibit immune responses. In addition, natural killer T-cells (NKT) and invariant natural killer T-lymphocytes (iNKT) and γδ T cells (γ/δT) are three numerically smaller subsets of T cells that express T-cell receptors (TCRs) with limited diversity, analogous to the antibodies made by B-1

cells, and are part of the natural immune system, which has both adaptive and innate features. These innate B cells and T cells use receptors encoded by somatically rearranged genes to recognize specific structures from microbes and self antigens. Functionally, innate-like lymphocytes mount quick effector responses such as cytolysis and the rapid secretion of cytokines, chemokines and antibodies (Lanier, 2013).

In humans, most naive T cells express a 200-kD isoform of a surface molecule called CD45RA. In contrast, most activated and memory T cells express a 180-kD isoform called CD45RO.

The ($CD16^+$ and 56^+) NK cells represent 5-15% of the mononuclear cells in blood. The NK cells are innate lymphoid cells and able to eliminate pathogens similar to T cells, but lacking T cell antigen receptors. These cells provide early defense against infectious pathogens, recognize and help to eliminate stressed and damaged host cells as well as tumor cells.

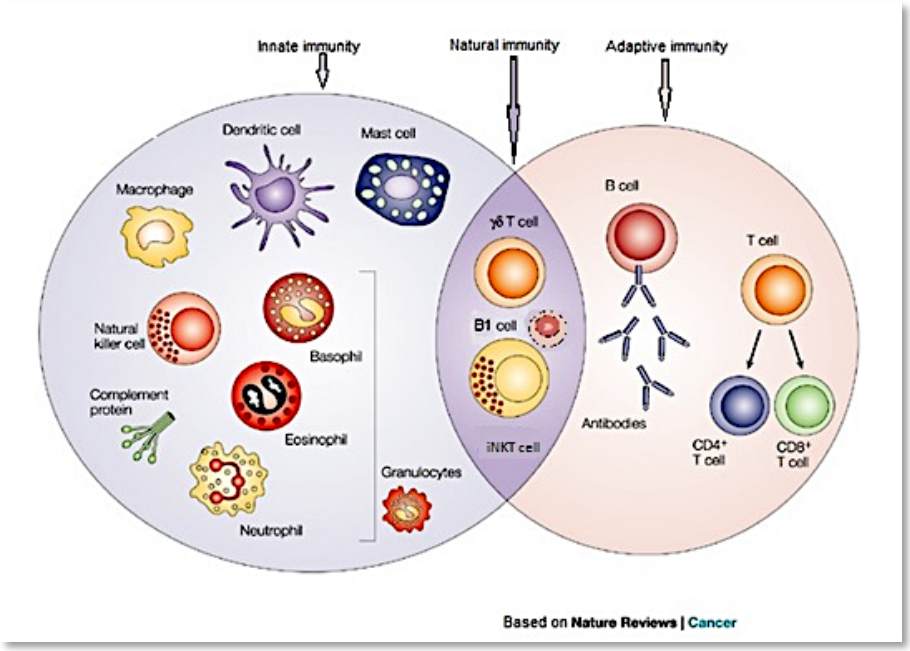

Figure 2. The cells of the immune system Based on:
http://www.nature.com/nrc/journal/v4/n1/fig_tab/nrc1252_F1.html

2.4.2. Cytokines

Cytokines are a large group of secreted proteins with diverse structures and functions, which regulate and coordinate many activities of the cells of innate and adaptive immunity. All cells of the immune system secrete at least some cytokines and express specific signaling receptors for several cytokines. The nomenclature for cytokines is inconsistent, with some named Interleukin followed by a number, and others named for a biological activity first attributed to them. The large subset of structurally related cytokines that regulate cell migration and movement are called chemokines. Some of the most effective drugs developed recently to treat immunologic diseases target cytokines, which reflects the importance of these (Abul K. Abbas, Andrew H. Lichtman, 2015).

Monocyte chemoattractant protein-1 (MCP1, also known as CCL2) is a pro-inflammatory mediator (Yao and Tsirka, 2014). MCP1 appears to play an additive and independent role during atherosclerosis (Hartmann, Schober and Weber, 2015).

IL8 is a chemotactic activator (particularly for neutrophils), promotes degranulation of neutrophils, and is also referred to as a neutrophil activating factor due to its property to induce local inflammatory responses. It is a strong stimulator for the oxidative processes in the neutrophils and for the release of enzymes.

IL6 is another important cytokine in acute inflammatory responses that has both local and systemic effects. It induces the synthesis of a variety of other inflammatory mediators in the liver, stimulates neutrophil production in the bone marrow, and promotes the differentiation of helper T cells. IL6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts (Abul K. Abbas, Andrew H. Lichtman, 2015). It is a potent inducer of cardiomyocyte hypertrophy. Studies demonstrated that the cytokines of the IL6 family might play a critical role in heart development and in muscle cell survival during the onset of heart failure during biochemical stress. Whereas serum IL6 levels are low (< 20 pg/ml) or undetectable in normal subjects, high levels have been reported in several inflammatory diseases, acute myocardial infarction, severe congestive heart failure, hematological diseases, and a variety of tumors including cardiac myxoma. IL6 has also been detected in other biological fluids such as synovial fluid and in the urine.

It has also been proved that pleural or peritoneal effusions contain large amounts of IL6 (Menet *et al.*).

The immune modulator pair CD40 and CD40 ligand (CD40L) [also termed CD154] has been proposed to be an important link between inflammation and thrombosis. The majority of the soluble fragment of CD40L (sCD40L) is derived from activated platelets and, hence, reflects platelet activation. On exposure to CD40-expressing vascular cells (or endothelial cells), platelet-associated CD40L or sCD40L, induces adhesion molecule expression, inflammatory cytokine release, and the procoagulant tissue. Increased sCD40L levels have been reported in patients with vascular diseases (Choudhury *et al.*, 2008).

Tissue-type plasminogen activator (tPA) is an extracellular proteolytic enzyme that was first described for its effects on blood coagulation and extracellular matrix homeostasis. However, during the last twenty years, tPA has been shown to have numerous functions in brain physiology and pathology. TPA is a glycoprotein belonging to the superfamily of serine proteases and is a member of the chymotrypsin family. TPA is now used in clinics to promote fibrinolysis, especially at the acute phase of ischemic stroke (Hébert *et al.*, 2016).

Selectins are plasma membrane carbohydrate-binding adhesion molecules that mediate an initial step of low affinity adhesion of circulating leukocytes to endothelial cells lining postcapillary venules. Selectins and their ligands are expressed on leukocytes and endothelial cells. Endothelial cells express two types of selectins, called P-selectin (CD62P) and E-selectin (CD62E). P-selectin, so called because it was first found in platelets, is stored in cytoplasmic granules of endothelial cells and is rapidly redistributed to the luminal surface in response to histamine from mast cells and thrombin generated during blood coagulation (Abul K. Abbas, Andrew H. Lichtman, 2015).

2.4.3. Characteristics of the investigated immunoglobulins

Antibodies or immunoglobulins (Ig) are circulating proteins that are produced in vertebrates in response to exposure to foreign structures known as antigens. Antibodies are synthesized only by cells of the B lymphocyte lineage and exist in

two forms: membrane-bound antibodies on the surface of B lymphocytes functioning as antigen receptors, and secreted antibodies neutralize toxins, prevent the entry and spread of pathogens, and eliminate microbes. The classes of antibody molecules are also called isotypes and are named IgA, IgD, IgE, IgG, and IgM. In humans, IgA and IgG isotypes can be further subdivided into closely related subclasses, or subtypes, called IgA1 and IgA2 and IgG1, IgG2, IgG3, and IgG4. Different isotypes and subtypes of antibodies perform different effector functions. When these B cells are activated by foreign antigens, typically of microbial origin, they may undergo a process called isotype (or class) switching, and therefore the antibody isotype produced by the B cell changes. Memory B lymphocytes may express certain classes (isotypes) of membrane Ig, such as IgG, IgE, or IgA, as a result of isotype switching, whereas naive B cells express only IgM and IgD. For example, the antibody response to many bacteria and viruses is dominated by IgG antibodies, which promote phagocytosis of microbes, and the response to helminths consists mainly of IgE. Switching to the IgG isotype also prolongs the effectiveness of humoral immune responses because of the long half-life of IgG antibodies.

The circulation of healthy individuals contains antibodies that react with self and non-self-antigens, and are generated without external antigen stimulation. These immunoglobulins are considered natural autoantibodies. (Zouali, 2009; Siloşi *et al*., 2016). Natural autoantibodies are produced by B1 cells (Kohler *et al*., 2003; Baumgarth, 2011; Lobo, 2016) without somatic hypermutation; most of them are of IgM isotype. Natural autoantibodies of IgG isotype have also been described (Mannoor, Xu and Chen, 2013; Nagele *et al*., 2013), which typically bind multiple self- and non-self-antigens. These autoantibodies are often directed against highly conserved molecules and bind various ligands with relatively low affinity, and therefore, they are called 'polyreactive'. Evidence from rodents deficient in natural antibody production suggests that natural autoantibodies serve homeostatic and housekeeping functions. Moreover, natural IgM autoantibodies serve as scavengers of damaged molecules and cells (Ehrenstein and Notley, 2010) Therefore, natural autoantibodies have been implicated in the control of inflammation and autoimmune diseases (Grönwall, Vas and Gregg J. Silverman, 2012; Grönwall and

Gregg J. Silverman, 2014). Natural antibodies may also serve as innate-like recognition receptors, recognizing various bacterial cell wall components or parasites (Gunti and Notkins, 2015; Panda and Ding, 2015). Antibodies produced by the adaptive immune system are generally derived from T-dependent B lymphocytes in response to exposure to microbes or vaccination. It has been shown previously that T cells are the dominant lymphocytes in the PF (Riemann *et al*., 1994), but less is known about the B1 and B2 cell composition of PF, which may reflect the source of natural and infection-related antibodies found in the pericardial space.

2.5 Role of the immune system in cardiovascular diseases and in the pericardial fluid

There is increasing evidence that the immune system plays an important role in the development of cardiovascular diseases, especially in atherosclerosis, one of the main underlying features of cardiovascular failure (Frostegård, 2013; Epelman, Liu and Mann, 2015; Ketelhuth and Hansson, 2016). Extravascular serous fluids (i.e. peritoneal, pleural, and pericardial) contain cellular and humoral components of the immune system to protect the organ from microbial invasion. The peritoneal and pleural cavities and the associated serous fluids have been investigated thoroughly regarding their biochemical and immunological composition (Montecino-Rodriguez and Dorshkind, 2006; Murphy *et al.*, 2012; Kopcinovic and Culej, 2014).

One would predict that components of the immune system may be involved in two major functions in the pericardium: (1) facilitation of the elimination of cell and tissue debris that may form as a result of the mechanical 'wear-and-tear' process and tissue homeostasis (Ehrenstein and Notley, 2010), and (2) protection of the heart and pericardium from microbial agents. However, as mentioned above, little is known about the immunological milieu in the pericardial space.

MI causes sterile inflammation, which is characterized by the recruitment and activation of immune cells of the innate and adaptive immune systems (Yan *et al.*, 2013). There is strong evidence that T lymphocytes play a central role in the pathogenesis of cardiac disease, either by direct cytotoxicity, by enhancing the inflammatory functions of other cells, or by helping B cells produce pathogenic antibodies (Lichtman, 2013). $CD4^+$ helper T (T_H) -, regulatory T- (Treg), natural killer T (NKT) cells, γ/δ T and iNKT cell populations were found to be crucial in the course of cardiovascular diseases including ACS and AMI (de Boer *et al.*, 2007; Mor *et al.*, 2007; Anane *et al.*, 2009; To *et al.*, 2009; Liu *et al.*, 2011; Hofmann *et al.*, 2012; Kimura *et al.*, 2012; Arslan, 2013; Homma *et al.*, 2013; Meng *et al.*, 2013; Napoleão *et al.*, 2016; Bonaventura, Montecucco and Dallegri, 2016). However, there is currently a lack of studies that focus on human samples and PF. MI also reactivates an embryonic program in the epicardium and factors in the PF could exert paracrine actions on certain epicardial cells. PF reflects the composition of cardiac interstitium; moreover, proteins with a molecular mass below 40 kDa can diffuse through the epicardium into the pericardial space (Mebazaa *et al.*, 1998; Winter *et al.*, 2007; Limana *et al.*, 2010).

Nonrheumatic CAS is the most common cause of aortic valve replacement in developed countries (Lindroos *et al.*, 1993). Studies suggest that valvular calcification in CAS is actively regulated and has inflammatory features such as $CD4^+$ and $CD8^+$ T cells infiltrate the aortic valve (Patrick Mathieu, Rihab Bouchareb, 2015). Investigations have shown a clonal expansion of T cells in mineralized aortic valves. A subset of memory T cells is activated in patients with CAS (Winchester *et al.*, 2011). Immunohistological analyses of explanted mineralized aortic valves showed that $CD8^+$ cells were present at the proximity of mineralized nodules (Mathieu, Bouchareb and Boulanger; Wu *et al.*, 2007).

Numerous heart diseases are associated with altered levels of cardiovascular markers and cytokines present in peripheral blood (Coats, 2001). Inflammatory cytokines are involved in the development and course of cardiovascular diseases including atherosclerosis, angina, acute myocardial infarction, and chronic heart failure; interleukin-6 (IL6), Interleukin-8 (IL8) concentrations of PF were shown to

be significantly higher than serum in all the above-named conditions (Gwechenberger *et al.*, 1999; Ege *et al.*, 2003). Abnormal levels of soluble CD40 ligand (sCD40L) have been reported in patients with hypertension, coronary artery disease, diabetes mellitus, and heart failure (Nannizzi-Alaimo *et al.*, 2002; Choudhury *et al.*, 2008; Vistnes *et al.*, 2010; Oemrawsingh *et al.*, 2011; Lowe and Rumley, 2014; Gremmel, Frelinger and Michelson, 2015). Other factors such as tPA, MCP-1, IL8, P-selectin also have noteworthy alternations in serum as well as in PF (Aukrust *et al.*, 2008; Lowe and Rumley, 2014). An increased level of soluble P-selectin (sP-sel) is a major predictive factor of cardiovascular events relating to platelet turnover and its activation and function. P-selectin plays a key role in diseases associated with injury and arterial thrombosis (Guo *et al.*, 2015). Moreover, there is growing evidence that epicardial adipose tissue could locally modulate the heart and vasculature through paracrine secretion of pro- and anti-inflammatory cytokines (TNF-alpha, Interleukin 1, IL6, MCP-1), thereby playing a possible role in the adiposity-related inflammation and atherosclerosis (Mazurek, 2003; Iacobellis and Barbaro, 2008). If so, PF investigation could also provide further insight into the pathophysiology of the coronary arteries, epicardial and pericardial fat tissue.

There is no information available on the presence and function of the natural immune system (an intermediate between the innate and adaptive immune systems) in the pericardial space. Based on previous studies, including those from our laboratory, it is known that citrate synthase (CS), a highly conserved mitochondrial inner membrane enzyme, is an autoantigen recognized by the natural immune system. We have documented previously that anti-CS IgM natural antibodies are continuously present in plasma of healthy controls and that anti-CS IgG levels increase in patients with autoimmune diseases and heart transplantation (Alter *et al*., 1990; Morgunov and Srere, 1998; Czömpöly *et al*., 2006, 2008); however, no data has been published regarding the anti-CS antibodies in PF. Mitochondria are the source of cellular energy production and are present in different types of cells. Heart muscle cells contain the highest number of mitochondria in body tissues, and

mitochondrial enzymes can become released in different cardiovascular diseases. Mitochondrial dysfunction is associated with the development of numerous cardiac diseases such as atherosclerosis, ischemia-reperfusion, injury, hypertension, diabetes, cardiac hypertrophy and heart failure, due to the uncontrolled production of reactive oxygen species (Brown *et al*., 2017).

Infection-related antibodies have also been found to have a potential role in the development of atherosclerosis, including *Chlamydia pneumoniae, Mycoplasma pneumoniae, Borrelia burgdorferi, Helicobacter pylori* and *Yersinia enterocolitica* (Danesh, Collins and Peto, 1997; Danesh *et al*., 1999; Ridker *et al*., 2001; Rugonfalvi-Kiss *et al*., 2002; Völzke *et al*., 2006; Rosenfeld and Ann Campbell, 2011; Chhibber-Goel *et al*., 2016).

2.6 Pericardial fluid is a relevant specimen to investigate cardiac pathology

As of today, PF analysis is important only to discern the etiology of pericarditis as recently recommended by ESC guidelines on the diagnosis and management of pericardial diseases (Adler *et al*., 2015). In each case of open-heart surgery, pericardiotomy always occurs, and PF is removed from the pericardial space as waste. In spite of the possible diagnostic and clinical relevance of the PF analysis, it is not investigated routinely; on the other hand, it is considered to be a reliable specimen due to its anatomical isolation and proximity to the heart (Burns *et al*., 1992; González-Herrera *et al*., 2013, 2016; Palmiere and Egger, 2014; Palmiere and Grabherr, 2019). Accumulating evidence shows that PF is a stable biofluid with low clearance rates and stocking heart-derived factors released from regions as deep as the myocardium (Trindade *et al*., 2019). An increasing number of studies indicate that intrapericardial drug administration is a safe route for therapeutic delivery, and cell transplantation and can result in better pharmacokinetics and higher drug efficiencies in conjunction with decreased side effects. (Filgueira *et al*., 2019)

PF composition may well influence the regeneration process following open heart surgery (Limana *et al*., 2007); moreover, PF is also proven to be a suitable vehicle for mesenchymal stem cells and the intrapericardial route provides an optimal retention and implantation of stem cells in animal models (Blázquez *et al.*, 2016; Zhang *et al.*, 2018). Immunomodulatory therapies can have the potential to limit the infarct volume and accelerate repair (Blázquez *et al*., 2016). Hence, the exploration of its diagnostic, prognostic, and therapeutic properties should draw more attention.

3 Aims

We hypothesized the existence of a unique immunologic milieu within the PF with its dedicated lymphocyte pool that is reactive to heart-related conditions.

1. Phenotype-analysis of lymphocyte subpopulations, found in the pericardial cavity and compare it to that in the serum samples within two different groups of patients: individuals who underwent CABG operation (groupA) and aortic valve repairment surgery group (groupB) as control.

2. Quantitative measurement of cardiovascular cytokines and markers in PF, and compare it to that in the serum samples within the above mentioned patient groups.

3. The other goal of this study was to determine whether natural autoantibodies to mitochondrial CS enzyme and antibodies to certain bacteria related to atherosclerosis are present in the plasma and PF of cardiac surgery patients.

4. We also aimed to investigate the relationship of the anti-CS antibodies to anti-bacterial antibodies and to the total immunoglobulin class levels in plasma and PF in patients with aortic valve replacement and CABG with or without previous MI.

4 Materials and methods

4.1 Ethics

The study was approved by the Institutional Review Board (Regional Research Ethics Committee of the Clinical Center, University of Pécs, 12 Szigeti Str. H-7643 Pécs, Hungary, reference number: 3415/2009). Patients gave a written, informed consent to all procedures.

4.2 Patients

Thirty-six consecutive patients who were admitted to the University of Pécs Heart Institute for elective open-heart surgery were selected in our study (Tbl.1.). The average age of patients was 58.81 ± 1.28 years, and the female:male ratio was 1:3. The patients were divided into two groups: coronary artery bypass graft surgery (groupA), and aortic valve replacement (groupB). Individuals in groupA had isolated CAD and patients in groupB had aortic valve stenosis without CAD. Prior to surgery, all patients underwent physical examination and wre checked for foci including urological, gynecological, dental, E-N-T examinations with chest X-ray, and abdominal ultrasound. The lab parameters the day before the surgery for each patient were in normal range (CRP, WBC and ESR included). Exclusion criteria were type1 DM, any kind of neoplasia, hepatitis, HIV, autoimmune disorders, any conditions treated by plasmapheresis, and indication for combined heart surgery.

	Aortic valve repair	CABG	All patients
N	12	24	36
female : male	4:8	5:19	9:27
age (mean +/- SEM)	60,50 (+/- 2,29)	57,96 (+/-1,55)	58,81 (+/- 1,28)
BMI (mean +/- SEM)	29,48 (+/- 1,58)	30,48 (+/- 0,97)	30,15 (+/- 0,83)
Type2 diabetic patients	4	9	13
Smokers	3	15	18

Table 1. Basic demographic characteristics of the investigated patient groups N: number of participants, SEM: standard error of mean, BMI: body mass index, CABG: coronary artery bypass graft

For the investigation of natural autoantibodies and bacterial antibodies, the CABG group was divided. Thus, three groups were compared: the aortic valve replacement group (AVR, n=10), coronary artery bypass grafting patients (CABG) with previous myocardial infarction (CABG with MI, n=10) ,and those who underwent CABG but had no previous myocardial infarction (CABG no MI, n=12). The diagnosis of MI was based on coronary angiography and echocardiography findings. All the no MI patients had coronary artery occlusion of one of the main coronary arteries (left anterior descending, circumflex, or right coronary artery) or significant coronary artery stenosis and hypo- or akinesis in the supplied segment, and lower ejection fraction of the left ventricle (EF≤35%). Due to lack of PF specimens, four patients could not be enrolled in this part of our project.

4.3 Sample collection and handling

Peripheral blood and PF samples were drawn simultaneously during elective open-heart surgery with careful bloodless preparation of the pericardium always under the same well-determined conditions. After median sternotomy (under general anesthesia) a small, bloodless incision was made at the anterior site of the pericardium with a high-frequency electric knife. At least 3ml of PF was sucked out by usage of a 20ml syringe connected to a sucking tube. (Fig.3.) At the same time, before the administration of heparin into the systemic circulation, 10ml peripheral blood was taken from the central venous catheter. Both specimens were kept in sterilized heparinized tubes (BD Vacutainer® CPT™ Cell Preparation Tube; BD Plymouth PL6 78P.UK) on ice for a maximum of 60 minutes. All samples were transferred on ice to the Department of Immunology and Biotechnology, where the immunological and serological analyses were performed. PF was checked for the possible presence of red blood cells and for the number of white blood cells with a hemocytometer.

