Immunopathology and Infectious Diseases

Rheumatic Heart Disease

Proinflammatory Cytokines Play a Role in the Progression and Maintenance of Valvular Lesions

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Heart lesions of rheumatic heart disease (RHD) patients contain T-cell clones that recognize heart proteins and streptococcal M peptides. To functionally characterize heart-infiltrating T lymphocytes, we evaluated their cytokine profile, both directly *in situ* and in T-cell lines derived from the heart (HIL). Interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-4, and IL-10 expressions were characterized in 20 heart tissue infiltrates from 14 RHD patients by immunohistochemistry. IFN- γ -, TNF- α -, and IL-10-positive cells were consistently predominant, whereas IL-4 was scarce in the valves. In agreement with these data, the *in vitro* experiments, in which 13 HILs derived from heart samples of eight patients were stimulated with M5 protein and the immunodominant M5 (81-96) peptide, IL-4 was detected in HIL derived from the atrium (three of six) but not from the valve (zero of seven). IFN- γ and IL-10 production were detected in culture supernatants in 11 of 13 and 6 of 12 HILs, respectively. The predominant IFN- γ and TNF- α expression in the heart suggests that Th1-type cytokines could mediate RHD. Unlike in reversible myocardium inflammation, the significantly lower IL-4 expression in the valvular tissue ($P = 0.02$) may contribute to the progression of the RHD leading to permanent valvular damage (relative risk, 4.3; odds ratio, 15.8). The lack of IL-4 *in vitro* production by valve-derived HIL also emphasizes the more severe tissue destruction in valves observed in RHD. (*Am J Pathol* 2004, 165:1583–1591)

The pathogenesis of rheumatic fever (RF) in susceptible individuals is related to autoimmune humoral and cellular responses directed toward human tissues, triggered by the response to β -hemolytic group A streptococci. Rheumatic heart disease (RHD) occurs in 30 to 45% of RF patients. Cardiac involvement induces pericardium, myocardium, and endocardium inflammation, leading to permanent damage of heart valves.¹ Mitral valve immunohistochemistry studies with acute RF/RHD patients show the predominance of CD4⁺ T cells and macrophages at the site of heart lesions.^{2,3} Apparently, adhesion molecules such as VCAM-1 facilitate the extravasation of CD4⁺ and CD8⁺ T cells into the valves, after being up-regulated on the vascular endothelium of the mitral valve of ARF patients.⁴

Our group has previously reported that intralesional CD4⁺ T-cell clones from surgical fragments of severe RHD patients crossreact with immunodominant M5 peptides and heart tissue proteins.⁵ These results indicate the existence of T-cell molecular mimicry between β -hemolytic streptococci and heart tissues, suggesting that this may be a relevant mechanism in the pathogenesis of RHD.⁵

Inflammatory cytokines as soluble mediators have been implicated in the immune response in several rheumatic diseases.⁶

Evaluation of the level of proinflammatory cytokines produced by peripheral blood and tonsillar mononuclear cells of RF/RHD patients without congestive heart failure, after streptococcal antigen and pokeweed mitogen stimulation, showed different patterns on peripheral blood mononuclear cells (PBMCs) and tonsillar cells. Tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-2 were overproduced by PBMC and were decreased in tonsillar

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mononuclear cells.⁷ The increased production of IL-2 in acute rheumatic fever (ARF) and in patients with active RHD has been reported. Additionally, these patients also exhibit high numbers of CD4⁺ and CD25⁺ cells, suggesting the expansion of activated T CD4⁺ cells in peripheral blood during the active phase of the disease.⁸ However, it should be mentioned that these cells could also be CD4⁺CD25⁺ regulatory T cells, more recently described.⁹ Other authors have confirmed these findings, also showing increased plasma levels of TNF- α in RF/RHD patients.^{10–12}

In heart lesions, the Aschoff nodule is considered as a pathognomonic sign of ARF. It is a granulomatous lesion localized mainly in the endocardium, subendocardium, or perivascular regions of myocardial interstitium. The Aschoff nodule has different stages, which involve different cells (Anitschkow cells, multinucleated cells, few lymphocytes, macrophages, plasma cells, and polymorphonuclear leukocytes).¹³ It has recently been described that the production of IL-1, TNF- α , and IL-2 in the valvular lesions of ARF patients was correlated with Aschoff nodule progression—IL-1 and TNF- α were secreted by monocytes/macrophages in stages 1 and 2; IL-2 was secreted by T lymphocytes in stage 3.¹⁴ These results suggest a major role for inflammatory cytokines in mediating heart lesions in RF.

