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Intramyocardial Synthesis of Pro- and Anti-Inflammatory Cytokines in Infants With Congenital Cardiac Defects

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OBJECTIVES We sought to test the hypothesis that cytokines would be expressed in the myocardium of infants with congenital cardiac defects and to identify the signaling pathways involved.

BACKGROUND Mechanical stress upregulates pro-inflammatory cytokines in the myocardium.

METHODS Fifteen infants with tetralogy of Fallot (TOF) (n = 7) or with ventricular septal defects (VSDs) (n = 8) were investigated. Concentrations of pro- and anti-inflammatory cytokines and of the inducible nitric oxide synthase (iNOS) were measured by enzyme-linked immunosorbent assay and/or Western blotting in the right ventricular myocardium taken during cardiac surgery. Activation of the nuclear factor-kappa-B (NF-kappa-B) and p38 mitogen-activated protein kinase (MAPK) pathways was assessed by electrophoretic mobility shift assay with supershift and/or Western blotting, respectively.

RESULTS The pro-inflammatory cytokines tumor necrosis factor (TNF)-alpha, interleukin (IL)-1-beta, and IL-6 and the anti-inflammatory cytokine IL-10 were detected in the myocardium of all patients. Concentrations of the pro-inflammatory cytokines and also of phosphorylated p38 MAPK were higher in patients with TOF than in those with VSD and correlated with the degree of pressure overload of the right ventricle. Levels of phosphorylated I-kappa-B-alpha, iNOS, and IL-10 were similar in patients with TOF and in those with VSD.

CONCLUSIONS Our results show intramyocardial synthesis of pro-inflammatory cytokines in infants with congenital cardiac defects. This is associated with activation of both the NF-kappa-B and p38 MAPK pathways. The latter could be particularly important for the transduction of mechanical signals in the infant's myocardium. Synthesis of IL-10 indicates an intramyocardial anti-inflammatory potential in this age group. (J Am Coll Cardiol 2003;41:2266–74)

Tumor necrosis factor (TNF)-alpha is an important pro-inflammatory cytokine that belongs to the repertoire of response to injury (1). The nuclear factor kappa-B (NF-kappa-B) family of nuclear transcription factors is critical for the synthesis of TNF-alpha and also for TNF-alpha–induced secondary mediators of inflammation, such as interleukin (IL)-1-beta, IL-6, and inducible nitric oxide synthase (iNOS) (2). The p38 mitogen-activated protein kinase (MAPK) signaling pathway is another pathway involved in intracellular signaling and in the regulation of the production of pro-inflammatory cytokines (3). Stimuli such as mechanical strain (4,5), hypoxia (6,7), and pro-inflammatory cytokines themselves (2,3) lead to the phosphorylation of the inhibitory protein of NF-kappa-B, inhibitory kappa-B (I-kappa-B), and p38 MAPK, thereby promoting the expression of inflammatory genes. In adults with heart failure, increased systemic and intramyocardial synthesis of TNF-alpha has been discussed as a contributor to sustained myocardial dysfunction (8). In contrast, the natural anti-inflammatory cytokine IL-10 could, by deactivation of macrophages and downregulation of pro-inflammatory cytokines, contribute to myocardial protection, as shown in animal models by our group (9) and others (10).

In infants with congenital cardiac defects, intramyocardial cytokine synthesis could be stimulated by mechanical stress (5), hypoxemia (11), and increased systemic cytokine levels (12), but this question has not been addressed so far. Therefore, this study was intended to test the hypothesis that cytokines would be upregulated in the ventricular myocardium of infants with congenital cardiac defects and to identify the signaling pathways involved.

METHODS

Clinical. PATIENTS. After approval by the Human Ethical Committee of the Aachen University of Technology and obtainment of the parents' informed consent, 15 infants age 1.5 to 11.5 months undergoing primary corrective surgery were investigated. Seven patients had tetralogy of Fallot (TOF) and eight had large ventricular septal defects (VSDs) with pulmonary hypertension. All patients with TOF had a mild degree of hypoxemia (arterial oxygen saturation [SaO_2]

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Abbreviations and Acronyms

EMSA	= electrophoretic mobility shift assay
IL	= interleukin
I-kappa-B	= inhibitory kappa-B
iNOS	= inducible nitric oxide synthase
LVP	= left ventricular pressure
MAPK	= mitogen-activated protein kinase
NF-kappa-B	= nuclear factor kappa-B
RVP	= right ventricular pressure
TNF	= tumor necrosis factor
TOF	= tetralogy of Fallot
VSD	= ventricular septal defect

<90%), and all patients with VSD had a left-to-right shunt >50% with volume overload of the left ventricle. Intracardiac pressures were measured at cardiac catheterization. The ratio between the right ventricular and left ventricular systolic pressure (RVP/LVP) was used as an index for RVP overload, independent of the patient's age and degree of sedation during cardiac catheterization. Three patients with VSD had Down syndrome. None of the patients was breastfed at the time of surgery. Patient data are shown in Table 1.

Cardiac operation and sampling of myocardial biopsies. In all cases, conventional general anesthesia consisted of midazolam, fentanyl sulfate, and pancuronium bromide. Dexamethasone (1 mg/m^2 body surface area) was given before the sternotomy. Before institution of cardiopulmonary bypass (CPB), a right atrial biopsy was taken. After institution of hypothermic CPB with a flow index of 2.7 l/min per m^2 body surface area for 15 to 20 min, the aorta was cross-clamped and cardiac arrest was instituted by intra-aortal injection of 4°C cold cardioplegic solution (Bretschneider, 30 ml/kg body weight), which was reaspirated in the right atrium. A right ventricular biopsy was taken from the outflow tract immediately after aortic clamping under deep hypothermia (22°C) and low-flow bypass (0.65 l/min per m^2 body surface area). The time interval between administration of dexamethasone and sampling of ventricular biopsies averaged 91 ± 14 min (mean ± SEM) and was similar in patients with TOF or VSD.