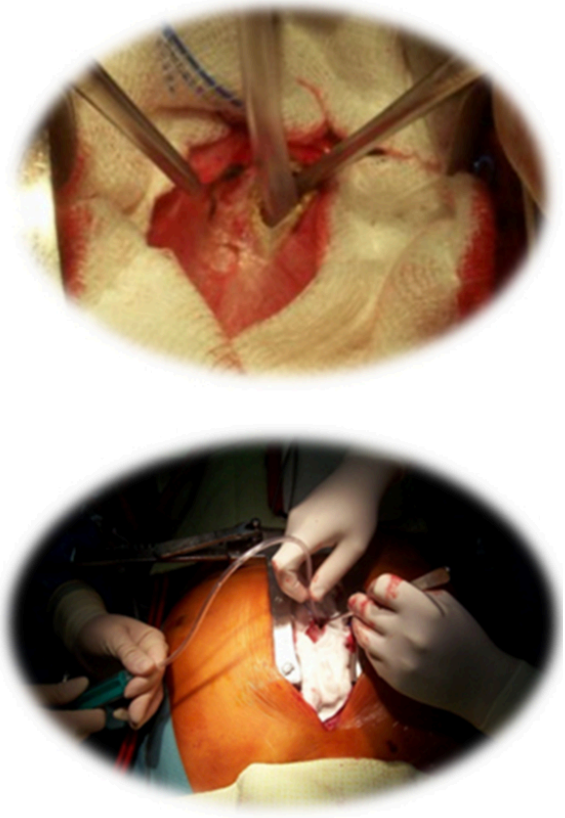

Figure 3. Bloodless preparation of the pericardium (top) **for the suction of PF** (bottom) (author's own picture)

4.4 Lymphocyte phenotype analysis

4.4.1. Immunofluorescent staining (IF)

In IF labelling techniques, monoclonal antibodies are chemically conjugated to fluorescent dyes (FITC, PE, PerCP, APC). These labeled antibodies bind directly to a specific molecule (antigen) on the surface of the target cell. Thus, the attached fluorochrome can be detected on the surface of the cell of interest via flow cytometry, which, depending on the messenger used, will emit a specific wavelength of light once excited, allowing us to identify the phenotype based on the presence of the cell surface molecule (Robinson, Bs and Kumar, 2009).

Buffers:

- PBS/BSA/azide buffer
  - 500 ml phosphate-buffered saline (PBS)
  - 0.5g bovine serum albumin (BSA)
  - 0.5g sodium-azide (NaN_3)
- BD FACS™ Lysing Solution (Beckton Dickson and Company Biosciences, San Jose)
  - The solution was used in a 1:10 dilution with room temperature (20°C–25°C), deionized water.
- FACS-FIX solution
  - 500ml 1x phosphate-buffered saline (PBS)
  - 1% (5 ml) paraformaldehyde
  - 0.02% (2ml of 2%) sodium azide
- Antibody cocktails (BD, San Jose and monoclonal antibodies produced by the Department of Immunology and Biotechnology) are mixture of monoclonal antibodies which identify the lymphocytes' phenotype by binding to the given cell surface (CD) marker (Tbl.2.)

Before the IF it was necessary to perform a cell count and viability analysis on Trypan Blue-treated PF using a hemocytometer under light microscope, in order to get ideal quality during staining and measuring.

Procedure:

1. Transfer 50 µl whole blood into the FACS tubes (1-7.).
2. Centrifuge whole blood as well as PF (2000 rpm, minutes, 4 °C) to separate cells.
3. Remove supernatants and store them in 500 µl units, at -20 °C until further usage.
4. Resuspend the cell pellet of PF in 700 µl PBS/BSA/azide buffer ($2x10^5$ – 10^6 cell/100µl – depending on the cell count), and dispense 100 µl to each PF test tube (8-14.) after vortexing.
5. Add Staining Cocktails (Table) and mix the suspensions well with vortex.
6. Antibody binding to the lymphocyte surface requires 25 minutes incubation in the dark in an ice bath.
7. Add 2ml lysis puffer to whole blood suspension and 1.5 ml to PF mixture tubes. Mix well with vortex.
8. Incubate samples for 15 minutes at room temperature. This step will dissolve RBCs.
9. Repeat step 7. with the usage of PBS/BSA/azide buffer for further washing.
10. Spin samples (1000 rpm, 5 minutes, 4 °C) to separate stained cells.
11. Aspirate supernatants.
12. Add 300 µl FACS-FIX solution to each tube.
13. Store samples at 4 °C until the measurement on a flow cytometer for maximum 24 hours.

Number of tube: volume of AB cocktail	Components of the AB cocktail (monoclonal AB – fluorochrome)	Volume of the components/tube
1.,8. tube: 30 µl cocktail	CD3-FITC CD8-PE CD56-PerCP	500 µl 250 µl 500 µl
2, 9. tube: 25 µl cocktail	CD3-FITC CD4-PE CD45-PerCP	500 µl 250 µl 250 µl
3., 10. tube: 15 µl cocktail	CD19-FITC CD5-PE	500 µl 250 µl
4., 11. tube: 25 µl cocktail	CD3-FITC CD25-PE CD4-PerCP	500 µl 250 µl 250 µl
5., 12. tube: 15 µl cocktail	HLA-DR-FITC CD8-PE	500 µl 250 µl
6., 13 tube: 35 µl/cocktail	CD3-FITC CD45-Ra-PE CD45-Ro-PerCP	500 µl 500 µl 500 µl
7., 14. tube: antibodies were added separately	iNKT-FITC γ/δ-FIC CD4-PerCP CD3-APC	20 µl/tube 20 µl/tube 2,5 µl/tube 5 µl/tube

Table 2. Antibody cocktails used in our experiments

4.4.2. Flow cytometry

Flow cytometry is precise and efficient method for measuring and analyzing multiple parameters (size, granularity, fluorescent signals) at single cell level in a mixed cell population at high speed.

Cells flow through a capillary tube system surrounded by the sheath fluid and counted as particles by the flow cytometer. Cells pass through a laser beam and cause diffraction of the light, which is detected by the flow cytometer. The forward scattered (FSC) light is proportional with cell size; the side scattered (SSC) light is

proportional with cell granularity. Detectors convert the light signals into digital signals and the computer analyzes the parameters of each cell. Each cell appears as a dot on a Dot Plot in the analyzing program (CellQuest).

Furthermore, the parallel detection and analysis of cell components becomes possible with multiple fluorescence labelling. Lasers of the flow cytometer (two or three lasers usually) excite the fluorescent molecules which in turn emit light at different wavelengths. The emitted light is collected by fluorescence detectors (FL-1, FL-2, FL-3, FL-4 channels).

10^5 cells were sorted by the flow cytometer in each antibody mixture except for the anti-iNKT and anti- γ/δ T antibody, where $2x10^5$ cells were analyzed due to the well-known low peripheral cell counts. Cell number results were expressed as a percentage. Flow cytometric analysis and sorting were performed on a BD FACSCalibur™ Flow Cytometer (BD Biosciences) and analyzed using BD CellQuest Pro 5.1 (BD Biosciences) software. The following cell types were investigated: $CD56^+CD3^-$ Natural killer cells (NK), $CD56^+CD3^+$ natural killer T-cells (NKT), $CD3^+CD8^+$ cytotoxic- and $CD3^+CD4^+$ helper T lymphocytes (Tc, Th), $CD19^+CD5^+$ B1- and $CD19^+CD5^-$ B2 B-lymphocytes (B1,B2); $CD4^+CD25^{high+}$ regulatory and $CD3^+CD25^{medium+}$ activated T-cells (Treg, Tact), $CD3^+CD45RA^+$ naive and $CD3^+CD45RO^+$ memory type T-cells (Tnaiv, Tmemo), $CD3^+iNKT^+$ invariant natural killer T-cells (iNKT) as well as as $CD3^+$ γ/δ TCR^+ gamma-delta T-cells (γ/δ T) (Fig.4.).

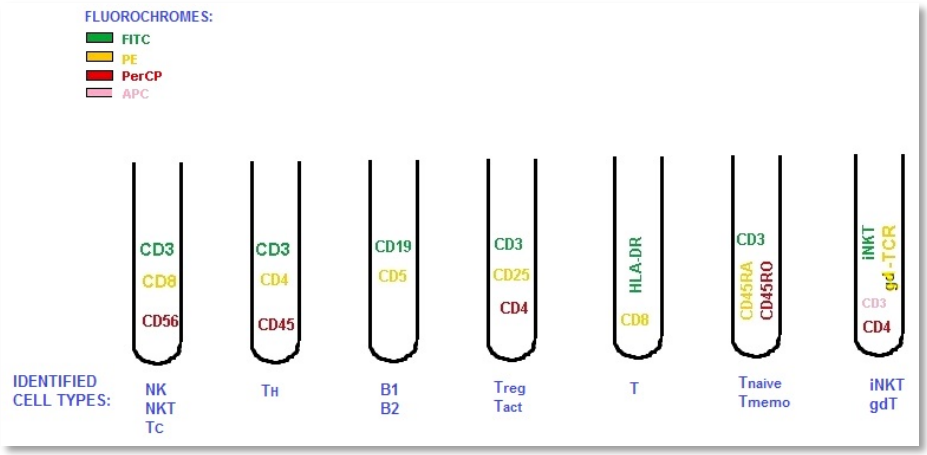

Figure 4. Antibody cocktails and the identified cell populations in PF and blood
gdT (gamma delta T cell), gd – TCR (gamma delta T cell receptor)

4.5 Measuring the concentration of cardiovascular markers and cytokines by multiplex microbead method

Multiplex microbead method is the use of fluorescently labeled microspheres as a solid phase instead of an ELISA plate that are individually identified by a flow cytometer based on their unique fluorescence labels. This was achieved by coating the microspheres with various concentrations of two fluorescent dyes: one identifies the analyzed biomarker, and the other is connected to the detection AB. The analytes present in the sample bind to the antibodies linked to the fluorescent beads. (Fig.5.)

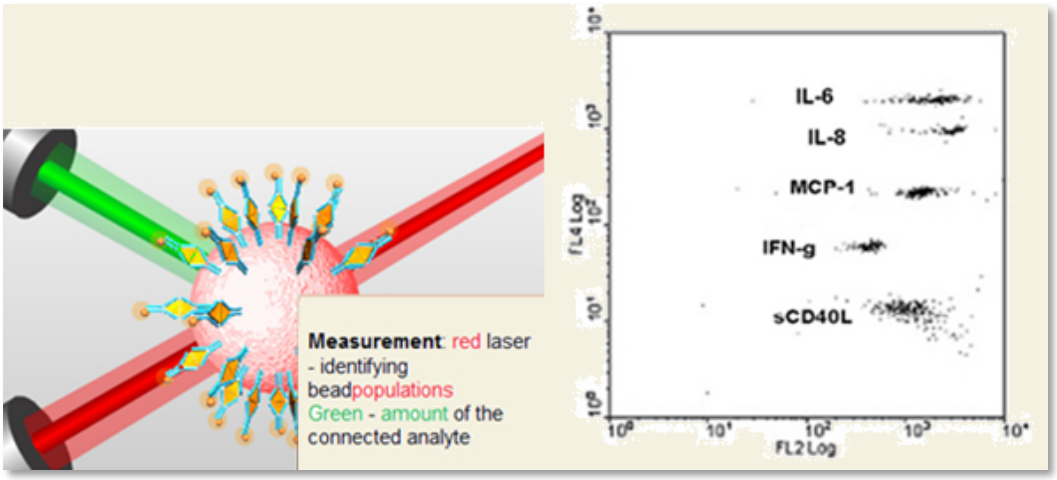

Figure 5. Beads used in the multiplex microbead method with the analytes connected to the detection ABs (left) and after measurements the beads appear as a dot on a **Dot Plot (**right) in the analyzing program where the x-axis represents the concentration, the y-axis helps to differentiate the analytes

Supernatants of PF samples and blood serums were stored at -20°C (and -80 °C when the specimen needed to be stored over longer periods of time) to avoid loss of bioactivity. The measurements were carried out according to the provider's manual.

Materials:

- Supernatant of PF (stored at -20 °C)

- Blood plasma (stored at -20 °C)
- Human Cardiovascular 6plex FlowCytomix Kit (Bender MedSystems GmbH Vienna, Austria) Contains:
    - Setup Beads (SB)
    - Fluorescent Beads coated with specific antibodies:
        - h sCD40L monoclonal
        - h IL6 monoclonal
        - h IL8 polyclonal
        - h MCP-1 monoclonal
        - h sP-selectin monoclonal
        - h t-PA polyclonal
    - Standard solutions for calibration
    - Assay buffer (10x) (PBS, 10% BSA)
    - Biotin-Conjugate (specific antibody conjugated to biotin)
    - Reagent Dilution Buffer (RDB)
    - Streptavidin-Phycoerythrin (Streptavidin-PE)

Procedure:

1. After thawing the sample pairs at room temperature, centrifuge samples (16000g, 5 minutes) to eliminate any debris.
2. Use 25 µl units of standard solution dissolved in Assay Buffer in given concentrations to calibrate the cytometer.
3. Subsequently, pipette 25 µl of sample then 25 µl of the bead containing reagent dilution buffer into the other tubes.
4. Then add 50 µl biotin-conjugate mixture and incubate the tubes at room temperature (18° to 25°C), protected from light for 2 hours.
5. Add 1000µl of Assay Buffer into each tube.
6. Afterwards separate the beads by centrifugation (200g, 5 minutes) and carefully remove the supernatant.
7. Repeat the last two steps.

8. Add 50 µl of Streptavidin-PE Solution; then, incubate for one hour at room temperature protected from light.
9. Finally, after two centrifugation steps (200g, 5 minutes) resuspend the beads in 500µl Assay Buffer.
10. Perform the flow cytometric analysis and sorting.

We carried out these experiments on a BD FACSCalibur™ Flow Cytometer (BD Biosciences), and analyzed the results using FlowCytomix Pro Software (Bender MedSystems GmbH Vienna, Austria)

The sensitivity of the analytes were the following: sCD40L 23,4 pg/ml; IL6 0,4 pg/ml; IL8 1,1 pg/ml; MCP1 1,3 pg/ml; sP-sel 1200 pg/ml; t-Pa 4,8 pg/ml.

4.6 Measurement of different immunoglobulin isotypes by Luminex method

Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a detection antibody conjugated to PE, the reported molecule, is introduced. The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals. The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. MILLIPLEX MAP Human Isotyping Kit (Millipore Corp) was used to measure human IgG subclasses (1, 2, 3, and 4), IgM, and IgA.

Materials:

- Supernatant of PF (stored at -20 ºC)
- Blood plasma (stored at -20 ºC)
- MILLIPLEX MAP Human Isotyping Kit (Millipore Corp) :
- MILLIPLEX® MAP Anti-Human κ and λ Light Chain,
- PE
- MILLIPLEX® MAP Human MultiImmunoglobulin Standard MILLIPLEX® MAP Human Immunoglobulin Positive Control MILLIPLEX® MAP Assay Buffer
- MILLIPLEX® MAP Wash Buffer
- 96-Well Plates
- Luminex Sheath Fluid

Procedure:

1. Add 50 µL of control, standard, or diluted sample to each well. (1:16000 and 1:10 dilutions of plasma and PF, respectively).
2. After vortexing the MILLIPLEX MAP Anti-Human MultiImmunoglobulin Beads at medium speed for 15 seconds, sonicate them for 15 seconds using a sonication bath
3. Add 25 µL of bead solution to each well and cover the plate with opaque plate cover and incubate 1 hour with agitation on plate shaker at room temperature.
4. Wash plate two times with 200 µL/well of Wash Buffer
5. Add 25 µL per well of diluted Anti-Human κ and λ light Chain, PE
6. Cover with opaque plate cover and incubate one hour with agitation on plate shaker at room temperature
7. Following the vacuum removal of the fluid, resuspend the beads in 150 µL/well of Sheath Fluid.
8. Proceed to reading results on an appropriate Luminex© instrument.

4.7 Measurement of anti-CS antibodies by enzyme-linked immunosorbent assay (ELISA)

The anti-CS IgM and IgG natural autoantibodies were measured by an in-house ELISA developed and validated by the Department of Immunology and Biotechnology (Nemeth *et al*.).

Materials:

- Supernatant of PF (stored at -20 ºC)
- Blood plasma (stored at -20 ºC)
- Ninety-six-well polystyrene plates (Nunc, Roskilde, Denmark)
- Citrate synthase (CS) from porcine heart (Sigma, St Louis, MO, USA)
- 0,1 M bicarbonate buffer (pH 9,6)
- 0,5% gelatin (Sigma) in phosphate- buffered saline (PBS) (pH 7,3)
- Washing buffer (PBS, 0,05% Tween 20)
- Horseradish peroxidase (HRP)-conjugated anti-human IgG- or IgM-specific secondary antibody (Dako, Glostrup, Denmark)
- Developing solution:
    - 8 mg o-phenylenediamine (OPD) (Sigma)
    - 10 ml ELISA substrate
    - 10 μl H_2O_2
- 4M H_2SO_4

Procedure:

1. Coat each well with 100 µl of 5 mg/ ml porcine heart CS in 0,1 M bicarbonate buffer at 4ºC overnight. Then wash plate three times with 300µl/well washing buffer.
2. Saturate the non-specific binding sites by pipetting 100 µl of 0,5% gelatin (Sigma) in PBS into each well.

3. Add 100 µl plasma samples in duplicate at 1:100 dilution and PF samples at 1:10 dilution in washing buffer into each well and incubate for one hour at room temperature.
4. Then, incubate plate with HRP-conjugated anti-human IgG- or IgM-specific secondary antibody (Dako, Glostrup, Denmark) for one hour at room temperature (anti-human IgG at 1:5000 dilution, anti-human at IgM 1:1000 dilution in washing buffer).
5. Develop the reaction by adding developing solution, then incubate for five minutes at room temperature protected from light.
6. Stop the reaction by adding 50 μl cc. H_2SO_4 to each well.
7. Measurement of the extinctions

An iEMS MF microphotometer (Thermo Labsystems, Waltham, MA, USA) was used to read extinctions at 492 nm. Cut-off values were calculated from the average of measured optical density (OD) 492 nm data. All measurements were standardized using a monoclonal anti-citrate synthase antibody (clone 4H3-E5) produced previously (Nemeth *et al*.) .

4.8 Serological tests for anti-bacterial antibodies

Antibodies against bacteria were measured by indirect sandwich ELISA tests.

Materials:

- Supernatant of PF (stored at -20 ºC)
- Blood plasma (stored at -20 ºC)
- Commercial ELISA kits
    - Wash buffer (phosphate buffer, NaCl, detergent and preservatives), had to be used in 1:10 dilution)
    - Dilution buffer (ready-for-use, contains protein detergent and blue dye)

    - Chromogenic substrate: tetramethylbenzidine (TMB, ready-for-use)
    - Stop solution: 24,9 % Phosphoric acid (H_3PO_4)
    - Positive control
    - Cutoff control
    - Negative control

- *B. burgdorferi*-specific IgG and IgM antibodies (Mikrogen GmBH, Neureid, Germany)
- anti-*H. pylori* IgA and IgG antibodies (Mikrogen GmBH, Neureid, Germany),
- anti-*Y. enterocolitica* IgA, IgM and IgG antibodies (Mikrogen GmBH, Neureid, Germany),
- anti-*C. Pneumoniae* IgA, IgG and IgM antibodies (Ani Labsystems Diagnostics, Vantaa, Finland)
- anti-*M. pneumoniae* IgA, IgG and IgM antibodies (Ani Labsystems Diagnostics, Vantaa, Finland)

Procedure:

1. Pipette 100 µl per well of the diluted samples (plasma samples at 1:100 dilution and PF samples at 1:20 dilution) and diluted controls.
2. Empty the wells completely, then wash four times, each time with 300 µl ready-to-use wash buffer per well.
3. Pipette 100 µl peroxidase conjugate per well. In manual processing, the microplate is carefully taped over with unused sealing tape and incubated for 30 minutes at 37°C.
4. Washing procedure
5. Pipette 100 µl TMB substrate solution per well. The microplate is incubated for 30 minutes at room temperature while protecting it from direct sunlight.
6. To stop the reaction, pipette 100 µl of stop solution per well.

7. Measurement of the extinctions

We measured the extinctions using a Siemens BEP- 2000 ELISA reader at 450 nm (Biocompare, San Francisco, CA, USA).

4.9 Statistical analysis

Values are presented as mean ± SEM. Data among multiple groups were compared using either the Kruskall–Wallis test followed by Spearman correlation and regression analysis, as appropriate. PF and blood data were analyzed using Wilcoxon test. A value of $P < 0.05$ was considered statistically significant when comparing CABG and AVR groups. For statistical evaluation of natural autoantibodies and bacterial antibodies among three groups, Spearman's correlation analysis and Mann–Whitney U-tests were used as appropriate. P-values < 0,05 were considered significant. Statistical analysis was performed with Origin Pro 2015 (Origin Lab Corporation, Northampton, USA) and SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA).

5 Results

5.1 Characterization of lymphocyte subpopulations and cardiovascular markers in pericardial fluid of cardiac surgery patients

5.1.1. Cellular composition

The absolute white blood cell count in PF was significantly lower (0.67±0.28 G/L) than in blood (9.70±11.88 G/L). Lymphocytes represented the dominant cell type in PF (61.94%) (Fig.6a). The granulocyte cell ratio was significantly lower, while monocyte and eosinophil cell ratio were both higher in PF than in blood. All the investigated lymphocyte subpopulations could be detected in the PF in different ratios than in blood (Figs. 6b, 6c, 6d). Based on the Spearman correlation, the levels of the following lymphocyte populations correlated in PF ($r \geq 0.4$; $p \leq 0.009$) with the same type of cell in blood: Tc (r=0.431; p=0.009), Treg (r=0.618; p=0.00006), Tnaive (r=0.581; p=0.0002), Tmemo (r=0.471; p=0,004), γ/δ T (r=0.447; p=0.003).