TNF- α has also been implicated in the pathogenesis of viral myocarditis.^{15–18} Recently, TNF- α was described as protective in the acute stages of viral myocarditis, contributing toward the elimination of infectious viruses, whereas in chronic stages TNF- α would have deleterious effects on the cardiovascular system.¹⁹ Proinflammatory cytokines also play an important role in Chagas' disease cardiomyopathy. In a recent study, CD4⁺ and CD8⁺ T cells from heart tissue-derived T-cell lines obtained from Chagas' disease patients produced a Th-1-type cytokine response, with increased production of TNF- α and interferon (IFN)- γ , when stimulated with *Trypanosome cruzi*-specific protein B13.²⁰ *In situ* determination of myocardium-infiltrating CD8⁺ T cells was positively correlated with the number of IFN- γ -producing cells.²¹ Increased levels of TNF- α in the sera of Chagas' disease cardiomyopathy patients suggests a role for this cytokine in the progression of the disease.²²

In RHD patients a persistent inflammatory process occurs in the heart tissue, in the absence of the infectious agent. Our group has been working on the hypothesis that crossreactive T cells have a crucial role in both triggering and maintaining inflammation in the heart. In the present study, we addressed the question of whether cytokine imbalances could contribute toward the perpetuation of inflammatory autoimmune reactions in RHD heart lesions. We analyzed the *in situ* cytokine profile of heart-infiltrating mononuclear cells from the heart tissue of severe RHD patients in the acute or chronic phases of the disease. The cytokine pattern produced *in vitro* by heart-infiltrating T-cell lines (HILs) derived from heart lesions of severe RHD patients in response to the M5 protein and to the immunodominant streptococcal M5(81-96) peptide^{5,23} was also evaluated, to better understand how these cells respond during the recurrence of throat infection episodes, reactivating RHD.

Patients and Methods

Patient Samples

The present study included 18 severe RHD patients seen at the Heart Institute, Clinical Hospital, University of São Paulo (HC-FMUSP). Patients were selected according to Jones' modified criteria.²⁴ RHD patients were followed by pediatric cardiologists for at least 5 years. Eight patients had more than one episode of ARF. Nine patients had recovered from disease reactivation (patients 1 to 5, 12, 14, and 16) and surgical fragments were collected during episodes of ARF recurrence for five of them; two patients (patients 6 and 7) had fragments collected 4 and 6 months after the ARF episode, respectively. For patients 8, 9, 10, 11, 13, 15, 17, and 18, heart surgical samples were collected during the chronic phase of RHD. Clinical and histopathological data are summarized in Table 1. Samples were surgical fragments obtained from the hearts of RHD patients during valve correction surgery. The surgical fragment collection procedures were approved by the Heart Institute Ethics Committee (HC-FMUSP).

HILs

T-cell lines were derived from mitral or aortic valve (Mi v, Ao v.) or left atrium (LA) surgical fragments. Tissue finely minced with injection needles and small scissors was placed in Falcon flat-bottom 96-multiwell plates (Becton Dickinson, Lincoln Park, NJ) with Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO), supplemented with 2 mmol/L L-glutamine (Sigma), 10% pooled normal human serum, 10 mmol/L Hepes (Sigma), antibiotics (gentamicin and Peflacyln) at the concentration of 40 μ g/ml and 20 μ g/ml, respectively, and 40 U/ml of human recombinant IL-2 (Biosource Inc., Camarillo, CA), on an HLA-DR-matched feeder layer of PBMCs at 10⁵ cells/well, irradiated at 5000 rads. HILs were maintained by two or three PHA-P stimulations in the presence of IL-2 and irradiated feeder cells.

Flow Cytometry Analysis—Fluorescence-Activated Cell Sorting

T-cell subpopulations from intralesional T-cell lines were analyzed using anti- $\alpha\beta$ TCR-fluorescein isothiocyanate (T10B9.1A-31) (Pharmingen, San Diego, CA), anti-CD3-fluorescein isothiocyanate (UCHL1), anti-CD4-phycoerythrin (MT310), and anti-CD8-fluorescein isothiocyanate (DK 25) monoclonal antibodies (Dakopatts, CA). Events (10 $\times$ 10³) gated on lymphocytes were analyzed using a FACScan flow cytometer and CellQuest software (Becton-Dickinson, Mountain View, CA).

Immunohistochemistry

Four- μ m sections were cut from cardiac tissue prepared from frozen surgical fragment specimens embedded in O.C.T. 4583 (Miles Laboratories Inc., Naperville, IL). Anti-

Table 1. Identification and Clinical and Histopathological Data of Severe RHD Patients