Biopsies taken for detection of messenger ribonucleic acid (mRNA), measurement of protein levels, and analysis of nuclear extracts were immediately snap-frozen in liquid nitrogen and stored at −80°C until processed. Biopsies taken for immunocytochemistry were fixed in B*5 buffer (0.6% $ZnCl_2$ and 0.1% acid acetate), embedded in paraffin, and sectioned into 3-μm thick sections.

Reverse transcriptase-polymerase chain reaction. Total ribonucleic acid (RNA) was extracted from the atrial and ventricular myocardium in all patients by using the RNeasy kit (QIAGEN Inc., Hilden, Germany). The RNA (2 μg) was reverse-transcribed to complementary deoxyribonucleic acid (DNA) with random hexamers. Complementary DNA products were amplified by polymerase chain reaction (35 cycles; 94°C for 45 s, 60°C for 45 s, 72°C for 1 min) with specific human primers for TNF-alpha, IL-10, and iNOS (Clontech, Heidelberg, Germany). The polymerase chain reaction products were subjected to electrophoresis in 1.8% agarose gel and stained with ethidium bromide and photographed. The predicted lengths of the amplification products for TNF-alpha, IL-10, and iNOS were 444, 289, and 259 base pairs, respectively.

Western blotting. Isolated human adult monocytes stimulated with 1 μg/ml endotoxin (lipopolysaccharide by Sigma, St. Louis, Missouri) (13) served as positive controls and nonstimulated monocytes as negative controls. Total protein homogenates (100 μg) from the ventricular myocardium taken in all patients and from monocytes were denatured and separated on 12% and 8% polyacrylamide gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Primary antibodies in immunoblotting were monoclonal mouse anti-human TNF-alpha and IL-10 (R&D Systems, Wiesbaden-Norderstadt, Germany);

Table 1. Epidemiologic Data and Preoperative Hemodynamics

	TOF Group (n = 7)	VSD Group (n = 8)	p Value
Gender (female/male)	2/5	4/4	NS
Age at cardiac catheterization (months)	4.7 ± 0.8	4.5 ± 0.6	NS
Age at operation (months)	6.8 ± 1.4	6.6 ± 0.8	NS
Weight at operation (g)	6,762.8 ± 741.2	5,198.7 ± 617.6	NS
Preoperative digoxin (n)	0	2	NS
RVP* (mm Hg)	69.1 ± 5.4	62.6 ± 5.5	NS
LVP* (mm Hg)	70.1 ± 5.3	78.1 ± 4.1	NS
RVP/LVP ratio*	0.98 ± 0.01	0.79 ± 0.05	0.005
PAP* (mm Hg)	15.0 ± 9.0	30.7 ± 3.3	< 0.1
L-R shunt* (%)	—	71.6 ± 3.6	—
LVEDP* (mm Hg)	8.00 ± 1.53	8.88 ± 0.72	NS
LAP* (mm Hg)	2.5 ± 0.50	6.1 ± 1.45	NS
RAP* (mm Hg)	2.2 ± 0.8	3.6 ± 0.7	NS
SaO_2* (%)	88.8 ± 2.55	95.2 ± 1.22	0.03

Data are presented as the mean value ± SEM. *At cardiac catheterization performed in patients breathing room air.

LAP = mean left atrial pressure; L-R = left to right; LVEDP = left ventricular end-diastolic pressure; LVP = systolic left ventricular pressure; NS = not significant; PAP = mean pulmonary arterial pressure; RAP = mean right atrial pressure; RVP = systolic right ventricular pressure; SaO_2 = arterial oxygen saturation; TOF = tetralogy of Fallot; VSD = ventricular septal defect.

monoclonal mouse anti-human phospho-I-kappa-B-alpha (Ser32/36) (Cell Signaling Technology, Inc., Frankfurt am Main, Germany); monoclonal mouse anti-human iNOS (BD Transduction Laboratories, Heidelberg, Germany), polyclonal rabbit anti-human phospho-p38 (Cell Signaling Technology, Inc.), and monoclonal mouse antihuman beta-actin (Sigma). The bands were detected by a chemiluminescence system according to the manufacturer's instructions (Amersham-Pharmacia, Freiburg, Germany). Protein signals for TNF-alpha, IL-10, iNOS, phospho-I-kappa-B-alpha, and phospho-p38 were normalized for beta-actin signals that were developed on the same blotting (National Institutes of Health [NIH] imaging 1.61b8. software, Bio-Rad Laboratories Inc., Hercules, California).

Enzyme-linked immunosorbent assay (ELISA). Cytokine concentrations in the ventricular myocardium were assessed in all patients by using commercially available ELISA kits for human TNF-alpha, IL-1-beta, IL-6, and IL-10. Frozen tissue was homogenized in ice-cold Tris buffer containing the following protease and phosphatase inhibitors: pepstatin A (2 μg/ml), leupeptin (5 μg/ml), aprotinin (5 μg/ml), and phenylmethylsulfonyl fluoride (PMSF) (1 mmol/l). After centrifugation at 13,000 rpm for 10 min, the samples were kept on ice for the duration of the assay. Total protein levels were quantified using the Bio-Rad protein assay (Bio-Rad Laboratories Inc.), with bovine serum albumin (Sigma) as a standard. Tissue samples were standardized to 1,000 μg total protein in 100 μl diluent buffer for each determination. Standard reference cytokines were provided by the manufacturer. Assays were done in duplicate on a microtiter plate reader (wavelength 450 nm). The assays have a high specificity for the measurement of natural and recombinant human TNF-alpha, IL-1-beta, IL-6 (Cytoscreen, BioSource International Inc., Camarillo, California), and IL-10 (Endogen Inc., Woburn, Massachusetts). Values are reported as pg/ml of standardized sample, which is equivalent to pg/1,000 μg total protein.