3.1.2. Basic lymphocyte populations

The $CD3^+$ T cells represented the major cell population in both PF and blood (72.05±1.9% and 69.15±1.43%).The level of CD8+TC cells (33.26±1.72% vs. 19.32±1,07%), and NK lymphocytes (13.80±1.54% vs. 9.51±1.27%) were significantly elevated in PF compared to blood, whereas the levels of $CD4^+T_H$ cells (38.74±1.9% vs. 49.24±1.97%), $CD5^+$B1 subgroup (1.35±0.18% vs. 2.33±0.30 %) and $CD19^+$B2 lymphocytes (4.07±0.63% vs. 5.42+/-0.09%) were significantly lower in PF than in blood (Fig. 6b)

The NK cell ratio was higher in the PF of both groups but was significant only in groupA. In groupA the $CD5^+$ B1 cell ratios were significantly lower in PF than in blood, unlike in groupB. There were significantly (3.45 times) less conventional B2 lymphocytes in PF of groupB compared to blood, while in groupA patients this difference was only 1.74 (Figs. 9a, 9d)

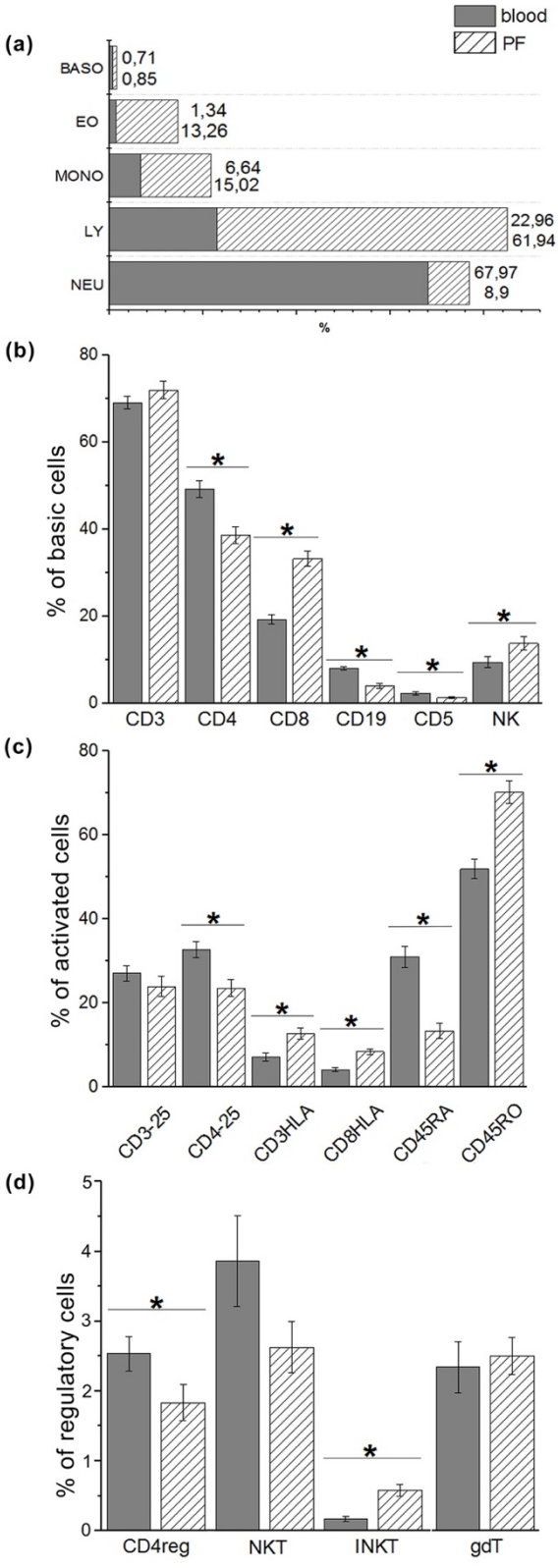

Figure 6. White blood cell counts and lymphocyte subpopulations of peripheral blood and pericardial fluid Automated differential white blood cell counts of blood and PF **(a)**, (N=12), Abbott Cell-Dyn 1700 automatic analyzer, WBC: white blood cell, NEU:neutrophil, LY: lymphocytes, MONO: monocytes, EO: eosinophil monocytes, BASO: basophil monocytes, bars represent the percentage of total WBC number, upper number shows results in blood, the lower number displays PF data. Comparison of basic **(b)**, activated **(c)** and regulatory **(d)** lymphocyte subpopulations in PF and blood of all investigated patients. *: significant difference between PF and blood, $p < 0,05$; N=36; data displayed as mean ± SEM; Error bars represent SEM. CD: cluster of differentiation, HLA: human leukocyte antigen A, CD3: T-cell, CD4: helper T-cell, CD8: cytotoxic T-cell, CD19: conventional/B2 lymphocyte, CD5: B1 lymphocytes, NK: CD56 positive natural killer cell; B: CD3-25: activated T-lymphocytes, CD4-25: activated helper T-cell, CD3-HLA:activated T-lymphocyte, CD8-HLA: activated cytotoxic T-cell, CD45-RA: naive cell, CD45-RO: memory cell; C: CD4reg: regulatory T-cells, NKT: natural killer T-cell, iNKT: invariant natural killer T-lymphocyte, γ/δ T: gamma-delta T-cell

5.1.3. Activated and naive/memory lymphocyte subpopulations

$CD45RO^+$Tmemo cells represented the dominant lymphocyte group in PF and blood (70.20±2.71%and 51,92±2,29%) (Figs. 6c, 7). Blood contained at least twice as many $CD45RA^+$ Tnaive cells as PF (13.38±1.89% vs. 31.06±2.5%). If we compare the Tact subpopulations, $CD3^+HLADR^+$, and $CD8^+HLADR^+$ T_c cell ratio is higher in PF than in blood ($CD3^+HLADR^+$: 12.69±1.4% vs. 7.19±0.96% ,$CD8^+HLADR^+$: 8,42±0.63% vs. 4.08±0.49%), while the $CD4^+CD25^+$ activated $T_н$-cell ratio is lower in PF than in blood (23.58±1.93% vs. 32.74±2.00%) (Fig. 6c).

$CD4^+CD25^+$ activated $T_н$ ratios were significantly higher in groupA both in PF (25.50±1.93% vs. 19.73±4.26%) and in blood (36.42±2.54% vs. 25.39±1.93%) compared to groupB. (Fig.7b).

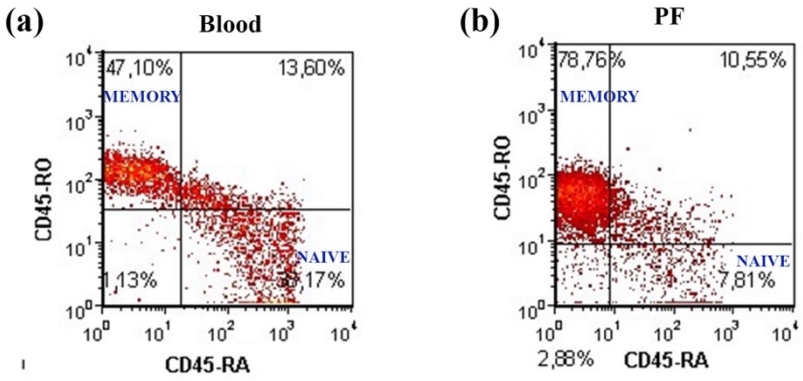

Figure 7. Flow cytometric dot plot example of naive and memory T cell distribution in blood (a) **and pericardial fluid** (b) Note the significantly higher number of CD45-RO+ memory T cell number in PF (b) in the upper left quadrant compared to blood (a) CD45-RA: naive cell, CD45-RO: memory cell, BD FACSCalibur™ Flow Cytometer (BD Biosciences, FlowCytomix Pro Software (Bender MedSystems GmbH Vienna, Austria)

5.1.4. Minor (regulatory) lymphocytes

In both groups PF contained at least 3.1 times more iNKT cells than blood. (groupA: 0.5±0.08% vs. 0.16±0.03%; groupB: 0.73±0.2% vs. 0.18±0.09%) (Figs.6d,8a,8b,9c). A significantly higher Treg ratio could be observed in both PF (1.99±0.29%) and blood (2.77±0.32%) of groupA compared to PF (1.51±0.50%) and blood (2.07±0.35%) of groupB (Figs. 9c, 9f). There were no significant differences in γ/δ T cell ratios at all.

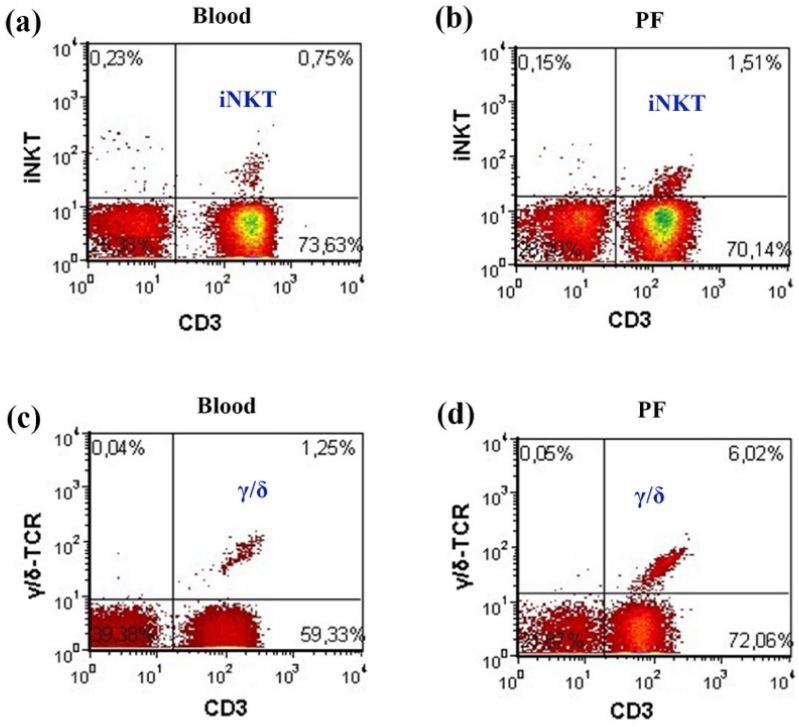

Figure 8. Flow cytometric dot plot examples of iNKT and γ/δ T cell distributions in blood and pericardial fluid in case one of the study participant (a) shows the significantly lower percentage of iNKT cells in blood in the upper right quadrant compared to the same lymphocyte population in PF **(b)** γ/δ T cells showed a tendency to reach higher levels in PF **(d)** then in blood **(c)** BD FACSCalibur™ Flow Cytometer (BD Biosciences, FlowCytomix Pro Software (Bender MedSystems GmbH Vienna, Austria)

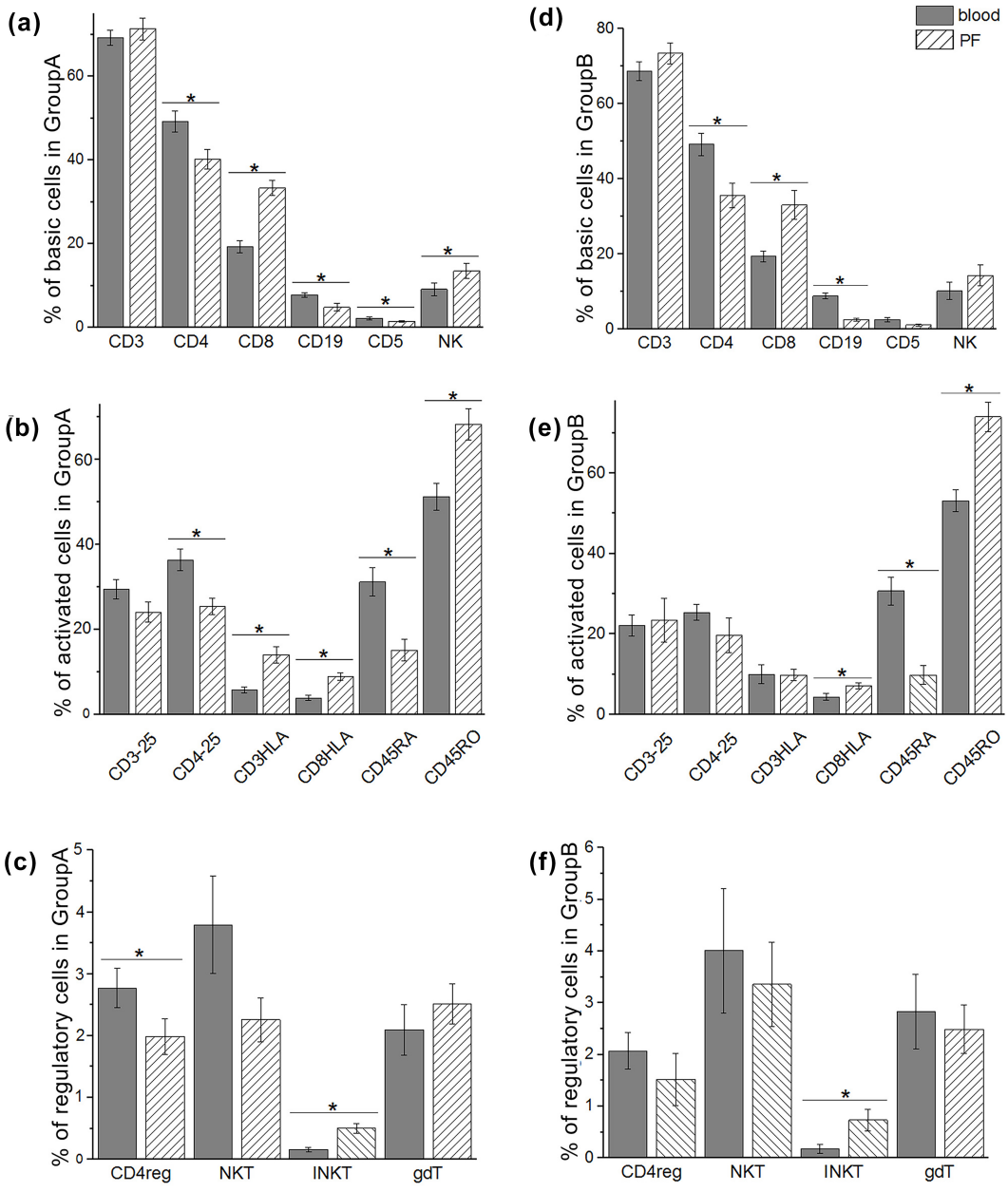

Figure 9. Investigated lymphocyte populations in PF and blood within groupA (CABG patients) (N=24) **and groupB** (aortic valve surgery patients) (N=12). Graph **(a)** shows the basic cell populations, graph **(b)** indicates the activated lymphocyte types, while graph **(c)** illustrates the regulatory cells within the CABG group. Graph **(d)** demonstrates the number of basic lymphocytes, graph **(e)** represents the amount of activated lymphocytes and graph **(f)** shows the regulatory cell number among aortic valve surgery patients. *: significant difference between PF and blood, $p < 0,05$; data displayed as mean ± SEM; Error bars represent SEM. CD:cluster of differentiation, HLA: human leukocyte antigen A, CD3: T-cell, CD4: helper T-cell, CD8:cytotoxic T-cell, CD19: conventional/B2 lymphocyte, CD5: B1 lymphocytes, NK: CD56 positive natural killer cell; B: CD3-25: activated T-lymphocytes, CD4-25: activated helper T-cell, CD3-HLA:activated T-lymphocyte, CD8-HLA: activated cytotoxic T-cell, CD45-RA: naive cell, CD45-RO: memory cell; C: CD4reg: regulatory T-cells, NKT: natural killer T-cell, iNKT: invariant natural killer T-lymphocyte, γ/δ T: gamma-delta T-cell

5.1.5. IgA, IgG, and IgM subclasses

We determined that IgA, IgG1-4, and IgM immunoglobulins were present in the PF. The levels of all the measured immunoglobulin classes were at least 3.5 times higher in the plasma than in the PF (Tbl. 3.), but the plasma/PF ratios were different. IgM, IgG2 and IgG3 were eight times higher in plasma than in PF, while the PF levels of IgA, IgG1 and IgG4 were 6.0-, 5.3- and 4.2-fold lower than in plasma. The average IgG subclass levels in PF was 6.2 times lower than in plasma (Tbl. 3.), which reflects a lower total IgM than total IgG class levels in PF.

Ig isotype	Plasma mean ± s.e.m.	PF mean ± s.e.m.	PF : plasma ratio	% plasma level
IgA(g/l)	0.41 ± 0.13	0.07 ± 0.02	1 : 6	16.6%
IgG1 (g/l)	1.62 ± 0.48	0.31 ± 0.10	1 : 5	18.9%
IgG2 (g/l)	4.89 ± 1.34	0.59 ± 0.18	1 : 8	12.5 %
IgG3 (g/l)	0.13 ± 0.05	0.02 ± 0.01	1 : 8	12.5 %
IgG4 (g/l)	0.15 ± 0.07	0.04 ± 0.02	1 : 4	23.8 %
IgM (g/l)	0.12 ± 0.07	0.014 ± 0.01	1 : 8	12.5 %

Table 3. Total immunoglobulin (Ig) subclass levels and their ratios in plasma and pericardial fluid (PF) of cardiac surgery patients s.e.m: standard error of mean

5.1.6. Inflammatory cytokines (MCP1, IL8, IL6)

IL6 and MCP1 had a significantly higher concentration in PF compared to plasma (IL6: 105.79±24.65ng/ml vs. 5.00±4.75ng/ml; MCP1: 3754.91±602.63ng/ml vs. 487.67±43.63ng/ml), unlike IL8 (13.91±9.89ng/ml vs. 23.05±11.22ng/ml) (Fig. 10a). The plasma level of MCP1 in groupA patients (575.01±51.71 ng/ml) was significantly elevated compared to groupB (313.01±53.21 ng/ml) (Figs. 10b, 10c).

5.1.7. Procoagulation markers (sCD40L, t-PA, sP-sel)

SCD40L, tPA, and sP-sel were present in the PF and reached significantly higher concentrations in plasma. The sCD40L concentration in plasma was on average 133.93 times higher than in PF (1744.185±478.95 ng/ml vs. 13.02±7.54 ng/ml). In both specimens sP-sel was the dominant marker (5368.60±12417.56 ng/ml in PF; 9286.47±2700.06 ng/ml in plasma). TPA was present in all of the samples (340.10±37.76 ng/ml vs. 12934.00±145.10 ng/ml) (Fig. 10b). When

correlating the cardiovascular factor concentrations within PF and plasma, only sP-sel showed positive correlation ($r=0.38$; $p<0.05$). No significant difference could be found between the patient groups (Figs. 10e, 10f)

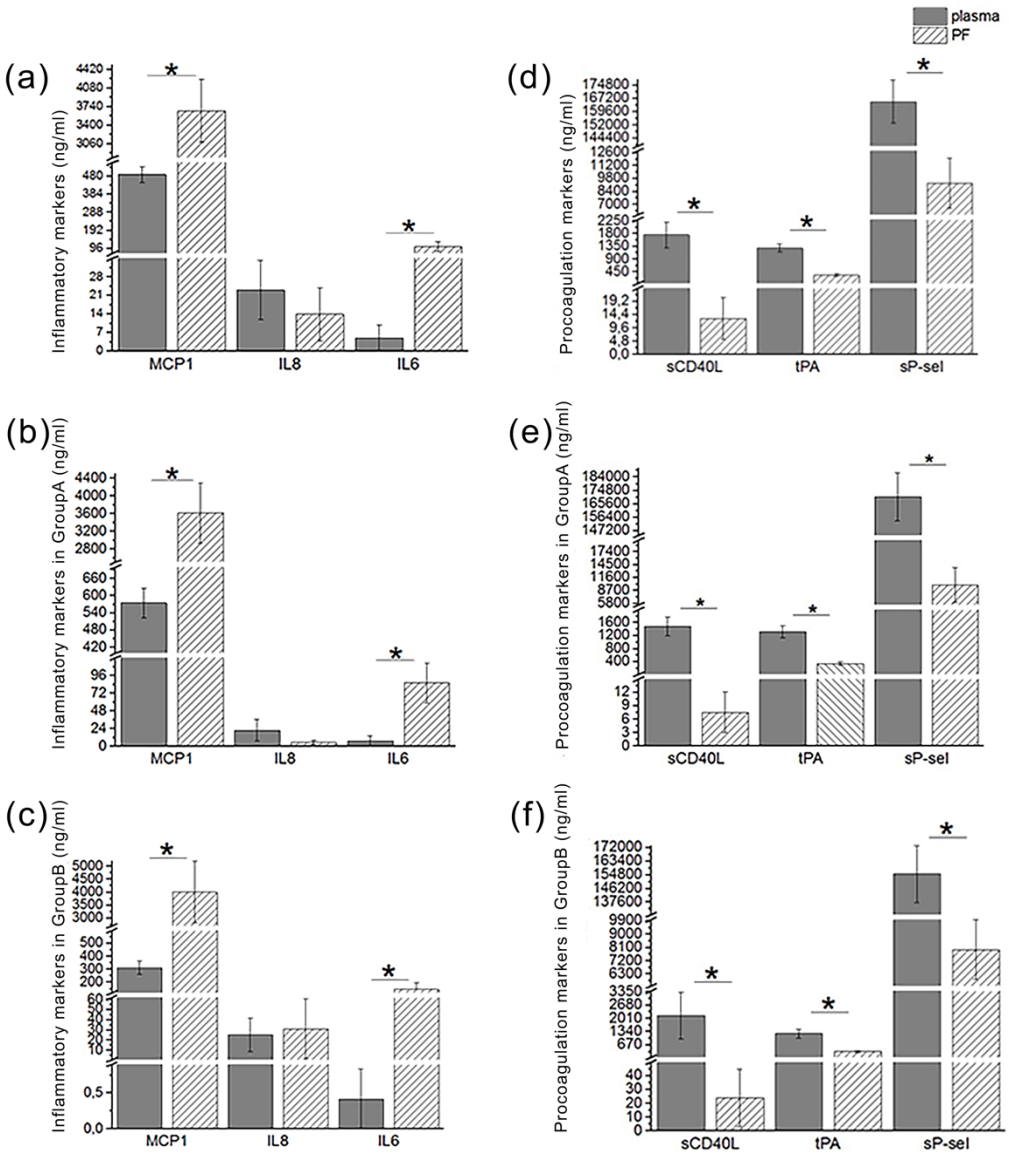

Figure 10. Inflammatory and procoagulation marker concentrations in the pericardial fluid and peripheral blood plasma Graph **(a)** shows the level of inflammatory markers within all participants (N=36), graph **(b)** indicates the procoagulation marker rates in the whole investigated population. Graph **(c)** represents the concentration of the investigated inflammatory markers in groupA (n=12). Graph **(d)** shows the level of the procoagulation markers in groupA. Graph **(e)** indicates the concentration of the investigated inflammatory markers in groupB, while graph **(f)** illustrates the procoagulation markers concentration among groupB. MCP1:

monocyte chemoattractant protein-1, IL8: I nterleukin-8, IL6: interleukin-6, sCD40L: soluble CD40-ligand, tPA: tissue plasminogen activator, sP-sel: soluble P-selectin, *: significant difference between PF and blood plasma, $p < 0,05$; data displayed as mean ± SEM; Error bars represent SEM

5.1.8. Possible interactions between the lymphocytes and biomarkers of PF and blood

When we correlated both lymphocyte subpopulations and cardiovascular biomarkers that revealed significant differences among the patient groups and were present in each sample in measurable amount (except for sCD40L), some notheworthy results were seen. (Fig. 11.) Circulating iNKT cells showed positive correlations with Tregs in blood ($r=0.401$; $p<0.05$), blood MCP1 levels ($r=0.349$; $p<0.05$), pericardial CD3-HLADR$^+$ activated T cells ($r=0.395$; $p<0.05$), Treg population in PF ($r=0.498$ $p<0.01$) and the amount of pericardial γ/δ T cells ($r=0.343$; $p<0.05$). Pericardial Treg lymphocytes correlated with circulating Treg cells ($r=0.618$; $p<0.01$), CD3-HLADR$^+$ activated T cells in blood ($r=-0.412$; $p<0.05$), MCP1 in blood ($r=0.489$; $p<0.01$), pericardial CD3-HLADR$^+$ lymphocytes ($r=-0.453$; $p<0.01$), activated T_H levels of PF ($r=0.404$; $p<0.05$), and with pericardial γ/δ T population ($r=0.382$; $p<0.01$). Treg cells of blood positively correlated with activated T_H population in blood ($r=0.362$; $p<0.05$), as well as with the pericardial CD3-HLADR$^+$ population ($r=0.36$; $p<0.05$). CD3-HLADR$^+$ lymphocytes in blood negatively correlated with pericardial Treg ($r=-0.412$; $p<0.05$) and with pericardial activated T_H levels ($r=-0.371$; $p<0.05$). Circulating MCP1 concetrations positively correlated with activated T_H cells of PF ($r=0.379$; $p<0.05$) and pericardial γ/δ T population ($r=0.333$; $p<0.05$). Pericardial sCD40L levels showed a positive correlation with CD3-HLADR$^+$ lymphocytes of PF ($r=0.395$; $p<0.05$) (Fig. 11.)