Patient no.	Sex	Age	ARF*	Clinical date	Histopathology†
1	M	12	01	Severe Mi v regurgitation	Mitral valvulitis, neutrophilic infiltrate, myocardium fiber hypertrophy
2	M	15	05	Severe Mi/Ao v regurgitation	Mitral valvulitis, neutrophilic infiltrate, myocardium fibrosis, and fiber hypertrophy
3	M	09	01	Severe Mi/Ao v regurgitation	Mitral and aortic valvulitis, neutrophilic infiltrate
4	M	11	01	Severe Mi/Ao v regurgitation	Valvulitis, neutrophilic infiltrate, Aschoff bodies in LA, histiolympocytic reactivity, fiber hypertrophy
5	M	11	02	Severe Mi/moderate Ao v regurgitation	Aschoff bodies in PM, histiolympocytic reactivity, pericarditis, myocardium hypertrophy
6	F	05	02	Severe Mi/moderate Ao v regurgitation	Valvulitis, neutrophilic infiltrate, Aschoff bodies in LA
7	M	06	01	Severe Mi/moderate Ao v regurgitation	Chronic valvulitis, histiolympocytic reactivity, Aschoff bodies in papillar muscle
8	M	10	01	Severe Mi/Ao v regurgitation	Chronic valvulitis, myocardium histiolympocytic reactivity, fibrosis, and fiber hypertrophy
9	M	13	02	Severe Mi/Ao v regurgitation	Aortic valve mucoid degeneration
10	M	11	02	Severe Mi/moderate Ao v regurgitation	Myocardium histiolympocytic reactivity, fibrosis, and fiber hypertrophy
11	M	20	01	Severe Mi stenosis, moderate Mi v regurgitation	Chronic valvulitis, lymphocytic infiltrate, myocardium calcification
12	F	13	01	Severe Mi v regurgitation	Valvulitis, neutrophilic infiltrate, and lymphocytic infiltrate
13	F	14	01	Severe Mi v regurgitation	Chronic valvulitis, interstitial fibrosis, histiolympocytic reactivity, myocardium hypertrophy
14	M	16	01	Severe Mi/Ao v regurgitation	Myocardium neutrophilic infiltrate
15	F	19	02	Severe Mi v regurgitation	Chronic valvulitis, interstitial fibrosis, focal histiolympocytic reactivity, myocardium hypertrophy
16	M	14	01	Severe Ao/moderate Mi v regurgitation	Mononuclear and neutrophilic infiltrate in Ao v. and aortic fibrosis
17	M	12	03	Severe Ao/moderate Mi v regurgitation	Valvular fibrosis, myocardium hypertrophy
18	M	14	02	Severe Mi/Ao v regurgitation	Chronic valvulitis, myocardium hypertrophy

M, male; F, female.

*Recurrence episodes.

†Data from H&E; LA, left atrium; Mi v, mitral valve; Ao v, aortic valve; PM, papillar muscle; acute rheumatic fever: presence of neutrophilic infiltrate and/or Aschoff bodies (granulomatous lesions)¹³; chronic rheumatic fever: presence of lymphocytic infiltrate and vascular proliferation; rare eosinophils were found in all tissue samples analyzed.

CD20 (L26), CD68 (KP1), anti-CD4 (MT 310), and anti-CD8 (DK 25) (DAKO, Carpinteria, CA) monoclonal antibodies were used to define B-, monocytes/macrophages, and T-cell subpopulations, respectively. For cytokine determination we used anti-IFN- γ (mAb 1004) and anti-IL-4 (mAb 45/6) (Chemicon Int., Inc., Temecula, CA) monoclonal antibodies and goat IgG polyclonal anti-IL-10 antibody (R&D Systems, Minneapolis, MN). For TNF- α we used rabbit polyclonal antibody (AB 1400) (Genzyme, Cambridge, MA). A secondary step was performed using biotinylated swine anti-mouse, -goat, and -rabbit immunoglobulins (E-0453; DAKO/S, Glostrup, Denmark). Peroxidase-coupled avidin (Dakopatts, CA) was later added for IFN- γ , IL-4, and TNF- α , and alkaline-phosphatase-coupled avidin for IL-10. Reactions were developed with diaminobenzidine (Sigma) and Fast Red (Sigma), respectively. Negative controls were performed with heart tissue samples without the primary antibodies and also with a nonspecific IgG monoclonal antibody (anti-HBs).²⁵ Positive cells were evaluated on 5 to 50 microscopic fields. We considered +, <10%; ++, 10 to 50%; and +++, >50% as positive mononuclear cells. Slides were also co-stained with hematoxylin and eosin to evaluate histological features. Figure 1, E and F, shows images of ARF in myocardium and mitral valve tissue, respectively, from one patient (patient 4).

In Vitro Cytokine Production Assays

Culture supernatants from 5×10^5 HILs and HLA-DR-matched irradiated PBMCs (5×10^5 /well), incubated with either 5 μ g/ml of the streptococcal M5(81-96) peptide (81-DKLKQQRDTLSTQKET-96, based on the sequence published by Manjula and colleagues²⁶) or with 10 μ g/ml of purified M5 protein (endotoxin <50 EU/mg of protein), kindly provided by Dr. Madeleine W. Cunningham (University of Oklahoma Health Sciences Center, Biomedical Research Center, Oklahoma City, OK), were harvested at 48 hours, centrifuged to eliminate cell debris, and kept at -80°C until use. The supernatants of 5×10^5 HILs and HLA-DR-matched irradiated PBMCs (5×10^5 /well) without added antigens were used as controls for baseline cytokine production. Antigen-induced cytokine production was determined by subtracting baseline production.