Electrophoretic mobility shift assay (EMSA). In six unselected patients (3 in each group), nuclear extracts from the ventricular myocardium were homogenized in hypotonic buffer A (10 mmol/l H EPES/KOH at pH 7.9, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 1 mmol/l Na_3VO_4, 0.2 mmol/l PMSF, 0.5 mmol/l dithiothreitol [DTT]) and incubated for 10 min at 4°C. After centrifugation, the pellet was resuspended with buffer C (20 mmol/l HEPES/KOH at pH 7.9, 420 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 25% (vol./vol.) glycerol, 1 mmol/l Na_3VO_4, 0.2 mmol/l PMSF, 0.5 mmol/l DTT), incubated for 20 min at 4°C, and centrifuged at 14,000 *g* for 2 min, yielding nuclear extracts for further processing. Protein concentrations of nuclear extracts were measured with the Bio-Rad protein assay. A double-stranded oligonucleotide containing the consensus binding motif for the transcription factor NF-kappa-B was used: 5′AGTTGAG*GGGACTTTCCC*AGG-3′, 5′-TGCCT*GGGAAAGTCCCC*TCA-3′ (MWG-Biotech AG, Ebersberg, Germany). The oligonucleotide probe was labeled by filling in 5′-protruding ends with the Klenow enzyme, using (alpha-^{32}P)-adenosine triphosphate (αATP) (10 mCi/ml, 3,000 Ci/nmol). For the supershift experiments, nuclear extracts were preincubated with 4 μg of NF-kappa-B-p50 or -p65 (Santa Cruz Biotechnology, Heidelberg, Germany). The protein-DNA complexes were resolved on a 5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mmol/l Tris HCl [pH 8.0], 20 mmol/l boric acid, 0.5 mmol/l EDTA). Gels were dried and autoradiographed.

Immunocytochemistry. Immunostaining was performed using the immunohistochemistry rabbit or mouse kit (InnoGenex, California) in four patients (2 in each group). Briefly, endogenous peroxidase was blocked by hydrogen peroxide. Sections were incubated with the primary antibodies for 2 h at room temperature. Immunoreaction sites were visualized with the use of the appropriate biotinylated secondary antibodies and horseradish peroxidase-steptavidin conjugate. Peroxidase activity was revealed with a solution of aminoethyl carbazole to produce a red reaction product, and sections were counterstained with Mayer's hematoxylin. Positive and negative controls were performed by staining inflamed and noninflamed human tonsils, respectively (not shown). In the myocardial probes, negative controls were obtained by omitting the primary antibodies. Typical morphologic characteristics for cardiomyocytes, macrophages, and endothelial cells were assessed by using oil microscopy at 1,000-fold magnification.

The following primary antibodies were used: monoclonal mouse anti-human TNF-alpha and anti-IL-10 (all from R&D Systems); polyclonal rabbit anti-human NF-kappa-B-p65 and -p50 (Santa Cruz Biotechnology); and monoclonal mouse anti-human iNOS (BD Transduction Laboratories).

Statistical analysis. Results are expressed as the mean value ± SEM. The Mann-Whitney *U* test was used to analyze differences between groups. Correlation of independent parameters was assessed by the Spearman rank correlation test. A p values <0.05 was considered significant. Data were analyzed with the Statistical Package for Social Sciences (SPSS Software GmbH, Munich, Germany).

RESULTS

Clinical results. Table 1 summarizes the patients' epidemiologic data. Patients with TOF had a significantly higher preoperative RVP/LVP ratio with significantly lower SaO_2 than patients with isolated VSD ($p = 0.005$ and $p = 0.03$, respectively).

Laboratory results. INTRAMYOCARDIAL SYNTHESIS OF PRO- AND ANTI-INFLAMMATORY CYTOKINES. Cytokine mRNA expression in the right atrium: both TNF-alpha and IL-10-mRNA were detected in seven of seven and five of seven patients with TOF and in seven of eight and four of eight patients with VSD, respectively.

Cytokine mRNA expression in the right ventricle: only

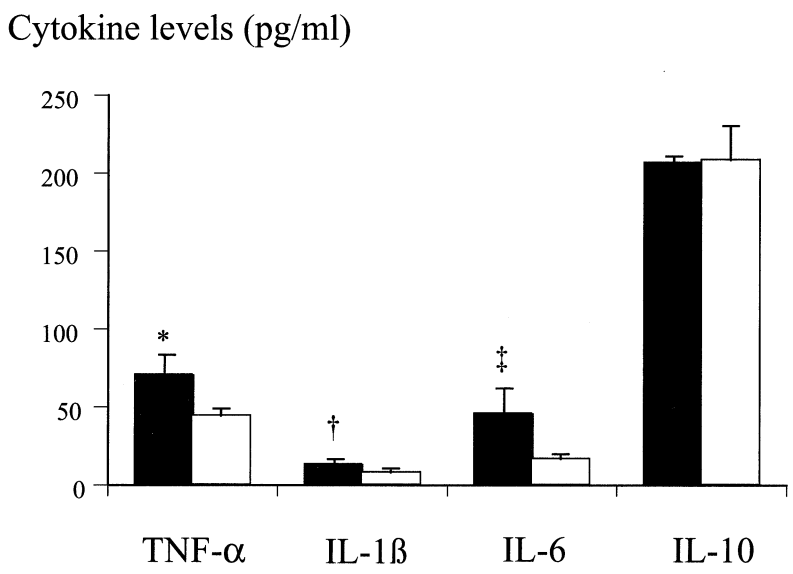

Figure 1. Levels of tumor necrosis factor (TNF)-alpha, interleukin (IL)-1-beta, IL-6, and IL-10 measured by enzyme-linked immunosorbent assay in the right ventricular myocardium of infants with tetralogy of Fallot (**solid bar**; n = 6) or ventricular septal defect (**open bar**; n = 6). Results are expressed as the mean value ± SEM. *$p < 0.02$; †$p < 0.05$; and ‡$p < 0.01$ between groups.