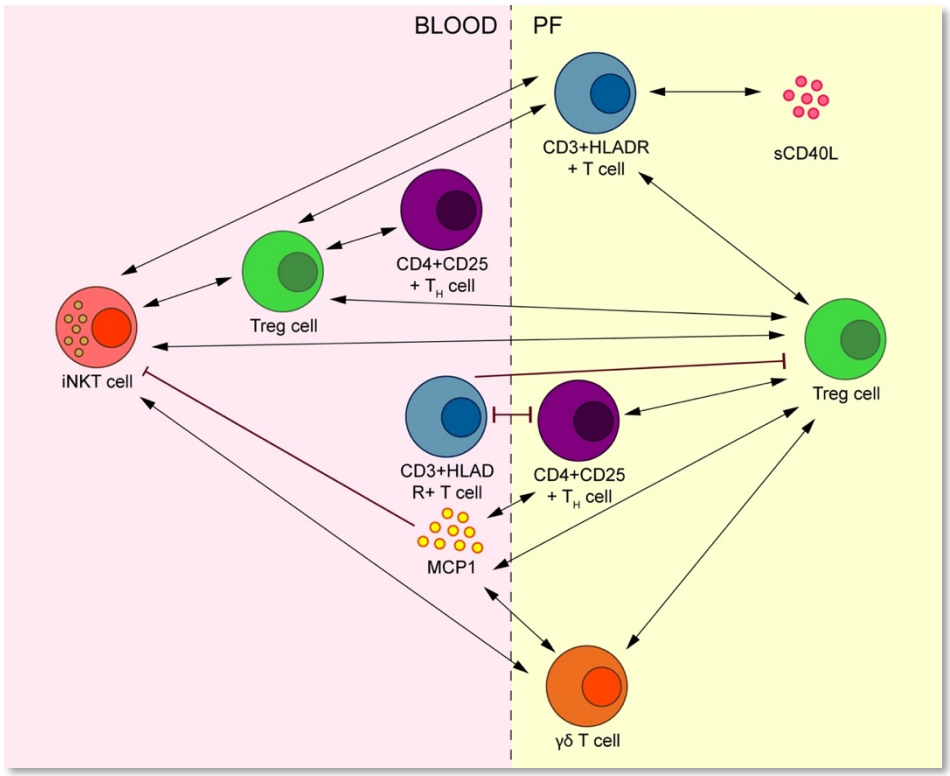

Figure 11. Possible interactions between the investigated biomarkers and lymphocyte subgroups based on statistically significant correlations among all investigated cardiac surgery patients Black arrow represents positive correlation, red whiskers indicate negative correlation Spearman correlation, N=36. Correlation is significant at the 0.05 level (2-tailed). PF:pericardial fluid, CD4-25 T_H cell: activated helper T-cell, CD3-HLADR$^+$ T cell: activated T-lymphocyte, Treg cell: regulatory T-cells, iNKT: invariant natural killer T-lymphocyte, γ/δ T: gamma-delta T-cell, MCP1: monocyte chemoattractant protein-1, sCD40L: soluble CD40-ligand. Author's original illustration.

5.2 Natural autoantibodies and cardiovascular disease-related anti-bacterial antibodies in PF

5.2.1. Relative excess of anti-CS IgM natural autoantibodies in the pericardial fluid

We detected anti-CS IgM and IgG antibodies both in the plasma and PF of patients undergoing cardiac surgery. The anti-CS IgG level was approximately 11-fold higher in plasma than in PF ($P < 0.001$) (Fig. 11b), whereas anti-CS IgM concentration was only approximately twofold lower in the PF than in the plasma ($P < 0.001$) (Fig. 11a). This reflects a relative excess of anti-CS IgM autoantibodies in the PF of cardiac surgery patients. Similar differences in the levels of anti-CS IgG and IgM autoantibodies were detected in the different disease groups (Tbl. 4.).

A statistically significant correlation of anti-CS IgM antibody concentrations ($P < 0.001$) (Fig. 12a.) and a strong correlation in the case of anti-CS IgG levels ($P < 0.001$) (Fig. 12b.) were found between the plasma and PF ($r^2 = 0.880$ and $r^2 = 0.997$, respectively).

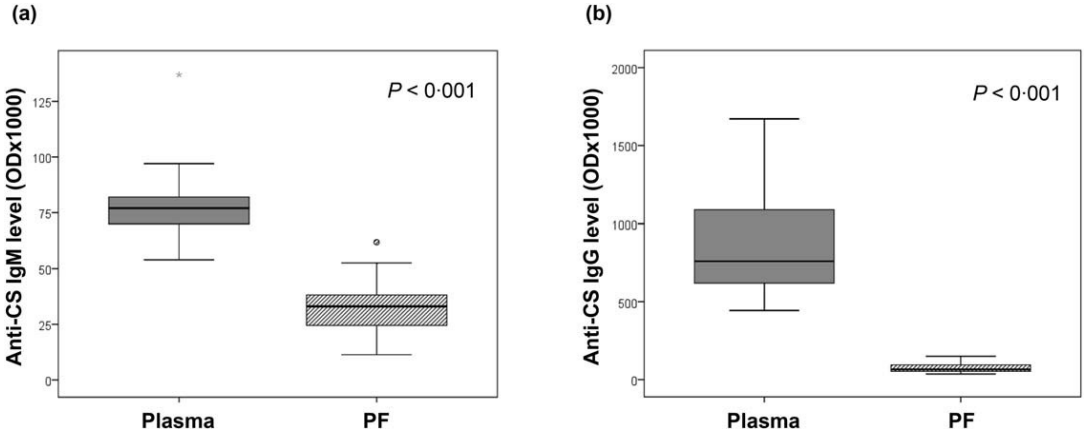

Figure 5. Anti-citrate synthase (anti-CS) natural autoantibody levels in the plasma and pericardial fluid (PF) of cardiac surgery patients The proportion of anti-CS immunoglobulin (Ig)M **(a)** antibody levels were only twofold higher ($P < 0.001$), while anti-CS IgG **(b)** antibody levels were 11-fold higher in the plasma than in the pericardial fluid ($P < 0.001$). Boxes show the interquartile ranges (IQR), whiskers indicate the lowest and highest values, horizontal lines represent medians, and dots indicate outliers of 1.5 X interquartile range (IQR). The antibody levels were measured using enzyme-linked immunosorbent assay (ELISA). OD: optical density; plasma, peripheral blood plasma; n=32 patients.

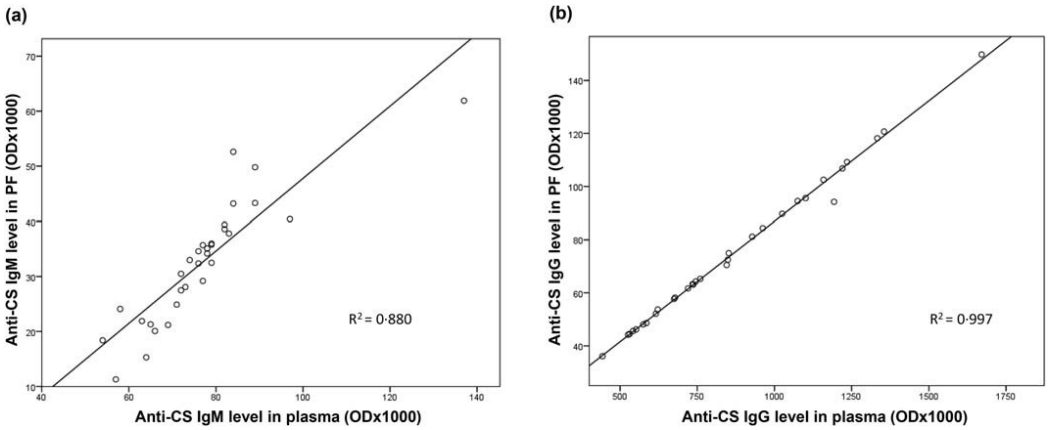

Figure 6. Correlation between the plasma and pericardial fluid (PF) levels of anti-citrate synthase (anti-CS) natural autoantibodies Significant correlations were detected in the levels of anti-CS immunoglobulin (Ig)M in the plasma versus PF ($P < 0.001$) **(a)**, as well as in the anti-CS IgG levels in plasma versus PF ($P < 0.001$) **(b)**

5.2.2. B1 cell frequencies are higher in pericardial fluid

In order to gain insight into the different Ig-producing cells in PF we investigated and compared the ratio of B cell subgroups in blood and PF. The percentage of $CD19^+$ B cells was significantly lower in PF than in blood ($P < 0.001$). Analyzing the B cell subsets, we found lower frequencies of both B1 and B2 B cells in PF compared to the blood ($P < 0.001$ and $P < 0.002$, respectively) (Fig. 13.), but the proportion of B1 cells in the $CD19^+$ B cell population was significantly higher in the PF than in the blood of cardiac surgery patients (n=32) (Tbl. 4.), which might be the source of natural anti-CS IgM autoantibody production in the PF.

Disease type	B1 cells in blood (% of $CD19^+$ B cells)	B1 cells in PF (% of $CD19^+$ B cells)	P-value
CABG no MI (n=12)	24 ± 8.6 %	36 ± 23.8 %	0.237
CABG with MI (n=10)	30 ± 26.1 %	48 ± 43.6 %	0.108
AVR (n=10)	27 ± 13.9 %	51 ± 34.5 %	0.078
Total (n=32)	27 ± 17.1 %	45 ± 34.1 ±	0.005

Table 4. Percentages of B1 cells in the $CD19^+$ B cell population in blood and PF of patients with different cardiac diseases Aortic valve replacement group (AVR, n=10), coronary artery bypass grafting patients (CABG)
with previous myocardial infarction (CABG MI, n=10), and those who underwent CABG but had no previous myocardial infarction (CABG no MI, n=12). PF = pericardial fluid

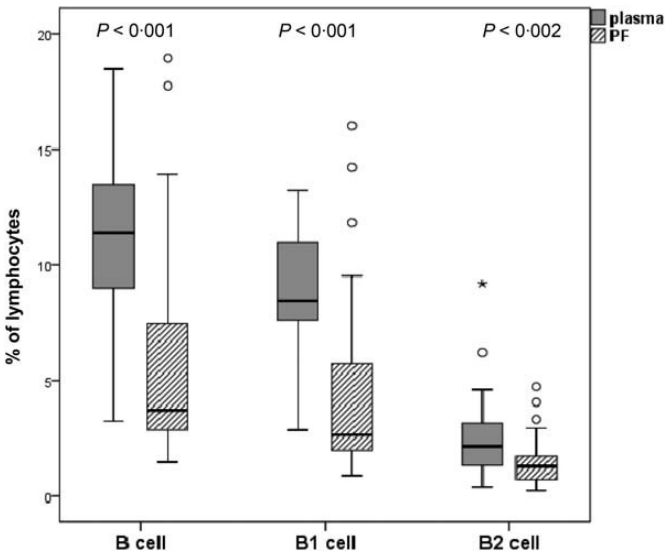

Figure 7. Frequencies of total CD19^{+} B cells and B1, B2 subpopulations in plasma and pericardial fluid (PF) The percentage of all investigated B cell groups were significantly lower in PF than in plasma samples (P < 0.001, P < 0.001 and P < 0.002, respectively). Boxes show the interquartile ranges (IQR), whiskers indicate the lowest and the highest values, horizontal lines represent medians, and dots indicate outliers of 1.5 X IQR. Frequencies of cells were measured with flow cytometer. plasma, peripheral blood plasma; n=32 patients

5.2.3. Anti-CS natural autoantibody levels correlate with anti-bacterial antibody titers in coronary artery bypass graft patients

Several infections are described as having a potential role in the development of atherosclerosis, therefore, we tested the plasma and PF samples for antibodies (IgG/IgA/IgM) against five different cardiovascular disease-associated bacteria, such as *M. pneumoniae, Y. enterocolitica, C. pneumoniae, H. pylori* and *B. burgdorferi*. All patients were positive for at least one anti-bacterial antibody (IgG and/or IgA) in the plasma, while in the PF we found fewer positive samples, even though their corresponding plasma sample was positive. The highest proportion of positivity for anti-bacterial antibodies in the plasma was against *M. pneumoniae* (74.2%) and *H. pylori* (74.2%), whereas in the PF it was against *H. pylori* (37.0%), *M. pneumoniae* (14.8%) and *Chlamydia pneumoniae* (14.8%). No IgM positivity could be detected either in plasma or PF, which indicates the absence of an early infection.

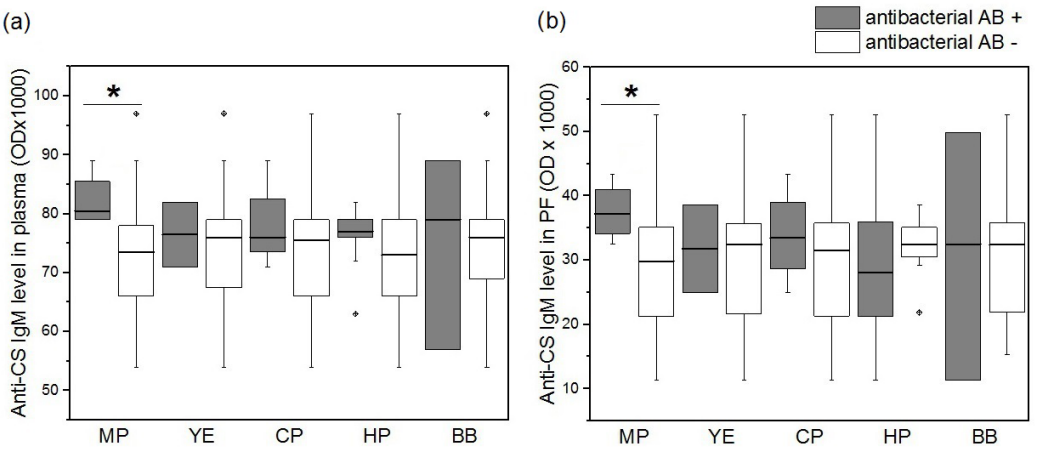

Figure 8. Anti-citrate synthase (anti-CS) immunoglobulin (Ig)M levels in plasma and pericardial fluid (PF) of patients negative or positive for various anti-bacterial antibodies Patients who were positive for *Mycoplasma pneumoniae* antibodies in the PF (and plasma) had significantly higher levels of anti-citrate synthase IgM in their plasma **(a)** and an elevated tendency in the PF **(b)** when compared to M. pneumoniae antibody-negative patients (P=0.031 and P=0.076, respectively). Boxes show the interquartile ranges (IQR), whiskers indicate the lowest and highest values, horizontal lines represent medians, dots indicate outliers of 1.5 X IQR, while the asterisks represent the significance (n=32). MP: *M. pneumoniae*; YE: *Yersinia enterocolitica*; CP:*Chlamydia pneumoniae*; HP: *Helicobacter pylori*; BB: *Borrelia burgdorferi*; plasma: peripheral blood plasma; AB: antibody

We tested whether there was a relationship between anti-CS IgM or IgG autoantibody levels and the presence of anti-bacterial antibody positivity for any of the five different bacterial strains, and we found significantly elevated anti-CS IgM levels in the plasma (P = 0.031) and an increased tendency in the PF (P = 0.076) of patients with positive M. pneumoniae antibodies (Fig. 14.).

We also tested the correlation of anti-CS IgG and IgM antibody levels with anti-bacterial antibody titers both in the plasma and PF of patients belonging to different disease groups. In CABG patients without previous MI there was a significant correlation between anti-CS IgG levels and MP antibody titers, while in CABG patients with previous MI *C. pneumoniae* and *B. burgdorferi* antibodies correlated significantly with anti-CS IgG levels. In patients with AVR, anti-CS IgG did not show a correlation with any of these anti-bacterial antibody levels. No clear-cut correlations were observed between anti-CS IgM and the anti-bacterial antibody titers investigated (Tbl. 5.).

Disease group →	CABG no MI	CABG with MI	AVR
Natural autoantibody ↓	Correlating antimicrobial antibody ↓		
Anti-CS IgG plasma	*MP* IgG (plasma) (rho=0.789; P=0.004) *MP* IgM (plasma) (rho=0.802; P=0.003) MP IgG (PF) (rho=0.579; P=0.062)	*CP* IgG (plasma) (rho=0.753; P=0.012) *CP* IgG (PF) (rho=0.769; P=0.026) *BB* IgG (plasma) (rho=0.632; P=0.049)	No correlation
Anti-CS IgG PF	*MP* IgG (plasma) (rho=0.797; P=0.003) *MP* IgM (plasma) (rho=0.829; P=0.002) *MP* IgG (PF) (rho=0.611; P=0.046)	*CP* IgG (plasma) (rho=0.754; P=0.012) *CP* IgG (PF) (rho=0.762; P=0.028) *BB* IgG (plasma) (rho=0.633; P=0.049)	No correlation
Anti-CS IgM plasma	No correlation	*YE* IgM (plasma)(rho=0.710; P=0.021)	*MP* IgG (plasma) (rho=0780;p=0.013)
Anti-CS IgM PF	No correlation	No correlation	No correlation

Table 6. Correlation of citrate synthase (anti-CS) IgG and immunoglobulin (Ig)M antibody levels with anti-bacterial antibody titers in plasma and PF of patients with cardiovascular diseases Aortic valve replacement group (AVR, n=10), coronary artery bypass grafting patients (CABG) with previous myocardial infarction (CABG with MI, n=10), and those who underwent CABG but had no previous myocardial infarction (CABG no MI, n=12). *MP: Mycoplasma pneumoniae; CP:Chlamydia pneumoniae; BB:Borrelia burgdorferi; YE:Yersinia enterocolitica*

Natural autoantibody	Disease group	PF : plasma ratio	% Plasma level
Anti-CS IgG	AVR	1 : 11.5 80.06 ± 8.45 : 921.60 ± 92.75	8.7 %
	CABG with MI	1 : 11.5 67.95 ± 7.54 : 787.60 ± 81.8	8.7 %
	CABG no MI	1 : 11.6 76.14 ± 9.73 : 886.64 ± 107.58	8.6 %
Anti-CS IgM	AVR	1 : 2.3 34.14 ± 2.60 : 78.90 ± 3.31	43.4 %
	CABG with MI	1 : 2.3 34.04 ± 4.48 : 78.10 ± 7.25	43.4 %
	CABG no MI	1 : 2.5 29.85 ± 3.21 : 74.00 ± 2.56	40.0 %

Table 5. Ratios of anti-citrate synthase (anti-CS) immunoglobulin (Ig)G and IgM autoantibodies in pericardial fluid and plasma of patients with different cardiac diseases AVR=aortic valve replacement; MI=myocardial infarction; CABG= coronary artery bypass graft

6 Discussion

6.1 Lymphocytes, cardiovascular markers, and cytokines in pericardial fluid

The present study describes a comprehensive immunological analysis of PF in humans for the first time. We showed that the investigated lymphocyte populations and cardiovascular markers in human PF have significantly different distributions compared to blood. The main finding of our study is that lymphocyte populations show different distributions in coronary disease and valvular aortic stenosis. Analysis of function immunomarkers and immunomodulation further extends this observation. The PF contained significantly higher amounts of activated lymphocyte subpopulations ($CD3^+HLA^+$ cells, $CD8^+$ HLA^+ lymphocytes and Tmemo cells, respectively) than blood.

The significantly elevated number of $CD25^+$ activated $CD4^+T_H$ cells in the PF of CABG patients compared to AVR patients is in line with the earlier observation in mice experiments that $CD4^+$ T-lymphocytes may be activated by cardiac ischemia, presumably driven by recognition of cardiac autoantigens, and facilitate wound healing of the myocardium (Hofmann *et al.*, 2012).