Enzyme-Linked Immunosorbent Assay Sandwich Assays

Supernatants from M5(81-96) peptide stimulation were tested for IL-4, IL-10 (Pharmingen), and IFN- γ (Endogen, Woburn, MA) through enzyme-linked immunosorbent assay sandwich assays. Briefly, 96-well plates (Easywash; Costar, Cambridge, MA), were coated with anti-cytokine capture antibodies and incubated overnight at 4°C (pH

Figure 1. Cytokines produced by infiltrating mononuclear cells from heart tissue fragments of RHD patients. Peroxidase (DAB) and alkaline phosphatase (Fast Red) immunohistochemistry were used for IFN- γ , TNF- α , IL-4, and IL-10 determination. The results were indicated by the number of positive cells by field. **A:** Positive cells (++) (10 to 50%) producing IFN- γ (patient 5); **B:** positive cells (++) (10 to 50%) producing TNF- α (patient 10); **C:** >50% of positive cells (+++) producing IL-4 with focal distribution (patient 4); **D:** 10 to 50% of positive cells (++) producing IL-10 indicated by **long arrows** on the **right** and >50% of positive cells (+++) in blood vessels indicated by **large** and **small arrows** on the **left** (patient 6). **E:** LA from patient 4; Aschoff bodies are indicated by **short arrows** and mononuclear infiltrating cells by **long arrows**. **F:** Mitral valve from patient 4; vegetations are indicated by **short arrows** and neutrophilic infiltrations by **long arrows**. H&E stain. Original magnifications: $\times 160$ (**A–D**); $\times 20$ (**E, F**).

9.6). After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.05% sodium azide and blocking with PBS-0.5% bovine serum albumin for 2 hours, plates with samples and standards in duplicates were incubated overnight at 4°C. Biotinylated secondary antibody specific for each cytokine was added to plates. After a 45-minute incubation, plates were washed and avidin-peroxidase was added for 30 minutes. Assays were developed with *o*-phenylenediamine and OD was measured at 490 nm using an enzyme-linked immunosorbent assay reader (model 1550; Bio-Rad Laboratories, Hercules, CA). Analysis was performed using Microplate Manager 4.0 software (Bio-Rad), based on a standard

concentration curve. Cytokine detection levels were 4 to 5000 pg/ml for IL-4 and IL-10 and 10 to 2000 pg/ml for IFN- γ .

Cytokine Expression at the RNA Level—Reverse Transcriptase-Polymerase Chain Reaction

We also analyzed cytokine mRNA for a re-evaluations from five intralesional T-cell lines (two Mi v and three LA), from patients 1, 3, and 9, at 12 and 48 hours after M5(81-96) peptide stimulation. RNA was extracted and cDNAs were prepared from 1 to 10 μ g of total RNA using AMV reverse transcriptase (cDNA cycle kit; Invitrogen, The

Table 2. Infiltrating Mononuclear Cells and Cytokine Profile in Heart Tissue of RHD Patients

Patient no.	Heart tissue	Mo	B	T cells		CD4/CD8	IFN- γ	TNF- α	IL-4	IL-10
				CD4+	CD8+					
3*	Mi v	NT	1.5	2.4	1.7	1.4	+++	++	+	++
4*	L A	1.6	0.3	2.6	2.0	1.3	+++	+++	+++	+++
5*	Mi v	0.9	2.7	1.5	0.4	3.7	++	+++	+	+/+++ [†]
6*	Mi v	0.9	2.2	1.6	0.6	2.7	+++	+++	+	+/+++ [†]
9	R A	NT	0	0.3	0.3	1.0	+++	+++	+	Neg
10	Mi v	NT	0	3.1	0.1	31.0	+++	++	Neg	+
11	Mi v	0.9	1.7	3.9	0.3	13.0	+++	+++	++	+++
12*	Mi v	56.0	1.4	2.5	0.8	3.1	+++	++	+	+/+++ [†]
	PM	0.9	0.3	0.3	0.2	1.5	++	++	+	+/+++ [†]
13	Mi v	1.1	0.02	0.2	0	—	+	+	+	+/+++ [†]
	PM	3.4	0.1	1.0	0	—	+++	++	++	+/+++ [†]
14*	LA	0.9	0.3	0.7	0.3	2.3	+++	+++	++	+/+++ [†]
15	Mi v	0.5	0.2	0.2	0.1	2.0	++	+++	+	+/+++ [†]
	LA	0.3	0	0.3	0	—	++	++	+/+++ [†]	+/+++ [†]
16*	Ao v	5.9	0	2.4	0.9	2.7	++	++	+/+++ [†]	+/+++ [†]
	LA	0.8	0	0	0.4	—	+++	++	++	+/+++ [†]
17	Ao v	0.03	0.2	NT	NT	—	++	Neg	Neg	+
	LA	0.4	0.1	0.1	0.03	3.3	+++	Neg	+/+++ [†]	+/+++ [†]
18	Mi v	20.0	0.3	0.9	0.5	1.8	+++	+++	+/+++ [†]	+/+++ [†]
	LA	0.3	0.3	NT	NT	—	+++	++	+/+++ [†]	+/+++ [†]

