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ORIGINAL PAPER

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A novel method of immunomodulation of endothelial cells using *Streptococcus pyogenes* and its lysate

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ABSTRACT

Introduction. Coronary artery diseases and autoimmune disorders are common in clinical practice.

Aim. In this study, *Streptococcus pyogenes* and its lysate were studied to modify the endothelial function.

Material and methods. HUVEC cells were seeded in the cell culture, and *Streptococcus pyogenes* were added to the cell culture, and the supernatant was studied for the secreted proteins. In the second phase, the bacterial lysate was synthesized, and the lysate was added to cell culture and studied.

Results. When *S. pyogenes* alone was added to culture, E Cadherin, Angiostatin, EpCAM and PDGF-AB were some of the biomarkers elevated significantly. HCC1, IGFBP2 and TIMP were some of the biomarkers which showed a reduction. When the lysate was added, the cell-culture was maintained for a longer time, and it showed the synthesis of immune regulatory cytokines. Heatmap analysis showed a significant number of proteins/cytokines concerning the immune/pathways, and toll-like receptors superfamily were modified. BLC, IL 17, BMP 7, PARC, Contactin2, IL 10 Rb, NAP 2 (CXCL 7), Eotaxin 2 were maximally increased. By principal component analysis, the results observed were significant.

Conclusion. There is potential for a novel method of immunomodulation of the endothelial cells, which have pleiotropic functions, using *S. pyogenes* and its lysates.

Keywords. biomarkers, endothelial cells, immune-regulation, *Streptococcus pyogenes*

Introduction

Immune-related disorders are common in clinical practice. Rheumatic heart diseases are common in the general population in the Asian countries with a prevalence of about 7.7 to 51/1000 community based on echocardiography screening in school in India.[1] *Streptococcus pyogenes* infections are associated with sore-throats, and they are also associated with rheumatic heart diseases. Rheumatic fever is associated with migratory joint pains and occasionally pan-carditis. In patients with rheumatic heart diseases, the incidence of coronary artery diseases is low.[2-5] This was observed in some stud-

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ies in India and neighboring countries. The incidence of rheumatic heart disease is showing a decreasing trend, and the incidence of coronary artery disease is rising in the recent days. The incidence in our center and other centers as well show a low association of coronary artery disease with rheumatic valvular heart diseases, irrespective of age and metabolic characteristics.[2,3] The incidence of mixed procedure i.e., combined valve surgeries and bypass surgeries, is <5%. And further analysis looking for rheumatic valvular etiology in these combined procedures would be much lesser, even in high volume centers like TUMS.[4]

S. pyogenes has anti-tumour activity which was studied in the early 1990's by Coley. *S. pyogenes* are known to produce a unique enzyme that is useful in cleaving immunoglobulin G in the blood (Ides).[6-8] Its usefulness therapeutically has been tested to cleave IgG antibodies.[9,10] *S. pyogenes* secrete serum optical factor, which shows increased uptake of HDL, and thereby it can reduce atherosclerosis.[11,12]

In this study, *S. pyogenes* was used to infect the endothelial cells, and the endothelial response was evaluated. Also, in the later part of the study the *S. pyogenes*' lysate was used on the endothelial cells, and the results were evaluated. Microorganisms can prevent autoimmune disorders though this phenomenon is not well studied.[13] Identifying the immunogenic potentials can identify potential uses of *S. pyogenes* to prevent cardiovascular, autoimmune, and tumor problems. The incidence of autoimmune disorders is common in the migrant populations especially of Asian ethnicity.[14,15] This is commonly attributed as the hygiene hypothesis, and the exact mechanism is speculative and not decisively studied.

In our center out of 650 consecutive valve replacement surgeries six cases underwent concomitant coronary artery bypass surgery. Out of these 6 cases a clear etiology of rheumatic heart disease was not established on any of the cases, and the primary etiologies were ischemia MR and degenerative sclerotic calcific aortic valve. The study was performed in search of novel applications of *S. pyogenes* in regulating immune functions, and its related effects on cardiovascular and its pleiotropic functions.

Aim

Bacterial infections can modulate immune functions though this phenomenon is poorly studied. In this study, the role of *S. pyogenes* and its lysate was evaluated for possible beneficial effects on the endothelial cells.

Material and methods

S. pyogenes were obtained from the laboratory (*Streptococcus pyogenes* Rosenbach – ATCC,19615TM Lancefield Group A). The bacteria were cultured by seeding, and the colonies were inoculated with endothelial cells. Serial observations about the endothelial cells were made at regular intervals by microscopy. The supernatant was collected, and various inflammatory markers were studied at serial time intervals.