Earlier investigations proved the ability of Treg cells to suppress proatherogenic effector T cells in atherosclerosis (Foks, Lichtman and Kuiper, 2015). Studies reported reduced circulating Treg cell numbers in patients with acute coronary syndrome compared to patients with stable angina (SA) (Han *et al.*, 2007; Zhang *et al.*, 2012). Our results also suggest that chronic myocardial ischemia with SA (our groupA) may lead to elevated Treg cell level, not only in the blood but also in the PF because the Treg population in CAD patients compared to AVR group was significantly higher in both specimens. Clinical experiments that investigated circulating Treg cells in patients with CAD showed conflicting results (To *et al.*, 2009). Pericardial Treg population seems to

orchestrate the experienced changes in heart surgery patients based on our investigations.

INKT subpopulation is activated by both self and exogenous lipid antigens and are considered to be proatherogenic (Getz and Reardon, 2017). INKT subset showed no significant difference when comparing the patient groups, but it reached significantly higher concentration in PF compared to blood in each case. This data supports that the pericardial space has its own, independent iNKT pool. Moreover, based on statistically significant correlations, circulating iNKT cells might influence the level of other lymphocyte subgroups in both blood and PF.

Tmemo lymphocytes conventionally are a feature of an immune response against foreign antigens. However, there is evidence in mice that iNKT cells can induce mature naive $CD8^+$ T cells to acquire innate characteristics in peripheral lymphoid tissue via IL4 (Jameson, Lee and Hogquist, 2015). This latter crosstalk could be a possible explanation for the high ratios of T_C and iNKT subpopulations.

Studies showed that B1 cells are atheroprotective mainly via the production of natural IgM antibodies that bind oxidized low-density lipoprotein and apoptotic cells. B2 cells are suggested to be proatherogenic (Tsiantoulas *et al.*, 2015). After AMI in mice, mature systemic B lymphocytes induce monocyte mobilization and recruitment to the heart, leading to enhanced tissue injury and deterioration of myocardial function (Zouggari *et al.*, 2013). We found an amount of B2 lymphocytes in PF of CABG patients 1.88 times higher than in the aortic valve group. The B2 cell number in systemic blood flow showed no such difference, suggesting that the B2 lymphocyte population of the PF might have a role in CAD related myocardial ischemia.

The distribution of PF immunoglobulin levels were similar to that of blood; however, the PF contained significantly less IgA, IgG1-4, and IgM compared to plasma. Here we uncover for the first time the Ig composition of the PF of cardiac surgery patients. It was earlier described that after ischemia and reperfusion of the heart, complement is activated by self-reactive natural IgM antibodies that bind to postischemic tissue (Atkinson *et al.*, 2015) .

The significantly different distribution of markers of immune activation in PF and plasma implies the presence of local factors that influence the concentration of cytokines in the PF. All of the investigated cytokines have a molecular weight under 40kDa, making diffusion through membranes possible, except for tPa (68 kDa). The half-life of MCP1, tPA, sCD40L, and IL8 are shorter (maximum 20 minutes) in the bloodstream, whereas sP-sel (100 minutes) and IL6 (maximum 6 hours) last longer. This could explain why sP-sel is the only factor with correlating concentrations between plasma and PF. Our data confirmed that PF reflects the composition of cardiac interstitium (Mebazaa *et al.*, 1998; Limana *et al.*, 2010).

The strong positive correlation between pericardial MCP1 and IL6 levels and the fact that, exclusively, these biomarkers had higher concentrations in PF when compared to blood demonstrates the local significance of these cytokines. We found significantly elevated plasma levels of MCP1 in CAD patients compared to the aortic valve group, which is in contradiction to the earlier observation of Shibasaki et al. who found no difference in plasma MCP1 mRNA levels when comparing open-heart surgery patients with and without CAD (Shibasaki *et al.*, 2010). Our data of elevated MCP1 in CABG patients congruent with the results of Yu et al. who described that shear stress (endothelial activation, first step to atherosclerosis) increases the secretion of MCP1 in human umbilical vein endothelial cells (Yu *et al.*, 2002). Although in another study shear stress increased the release of IL8 from endothelial cells (Urschel *et al.*, 2012), we did not detect any difference of IL8 level in the patient groups, nor any difference in blood and PF. Our results also revealed that circulating MCP1 also seems to act as an important link between PF and plasma lymphocytes.

In accordance with previous results (Menet *et al.*, 2002), pericardial IL6 levels were at least five times higher than plasma levels, with no correlation between them.

Although plasma sCD40L (platelet activation marker) was considered to have a positive predictive value for ischemic events in patients with ACS in systemic and coronary artery blood, our investigation showed no significant

difference in sCD40L levels between CAD and CAS group (Gremmel, Frelinger and Michelson, 2015). This is in line with Fong et al. who also found no association between sCD40L levels and long-term ischemic outcomes in unselected aspirin-treated patients undergoing cardiac catheterization (Fong *et al.*, 2015).

Circulating sP-sel as a marker for platelet hyperactivity, inflammation, and endothelial dysfunction were previously found to be significantly elevated in patients with diastolic dysfunction, and play a role in the initiation of atherosclerosis and AMI (Bielinski *et al.*, 2015; Guo *et al.*, 2015). Our present research showed the presence of sP-sel in PF but had no correlation with CAD or CAS, both in PF and serum.

Even though plasma inflammatory biomarkers may not adequately reflect local tissue levels, earlier studies measured the concentrations of peripheral venous or coronary blood (Menet *et al.*, 2002; Limana *et al.*, 2007, 2010) and suggest that compounds with key roles in plaque-destabilization and MI are also released from non-coronary sources; therefore, further PF analysis is needed.

6.2 Natural autoantibodies and anti-bacterial antibodies in pericardial fluid

The goal of this study was to determine whether natural autoantibodies to mitochondrial CS enzyme and antibodies to certain bacteria related to atherosclerosis are present in the plasma and in the PF of cardiac surgery patients, and to determine the relationship of these antibodies to each other and to the total immunoglobulin class levels in plasma and PF. Our study has revealed that anti-CS natural autoantibodies and anti-bacterial antibodies are present not only in the plasma, but also in the PF. We also wanted to examine the frequencies of B1 and B2 B lymphocytes in PF and compare them to the percentages found in blood to gain insight into the source of natural autoantibodies in PF.

First, we determined the total immunoglobulin (IgA, IgM and IgG1–4) subclass concentrations in PF and plasma of cardiac surgery patients and found that in PF their level is four- to eight-fold lower than that in plasma. In contrast, when we compared the anti-CS natural antibody levels, the anti-CS IgM was only twofold lower, whereas the anti-CS IgG levels were tenfold lower in PF than in plasma in all study groups. The increased level of anti-CS IgM may be due to the presence of increased frequencies of B1 cells in the PF. Although the total $CD19^+$ B cell ratio was significantly lower in PF compared to blood, the proportion of B1 cells in the $CD19^+$ B cell population was significantly higher in the PF than in the blood of cardiac surgery patients, which suggests that B1 cells are the probable source of IgM natural autoantibodies (Riemann *et al*., 1994; Cornfield and Gheith, 2009). The tissue specific signals in different tissue compartments may affect the functionally heterogeneous B1 cells differentially (Baumgarth, 2016), but their shared function might be to ensure a prompt and effective local defense.

Natural antibodies may exert distinct effector functions according to their specificity and respective isotypes (Epelman, Liu and Mann, 2015). Our data suggests that the pericardial space containing the PF is protected by the natural immune system, which may play a crucial role in tissue homeostasis by the elimination of cellular and molecular debris that needs to be cleared in order to

prevent the accumulation of apoptotic material (Ehrenstein and Notley, 2010) to control inflammation and autoimmune mechanisms (Grönwall, Vas and Gregg J Silverman, 2012; Nagele *et al.*, 2013; Grönwall and Gregg J Silverman, 2014). Another key and overarching function of natural antibodies is to serve as a bridge between the innate and adaptive immune systems (Panda and Ding, 2015) by pathogen neutralization, complement activation and antigen recruitment to secondary lymphoid organs, and for immune regulation and homeostasis (Panda and Ding, 2015; Lobo, 2016). Our data may support the notion that anti-CS natural autoantibodies, similarly to other polyreactive natural antibodies, may serve as scavengers of dead or dying cells and microbial antigens both in plasma and in the PF (Nimmerjahn and Ravetch, 2008; Nagata, Hanayama and Kawane, 2010). It is probable that CS is not the only molecular constituent cleared by such mechanisms, but there could be a plethora of other self- and pathogen- related molecules recognized by the natural autoantibodies destined for subsequent clearance to prevent their 'toxicity' to cardiomyocytes (Nagele *et al.*, 2013; McLendon and Robbins, 2015). In mouse models of atherosclerosis, natural IgM antibodies produced by serosal B1 cells have been shown to protect from atherosclerosis, whereas antibodies produced by B2 cells promote atherosclerosis (Kyaw *et al.*, 2012). Clinical data obtained from patients with systemic lupus erythematosus also support the notion that natural IgM autoantibodies may inhibit carotid atherogenesis (Grönwall *et al.*, 2014).

The strong correlation of anti-CS IgG in plasma versus PF may suggest that these antibodies may not be produced exclusively in the PF, but they could be transported from the plasma or lymphatic fluid into the pericardial space. A receptor-mediated transcytosis of immunoglobulins (Rojas and Apodaca, 2002) may underlie the transport of anti-CS and other IgG antibodies into the PF. In addition to transcytosis, some antibodies may be synthesized locally in the pericardial space. Our data showing the relative increase of anti-CS IgM antibody in the PF is consistent with the notion that B1 cells produce anti-CS IgM (but not anti-CS IgG) locally in the pericardial space.

Using immunoserological methods we measured *M. pneumoniae-, Y. enterocolitica-, C. pneumoniae-, H. pylori-* and *B. burgdorferi*-specific antibodies in PF and plasma because data has been published on the possible involvement of these microbes in the development of cardiovascular diseases (Rosenfeld and Campbell, 2011). Of note, all patients were positive for antibodies to at least one bacterial species; however, some patients were negative for some of these antibodies in the PF. This may suggest that the levels of anti-bacterial antibodies in the plasma may be generally higher than in the PF (the PF is more sequestered from the immune system than blood), resulting in antibody levels below the limit of detection in the PF. Interestingly, *M. pneumoniae* has been found in atherosclerotic plaques and in normal arteries and veins of patients (Rosenfeld and Campbell, 2011), and this bacterium was also shown to be present in the PF of atherosclerotic patients (Prasad *et al.*, 2002).

H. pylori has also been found in human atherosclerotic plaques, and a significant association of antibodies to this pathogen has been found in patients with coronary arteriosclerosis when compared to control subjects (Prasad *et al.*, 2002). *C. pneumoniae* has been detected in atherosclerotic plaques by quantitative polymerase chain reaction (qPCR) (Koren *et al.*, 2011), and viable *C. pneumoniae* organisms were isolated from plaques (Rosenfeld and Campbell, 2011). Our current data on the high frequency of anti-microbial antibodies in the plasma and PF of patients undergoing heart surgery (atherosclerosis-related coronary heart disease) is consistent with the potential pathogenic role of the above-mentioned bacteria in these diseases.

Our data suggests an interrelationship between natural and anti-bacterial antibodies. We found that all patients whose plasma and/or PF were positive for antibodies to *M. pneumoniae* had significantly higher levels of anti-CS IgM, both in their plasma and PF. Investigating the different disease groups, in CABG patients without previous MI we found a significant correlation between anti-CS IgG levels and *M. pneumoniae* antibody titers, while in CABG patients with previous MI, *C. pneumoniae* and *B. burgdorferi* antibody concentrations showed correlation with anti-CS IgG levels. No such correlation with any of these anti-

bacterial antibody levels was observed in patients with AVR. Of note *M. pneumoniae*, as an intracellular microbe and an obligate parasite, requires the essential metabolites from the host cells. This bacterium can cause cell death and inflammation in the respiratory system (Waites and Talkington, 2004; Krishnan, Kannan and Baseman, 2013), resulting in the release of CS molecules from damaged cells, which then could lead to increased production of anti-CS antibodies by the immune system. Interestingly, during its evolution *M. pneumoniae* lost many of its genes, including citrate synthase (Himmelreich *et al.*, 1996); therefore, it is not the bacterial CS that causes the elevation of the natural anti-CS IgM and IgG autoantibody levels. *M. pneumoniae* infection can have extrapulmonary manifestations, including infection of the pericardium (Kyaw *et al.*, 2012), which may result in increased levels of anti-CS antibodies in the plasma and PF. Our results provide evidence that the PF of cardiac surgery patients contains functionally heterogeneous anti-CS IgM and IgG natural autoantibodies. The correlation of elevated anti-CS IgM levels with the *M. pneumoniae* infection was independent from the disease groups. This may reflect its general protective role as a natural autoantibody, independent of the presence or absence of atherosclerosis. In contrast, anti-CS IgG level showed a strong correlation with higher levels of atherosclerosis-related anti-bacterial antibodies only in patients with coronary artery disease, which reflects its potential protective role in these infections. This data suggests that the PF is being 'surveyed' by the humoral components of the natural and adaptive immune systems in strong cooperation to protect the pericardial space from accumulation of toxic molecules and various pathogenic microbes. Further studies will be needed to investigate the network of natural and anti- bacterial autoantibodies and their role in the pathogenesis of cardiovascular diseases.

7 Conclusions

Our experiments confirmed that the PF is an immunologically active space which can play a crucial role in the course of MI or any disease involving the myocardium. We showed that the investigated lymphocyte populations and cardiovascular markers in human PF have significantly different distribution compared to blood. This study proved that lymphocyte populations show different distribution in coronary disease and valvular aortic stenosis. Analysis of function immunomarkers and immunomodulation further extends this observation. The PF contained significantly higher amounts of activated lymphocyte subpopulations ($CD3^+HLA^+$ cells, $CD8^+$ HLA^+ lymphocytes and Tmemo cells, respectively) than blood.

Our data revealed that the PF of heart surgery patients contains anti-CS IgM and IgG natural autoantibodies and atherosclerosis-related anti-bacterial antibodies. Interestingly, anti-CS IgG level showed a strong correlation with higher levels of atherosclerosis-related anti-bacterial antibodies in patients with coronary artery disease, which reflects its potential protective role in these infections..Further studies will be needed to investigate the network of natural and adaptive immune systems and their role in the pathogenesis of cardiovascular diseases.

For these above-mentioned reasons, immunomodulatory therapies can have the potential to limiting the infarct volume and accelerate repair after MI, as well as improve the healing process in other heart diseases or even following cardiac surgery. The pericardial space seems to be a promising medium for such approaches with less systemic effects. Therefore, the extensive collection of PF during open-heart surgery, PF investigation, and analysis for possible diagnostic and therapeutic usage would be necessary. We hope our study widens the outlook on the possible clinical usefulness of PF.

8 Novelties

- The present study describes a comprehensive immunological analysis of PF in humans for the first time.
- The investigated lymphocyte populations and cardiovascular markers in human PF have significantly different distribution compared to blood.
- Lymphocyte populations show different distribution in coronary disease and valvular aortic stenosis.
- We found significantly elevated plasma levels of MCP1 in CAD patients compared to the aortic valve group.
- We determined for the first time that IgA, IgG1-4, and IgM immunoglobulins were present in the PF.
- Anti-CS natural autoantibodies and anti-bacterial antibodies are in the PF.
- The correlation of elevated anti-CS IgM levels with the *M. pneumoniae* infection was independent from the disease groups.
- Anti-CS IgG level showed a strong correlation with higher levels of atherosclerosis-related anti-bacterial antibodies only in patients with coronary artery disease.

9 References

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10 Publications and presentations related to this dissertation

10.1 Publications related to this dissertation

Gilicze, O., Simon, D., Farkas, N., Lantos, M., Jancso, G., Berki, T., & Lenard, L. (2019). Characterization of lymphocyte subpopulations and cardiovascular markers in pericardial fluid of cardiac surgery patients. *Clinical Hemorheology and Microcirculation*, 1–12. https://doi.org/10.3233/ch-190594 IF: 1.642

Simon D, **Gilicze O**, Farkas N, Najbauer J, Németh P, Lénárd L, Berki T (2018) 'Correlation of natural autoantibodies and cardiovascular disease-related anti-bacterial antibodies in pericardial fluid of cardiac surgery patients', *Clinical & Experimental Immunology*. John Wiley & Sons, Ltd (10.1111), 193(1), pp. 55–63. doi: 10.1111/cei.13127. IF: 2.09

10.2 Abstracts in peer-reviewed journals

Gilicze O., Lenard L., Kvell V., Farkas N., Szabados S., Czirjak L. S (2012) 'Autoantibody and cardiovascular marker quantification in pericardial fluid and blood samples - Plenary Lectures and Subspecialty Poster Sessions', *European Journal of Clinical Investigation*. John Wiley & Sons, Ltd (10.1111), 42, pp. 1–11. doi: 10.1111/j.1365-2362.2012.02649.x.

Gilicze O, Simon D, Berki T, Czirják L, Szabados S, Lénárd L (2012) 'PP-093 Identification of lymphocyte populations and cardiovascular factors in pericardial fluid and blood samples among patients undergoing open heart surgery', *International Journal of Cardiology*. Elsevier, 155, pp. S129–S130. doi: 10.1016/S0167-5273(12)70313-3.

Gilicze O, Lénárd L, Simon D, Berki T, Czirják L, Szabados S: Kardiovaszkuláris markerek eltérő koncentrációja infarktuson átesett betegek perikardiális folyadékában. *Cardiologia Hungarica* 2012, 42: K14

Gilicze O, Simon D, Donauer E, Hejjel L, Pintér Ö, Szabados S, Berki T, Hegyi A, Lénárd L: Limfocita csoportok és kardiovaszkuláris markerek vizsgálata perikardiális folyadék és vérmintákban szívműtétre kerülő betegcsoportokban. *Cardiologia. Hungarica* 2011, 41: N5

Other abstartcs in peer-reviewed journals

Lénárd L, **Gilicze O**, Hejjel L, Pintér Ö, Donauer E, Berki T, Czirják L, Szabados S: Myxoma miatt operált betegeink klinikai elemzése. Cardiol. Hung. 2013; 43: H12

10.3 Presentations related to the dissertation

Hungarian

Gilicze O, Simon D, Donauer E, Hejjel L, Pintér Ö, Szabados S, Berki T, Hegyi A, Lénárd L Kardiovaszkuláris markerek eltérő koncentrációja infarktuson átesett betegek perikardiális folyadékában *-18th Scientific Conference of the Hungarian Association of Cardiac Surgery,* 11. 2011. (Budapest) - oral

Gilicze O., Lenard L., Kvell V., Farkas N., Szabados S., Czirjak L. S*:* Autoantibody and cardiovascular marker quantification in pericardial fluid and blood samples- *42nd Membrantransport Conference*, 05. 2012. (Sümeg) - poster

Gilicze O, Lénárd L, Simon D, Berki T, Czirják L, Szabados S: Limfocita csoportok és kardiovaszkuláris markerek vizsgálata perikardiális folyadék és vérmintákban szívműtétre kerülő betegcsoportokban - *19th Scientific Conference of the Hungarian Association of Cardiac Surgery*, 11. 2012. (Héviz) – oral

Gilicze O, Lénárd L, Simon D, Berki T, Czirják L, Szabados S: Alternating level of cardiovascular factors in pericardial fluid of myocardial infarction patients – *Magyar Immunológiai Társaság 41. Vándorgyűlése*, 2012. (Debrecen) - poster

International

Gilicze O., Lenard L., Kvell V., Farkas N., Szabados S., Czirjak L., Simon D., Berki T.: Autoantibody and cardiovascular marker quantification in pericardial fluid and blood samples, *46th Annual Scientific Meeting of the European Society for Clinical Investigation* 03. 2012. (Budapest) - poster

Gilicze O., Lenard L., Kvell V., Farkas N., Szabados S., Czirjak L. Simon D., Berki T.: Detection of autoantibodies and cardiovascular markers in pericardial fluid and blood samples- *The 8th International Congress on Autoimmunity,* 05. 2012. (Granada) - poster

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12 Appendix (Publications related to this Ph.D. dissertation)

Clinical Hemorheology and Microcirculation pre-Press (2019) 1-12
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Characterization of lymphocyte subpopulations and cardiovascular markers in pericardial fluid of cardiac surgery patients

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Abstract.
BACKGROUND: Composition of pericardial fluid (PF) may reveal immunological processes influencing oxidative stress and microcirculation of different tissues of the heart and may play a role in the course of myocardial infarction, atherosclerosis, and aortic stenosis.
PATIENTS AND METHODS: We investigated lymphocyte populations, cardiovascular markers and immunoglobulin composition in PF and blood samples of patients undergoing CABG operation and compared them to those who had aortic valve surgery.
RESULTS: The amount of CD8 + T, NK, memoT and activated T-cytotoxic cells were elevated in PF compared to blood, but naiveT and activated T-helper cell ratio were lower in PF. Amount of activated T-helper cells and regulatory T-lymphocytes were elevated in CABG participants in both PF and blood. INKT cells represented the only regulatory lymphocyte population reaching significantly higher concentration in PF than in blood. IL-6 and MCP1 level were elevated in PF compared to blood and MCP1 plasma level was markedly elevated in CABG group.
CONCLUSIONS: Our study describes a comprehensive immunological analysis of PF in humans for the first time. We showed that the investigated lymphocyte populations and cardiovascular markers in PF have significantly different distribution compared to blood, and lymphocyte populations show different compartmentization in coronary disease and aortic stenosis.

Keywords: Lymphocyte populations, cardiovascular markers, immunoglobulin, pericardial fluid, ischemic heart disease, calcific aortic stenosis

1. Introduction

Atherosclerotic coronary artery disease (CAD) is one of the leading causes of death [1, 2] and nonrheumatic, calcificating aortic valve stenosis (CAS) is the most common cause of aortic valve operations in developed countries [3]. Preceding aortic valve replacement (AVR) coronary artery bypass graft surgery (CABG) is the first most common open heart operation in adults.

During open heart surgery 3–20 ml of pericardial fluid (PF) is removed from the pericardial space. However, despite its possible diagnostic and clinical relevance due to its anatomical isolation and

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proximity to the heart, PF is not routinely investigated [4]. PF is mainly an ultrafiltrate of plasma that includes some overflow of myocardial interstitial fluid and myocardial lymph drainage [5].