TNF-alpha-mRNA was detected in seven of seven patients with TOF and in seven of eight with VSD.

Cytokine concentrations in the right ventricle: in all patients, TNF-alpha, IL-1-beta, and IL-6 could be detected by ELISA. Their concentrations were higher in infants with TOF than in those with VSD ($p < 0.02$, $p < 0.05$, and $p < 0.01$, respectively) (Fig. 1). Western blotting confirmed this by showing higher levels of TNF-alpha in patients with TOF than in the others ($p < 0.05$) (Fig. 2). In contrast, levels of IL-10 measured by ELISA and Western blotting were similar in both groups (Figs. 1 and 2). Considering all patients, concentrations of TNF-alpha, IL-1-beta, and IL-6 measured by ELISA correlated with the RVP/LVP ratio (Spearman coefficient: 0.66 [$p < 0.05$], 0.64 [$p < 0.05$], and 0.68 [$p < 0.05$], respectively). The RVP/LVP ratio also correlated with the levels of TNF-alpha measured by Western blotting (Spearman coefficient: 0.73 [$p = 0.002$]) (Fig. 3). There was no correlation between cytokine levels and left ventricular end-diastolic pressure, left atrial pressure, degree of left-to-right shunt, or SaO_2.

Immunocytochemistry showed the presence of TNF-alpha and IL-10 in cardiomyocytes, macrophages, and endothelial cells. Figure 4 illustrates the localization of TNF-alpha in cardiomyocytes and that of IL-10 in cardiomyocytes, macrophages, and endothelial cells.

Intramyocardial synthesis of iNOS. Inducible NOS-mRNA was detected in the right atrial myocardium of all patients. Levels of iNOS measured by Western blotting in the ventricular myocardium were similar in patients with TOF and in those with VSD (1.01 ± 0.28 vs. 0.83 ± 0.15, respectively [p = NS]). Immunocytochemistry showed positive staining for iNOS in many cardiomyocytes (Fig. 4).

Activation of NF-kappa-B in the myocardium. The DNA binding activity of NF-kappa-B was detected by EMSA with supershift in the ventricular myocardium in all patients investigated (Fig. 5). Levels of phosphorylated I-kappa-B-alpha measured by Western blotting were similar in patients with TOF and in those with VSD (Fig. 6).

Positive staining for NF-kappa-B-p50 and NF-kappa-B-p65 was present in the cytoplasma and cell nucleus of numerous cardiomyocytes (Fig. 4).

Phosphorylation of p38 MAPK in the ventricular myocardium. Levels of phospho-p38 MAPK detected by Western blotting were higher in patients with TOF than in those with VSD ($p = 0.03$) (Fig. 7). In all patients investigated, they correlated with RVP/LVP (Spearman coefficient: 0.58 [$p < 0.1$]).

DISCUSSION

Presence of pro-inflammatory cytokines in the myocardium of infants with congenital cardiac defects. Our results provide evidence for the intramyocardial expression of pro-inflammatory cytokines in infants with congenital cardiac defects. The presence of cytokine-mRNA in the atrial samples taken before CPB and the presence of cytokines in the ventricular myocardium sampled after a short period of hypothermic CPB, as well as after pretreatment with dexamethasone (14), support the view that the intramyocardial cytokine synthesis reported here was not simply due to cardiac surgery.

In the present series, all patients showed chronic RVP overload. Numerous experimental studies indicate that mechanical stress to the myocardium can induce the expression of a variety of genes to which TNF-alpha belongs (5). This supports our data suggesting an association between pres-

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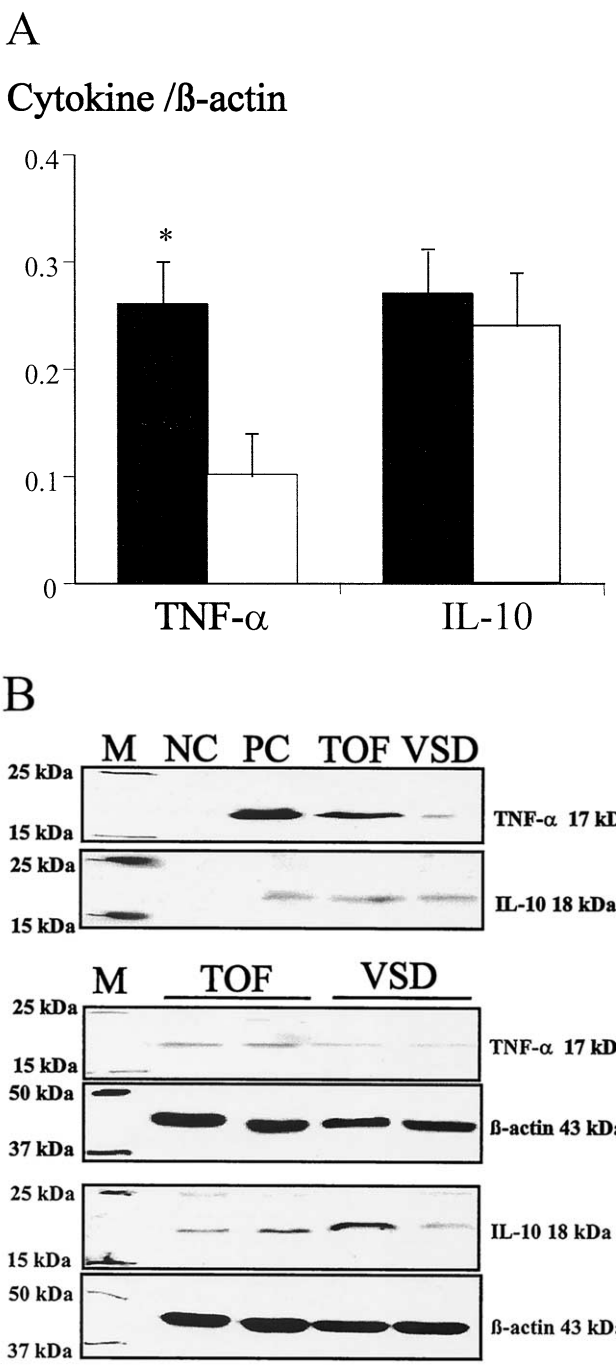