Colony forming unit (CFU) assay

S. pyogenes was grown on of BD Bacto-Brain Heart Infusion agar plates with 5% CO_2 at 37 ^{0}C overnight. Streptococcus pyogenes was collected into 5 ml of Brain Heart Infusion broth. 10-fold serial dilutions with Brain Heart Infusion broth was then run. For each dilution, plate 100ul of cells on Brain Heart Infusion agar plates was added. Incubation of plates was performed with 5% CO_2 at 37^{0}C overnight. The calculation was made- CFU: 2 x 10^9 CFU/ml.

Cell treatment

Trypsin digested HUVEC cells was mixed with fresh complete medium in 50 ml tubes and aliquot the cells in two 6-well plates. The cells were grown to 80% confluence (9 cm^2 surface area/well, 2 ml culture medium/well). The cells were washed with 2 ml PBS/well, twice. *S. pyogenes* stock dilution: the Streptococcus stock was 2 x 10^9 CFU/ml. 100-fold dilution was made using 2 x 10^7 CFU/ml and sterile PBS, and 10ul *Streptococcus* stock, and 990ul sterile PBS. The infection dose was 100,000 CFU/well. The Streptococcus inoculation amount was 100,000 CFU / 2 x 10^7 CFU/ml (100-diluted) = 5 x 10^{-3} ml = 5µl per well. 30µl of 100-fold diluted *Streptococcus* stock was added into 24 ml new complete medium without any antibiotics and mixed well. 2 ml/well mixture was distributed into each well. The samples were collected at following timepoints (Table 1). At each time point, the cell culture supernatants were collected and centrifuged at 500 g for 10 min at 4^{0}C. The supernatants were transferred (~ 1.9 ml) to new tubes. The samples were stored at -80^{0}C till use. Each time point, the untreated controls were also collected. Total 10 samples were collected.

Table 1. Study plan

Time Point	Starting	Ending	*Streptococcus pyogenes* infection	
			Treated	Untreated
0-hr	8:00 AM	8:00 AM	100,000 CFU + **PBS**	**PBS**
2-hrs	8:00 AM	10:00 AM	100,000 CFU + **PBS**	**PBS**
6-hrs	8:00 AM	2:00 PM	100,000 CFU + **PBS**	**PBS**
10-hrs	8:00 AM	6:00 PM	100,000 CFU + **PBS**	**PBS**
14-hrs	8:00 AM	10:00 PM	100,000 CFU + **PBS**	**PBS**

In the second phase of the study, the *S. pyogenes*' lysate was prepared from the same bacteria. The lysate

Table 2. Elevation in MIF, lymphotactin, LIF, OPN, MSP, IL-9, Lymphotactin, IL18 BPa and TECK