*Patients with acute rheumatic fever (ARF) episodes; RA, right atrium; Mi v, mitral valve; Ao v, aortic valve; L A, left atrium; Immunohistochemistry was performed in valve tissue for 5 patients (nos. 3, 5, 6, 10, 11); only in myocardium for patients 4, 9, and 14; in both valve tissue and myocardium for patients 12, 13, 15, 16, 17, and 18; Mo, monocyte/macrophage; B, B-lymphocytes; Neg, negative; NT, not tested. Positive cells were evaluated on 5 to 50 microscopic fields as +, <10%; ++, 10 to 50%; and +++, >50% positive mononuclear cells.

[†]Cytokines detected in the vessel.

Netherlands). IL-4, IL-10, and IFN- γ mRNA detection was done by polymerase chain reaction using specific primers (IL-4: 5'-TTC TAC AGC CAC CAT GAG AAG and 3'-CAG CTC GAA CAC TTT GAA TAT; IL-10: 5'-ATG CCC CAA GCT GAG AAC CAA GAC CCA and 3'-TCT CAA GGG GCT GGG TCA GCT ATC CCA; and IFN- γ : 5'-CCA GAG CAT CCA AAA GAG TGT and 3'-CTA GTT GGC CCC TGA GAT AAA-5') (Genset).

Flow Cytometry Analysis—Fluorescence-Activated Cell Sorting

Supernatants of HILs stimulated with purified M5 protein were tested by flow cytometry analysis, using a cytometric bead array (CBA) kit (BD Biosciences Pharmingen). Briefly, human Th1/Th2 (IL-4, IL-5, IL-10, IFN- γ) phycoerythrin-coated cytokines were incubated (20 μ l) with a mixture of capture beads (20 μ l) and 50 μ l of culture supernatants for 3 hours at room temperature, protected from light, and then washed with sample buffer. Negative and positive controls were 50 μ l of fluorescein isothiocyanate/phycoerythrin and amounts of recombinant cytokines determined by a standard curve, respectively. CellQuest software was used for sample analysis and data were formatted using BD CBA software. Results were based on a standard concentration curve. Cytokine detection levels were 2.6 to 5000 pg/ml for IL-4; 2.8 to 5000 pg/ml for IL-10; and 7.1 to 5000 pg/ml for IFN- γ .

Statistical Analysis

Fisher's exact test was used to compare frequencies of cytokine production by heart tissue-infiltrating mononu-

clear cells. *P* value, relative risk (RR), and odds ratio (OR) were determined.

Results

T-Cell Subsets and Cytokine Profile in Heart Tissue

Immunohistochemical determination of mononuclear cell subsets and cytokine profile was performed in 20 heart-tissue fragments obtained from 14 patients: 11 fragments were from valves (nine Mi v and two Ao v) and 9 from the myocardium (one right atrium, six left atrium, and two papillary muscle). Both myocardium and valve samples were analyzed in six patients (patients 12, 13, 15, 16, 17, and 18). CD4⁺ T cells were predominant among infiltrating mononuclear cells mainly in the valvular tissue (8 of 10 valve fragments). CD8⁺ T cells, B cells, and macrophages were also observed (Table 2).

IFN- γ and TNF- α were detected in 50% or more infiltrating mononuclear cells in all myocardium (9 of 9) and 10 of 11 valvular tissue fragments analyzed (Table 2, Figure 2). Only one patient (patient 13) had less than 10% of mitral valve tissue-infiltrating mononuclear cells producing IFN- γ and TNF- α . Patient 17 did not present mononuclear cells positive for TNF- α in either LA or mitral valve tissues (Table 2). IFN- γ - and TNF- α -positive cells are indicated in Figure 1, A and B, respectively.

IL-4 was produced by large numbers of myocardium-infiltrating cells (at least 50%) in seven of nine (78%) LA samples (Table 2 and Figure 2), showing a focal pattern for most samples, as illustrated in Figure 1C. In contrast, few mitral valve-infiltrating cells produced IL-4. Only 2 of

Figure 2. Summary of Th1 and Th2 cytokines detected in infiltrating mononuclear cells from heart tissue samples of RHD patients. IFN- γ , TNF- α , IL-4, and IL-10 were determined in heart tissue samples by immunohistochemistry as shown in Figure 1. The frequencies of each cytokine were determined based on data from Table 2. *, <10% of positive cells, valve versus myocardium, $P = 0.02$, RR = 4.3, OR = 15.8, indicates the probability of valvular RHD lesion progression; **, >10 of positive cells, valve versus myocardium, $P = 0.02$, RR = 0.2, OR = 0.06, indicates a regulatory effect of IL-4 secretion leading to myocarditis healing.