Cellular component of PF contains remarkable predominance of lymphocytes, especially memory T cells, yet information is limited regarding of its physiological composition and possible role in cardiac diseases. [6–10]. Lymphocytes including helper T (T_H) cells, regulatory T (Treg) lymphocytes, natural killer T (NKT) cells, gammadelta T (gdT) cells and invariant natural killer T (iNKT) lymphocytes are considered to have a key role in the development of atherosclerosis and acute myocardial infarction (AMI) [11–17]. Valvular calcification in CAS is actively regulated, and CD4 + and CD8 + T cells infiltrate the valve [18–20].

In subjects with myocardial ischemia several biomarkers are detectable in PF before they reach measurable concentration in circulation [21, 22]. Numerous heart diseases are associated with altered blood levels of markers and cytokines such as soluble CD40 ligand (sCD40 L), tissue plasminogen activator (tPA), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and soluble *P*-selectin (sPsel) [23–25].

We hypothesized the existence of a unique immunologic milieu within the PF with its dedicated lymphocyte pool that is reactive to heart-related conditions. Our goal was the immune characterization of PF within the most common groups of cardiac surgery patients: individuals who underwent CABG operation and those who had AVR operation. We focused on the phenotype-analysis of lymphocyte subpopulations and quantitative measurement of cardiovascular markers found in PF. Considering the difficulties of obtaining PF from healthy subjects we aimed to characterize compartmentation comparing to blood/plasma samples between the two most frequent groups undergoing open heart surgery.

2. Patients and methods

2.1. Patients

Patients admitted for elective open heart surgery were recruited into our study. Individuals were divided into two groups: CABG surgery (GroupA), and AVR (GroupB) (Table 1). GroupA had isolated CAD; GroupB had CAS without CAD. All patients underwent preoperative physical examination and excluding infections including urological, gynecological, dental, E-N-T examinations with chest X-ray, and abdominal ultrasound. The lab parameters preoperative were in normal range (CRP, WBC, ESR included). Exclusion criteria were: type1 diabetes mellitus, any kind of neoplasia, hepatitis, HIV, autoimmune disorders, any conditions treated by plasmapheresis, indication for combined heart

Table 1
Basic demographic characteristics of the investigated patient groups (GroupA: CABG, GroupB: aortic valve). The data are displayed as mean and standard error of mean (SEM) in case of age and body mass index (BMI). Diabetes mellitus (DM)

	GroupA	GroupB	All patients
Number of patients	24	12	36
female:male	5:19	4:8	9:27
age (mean+/–SEM)	57,96(+/–1,55)	60,50(+/–2,29)	58,81(+/–1,28)
BMI (mean+/–SEM)	30,48(+/–0,97)	29,48(+/–1,58)	30,15(+/–0,83)
Type2 DM	9	4	13
Smokers	15	3	18

surgery. All procedures were in accordance with the 1964 Helsinki declaration and its later amendments. The study was approved by the Regional Research Ethics Committee of the Clinical Center, University of Pécs, (reference number: 3415/2009). Patients gave written informed consent for all procedures.

2.2. Pericardial fluid and blood samples

PF samples were drawn with careful, bloodless preparation of the pericardium under the same well-determined conditions. At least 3 ml of PF was obtained with gentle suction into heparinized tubes (BD Biosciences) preceding the administration of heparin into the systemic circulation. Simultaneously 10 ml peripheral blood was taken from the central venous catheter. Both specimens were kept in sterilized on ice for a maximum of 60 minutes, until the beginning of the immunological and serological analyses.

2.3. Cell preparation for flow cytometry

Lymphocyte subpopulations in blood and PF were analyzed based on surface marker immunofluorescent staining by flow cytometry. 50μl of blood and 100μl of $2x10^6$ cell/100 μl in PBS/BSA/azide buffer resuspended PF pellet were processed (BD Biosciences). The cells were stained with a set of antibodies on ice for 25 minutes. Subsequently, 2 ml lysis buffer was added and incubated at room temperature for 10 minutes to eliminate the red blood cells. After PBS/BSA/azide washing and centrifugation (1000 rpm, 5 minutes) the supernatant was removed and the cells were fixed with 300μl FACS-FIX solution. The samples were kept at 4°C until flow cytometric detection was performed on a FACSCalibur™ Flow Cytometer (BD Biosciences). Results were analyzed using the CellQuest Pro 5.1 (BD Biosciences) software. 10^5 cells were collected form the lymphocyte gate and the lymphocyte subpopulations were analyzed and expressed as percentages of total lymphocytes. The flow cytometric analysis included the identification of the following cell types: CD56 + natural killer cells (NK), CD3 + CD56 + NKT, CD3 + CD8 + cytotoxic T cells (Tc), CD3 + CD4 + T_H lymphocytes, CD19 + CD5 + B1 population, CD19 + CD5- B2 B lymphocytes (B1,B2); CD4 + CD25high + Treg, CD3 + CD25 medium+activated T cells (Tact), CD3 + HLA-DR+Tact lymphocytes, CD8 + HLA-DR + Tact cell, CD3 + CD45RA + naive and CD3 + CD45RO + memory T cells (Tnaive, Tmemo), CD3 + iNKT + iNKT lymphocytes, CD3 + gdTCR + gdT cells.

2.4. Multiplex microbead technique

We used Human Cardiovascular 6plex FlowCytomix Kit (Bender MedSystems) to measure the concentration of sCD40L, IL-8, IL-6, MCP-1, sPsel, tPA in plasma and PF. The samples were measured with a FACSCalibur flow cytometer. Supernatants of PF samples and plasma were stored at –20°C. The measurements were carried out according to the manufacturer's manual. After thawing, the sample pairs were centrifuged (16000g, 5 minutes), then mixed with 1000 beads and incubated with 50μl biotin-conjugate mixture for 2 hours at room temperature (RT). To create a calibration curve, standards were diluted by subsequent dilution steps in Assay Buffer mixed with the beads, then 50μl biotin-conjugate mixture was added and the tubes were incubated at RT for 2 hours. After 2 washing steps, 50 μ l Streptavidin-PE was added to the samples. Finally, following two centrifugation steps (200g, 5 minutes), the beads were resuspended in 500 μ l Assay Buffer for flow cytometric acquisition and analysis using FlowCytomix Pro Software (Bender MedSystems GmbH).

2.5. Measurement of immunoglobulin isotypes

MILLIPLEX MAP Human Isotyping Kit (Millipore Corporation) was used to measure human IgG subclasses (1, 2, 3, and 4), IgM and IgA in plasma and PF samples (1:16000 and 1:10 dilutions of plasma and PF, respectively) according to the manufacturer's instructions. The samples and the Anti-Human Multi-Immunoglobulin Beads were added to the wet Microtiter Filter Plate and incubated for 1 hour on a plate shaker at RT. After 2 washing steps the samples were incubated with anti-human kappa or lambda light chain antibodies. Following the vacuum removal of the fluid, the beads were suspended in Sheath Fluid and the reaction was read on a Luminex 100™ IS instrument.

2.6. Statistical analysis

Values are presented as mean ± standard error of mean (SEM). Data among groups were compared using the Kruskall–Wallis test followed by Spearman correlation and regression analysis, as appropriate. PF and blood data was analyzed using Wilcoxon test. A value of $P < 0.05$ was considered

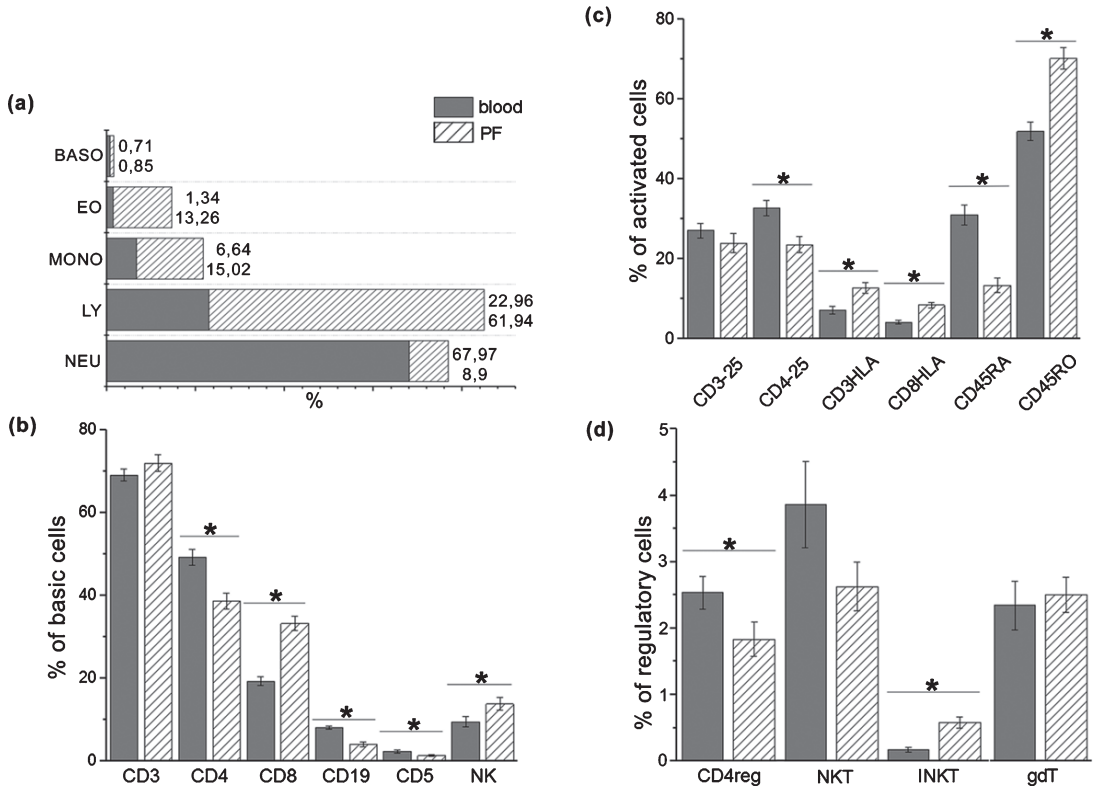

Fig. 1. White blood cell counts and lymphocyte subpopulations of peripheral blood and pericardial fluid. Automated differential white blood cell counts of blood and PF (a), ($N = 12$), Abbott Cell-Dyn 1700 automatic analyzer, WBC: white blood cell, NEU:neutrophil, LY : lymphocytes, MONO: monocytes, EO: eosinophil monocytes, BASO: basophil monocytes, bars represent the percentage of total WBC number, upper number shows results in blood, the lower number displays PF data. Comparison of basic (b), activated (c) and regulatory (d) lymphocyte subpopulations in PF and blood of all investigated patients. *: significant difference between PF and blood, $p < 0{,}05$; $N = 36$; data displayed as mean ± SEM; Error bars represent SEM.

statistically significant. Statistical analysis was performed with IBM SPSS Statistics 22 and OriginPro softwares.

3. Results

3.1. Cellular composition

The absolute white blood cell count in PF was significantly lower ($0{,}67 \pm 0{,}28$G/L) than in blood ($9{,}70 \pm 11{,}88$G/L). Lymphocytes represented the dominant cell type in PF (61,94%) (Fig. 1a). The granulocyte cell ratio was significantly lower, while monocyte and eosinophil cell ratio were both higher in PF than in blood. All the investigated lymphocyte subpopulations could be detected in the PF in different ratios than in blood (Fig. 1b,1c,1d).

Based on Spearman correlation, the levels of the following lymphocyte populations correlated in PF ($r \geq 0{,}4$; $p \leq 0{,}009$) with the same type of cell in blood: Tc ($r = 0{,}431$; $p = 0{,}009$), Treg ($r = 0{,}618$; $p = 0{,}00006$), Tnaive ($r = 0{,}581$; $p = 0{,}0002$), Tmemo ($r = 0{,}471$; $p = 0{,}004$), gdT ($r = 0{,}447$; $p = 0{,}003$).

3.2. Basic lymphocyte populations

The CD3 + T cells represented the major cell population in both PF and blood ($72{,}05 \pm 1{,}9\%$ and $69{,}15 \pm 1{,}43\%$).The level of CD8 + T_C cells ($33{,}26 \pm 1{,}72\%$ vs. $19{,}32 \pm 1{,}07\%$), and NK lymphocytes ($13{,}80 \pm 1{,}54\%$ vs. $9{,}51 \pm 1{,}27\%$) were significantly elevated in PF compared to blood. Whereas the levels of CD4 + T_H cells ($38{,}74 \pm 1{,}9\%$ vs. $49{,}24 \pm 1{,}97\%$), CD5 + B1 subgroup ($1{,}35 \pm 0{,}18\%$ vs. $2{,}33 \pm 0{,}30$ %) and CD19 + B2 lymphocytes ($4{,}07 \pm 0{,}63\%$ vs. 5,42 + /-0,09%) were significantly lower in PF than in blood (Fig. 1b)

The NK cell ratio was higher in the PF of both groups, but was significant only in groupA. In groupA the CD5 + B1 cell ratios were significantly lower in PF than in blood, unlike in groupB. The conventional B2 cell ratios between PF and blood, there were significantly (3,45 times) less B2 lymphocytes in PF of groupB compared to blood, while in groupA patients this difference was only 1,74 (Fig. 2a, 2d)

3.3. Activated and naive/memory lymphocyte subpopulations

$CD45RO^+$ Tmemo cells represented the dominant lymphocyte group in PF and blood ($70{,}20 \pm 2{,}71\%$and $51{,}92 \pm 2{,}29\%$) (Fig. 1c). Blood contained at least twice as many $CD45RA^+$ Tnaive cells as PF ($13{,}38 \pm 1{,}89\%$ vs. $31{,}06 \pm 2{,}5\%$). If we compare the Tact subpopulations, CD3 + HLADR+, and CD8 + HLADR+T_C cell ratio is higher in PF than in blood (CD3 + HLADR+: $12{,}69 \pm 1{,}4\%$ vs. $7{,}19 \pm 0{,}96\%$,CD8 + HLADR+: $8{,}42 \pm 0{,}63\%$ vs. $4{,}08 \pm 0{,}49\%$), while the CD4 + CD25 + activated T_H cell ratio is lower in PF than in blood ($23{,}58 \pm 1{,}93\%$ vs. $32{,}74 \pm 2{,}00\%$) (Fig. 1c).

CD4 + CD25 + activated T_H ratios were significantly higher in groupA both in PF ($25{,}50 \pm 1{,}93\%$ vs. $19{,}73 \pm 4{,}26\%$) and in blood ($36{,}42 \pm 2{,}54\%$ vs. $25{,}39 \pm 1{,}93\%$) compared to groupB. (Fig. 2b).

3.4. Minor (regulatory) lymphocytes

In both groups PF contained at least 3,1 times more iNKT cells than blood. (groupA: $0{,}5 \pm 0{,}08\%$ vs. $0{,}16 \pm 0{,}03\%$; groupB: $0{,}73 \pm 0{,}2\%$ vs. $0{,}18 \pm 0{,}09\%$) (Figs. 1d, 2c). A significantly higher Treg

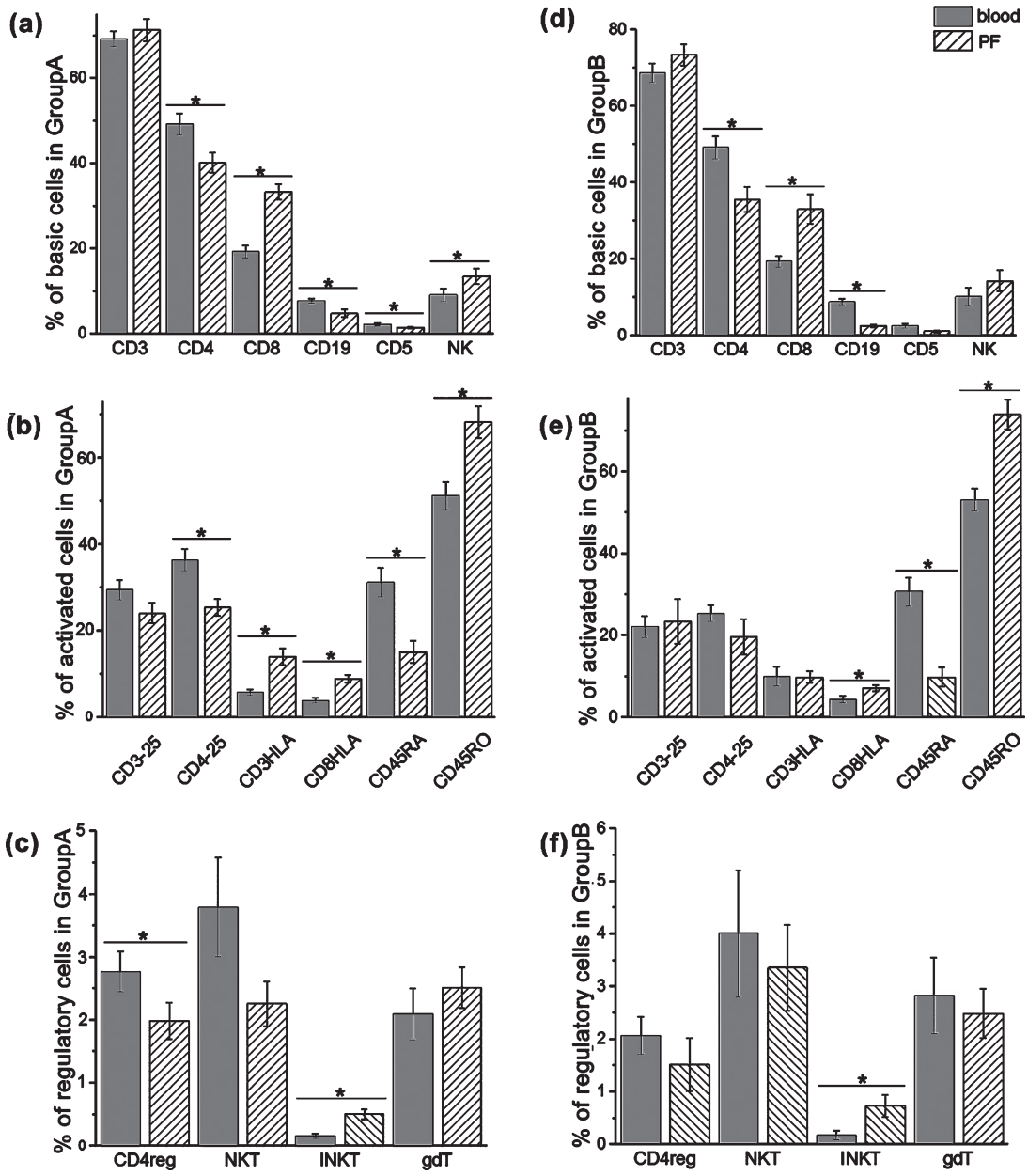

Fig. 2. Investigated lymphocyte populations in PF and blood within groupA (CABG patients) ($N = 24$) and groupB (aortic valve surgery patients) ($N = 12$). Graph (a) shows the basic cell populations, graph (b) indicates the activated lymphocyte types, while graph (c) illustrates the regulatory cells within the CABG group. Graph (d) demonstrates the number of basic lymphocytes, graph (e) represents the amount of activated lymphocytes and graph (f) shows the regulatory cell number among aortic valve surgery patients. *: significant difference between PF and blood, $p < 0{,}05$; data displayed as mean ± SEM; Error bars represent SEM.

ratio could be observed in both PF ($1{,}99 \pm 0{,}29\%$) and blood ($2{,}77 \pm 0{,}32\%$) of groupA compared to PF ($1{,}51 \pm 0{,}50\%$) and blood ($2{,}07 \pm 0{,}35\%$) of groupB (Fig. 2c, 2f). There were no significant differences in gdT cell ratios at all.

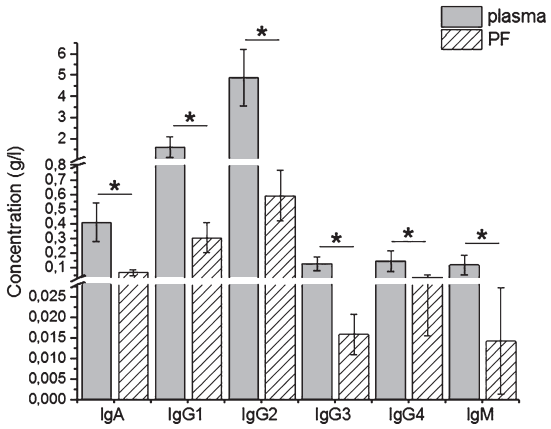

Fig. 3. Immunoglobulin isotypes in plasma and pericardial fluid samples of cardiac surgery patients.*: significant difference between PF and blood, $p < 0{,}05$; $N = 12$; data displayed as mean ± SEM; Error bars represent SEM.

3.5. IgA, IgG and IgM subclasses

We determined that IgA, IgG1-4, and IgM immunoglobulins were present in the PF. The levels of all the measured immunoglobulin classes were at least 3,5 times higher in the plasma than in the PF (Fig. 3). IgG2 was the dominant subclass, whereas the levels of IgM were the lowest in both plasma and PF.

3.6. Inflammatory cytokines (MCP1, IL8, IL6)

IL6 and MCP1 had significantly higher concentration in PF compared to plasma (IL6:105,79 ± 24,65 ng/ml vs. 5,00 ± 4,75 ng/ml; MCP1:3754,91 ± 602,63 ng/ml vs. 487,67 ± 43,63 ng/ml), unlike IL8 (13,91 ± 9,89 ng/ml vs. 23,05 ± 11,22 ng/ml). (Fig. 4a) The plasma level of MCP1 in groupA patients (575,01 ± 51,71 ng/ml) was significantly elevated compared to groupB (313,01 ± 53,21 ng/ml) (Fig. 4b, 4c).