LOD	MAX	(pg/ml)	0 hour treated	2 hour treated	6 hour treated	10 hour treated	14 hour treated	(pg/ml)	14 hour untreated
14.2	40,000.0	6Ckine	1.9	1.4	6.2	0.3	0.0	6Ckine	2.0
10.9	4,000.0	Axl	0.0	0.0	0.0	16.1	14.8	Axl	11.8
17.8	20,000.0	BTC	0.0	0.0	0.0	0.0	0.0	BTC	0.0
26.2	40,000.0	CCL28	0.0	0.0	0.0	0.0	0.0	CCL28	0.0
26.7	50,000.0	CTACK	0.0	0.0	0.0	0.0	0.0	CTACK	0.0
11.7	20,000.0	CXCL16	0.0	6.6	0.4	6.4	19.2	CXCL16	36.4
13.7	10,000.0	ENA-78	2.1	0.0	0.0	0.0	9.0	ENA-78	2.0
62.4	20,000.0	Eotaxin-3	0.0	0.0	0.0	0.0	0.0	Eotaxin-3	0.0
4.2	10,000.0	GCP-2	0.0	0.0	0.0	0.0	0.0	GCP-2	2.8
2.9	1,000.0	GRO	1.4	3.2	4.1	6.9	8.4	GRO	25.0
6.7	4,000.0	HCC-1	0.0	7.4	44.9	69.6	90.9	HCC-1	213.1
4.0	3,333.3	HCC-4	0.0	0.1	0.0	0.4	0.4	HCC-4	0.2
800.0	200,000.0	IL-9	0.0	206.2	28.9	0.0	109.6	IL-9	0.0
49.6	100,000.0	IL-17F	0.1	0.1	0.3	0.1	0.4	IL-17F	0.0
52.0	60,000.0	IL-18 BPa	4.0	3.4	0.0	0.0	33.3	IL-18 BPa	0.0
7.7	10,000.0	IL-28A	0.0	0.0	0.5	0.0	0.0	IL-28A	0.0
302.0	100,000.0	IL-29	0.0	30.5	0.0	0.0	0.0	IL-29	0.0
97.8	40,000.0	IL-31	0.0	0.0	0.0	0.0	7.8	IL-31	0.0
13.7	10,000.0	IP-10	0.5	0.0	1.8	0.0	0.9	IP-10	0.1
16.8	10,000.0	I-TAC	0.0	2.4	0.0	0.0	5.9	I-TAC	9.7
28.4	13,000.0	LIF	0.0	16.4	18.8	0.2	47.9	LIF	0.0
15.6	10,000.0	LIGHT	0.0	0.0	0.0	0.0	0.9	LIGHT	0.0
47.5	100,000.0	Lymphotactin	31.3	43.7	45.7	0.0	78.8	Lymphotactin	49.3
5.7	2,000.0	MCP-2	0.9	0.0	0.7	0.0	1.7	MCP-2	1.7
4.2	4,000.0	MCP-3	0.0	0.0	0.0	0.0	0.0	MCP-3	0.0
4.6	1,111.1	MCP-4	0.0	0.1	0.4	0.0	0.1	MCP-4	0.5
4.9	1,111.1	MDC	0.0	0.0	0.0	0.0	0.3	MDC	0.2
13.6	4,000.0	MIF	72.4	70.9	323.2	709.2	1,417.9	MIF	74.6
4.5	4,000.0	MIP-3a	0.0	0.5	0.7	0.5	2.5	MIP-3a	0.8
17.8	20,000.0	MIP-3b	0.0	0.0	0.0	0.0	1.6	MIP-3b	0.0
15.0	10,000.0	MPIF-1	0.0	0.0	1.7	2.0	0.1	MPIF-1	0.7
213.2	100,000.0	MSP	0.0	2.4	39.5	17.7	61.1	MSP	21.1
1.9	444.4	NAP-2	0.0	0.0	0.3	0.0	0.1	NAP-2	0.1
28.6	100,000.0	OPN	37.1	52.9	36.9	1.8	36.8	OPN	14.2
4.9	4,000.0	PARC	0.0	0.2	0.4	0.6	0.5	PARC	1.2
219.7	100,000.0	PF4	0.0	0.0	0.0	0.0	0.0	PF4	0.4
2.8	10,000.0	SDF-1a	0.1	0.2	0.3	0.1	2.8	SDF-1a	1.4
6.9	10,000.0	TARC	0.0	0.0	0.0	0.0	0.0	TARC	0.9
85.2	100,000.0	TECK	0.0	0.0	0.0	0.0	38.0	TECK	0.0
7.3	10,000.0	TSLP	0.0	0.0	0.4	0.0	2.9	TSLP	10.2

was added to the endothelial cells, and the endothelial cell response was studied at serial intervals -0, 36, 138, 336, 672 hours, and control samples were studied. The biomarkers secreted were studied, and the results were achieved.

HUVEC Cells and Lysate preparation

Normally subculture of cells is performed, when the culture has reached approximately 80% confluence, new flasks are seeded at a density of 2,500 cells per cm^2. Cells are typically ready to passage after 4 to 7 days in culture, when inoculated with 2,500 cells/cm^2.

Culture medium was prepared and bacteria was grown at CO_2 incubator. Fresh cells were collected and cell lysate was prepared by lysozyme digestion and DNAase digestion. Then centrifugation was performed to obtain the clarified supernatant. The lysate was passed through 0.22 μm filter. The cell lysate protein concentration was then measured. Aliquot lysate protein was in small size and it was stored at -80°C till use.

HUVEC stimulation

HUVEC cells were thawed and grown in 6-well plate. 2 wells (A, B well): Well A is negative control without adding cell lysate. Well B is test well with adding cell lysate 40μg/ml. The cells were passed twice a week. With every cell pass, the same amount of cell lysate 40μg/ml was added to cell well. Each time one vial was used and left over lysate was discarded. The cell culture supernatants was collected (~1.5 ml) at following time points. Totally 10 samples will be collected (A1-5, B1-5). For array test, 5 samples were used - i.T0 ii. T36hrs (1.5 days) iii.168hrs (7 days) iv. 336hrs (14 days) v. 672hrs (28 days). Centrifugation of the supernatants at 500 g for 10 min to remove any cell debris was performed, and the samples were stored at -80°C immediately.