11 (18%) valve fragments showed at least 50% mononuclear cells positive for IL-4, (patients 11 and 18), whereas 9 of 11 valve fragments (82%), showed less than 10% IL-4-positive cells (Table 2, Figure 2). The difference in the frequencies of valve and myocardium fragments bearing IL-4-positive infiltrating cells was significant ($P = 0.02$). This pattern was confirmed by an analysis of both myocardium and valvular tissue from the same patients (patients 12, 13, 15, 16, 17, and 18). The single exception was patient 18 who showed large amounts of IL-4 in both myocardium and mitral valve. Nine patients had large proportions of IL-10-producing cells (Table 2, Figure 2). Most samples analyzed (14 of 20) also revealed the presence of IL-10 on the vessel surface (Table 2, Figure 1D). Immunohistochemical analysis showed no differ-

ence between samples from ARF or chronic RHD patients regarding the cytokine pattern produced by infiltrating cells.

Cytokine Profile of HILs after Stimulation with Purified M5 Protein and M5(81-96) Peptide

HILs were derived from five mitral valve, two aortic valve, and six LA fragments of eight patients. Ten of twelve HILs (83%) showed a predominance of CD4⁺ T cells (Table 3). Only patient 2 showed more than 15% CD8⁺ T cells in both mitral valve-derived and LA-derived T-cell lines (29% and 38.5%, respectively) (Table 3).

Cytokine production by HILs was first analyzed after stimulation with purified M5 protein and with the M5(81-96) immunodominant and cross-reactive epitope of the M5 protein.^{5,23} Of the HILs, eight were stimulated with the purified M5 protein and 13 with the M5(81-96) peptide. IFN- γ was produced by all T-cell lines stimulated with the M5 protein and by 9 of 13 HILs (69%) stimulated with the M5(81-96) peptide. As a whole, IFN- γ production was detected in the culture supernatants of 11 of 13 HILs (85%) with a concentration ≤ 90 pg/ml, with the exception of three HILs (Ao v no. 3, 73 pg/ml; Ao v no. 16, 23 pg/ml; and LA no. 2, 10 pg/ml) (Table 3). IL-4 and/or IL-10 were produced by LA-HILs (five of six) stimulated with the M5(81-96) peptide. When cells were stimulated with purified M5 only, IL-10 was detected in both myocardium (three of six) (LA no. 2, 8, and 17) and valve (two of seven) [Ao v no. 3 (low concentration) and Ao v no. 16]. Interestingly, IL-4 was not detected in the culture supernatants of valve-derived T-cell lines when stimulated with either the M5(81-96) peptide or with purified M5 protein (zero of seven) (Table 3).

We also determined cytokine production at the RNA level, in five HILs (from patients 1, 3, and 9), at 12 and 48

Table 3. Cytokine Profile of Heart-Infiltrating T-Cell Lines (HILs) Stimulated with Purified M5 Protein and M5(81-96) Peptide

Patient no.	RHD clinical presentation	HIL	Cytokines (pg/ml)							
			T cells		IFN- γ		IL-4		IL-10	
			CD4 ⁺	CD8 ⁺	M5 protein	M5 (81-96)	M5 protein	M5 (81-96)	M5 protein	M5 (81-96)
1	ARF	Mi v	97.4	1.5	NT	390	NT	<4	NT	<4*
		LA	99.1	0	NT	219	NT	<4	NT	726
2	ARF	Mi v	22.0	29.0	NT	<10	NT	<4	NT	<4
		LA	49.2	38.5	123	10	<2.6	220	6.5	30
3	ARF	Mi v	79.1	0.6	NT	<10	NT	<4	NT	<4*
		Ao	86.4	0.6	73	<10	<2.6	<4	2.8	<4
4	ARF	v								
4	ARF	LA	59.7	13.0	NT	265	NT	35	NT	<4
7	Post ARF	Mi v	87.2	7.4	3096	100	<2.6	<4	<2.8	<4
8	Ch RHD	Mi v	91.0	6.0	520	<10	<2.6	<4	<2.8	<4
		LA	76.6	8.5	1516	490	<2.6	<4	5.4	<4
9	Ch RHD	LA	98.6	1.2	132	90	<2.6	NT*	<2.8	NT*
17	Ch RHD	Ao	NT	NT	2604	23	<2.6	<2.6	56	<4
		V								
		LA	98.7	0.4	1848	930	<2.6	<2.6	7.5	<4

ARF, acute rheumatic fever; Ch RHD, chronic rheumatic heart disease; HIL, heart-infiltrating T-cell lines; Mi v, mitral valve; Ao v, aortic valve; LA, left atrium; NT, not tested.