3.7. Procoagulation markers (sCD40L, t-PA, sP-sel)

SCD40L, tPA, and sPsel were present in the PF and reached significantly higher concentrations in plasma. The sCD40L concentration in plasma was on average 133,93 times higher than in PF (1744,185 ± 478,95 ng/ml vs. 13,02 ± 7,54 ng/ml). In both specimen sP-sel was the dominant marker (5368,60 ± 12417,56 ng/ml in PF;9286,47 ± 2700,06 ng/ml in plasma). TPA was present in all of the samples (340,10 ± 37,76 ng/ml vs. 12934,00 ± 145,10 ng/ml) (Fig. 4b). When correlating the cardiovascular factor concentrations within PF and plasma, only sP-sel showed positive correlation ($r = 0{,}38$; $p < 0{,}05$). No significant difference could be found between the patient groups (Fig. 4e, 4f)

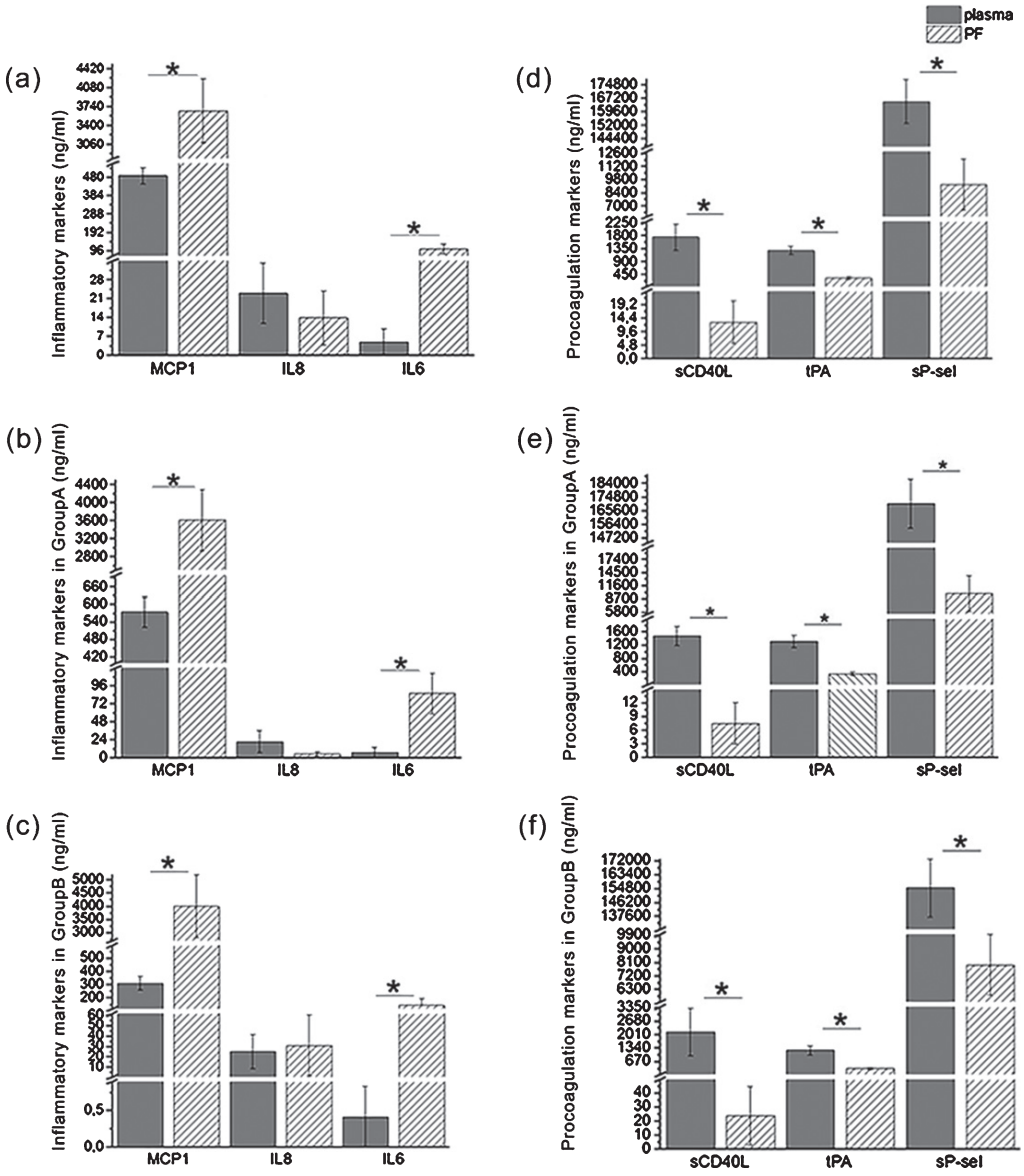

Fig. 4. Inflammatory and procoagulation marker concentrations in PF and blood plasma of all participants (a, d; $N = 36$) and within groupA (b, e; $N = 24$) and groupB (c, f; $N = 12$). *: significant difference between PF and blood, $p < 0{,}05$; data displayed as mean ± SEM; Error bars represent SEM.

4. Discussion

The present study describes a comprehensive immunological analysis of PF in humans for the first time. We showed that the investigated lymphocyte populations and cardiovascular markers in human PF have significantly different distribution compared to blood. The main finding of our study is that lymphocyte populations show different compartmentization in coronary disease and valvular aortic stenosis. Analysis of function immunomarkers and immunomodulation further extends this

observation. The PF contained significantly higher amounts of activated lymphocyte subpopulations (CD3 + HLA+cells, CD8 + HLA + lymphocytes and Tmemo cells, respectively) than blood.

The significantly elevated number of CD25 + activated CD4 + T_H cells in the PF of CABG patients compared to AVR patients is in line with the earlier observation in mice experiments that CD4 + T-lymphocytes may be activated by cardiac ischemia, presumably driven by recognition of cardiac autoantigens, and facilitate the wound healing of the myocardium [11].

Earlier investigations proved the ability of Treg cells to suppress proatherogenic effector T cells in atherosclerosis [12]. Studies reported reduced circulating Treg cell numbers in patients with acute coronary syndrome compared to patients with stable angina (SA) [26, 27]. Our results also suggest that chronic myocardial ischemia with SA (our groupA) may lead to elevated Treg cell level not only in the blood but also in the PF because the Treg population in CAD patients compared to AVR group was significantly higher in both specimens. Clinical experiments that investigated circulating Treg cells in patients with CAD showed conflicting results [13].

INKT subpopulation is activated by both self and exogenous lipid antigens and are considered to be proatherogenic [28]. INKT subset showed no significant difference when comparing the patient groups, but it reached significantly higher concentration in PF compared to blood in each case. These data support that the pericardial space has its own, independent iNKT pool.

Tmemo lymphocytes conventionally are a feature of an immune response against foreign antigens. However, there is evidence in mice that iNKT cells can induce mature naive CD8 + T cells to acquire innate characteristics in peripheral lymphoid tissue via IL-4 [29]. This latter crosstalk could be a possible explanation for the high ratios of T_C and iNKT subpopulations.

Studies showed that B1 cells are atheroprotective mainly via the production of natural IgM antibodies that bind oxidized low-density lipoprotein and apoptotic cells. B2 cells are suggested to be proatherogenic [30]. After AMI in mice, mature systemic B lymphocytes induce monocyte mobilization and recruitment to the heart, leading to enhanced tissue injury and deterioration of myocardial function [31]. We found an amount of B2 lymphocytes in PF of CABG patients 1,88 times higher than in the aortic valve group. The B2 cell number in systemic blood flow showed no such difference, suggesting that the B2 lymphocyte population of the PF might have a role in CAD related myocardial ischemia.

The distribution of PF immunoglobulin levels were the similar to that of in blood; however, the PF contained significantly less IgA, IgG1-4, and IgM compared to plasma. Here we uncover for the first time the Ig composition of the PF of cardiac surgery patients. It was earlier described that after ischemia and reperfusion of heart, complement is activated by self-reactive natural IgM antibodies that bind to postischemic tissue [32]. Furher investigation of pericardial Ig subclasses could open new paths in the field of heart-related disorders.

The significantly different distribution of markers of immunactivation in PF and plasma implies the presence of local factors that influence the concentration of cytokines in the PF. All of the investigated cytokines have a molecular weight under 40kDa, making diffusion through membranes possible, except for tPa (68 kDa). The half-life of MCP1, tPA, sCD40L, and IL8 are shorter (maximum 20 minutes) in bloodstream, whereas sPsel (100 minutes) and IL6 (maximum 6 hours) last longer. This could explain why sPsel is the only factor with correlating concentrations between plasma and PF. Our data confirmed that PF reflects the composition of cardiac interstitium [5, 33].

The strong positive correlation between pericardial MCP1 and IL6 levels and the fact that exclusively these biomarkers had higher concentrations in PF compared to blood demonstrates the local significance of these cytokines. We found significantly elevated plasma level of MCP1 in CAD patients compared to the aortic valve group, which is in contradiction to the earlier observation of Shibasaki et al., who found no difference in plasma MCP1 mRNA levels when comparing open heart surgery patients with and without CAD [34]. Our data of elevated MCP 1 in CABG patients congruent with the results of Yu et al. who described that shear stress (endothelial activation, first step to atherosclerosis) increase

the secretion of MCP 1 in human umbilical vein endothelial cells [35]. Although in other study shear stress increased the release of IL-8 from endothelial cells [36], we did not detect any difference of IL-8 level in the patient groups, nor any difference in blood and PF.

In accordance with previous results [37] pericardial IL-6 levels were at least 5 times higher than plasma levels, with no correlation between them.

Although plasma sCD40 L (platelet activation marker) was considered to have a positive predictive value for ischemic events in patients with ACS in systemic and coronary artery blood [38], our investigation showed no significant difference in sCD40L levels between CAD and CAS group. This is in line with Fong et al. also found no association between sCD40L levels and long-term ischemic outcomes in unselected aspirin-treated patients undergoing cardiac catheterization [24].

Circulating sP-sel as a marker for platelet hyperactivity, inflammation, and endothelial dysfunction were previously found to be significantly elevated in patients with diastolic dysfunction, and play a role in the initiation of atherosclerosis and AMI [39, 40]. Our present research showed the presence of sPsel in PF but had no correlation with CAD or CAS, both in PF and serum.

Even though plasma inflammatory biomarkers may not adequately reflect local tissue levels, earlier studies measured the concentrations of peripheral venous or coronary blood [33, 37, 41] and suggest that compounds with key roles in plaque-destabilization and MI are also released from non-coronary sources; therefore, further PF analysis is needed.

Our experiments revealed that the PF is an immunologically active space which can play a crucial role in the course of MI or any disease involving the myocardium, differences of PF composition may as well as influence the regeneration process following open heart surgery. Moreover, pericardial sac integrity can affect the cardiac regeneration after cardiac surgery [41]. Hopefully future PF analyses will change the scientific and clinical aspects of the PF, since immunomodulatory therapies can have the potential to limit the infarct volume and accelerate repair [42]. The pericardial space seems to be a promising medium for such approaches with less systemic effects.

Acknowledgments

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Correlation of natural autoantibodies and cardiovascular disease-related anti-bacterial antibodies in pericardial fluid of cardiac surgery patients

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Summary

Our previous studies showed that anti-citrate synthase (anti-CS) immunoglobulin (Ig)M natural autoantibodies are present in healthy individuals without previous antigen stimulation, but no studies have investigated their presence in the pericardial fluid (PF). Therefore, we detected the natural anti-CS IgG/M autoantibody levels in plasma and PF of cardiac surgery patients and investigated their relationship with cardiovascular disease-associated bacterial pathogens. PF and blood samples of 22 coronary artery bypass graft (CABG) and 10 aortic valve replacement (AVR) patients were tested for total Ig levels, natural autoantibodies and infection-related antibodies using enzyme-linked immunosorbent assay (ELISA) and Luminex methods. The B cell subsets were measured by flow cytometry. The total Ig subclass levels were four to eight times lower in PF than in plasma, but the natural anti-CS IgM autoantibodies showed a relative increase in PF. The frequency of $CD19^+$ B lymphocytes was significantly lower in PF than in blood ($P = 0{\cdot}01$), with a significant relative increase of B1 cells ($P = 0{\cdot}005$). *Mycoplasma pneumoniae* antibody-positive patients had significantly higher anti-CS IgM levels. In CABG patients we found a correlation between anti-CS IgG levels and *M. pneumoniae, Chlamydia pneumoniae* and *Borrelia burgdorferi* antibody titres. Our results provide the first evidence that natural autoantibodies are present in the PF, and they show a significant correlation with certain anti-bacterial antibody titres in a disease-specific manner.

Keywords: anti-bacterial antibodies, cardiovascular disease, citrate synthase, natural autoantibodies, pericardial fluid

Introduction

There is increasing evidence that the immune system plays an important role in the development of cardiovascular diseases, especially in atherosclerosis, one of the main underlying features of cardiovascular failure [1–4]. However, little is known about the immunological milieu of the pericardial fluid produced by the serous membranes surrounding the heart [2,5]. Extravascular serous fluids (i.e. peritoneal, pleural and pericardial) contain cellular and humoral components of the immune system to protect the organ from microbial invasion. The peritoneal and pleural cavities and the associated serous fluids have been investigated thoroughly regarding their biochemical and immunological composition [5–7]. It is not surprising that the pericardial fluid is not so well studied, because of the invasive nature of obtaining pericardial fluid during open-heart surgery [8,9]. Thus, our goal was to study the presence of antibodies of the natural and adaptive immune systems and the corresponding B cell subsets in the pericardial fluid (PF) of patients with cardiovascular diseases undergoing cardiac surgery.

The PF lubricates the surface of the heart, and its cellular and molecular immune components may function to: (i) protect the heart and pericardium from microbial agents and (ii) facilitate of the elimination of cell and tissue debris to ensure tissue homeostasis [10,11]. Recently numerous studies have supported the role of the natural immune system, including natural autoantibodies, in the above functions as key players in the first line of defence against

infections and clearance of apoptotic cells. Natural autoantibodies are present in the circulation of humans and other mammalian species without previous exposure to antigens [12,13], and are produced by B1 cells [14–16] without somatic hypermutation; most of them are of IgM isotype. Natural autoantibodies of immunoglobulin (Ig)G isotype have also been described [17,18], which typically bind multiple self- and non-self-antigens. These autoantibodies are often directed against highly conserved molecules and bind various ligands with relatively low affinity, and therefore they are called 'polyreactive'. Evidence from rodents deficient in natural antibody production suggests that natural autoantibodies serve homeostatic and housekeeping functions and natural IgM autoantibodies serve as scavengers of damaged molecules and cells [10], and therefore have been implicated in the control of inflammation and autoimmune diseases [19,20]. Natural antibodies may also serve as innate-like recognition receptors, recognizing various bacterial cell wall components or parasites [21,22]. Antibodies produced by the adaptive immune system are generally derived from T-dependent B lymphocytes, in response to exposure to microbes or vaccination. It has been shown previously that T cells are the dominant lymphocytes in the PF [23] but less is known about the B1 and B2 cell composition of PF, which may reflect on the source of natural and infection-related antibodies found in the PF space.

Here we investigated whether IgM and IgG natural autoantibodies are present in the PF of patients with different cardiovascular diseases undergoing open-heart surgery. Based on previous studies, including those from our laboratory, it is known that citrate synthase (CS), a highly conserved mitochondrial inner membrane enzyme, is an autoantigen recognized by the natural immune system [24–27]. Heart muscle cells contain the highest number of mitochondria in body tissues, and mitochondrial enzymes can become released in different cardiovascular diseases. For this reason, we chose to investigate anti-CS natural autoantibodies in patients with cardiovascular diseases and the levels of infection-related antibodies with a potential role in the development of atherosclerosis, including *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Borrelia burgdorferi*, *Helicobacter pylori* and *Yersinia enterocolitica* [28–33]. We compared the plasma/PF ratios of anti-CS IgG and IgM antibodies and correlated their levels with total and anti-bacterial IgG/IgM antibody titres in patients with aortic valve replacement (AVR) and coronary artery bypass graft (CABG) with or without previous myocardial infarction (MI). We also investigated the frequencies of B1 and B2 cells to gain insight into the source of natural antibody production in PF. Our data provide evidence that natural autoantibodies and anti-bacterial antibodies are present in the PF, and their correlation may suggest their importance in the development of cardiovascular diseases.

Materials and methods

Patients

Thirty-two patients who were admitted to University of Pécs Heart Institute for elective open-heart surgery (due to coronary or aortic valve disease) during a 1-year period were selected for our study. Patients with known autoimmune disease and patients who underwent combined open-heart surgery and patients whose samples could not be used due to technical issues were excluded from the study. The average age of patients was 58·8 ± 7·5 years, and the female : male ratio was 8 : 24. The patients were divided into three groups: aortic valve replacement group (AVR, $n = 10$), coronary artery bypass grafting patients (CABG) with previous myocardial infarction (CABG with MI, $n = 10$) and those who underwent CABG but had no previous myocardial infarction (CABG no MI, $n = 12$). The diagnosis of myocardial infarction was based on coronary angiography and echocardiography findings. Prior to surgery, all patients underwent physical examination and search for inflammatory foci, including urological, gynaecological, dental and ear, nose and throat examinations, together with chest X-ray and abdominal ultrasound scans. The laboratory parameters the day before surgery were within the normal range [C-reactive protein (CRP); white blood cells count (WBC); erythrocyte sedimentation rate (ESR) were included]. The study was approved by the Regional Review Board (RIGEB 3415/2009). Patients gave written informed consent to all procedures.

Pericardial fluid and peripheral blood samples

PF and blood samples were drawn simultaneously during elective open-heart surgery. At least 3 ml of PF was drained using a syringe connected to a Vacutainer tube. At the same time, peripheral blood samples were taken in sterile, heparinized tubes (BD Vacutainer CPT^{TM} Cell Preparation Tube; Becton Dickinson, Franklin Lakes, NJ, USA). The samples were processed within 60 min by separating the cells by centrifugation and the plasma and PF samples were aliquoted promptly and stored subsequently at −80°C until analysis.

Immunological and serological analysis

Measurement of immunoglobulin isotypes by the Luminex method. The Milliplex Map Human Isotyping Kit (Millipore Corp., Burlington, MA, USA) was used to measure human IgG subclasses (1, 2, 3 and 4), IgM and IgA in the plasma and PF samples (1 : 16 000 and 1 : 10 dilutions of plasma and PF, respectively), according to the manufacturer's instructions. Briefly, the samples and the anti-human multi-immunoglobulin beads were added to the wet microtitre filter plate. The plate was covered and

incubated for 1 h on a plate shaker at room temperature. The plate was then incubated with anti-human antibodies to kappa or lambda light chains. Following the vacuum removal of the fluid, the beads were suspended in sheath fluid. The reaction was measured on a Luminex 100^{TM} IS (Luminex, Austin, TX, USA) instrument.

Measurement of anti-CS antibodies by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml citrate synthase (CS) from porcine heart (Sigma, St Louis, MO, USA) in 0·1 M bicarbonate buffer, pH 9·6 at 4°C overnight. Following the saturation of non-specific binding sites with 0·5% gelatin (Sigma) in phosphate-buffered saline (PBS) (pH 7·3), plasma samples were incubated in duplicate at 1 : 100 dilution and PF samples at 1 : 10 dilution in washing buffer (PBS, 0·05% Tween 20) for 1 h at room temperature. Finally, the plate was incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG- or IgM-specific secondary antibody (Dako, Glostrup, Denmark) for 1 h at room temperature. The reaction was developed with o-phenylenediamine (Sigma) and measured using an iEMS MF microphotometer (Thermo Labsystems, Waltham, MA, USA) at 492 nm. Cut-off values were calculated from the average of measured optical density (OD) 492 nm data. All measurements were standardized using a monoclonal anti-citrate synthase antibody (clone 4H3-E5) produced previously [34].

Serological tests for anti-bacterial antibodies. Commercial ELISA kits were used to determine the anti-bacterial antibody concentrations in the plasma and PF samples. *B. burgdorferi*-specific IgG and IgM, anti-*H. pylori* IgA and IgG, anti-*Y. enterocolitica* IgA, IgM and IgG antibodies (Mikrogen GmBH, Neureid, Germany), anti-*C. pneumoniae* and anti-*M. pneumonia* IgA, IgG and IgM antibodies (Ani Labsystems Diagnostics, Vantaa, Finland) were measured by indirect ELISA tests, according to the manufacturer's instructions. Briefly, plasma samples at 1 : 100 dilution and PF samples at 1 : 20 dilution were incubated for 1 h at 37°C. The plate was then incubated with HRP-conjugated anti-human IgA/IgG/IgM antibodies for 30 min at 37°C. The reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) and measured using a Siemens BEP-2000 ELISA reader at 450 nm Biocompare, San Francisco, CA, USA).

Measurement of B1 and B2 B cells in PF and plasma by flow cytometry. Multi-parametric flow cytometry was performed on PF and plasma samples with antibodies specific for CD19, CD5 and CD3. To identify B cells, fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (4G7; Becton Dickinson) were used to distinguish between B1 and B2 B cell phycoerythrin (PE)-conjugated anti-CD5 antibodies (L17F12; Becton Dickinson). Stained cells were detected and data acquired using a fluorescence activated cell sorter (FACS)Calibur flow cytometer (Becton Dickinson) and analysed with FCS Express 4 software (DeNovo Software, Glendale, CA, USA).

Statistical analysis

Statistical evaluation was performed using SPSS version 20.0 statistics package (IBM, Armonk, NY, USA). Spearman's correlation analysis and Mann–Whitney *U*-tests were used as appropriate. *P*-values $< 0·05$ were considered significant.

Results

Relative excess of anti-CS IgM natural autoantibodies in the pericardial fluid

CS has been characterized as an autoantigen recognized by the natural immune system in humans. We have documented previously that anti-CS IgM natural antibodies are continuously present in plasma of healthy controls and that anti-CS IgG levels increase in patients with autoimmune diseases and heart transplantation [25,26,35]; however, no data have been published regarding the anti-CS antibodies in PF. The anti-CS IgM and IgG natural autoantibodies were measured by an in-house ELISA developed and validated in our laboratory. We detected anti-CS IgM and IgG antibodies both in the plasma and PF of patients undergoing cardiac surgery. The anti-CS IgG level was approximately 11-fold higher in plasma than in PF ($P < 0·001$) (Fig. 1b), whereas anti-CS IgM concentration was only approximately twofold lower in the PF than in the plasma ($P < 0·001$) (Fig. 1a). This reflects a relative excess of anti-CS IgM autoantibodies in the PF of cardiac surgery patients. Similar differences in the levels of anti-CS IgG and IgM autoantibodies were detected in the different disease groups (Table 1).