Figure 2. **(A)** Levels of tumor necrosis factor (TNF)-alpha and interleukin (IL)-10 measured by Western blotting in the right ventricular myocardium of infants with tetralogy of Fallot (TOF) (**solid bar**; n = 7) or ventricular septal defect (VSD) (**open bar**; n = 8). Band intensities for TNF-alpha and IL-10 are normalized for bands of beta-actin. Results are expressed as the mean value ± SEM. *$p < 0.01$ between groups. **(B)** Exemplary gels obtained after Western blotting representative of seven experiments in infants with TOF and eight experiments in infants with VSD, showing a higher expression of TNF-alpha but not IL-10 in the former group. The **upper panel** includes positive controls (PC) (human monocytes stimulated with lipopolysaccharide) and negative controls (NC) (nonstimulated human monocytes). M = marker.

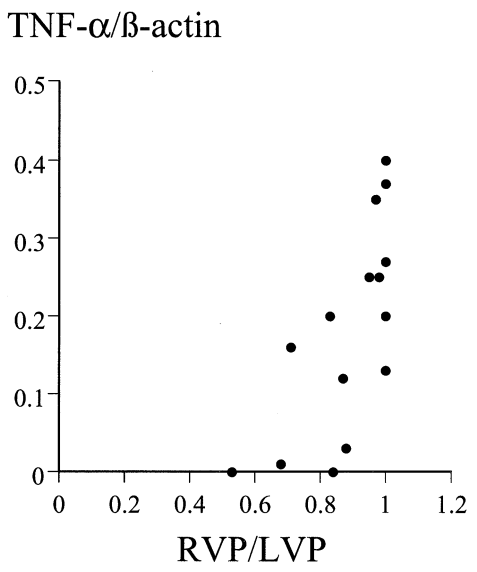

Figure 3. Relationship between the right ventricular pressure (RVP)/left ventricular pressure (LVP) ratio and protein levels of tumor necrosis factor (TNF)-alpha measured by Western blotting in all patients (n = 15). Spearman coefficient: 0.73 ($p = 0.002$).

sure overload of the right ventricle and upregulation of TNF-alpha in the myocardium. However, the role of other stimuli in the induction of pro-inflammatory cytokines in the myocardium is not excluded. Indeed, hypoxemia in patients with TOF or congestive heart failure in patients with VSD might also have contributed to cytokine upregulation. Indeed, we have shown recently that the systemic levels of pro-inflammatory cytokines are elevated in infants with congenital cardiac defects compared with healthy infants (12). In our patients, downstream cytokines such as IL-1-beta and IL-6 were also over-expressed, suggesting the activation of a hierarchical cytokine cascade initiated by TNF-alpha and then sustained by IL-1-beta and IL-6. Because these pro-inflammatory cytokines play a regulatory role in cardiac remodeling (8,15) and possess strong cardiac-depressant properties, they may contribute to the pathophysiology of myocardial hypertrophy and failure in infants with congenital cardiac defects. Furthermore, the presence of these cytokines in the myocardium at the time of cardiac surgery could well enhance the postoperative cytokine-related myocardial damage we recently described (16).

Potential for anti-inflammatory balance in the infant myocardium. A further major new finding of this study is the presence of the anti-inflammatory cytokine IL-10 in the myocardium of infants with congenital cardiac defects. This indicates a potential for cytokine balance in this age group. The fact that patients with TOF had similar intramyocardial production of IL-10 despite of higher levels of pro-inflammatory cytokines than patients with VSD suggests altered cytokine balance in the former. This is in line with our previous results showing that infants with TOF have higher plasma levels of IL-6 but lower concentrations of IL-10 than patients with heart failure (12). Inadequate cytokine balance in patients with TOF might be the result of the repression of IL-10 by chronic hypoxemia (17). In a model of myocardial infarction, IL-10 was shown to be predominantly produced by CD5-positive lymphocytes