*Positive by RT-PCR (Figure 3); M5 protein-induced cytokines analyzed by FACS using CBA kit; M5 (81-96) peptide induced cytokines analyzed through ELISA assays. Detection limits by FACS: <2.6 pg/ml for IL-4, <2.8 pg/ml for IL-10, and <7.1 pg/ml for IFN- γ ; by ELISA: 4 pg/ml for IL-4 and IL-10, and 10 pg/ml for IFN- γ .

A: RT-PCR product for one representative patient

Patient # 1

B: Summary of cytokine pattern at RNA level of 5 HIL from patients # 1, 3 and 9

Patients #	HIL	IFN- γ		IL-4		IL-10	
		12h	48h	12h	48h	12h	48h
1	Mi v	+	-	-	-	+	-
1	LA	+	+	-	-	+	+
3	Mi v	-	-	-	-	+	+
3	Ao v	-	-	-	-	-	-
9	LA	-	-	+	-	+	+
		2/5	1/5	1/5	0/5	4/5	3/5

Figure 3. Cytokine expression at the RNA level in five HILs after stimulation with the M5(81-96) peptide. Reverse transcriptase-polymerase chain reaction detection of IFN- γ , IL-4, and IL-10 mRNA from LA- and mitral and aortic valve (Mi v and Ao v)-derived T-cell lines at 12 and 48 hours after M5(81-96) stimulation. **A:** Reverse transcriptase-polymerase chain reaction product of patient 1 as an example of the pattern of cytokine detection. **B:** Cytokines produced were indicated by plus signs; negative means that no cytokines were detected.

hours after stimulation with the M5(81-96) peptide. In patient 1 we were able to detect the production of IFN- γ in Mi v-HIL at 12 hours and in LA-HIL at 12 and 48 hours (Figure 3), which matched the results obtained with the culture supernatants (Table 3). MRNA for IL-10 was detected in Mi v-HILs from patients 1 and 3 at 12 hours, and also at 48 hours for patient 3 (Figure 3), although it was not detected in culture supernatants (Table 3). IL-4 and IL-10 were tested only at the RNA level for LA-HIL no. 9, and both cytokines were detected after 12 hours, with IL-10 being also detected at 48 hours after M5(81-96) peptide stimulation (Figure 3). When adding these results to those obtained for IL-4 and/or IL-10 detection in culture supernatants, the above-mentioned regulatory cytokines were produced by six of six LA-derived HILs and IL-10 by four of seven valve-derived HILs.

Discussion

The data presented in this study show that intralesional mononuclear cells from heart lesions predominantly secrete IFN- γ and TNF- α , Th1-type cytokines, in both ARF and chronic RHD patients. This indicates that, even during the chronic phase, these mononuclear cells still produce inflammatory cytokines. The correlation between the scarce production of IL-4 by valve-infiltrating cells and the large numbers of IL-4-positive cells in the myocardium, suggests that deficiencies in IL-4-producing cells in the valvar tissue may contribute to the progression of valvar RHD lesions (RR, 4.3; OR, 15.8). On the other hand, the production of IL-4 in the myocardium seems to play a protective role (RR, 0.2; OR, 0.06). IL-10, a predominant regulatory cytokine was also secreted by

a large numbers of cells in both valve and myocardium tissue. This finding suggests that the regression of myocardium inflammation does not depend solely on the regulatory function of IL-10-producing cells. Likewise, all myocardium-infiltrating T-cell lines produced IL-4 and IL-10, whereas valve-derived T-cell lines did not produce IL-4 and produced less IL-10 than myocardial-derived lines. On the other hand, streptococcal M5 antigen-stimulated T-cell lines produced IFN- γ in 85% of cases, regardless of their origin. These observations reinforce the putative role of these regulatory cytokines in myocardium healing in RHD and in the induction of progressive and permanent valve damage. The differential cytokine polarization in the heart in RHD is probably related to immigrant autoreactive T cells, local chemokines produced by inflammatory cells, and adhesion molecules. The heterogeneity of endothelial cells in different sites was recently described in a murine model, in which VCAM-1 is constitutively expressed in the heart, but not in the lung endothelium. However, it was up-regulated on TNF- α stimulation in both endothelia. Another chemokine (RANTES) is up-regulated only in the heart. Both endothelia show CD4⁺ T-cell interactions. Blocking studies showed that the capture of T cell was mediated both by constitutive VCAM-1 and by TNF- α -induced RANTES.²⁷ In another animal model of destructive autoimmune myocarditis, certain chemokines, including MIP-1 α and RANTES, polarized the recruitment of Th1 and Th2 subsets.²⁸ In RHD, T-cell infiltration probably takes place after the binding of crossreactive antibodies to the endothelial surface, leading to inflammation and valve scarring.²⁹ The up-regulation of certain adhesion molecules such as VCAM-1 promotes lymphocyte adhesion to the endothelium.⁴ Given these data, one possible explanation for the low numbers of T cells secreting IL-4 in the valve samples from RHD patients could be related to the differential secretion of specific chemokines.