We also tested the correlation of anti-CS IgM and IgG levels between plasma and PF in individual patients. A statistically significant correlation of anti-CS IgM antibody concentrations ($P < 0·001$) (Fig. 2a) and a strong correlation in the case of anti-CS IgG levels ($P < 0·001$) (Fig. 2b) was found between the plasma and PF ($r^2 = 0.880$ and $r^2 = 0.997$, respectively).

We wanted to compare the anti-CS IgG/IgM levels to the total immunoglobulin subclass levels in plasma and PF. We found that the concentrations of all the investigated immunoglobulin isotypes were significantly lower in PF than in plasma (Table 2), but the plasma/PF ratios were different. IgM, IgG2 and IgG3 were eight times higher in plasma than in PF, while the PF levels of IgA, IgG1 and IgG4 were 6·0-, 5·3- and 4·2-fold lower than in plasma. The average IgG subclass levels in PF was 6·2 times lower than in

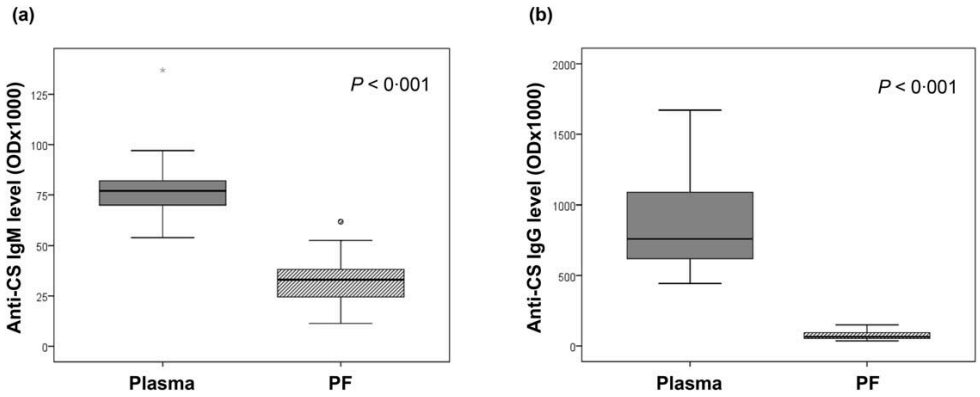

Fig. 1. Anti-citrate synthase (anti-CS) natural autoantibody levels in the plasma and pericardial fluid (PF) of cardiac surgery patients. The proportion of anti-CS immunoglobulin (Ig)M (a) antibody levels were only twofold higher ($P < 0{\cdot}001$), while anti-CS IgG (b) antibody levels were 11-fold higher in the plasma than in the pericardial fluid ($P < 0{\cdot}001$). Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians, dots indicate outliers of 1.5 × interquartile range (IQR). The antibody levels were measured using enzyme-linked immunosorbent assay (ELISA). OD = optical density; plasma, peripheral blood plasma; $n = 32$ patients.

plasma (Table 2), which reflects a lower total IgM than total IgG class levels in PF.

B1 cell frequencies are higher in pericardial fluid

In order to gain insight into the different Ig class-producing cells in PF we investigated and compared the ratio of B cell subgroups in blood and PF. The percentage of $CD19^+$ B cells was significantly lower in PF than in blood ($P < 0{\cdot}001$). Analysing the B cell subsets, we found lower frequencies of both B1 and B2 B cells in PF compared to the blood ($P < 0{\cdot}001$ and $P < 0{\cdot}002$, respectively) (Fig. 3), but the proportion of B1 cells in the $CD19^+$ B cell population was significantly higher in the PF than in the blood of cardiac surgery patients ($n = 32$) (Table 3), which might be the source of natural anti-CS IgM autoantibody production in the PF.

Anti-CS natural autoantibody levels correlate with anti-bacterial antibody titres in coronary artery bypass graft patients

Several infections are described to have a potential role in the development of atherosclerosis, therefore we tested the plasma and PF samples for antibodies (IgG/IgA/IgM) against five different cardiovascular disease associated bacteria, such as *M. pneumoniae*, *Y. enterocolitica*, *C. pneumoniae*, *H. pylori* and *B. burgdorferi*. All patients were positive for at least one anti-bacterial antibody (IgG and/or IgA) in the plasma, while in the PF we found fewer positive samples, although their corresponding plasma sample was positive. The highest proportion of positivity for anti-bacterial antibodies in the plasma was against *M. pneumoniae* (74·2%) and *H. pylori* (74·2%), whereas in the PF it was against *H. pylori* (37·0%), *M. pneumoniae* (14.8%) and *Chlamydia pneumoniae* (14·8%). No IgM positivity could

Table 1. Ratios of anti-citrate synthase (anti-CS) immunoglobulin (Ig)G and IgM autoantibodies in pericardial fluid and plasma of patients with different cardiac diseases

Natural autoantibody	Disease group	PF : plasma ratio	% Plasma level
Anti-CS IgG	AVR	1 : 11·5	8·7%
		80·06 ± 8·45 : 921·60 ± 92·75	
	CABG with MI	1 : 11·5	8·7%
		67·95 ± 7·54 : 787·60 ± 81·80	
	CABG no MI	1 : 11·6	8·6%
		76·14 ± 9·73 : 886·64 ± 107·58	
Anti-CS IgM	AVR	1 : 2·3	43·4%
		34·14 ± 2·60 : 78·90 ± 3·31	
	CABG with MI	1 : 2·3	43·4%
		34·04 ± 4·48 : 78·10 ± 7·25	
	CABG no MI	1 : 2·5	40·0%
		29·85 ± 3·21 : 74·00 ± 2·56	

AVR = aortic valve replacement; MI = myocardial infarction; CABG = coronary artery bypass graft.

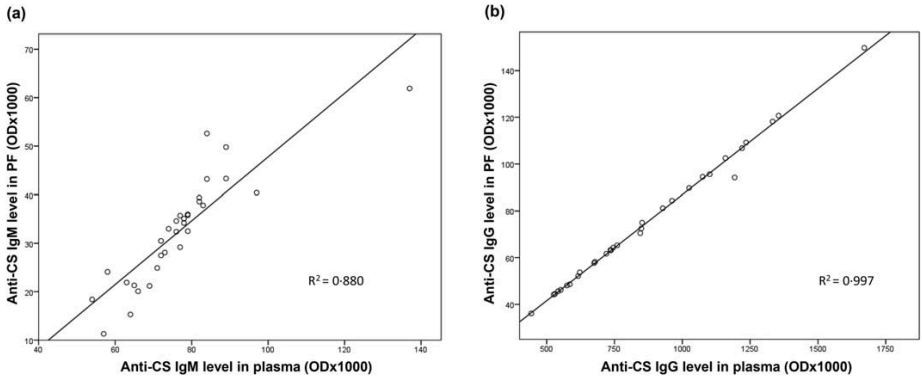

Fig. 2. Correlation between the plasma and pericardial fluid (PF) levels of anti-citrate synthase (anti-CS) natural autoantibodies. Significant correlations were detected in the levels of anti-CS immunoglobulin (Ig)M in the plasma *versus* PF ($P < 0{\cdot}001$) (a), as well as in the anti-CS IgG levels in plasma versus PF ($P < 0{\cdot}001$) (b).

be detected either in plasma or PF, which indicates the absence of an early infection.

We tested whether there was a relationship between anti-CS IgM or IgG autoantibody levels and the presence of anti-bacterial antibody positivity for any of the five different bacterial strains, and we found significantly elevated anti-CS IgM levels in the plasma ($P = 0{\cdot}031$) and an increased tendency in the PF ($P = 0{\cdot}076$) of patients with positive *M. pneumoniae* (MP) antibodies (Fig. 4).

We also tested the correlation of anti-CS IgG and IgM antibody levels with anti-bacterial antibody titres both in the plasma and PF of patients belonging to different disease groups. In CABG patients without previous MI there was a significant correlation between anti-CS IgG levels and *M. pneumoniae* antibody titres, while in CABG patients with previous MI *C. pneumoniae* and *B. burgdorferi* antibodies correlated significantly with anti-CS IgG levels. In patients with AVR, anti-CS IgG did not show a correlation with any of these anti-bacterial antibody levels. No clear-cut correlations were observed between anti-CS IgM and the anti-bacterial antibody titres investigated (Table 4).

Discussion

The goal of this study was to determine whether natural autoantibodies to mitochondrial citrate synthase enzyme (CS) and antibodies to certain bacteria related to atherosclerosis are present in the plasma and PF of cardiac surgery patients, and to determine the relationship of these antibodies to each other and to the total immunoglobulin class levels in plasma and PF. Our study has revealed that anti-CS natural autoantibodies and anti-bacterial antibodies are present not only in the plasma, but also in the PF. We also wanted to examine the frequencies of B1 and B2 B lymphocytes in PF and compare them to the percentages found in blood to gain insight into the source of natural autoantibodies in PF.

First, we determined the total immunoglobulin (IgA, IgM and IgG1–4) subclass concentrations in PF and plasma of cardiac surgery patients, and found that in PF their level is four- to eightfold lower than that in plasma. In contrast, when we compared the anti-CS natural antibody levels, the anti-CS IgM was only twofold lower, whereas the anti-CS IgG levels were 10-fold lower in PF than in plasma in all study groups. The increased level of anti-CS IgM may be due to the presence of increased frequencies of B1 cells in the PF. Although the total $CD19^+$ B cell ratio is significantly lower in PF compared to blood, the proportion of B1 cells in the $CD19^+$ B cell population was significantly higher in the PF than in the blood of cardiac surgery patients, which suggests that B1 cells are the probable source of IgM natural autoantibodies [23,36]. The tissue-

Table 2. Total immunoglobulin (Ig) subclass levels and their ratios in plasma and pericardial fluid (PF) of cardiac surgery patients

Ig isotype	Plasma mean ± s.e.m.	Pericardial fluid mean ± s.e.m.	PF : plasma ratio	% Plasma level
IgA (g/l)	0·41 ± 0·13	0·07 ± 0·02	1 : 6	16·6%
IgG1 (g/l)	1·62 ± 0·48	0·31 ± ·10	1 : 5	18·9%
IgG2 (g/l)	4·89 ± 1·34	0·59 ± 0·18	1 : 8	12·5%
IgG3 (g/l)	0·13 ± ,05	0·02 ± 0·01	1 : 8	12·5%
IgG4 (g/l)	0·15 ± 0·07	0·04 ± 0·02	1 : 4	23·8%
IgM (g/l)	0·12 ± 0·07	0·014 ± 0·01	1 : 8	12·5%

s.e.m. = standard error of the mean.

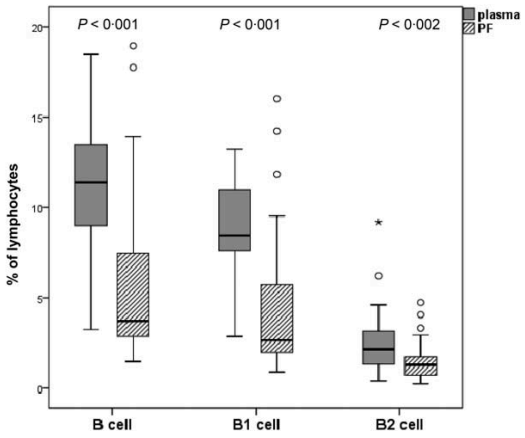

Fig. 3. Frequencies of total CD19$^+$ B cells and B1, B2 subpopulations in plasma and pericardial fluid (PF). The percentage of all investigated B cell groups were significantly lower in PF than in plasma samples ($P < 0{\cdot}001$, $P < 0{\cdot}001$ and $P < 0{\cdot}002$, respectively). Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians, dots indicate outliers of 1·5 × IQR. Frequencies of cells were measured with flow cytometer. plasma, peripheral blood plasma; $n = 32$ patients.

specific signals in different tissue compartments may affect the functionally heterogeneous B1 cells differentially [37], but their shared function might be to ensure a prompt and effective local defence.

Natural antibodies may exert distinct effector functions according to their specificity and respective isotypes [2]. Our data suggest that the pericardial space containing the PF is protected by the natural immune system, which may play a crucial role in tissue homeostasis by elimination of cellular and molecular debris that needs to be cleared in order to prevent the accumulation of apoptotic material [10] to control inflammation and autoimmune mechanisms [17,19,20]. Another key and overarching function of natural antibodies is to serve as a bridge between the innate and adaptive immune systems [22] by pathogen neutralization, complement activation and antigen recruitment to secondary lymphoid organs, and for immune regulation and homeostasis [16,22]. Our data may support the notion that anti-CS natural autoantibodies, similarly to other polyreactive natural antibodies, may serve as scavengers of dead or dying cells and microbial antigens both in plasma and in the PF [38,39]. It is probable that CS is not the only molecular constituent cleared by such mechanisms, but there could be a plethora of other self- and pathogen-related molecules recognized by the natural autoantibodies destined for subsequent clearance to prevent their 'toxicity' to cardiomyocytes [11,17]. In mouse models of atherosclerosis, natural IgM antibodies produced by serosal B1 cells have been shown to protect from atherosclerosis, whereas antibodies produced by B2 cells promote atherosclerosis [40]. Clinical data obtained from patients with systemic lupus erythematosus also support the notion that natural IgM autoantibodies may inhibit carotid atherogenesis [41].

The strong correlation of anti-CS IgG in plasma *versus* PF may suggest that these antibodies may not be produced exclusively in the PF, but they could be transported from the plasma or lymphatic fluid into the pericardial space. A receptor-mediated transcytosis of immunoglobulins [42] may underlie the transport of anti-CS and other IgG antibodies into the PF. In addition to transcytosis, some antibodies may be synthesized locally in the pericardial space. Our data showing the relative increase of anti-CS IgM antibody in the PF is consistent with the notion that B1 cells produce anti-CS IgM (but not anti-CS IgG) locally in the pericardial space.

Using immunoserological methods we measured *M. pneumoniae*-, *Y. enterocolitica*-, *C. pneumoniae*-, *H. pylori*- and *B. burgdorferi*-specific antibodies in PF and plasma because data have been published on the possible involvement of these microbes in the development of cardiovascular diseases [31]. Of note, all patients were positive for antibodies to at least one bacterial species; however, some patients were negative for some of these antibodies in the PF. This may suggest that the levels of anti-bacterial antibodies in the plasma may be generally higher than in the PF (the PF is more sequestered from the immune system than blood), resulting in antibody levels below the limit of detection in the PF. Interestingly, *M. pneumoniae* has been found in atherosclerotic plaques and in normal arteries and veins of patients [31], and this bacterium was also shown to be present in the PF of atherosclerotic patients [43]. *H. pylori* has also been found in human atherosclerotic plaques, and a significant association of antibodies to this pathogen has been found in patients with coronary

Table 3. Percentages of B1 cells in the CD19$^+$ B cell population in blood and PF of patients with different cardiac diseases

Disease type	B1 cells in blood (% of CD19$^+$ B cells)	B1 cells in PF (% of CD19$^+$ B cells)	*P*-value
CABG no MI (n = 12)	24 ± 8·6%	36 ± 23·8%	0·237
CABG with MI (n = 10)	30 ± 26·1%	48 ± 43·6%	0·108
AVR (n = 10)	27 ± 13·9%	51 ± 34·5%	0·078
Total (n = 32)	27 ± 17·1%	45 ± 34·1%	0·005

Aortic valve replacement group (AVR, $n = 10$), coronary artery bypass grafting patients (CABG) with previous myocardial infarction (CABG with MI, $n = 10$), and those who underwent CABG but had no previous myocardial infarction (CABG no MI, $n = 12$). PF = pericardial fluid.

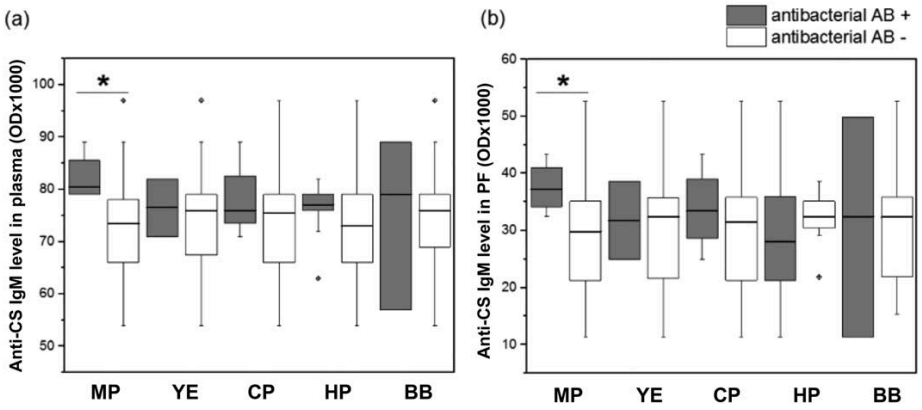

Fig. 4. Anti-citrate synthase (anti-CS) immunoglobulin (Ig)M levels in plasma and pericardial fluid (PF) of patients negative or positive for various anti-bacterial antibodies. Patients who were positive for *Mycoplasma pneumoniae* antibodies in the PF (and plasma) had significantly higher levels of anti-citrate synthase IgM in their plasma (a) and an elevated tendency in the PF (b) when compared to *M. pneumoniae* antibody-negative patients ($P = 0{\cdot}031$ and $P = 0{\cdot}076$, respectively). Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians, dots indicate outliers of $1{\cdot}5 \times$ IQR, while the asterisks represent the significance ($n = 32$). MP = *M. pneumoniae*; YE = *Yersinia enterocolitica*; CP = *Chlamydia pneumoniae*; HP = *Helicobacter pylori*; BB = *Borelia burgdorferi*; plasma = peripheral blood plasma; AB = antibody.

arteriosclerosis when compared to control subjects [43]. *C. pneumoniae* has been detected in atherosclerotic plaques by quantitative polymerase chain reaction (qPCR) [44], and viable *C. pneumoniae* organisms were isolated from plaques [31]. Our current data on the high frequency of anti-microbial antibodies in the plasma and PF of patients undergoing heart surgery (atherosclerosis-related coronary heart disease) are consistent with the potential pathogenic role of the above-mentioned bacteria in these diseases.

Our data suggest an inter-relationship between natural and anti-bacterial antibodies. We found that all patients whose plasma and/or PF were positive for antibodies to *M. pneumoniae* had significantly higher levels of anti-CS IgM, both in their plasma and PF. Investigating the different disease groups, in CABG patients without previous MI we found a significant correlation between anti-CS IgG levels and *M. pneumoniae* antibody titres, while in CABG patients with previous MI, *C. pneumoniae* and *B.*

Table 4. Correlation of citrate synthase (anti-CS) IgG and immunoglobulin (Ig)M antibody levels with antibacterial antibody titres in plasma and PF of patients with cardiovascular diseases

Disease group	CABG no MI	CABG with MI	AVR
Natural autoantibody	Correlating anti-microbial antibody		
Anti-CS IgG plasma	MP IgG (plasma) (rho = 0·789; $P = 0{\cdot}004$)	CP IgG (plasma) (rho = 0·753; $P = 0{\cdot}012$)	No correlation
	MP IgM (plasma) (rho = 0·802; $P = 0{\cdot}003$)	CP IgG (PF) (rho = 0·769; $P = 0{\cdot}026$)	
	MP IgG (PF) (rho = 0·579; $P = 0{\cdot}062$)	BB IgG (plasma) (rho = 0·632; $P = 0{\cdot}049$)	
Anti-CS IgG PF	MP IgG (plasma) (rho = 0·797; $P = 0{\cdot}003$)	CP IgG (plasma) (rho = 0·754; $P = 0{\cdot}012$)	No correlation
	MP IgM (plasma) (rho = 0·829; $P = 0{\cdot}002$)	CP IgG (PF) (rho = 0·762; $P = 0{\cdot}028$)	
	MP IgG (PF) (rho = 0·611; $P = 0{\cdot}046$)	BB IgG (plasma) (rho = 0·633; $P = 0{\cdot}049$)	
Anti-CS IgM plasma	No correlation	YE IgM (plasma) (rho = 0·710; $P = 0{\cdot}021$)	MP IgG (plasma) (rho = 0·780; $P = 0{\cdot}013$)
Anti-CS IgM PF	No correlation	No correlation	No correlation

Aortic valve replacement group (AVR, $n = 10$), coronary artery bypass grafting patients (CABG) with previous myocardial infarction (CABG with MI, $n = 10$), and those who underwent CABG but had no previous myocardial infarction (CABG no MI, $n = 12$). MP = *Mycoplasma pneumoniae*; CP = *Chlamydia pneumoniae*; BB = *Borrelia burgdorferi*; YE = *Yersinia enterocolitica*.

burgdorferi antibody concentrations showed correlation with anti-CS IgG levels. No such correlation with any of these anti-bacterial antibody levels was observed in patients with AVR. Of note *M. pneumoniae*, as an intracellular microbe and an obligate parasite, requires the essential metabolites from the host cells. This bacterium can cause cell death and inflammation in the respiratory system [45,46], resulting in the release of CS molecules from damaged cells, which then could lead to increased production of anti-CS antibodies by the immune system. Interestingly, during its evolution *M. pneumoniae* has lost many of its genes, including citrate synthase [47]; therefore, it is not the bacterial CS that causes the elevation of the natural anti-CS IgM and IgG autoantibody levels. *M. pneumoniae* infection can have extrapulmonary manifestations, including infection of the pericardium [40], which may result in increased levels of anti-CS antibodies in the plasma and PF. Our results provide evidence that the pericardial fluid (PF) of cardiac surgery patients contains functionally heterogeneous anti-CS IgM and IgG natural autoantibodies. The correlation of elevated anti-CS IgM levels with the *M. pneumoniae* infection was independent from the disease groups. This may reflect its general protective role as a natural autoantibody, independent of the presence or absence of atherosclerosis. In contrast, anti-CS IgG level showed a strong correlation with higher levels of atherosclerosis-related anti-bacterial antibodies only in patients with coronary artery disease, which reflects its potential protective role in these infections. These data suggest that the PF is being 'surveyed' by the humoral components of the natural and adaptive immune systems in strong co-operation to protect the pericardial space from accumulation of toxic molecules and various pathogenic microbes. Further studies will be needed to investigate the network of natural and anti-bacterial autoantibodies and their role in the pathogenesis of cardiovascular diseases.

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Disclosure

The authors declare no conflicts of interest.

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