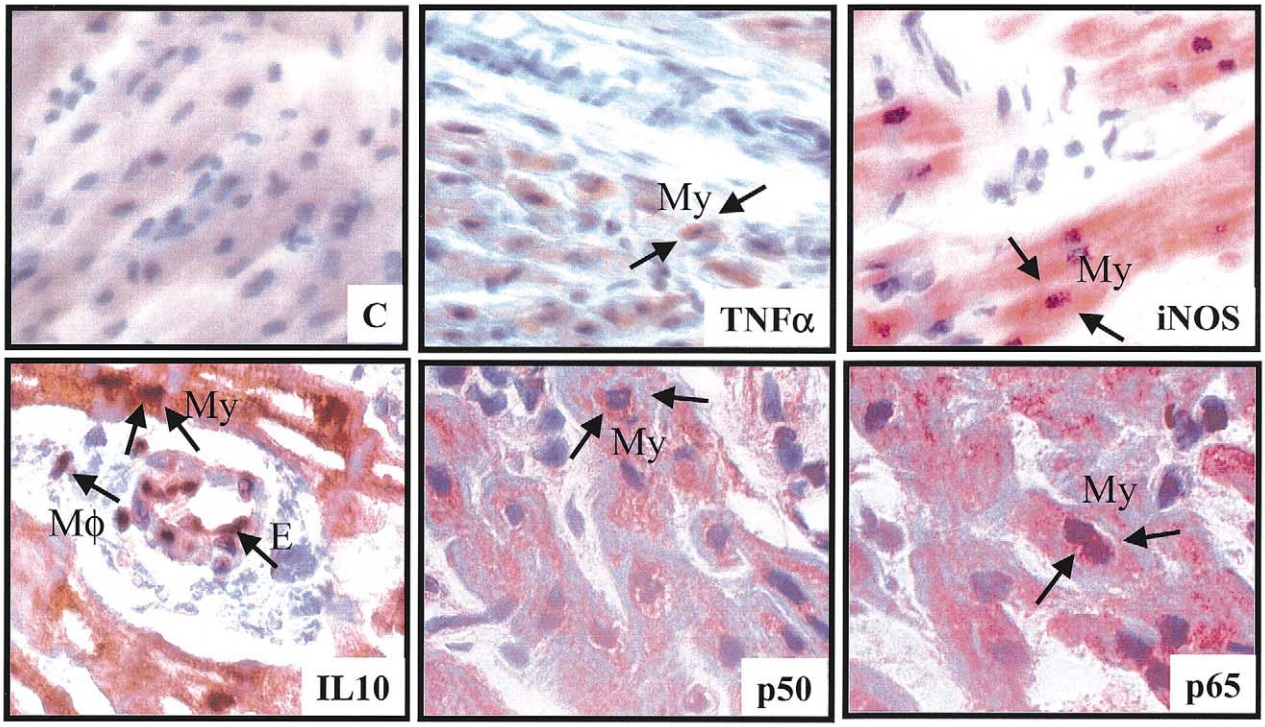

Figure 4. Immunocytochemistry of the right ventricular myocardium in one infant with tetralogy of Fallot, showing the presence of tumor necrosis factor (TNF)-alpha in cardiomyocytes (My), interleukin (IL)-10 in cardiomyocytes, macrophages (Mϕ), and endothelial cells (EC), inducible nitric oxide synthase (iNOS) in cardiomyocytes, and nuclear factor (NF)-kappa-B p50 and p65 subunits in the cytoplasma and nuclei of cardiomyocytes. C = negative control (no primary antibody). Magnification: ×400 for control, cytokines, and iNOS and ×1,000 for NF-kappa-B p50 and p65 subunits. These stains are representative of those obtained in four patients (two in each group).

(18). In our series, cardiomyocytes were overtly involved in IL-10 synthesis. Because IL-10 inhibits TNF-alpha, IL-1-beta, and IL-6 at the transcriptional level, mainly by stimulating the production of I-kappa-B (19), and because IL-10 may inhibit the activation of p38 MAPK (20), the presence of IL-10 in the myocardium of infants with congenital cardiac defects might indicate an adaptive mechanism to limit inflammation-related damage and/or dysfunction.

Activation of the NF-kappa-B and p38 MAPK pathways in the infant myocardium. Nuclear factor-kappa-B is a transcription factor regulating numerous inflammatory genes (2). Recent data suggest that, in adult patients, it might play a key role in the pathophysiology of myocardial ischemia/reperfusion injury and congestive heart failure (2,21). The p38 MAPK cascade belongs to the signal transduction pathways regulating stress responses, inflammation, and apoptosis (3). Mechanical cell stress is one of the common potential stimuli for the activation of both the NF-kappa-B and p38 MAPK pathways (4). On stimulation, several interactions take place between both pathways, which could enhance the inflammatory response (3). In our study, degradation of I-kappa-B-alpha and nuclear translocation of NF-kappa-B in the ventricular myocardium suggest NF-kappa-B activation, confirming the results of a recent study performed in children with congenital cardiac defects (22). Furthermore, our results give the first evidence that the p38 MAPK cascade is also activated in the infant myocardium. The fact that patients with TOF, who had the highest production of pro-inflammatory cytokines, also had the highest levels of phospho-p38 MAPK, as well as the positive correlation between pressure overload of the RV and levels of phospho-p38 MAPK, supports the view that p38 MAPK might be of particular importance for the signal transduction regulating inflammatory processes in response to a mechanical stimulus to the infant myocardium.

Synthesis of iNOS in the myocardium of infants with congenital cardiac defects. Because NF-kappa-B is responsible for TNF-alpha and IL-1-beta–mediated gene expression of iNOS (23) and NO production in cardiomyocytes is enhanced through p38 MAPK-mediated NF-kappa-B activation (24), we expected synthesis of iNOS in our patients. This was confirmed by our results, which go along with those of a recent study where iNOS activity was detected in the right atrial myocardium of children with congenital cardiac defects (25). In that study, iNOS activity was higher in cyanotic than in acyanotic children. In contrast, in our series, the myocardial concentrations of iNOS were not influenced by hypoxemia. The clinical significance of upregulation of iNOS in the myocardium of infants with congenital cardiac defects remains to be established. However, a large body of evidence suggests that high