Rare eosinophils were observed in all heart tissue fragments of RHD patients analyzed. The role of these cells in the rheumatic heart lesion has not been described. These cells may have been activated by IL-5, a Th2-type cytokine. Once activated, they could contribute to local inflammation through the production of several proinflammatory cytokines (IL-6, IL-8, TNF- α , IL-1 α) and chemokines. In the present study, we found small amounts of IL-5 being produced by intralesional T cells when stimulated with purified M5 protein through flow cytometry analysis (data not shown). However, the majority of heart tissue fragments analyzed showed only a few IL-4-producing cells, and large numbers of IL-10-producing cells (Th-2/regulatory cytokines), suggesting that despite the presence of Th2 cells, they are probably not sufficient to down-regulate the deleterious effect of Th-1 cytokines, predominant mainly in the valves. The *in vitro* pattern of cytokines detected in intralesional T-cell lines when stimulated with streptococcal antigens is in line with these findings.

The observation, by other groups, that activated T cells from the periphery of RF patients produced, after *in vitro* streptococcal antigen stimulation, high levels of proinflammatory cytokines^{7,8} is in agreement with our results.

It should be pointed out, however, that only proinflammatory cytokines were analyzed. It is known that Th1 cytokines play an important role in certain organ-specific autoimmune diseases^{30,31} and that IFN- γ plays a key role in the progression of the pathological process, probably by inducing increased uptake and presentation of autoantigens from target organs.³² The results here presented are in line with these findings and suggest that, in both ARF and chronic RHD patients, the high number of infiltrating T cells secreting IFN- γ , along with the down-regulation because of the low number of cells producing regulatory cytokines, may be determinant in maintaining tissue damage in the valves.

In a previous work, we described the presence of activated intralesional T-cell clones that crossreactively recognized heart proteins and streptococcal M peptides. We also showed that the frequencies of streptococcal and/or heart protein-reactive intralesional T cells were similar in RF patients with ongoing recurrence episodes of the disease and in chronic RHD patients.^{4,33} The high frequency of intralesional oligoclonal T-cell expansions (more than 10%) in both ARF and chronic RHD patients has also been demonstrated by our group.^{33,34} CD4⁺ heart-infiltrating T cells have been predominantly found in the lesions, as demonstrated by us^{5,34} and others.^{2,3}

The role of proinflammatory cytokines such as IL-1, IL-6, and TNF- α has been evaluated in several cardiovascular diseases. Increased plasma levels of IL-1, IL-6, and TNF- α in patients with heart failure, as well as the presence of TNF- α mRNA in the heart in several animal models have been described.^{15–18} These studies suggest that TNF- α could be responsible for the development and progression of heart failure. In these patients, the production of TNF- α could be the result of immune response activation because of tissue injury, stress, or to the underperfusion of systemic circulation, but could also be locally produced by myocytes.^{35–38} Recently, Oral and colleagues³⁹ showed that patients with chronic mitral valve regurgitation showed high production of TNF- α in the plasma and increased expression in the myocardium. Their results suggest that TNF- α plays a role in the remodeling of left ventricular volume.

In RHD patients, our results suggest that TNF- α and IFN- γ produced by heart-infiltrating mononuclear cells could act by amplifying local inflammation triggered by a heart-driven autoimmune reaction. It has been reported that activated macrophages and B lymphocytes from Aschoff bodies express large amounts of HLA class II molecules on their surface,³ suggesting that the development of Aschoff bodies may favor antigen presentation by these antigen-presenting cells in the heart tissue. In addition, the observation that Aschoff bodies underneath activated valvular endothelium favors the idea that T-cell infiltration may allow for the formation of Aschoff bodies in the valves.⁴⁰ These infiltrating T cells, apparently, are peripheral primed T lymphocytes that migrate to the heart and then recognize heart tissue antigens, probably presented by local antigen-presenting cells. This hypothesis is in agreement with the idea of the antigen-presenting cell role of cells derived from Aschoff bodies. In the ARF patients studied, it was possible to identify Aschoff bod-

ies in heart tissue samples from only 4 of 18 patients (patients 4, 5, 6, and 7) as illustrated in Figure 2E (patient 4). However, the presence of Aschoff bodies was not related to any particular pattern of cytokine expression.

In conclusion, our present data, which shows a mixed pattern of cytokines with significantly lower numbers of IL-4-secreting cells, both *in situ* and in valve-derived T-cell lines, suggest the co-existence of intralesional inflammatory and regulatory T lymphocytes. However, such regulatory cells are probably not efficient in neutralizing the aggressive autoreactive T cells and, consequently, in maintaining immunological self-tolerance. Therefore, the search for intralesional regulatory T cells⁹ and the identification of specific chemokines involved in T-cell polarization and/or differential migration⁴¹ will certainly contribute toward a better understanding the immunopathogenesis of RHD and toward the introduction of novel therapies, aimed at controlling the heart damage in RHD patients.

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