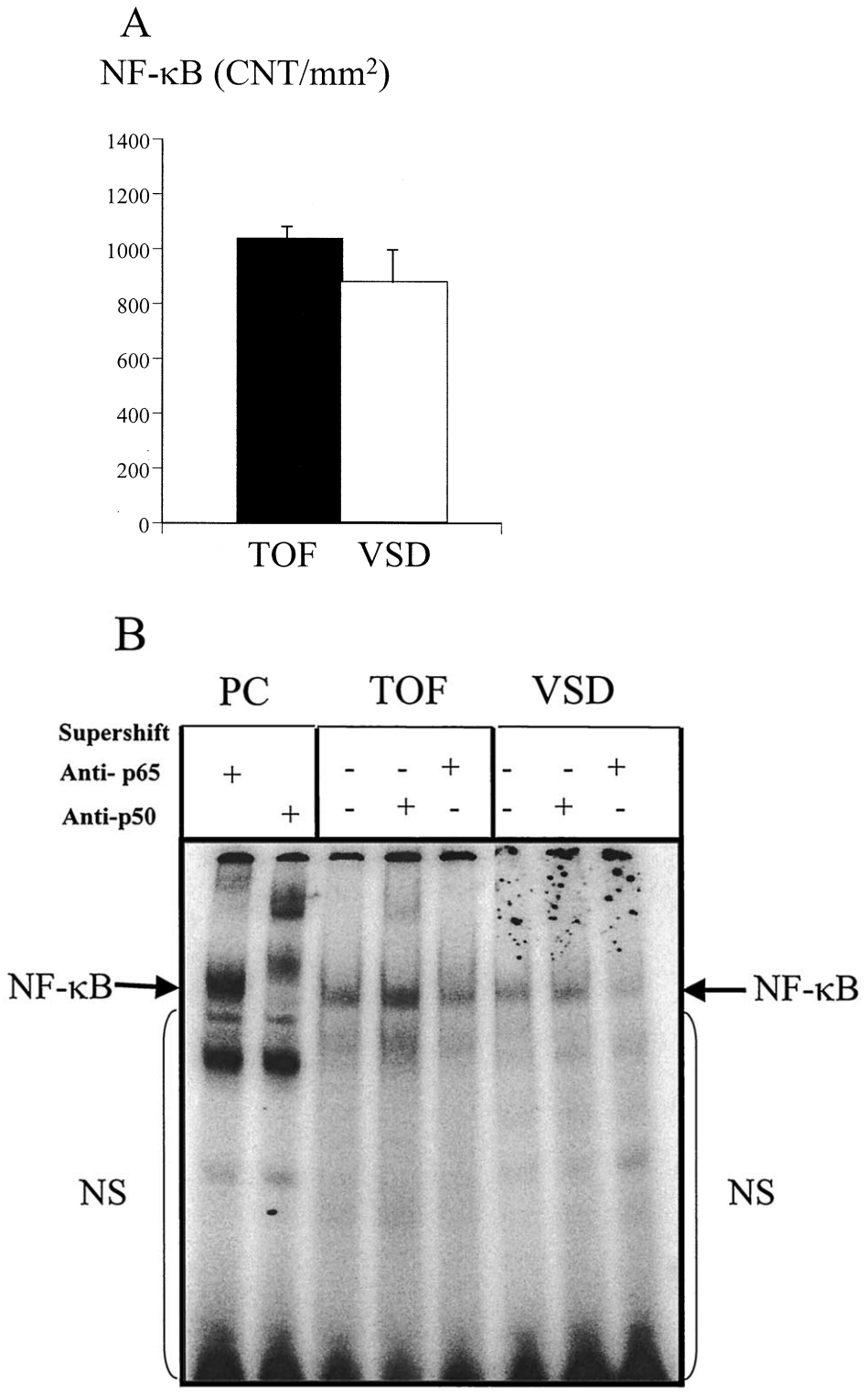

Figure 5. **(A)** Deoxyribonucleic acid (DNA)-binding activity of nuclear factor (NF)-kappa-B measured by EMSA with supershift in the right ventricular myocardium of infants with tetralogy of Fallot (TOF) **(solid bar)** or ventricular septal defect (VSD) **(open bar)**. Results are expressed as counts (CNT)/mm^2. **(B)** Exemplary gel showing the presence NF-kappa-B **(arrow)** detected by electrophoretic mobility shift assay and supershift with anti-p65 and anti-p50 in nuclear extracts of the right ventricular myocardium of one patient with TOF and one patient with VSD. This gel is representative of those obtained in three patients from each group. PC = positive control (human HepG2 cells stimulated by TNF-alpha); NS = nonspecific.

local concentrations of nitric oxide related to induction of iNOS are associated with myocardial cell damage and cell death (26). Nitric oxide mediates the so-called nitric oxide-dependent acute negative inotropic and cytotoxic effects of TNF-alpha, including disruption of calcium handling (27). On the other hand, iNOS could also mediate protective

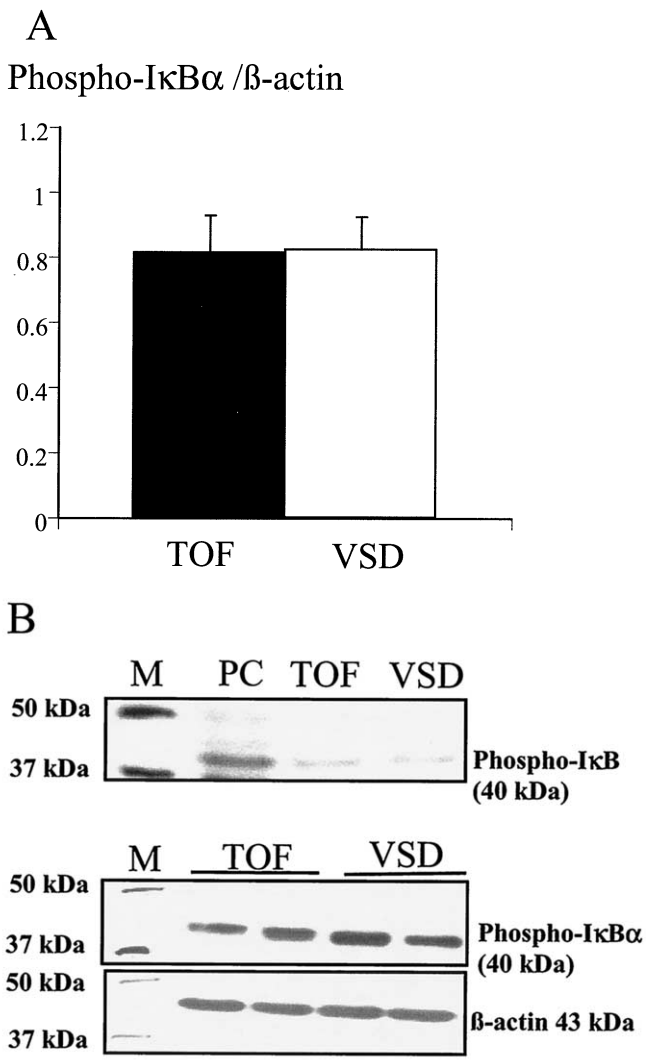

Figure 6. (A) Levels of phospho-I-kappa-B-alpha measured by Western blotting in the right ventricular myocardium of infants with tetralogy of Fallot (TOF) (**solid bar**; n = 7) or ventricular septal defect (VSD) (**open bar**; n = 8). Band intensities for phospho-I-kappa-B-alpha are normalized for bands of beta-actin. Results are expressed as the mean value ± SEM. **(B)** Exemplary gel obtained after Western blotting representative of independent experiments in seven patients with TOF and eight with VSD, showing phosphorylation of I-kappa-B-alpha in all patients. The **upper panel** includes positive controls (PC) (human monocytes stimulated with endotoxin). M = marker.

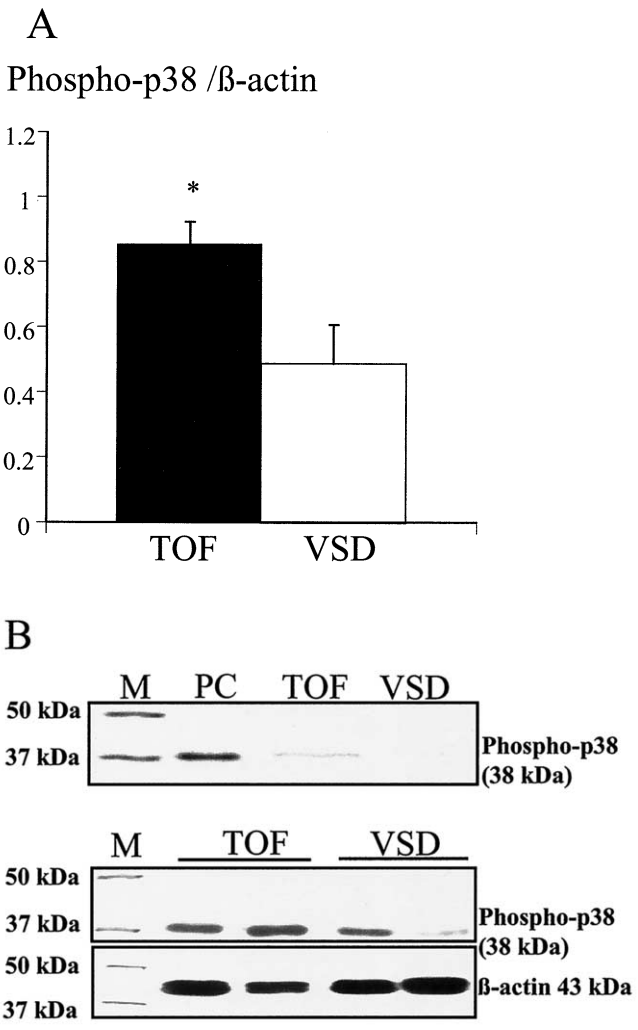

Figure 7. (A) Levels of phospho-p38 mitogen-activated protein kinase (MAPK) measured by Western blotting in the right ventricular myocardium of infants with tetralogy of Fallot (TOF) (**solid bar**; n = 5) or ventricular septal defect (VSD) (**open bar**; n = 5). Results of phospho-p38 MAPK are normalized for the bands of beta-actin and are expressed as the mean value ± SEM. *p < 0.05 between groups. **(B)** Exemplary gel obtained after Western blotting representative of five experiments in each group, showing higher phosphorylation of p38 MAPK in the right ventricular myocardium of patients with TOF than of those with VSD. The **upper panel** includes the positive control (PC; human monocytes stimulated with lipopolysacchardide). M = marker.

effects, as demonstrated in late myocardial ischemic preconditioning (28), suggesting the importance of the nature and timing of the inflammatory stress as to whether iNOS will harm or protect the myocardium.

Further studies are therefore imperative to clarify the role of chronic intramyocardial iNOS synthesis in infants with congenital cardiac defects.

Conclusions. This study demonstrates, for the first time, the synthesis of cytokines in the myocardium of infants with congenital cardiac defects. Stimuli such as pressure overload of the myocardium could upregulate pro-inflammatory cytokines by activating, at least in part, the NF-kappa-B and p38 MAPK pathways. Synthesis of the natural anti-inflammatory cytokine IL-10 in the infant myocardium might counterbalance the inflammatory pathways and could be a potential target for future therapeutic interventions.

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