Statistical analysis - data filtration

The biomarkers demonstrating no variation among all the samples (zero variance) were excluded from the data profile analysis since they do not contribute regarding distinguishing samples from each other.

Table 3. Reduction in BMP-7, FGF-7, IGFBP 1-2 and 6, OPG, PDFG AA, PIGF and GDF15

LOD	MAX	(pg/ml)	0 hour treated	2 hour treated	6 hour treated	10 hour treated	14 hour treated	(pg/ml)	14 hour untreated
39.7	10,000.0	AR	15.3	14.2	1.9	8.6	0.6	AR	0.0
2.8	2,000.0	BDNF	0.6	0.7	1.7	1.3	0.8	BDNF	1.8
21.7	6,666.7	bFGF	14.4	19.3	15.4	15.2	6.7	bFGF	12.0
103.6	33,333.3	BMP-4	0.4	1.0	0.6	0.0	0.0	BMP-4	0.9
551.9	100,000.0	BMP-5	189.0	0.0	3.5	0.0	0.0	BMP-5	0.0
151.5	40,000.0	BMP-7	93.5	30.6	78.1	8.0	11.9	BMP-7	56.3
4.3	10,000.0	b-NGF	0.1	0.0	0.0	0.0	0.0	b-NGF	0.0
0.2	200.0	EGF	42.2	35.8	38.5	37.8	36.6	EGF	38.9
6.5	10,000.0	EGF R	0.9	0.0	1.1	0.6	0.1	EGF R	3.0
26.0	10,000.0	EG-VEGF	0.0	0.0	0.0	0.0	0.0	EG-VEGF	0.0
184.2	33,333.3	FGF-4	8.9	35.4	0.0	0.0	0.0	FGF-4	0.0
28.2	10,000.0	FGF-7	35.0	0.0	0.0	54.9	18.1	FGF-7	41.9
1.2	2,000.0	GDF-15	0.5	0.8	9.0	16.0	37.0	GDF-15	47.9
10.2	4,000.0	GDNF	0.0	0.0	0.0	0.2	0.0	GDNF	1.0
23.1	10,000.0	GH	0.0	10.7	0.0	5.4	0.0	GH	1.9
10.3	10,000.0	HB-EGF	1.3	0.0	0.0	10.2	2.5	HB-EGF	0.1
10.9	4,000.0	HGF	0.0	2.6	0.0	4.7	0.0	HGF	2.5
6.5	5,000.0	IGFBP-1	11.6	5.1	3.8	8.3	1.2	IGFBP-1	12.7
46.8	20,000.0	IGFBP-2	4.0	67.3	305.9	442.8	443.7	IGFBP-2	1,074.7
485.5	200,000.0	IGFBP-3	131.5	288.2	0.0	313.8	0.0	IGFBP-3	18.5
719.3	200,000.0	IGFBP-4	648.4	259.4	166.1	99.8	0.0	IGFBP-4	40.8
138.8	100,000.0	IGFBP-6	122.6	28.3	99.1	123.4	75.7	IGFBP-6	132.7
65.6	20,000.0	IGF-1	48.4	15.9	0.0	23.5	0.0	IGF-1	12.5
78.3	20,000.0	Insulin	5,894.2	4,512.6	4,837.5	4,683.9	4,757.0	Insulin	4,939.6
30.3	40,000.0	MCSF R	6.1	0.0	0.0	0.0	0.0	MCSF R	0.0
18.5	10,000.0	NGF R	0.0	0.0	0.0	0.0	0.0	NGF R	0.0
63.0	40,000.0	NT-3	0.0	0.0	0.0	0.0	0.0	NT-3	0.0
13.2	10,000.0	NT-4	0.0	0.0	0.0	1.0	0.0	NT-4	0.0
9.0	4,000.0	OPG	0.0	2.8	10.9	16.0	9.3	OPG	35.5
11.5	10,000.0	PDGF-AA	2.9	1.2	35.8	44.1	48.9	PDGF-AA	91.6
3.1	4,000.0	PIGF	0.9	3.6	10.5	9.9	12.6	PIGF	24.9
8.8	10,000.0	SCF	6.8	0.9	4.7	1.9	0.0	SCF	3.7
28.1	20,000.0	SCF R	2.2	3.7	2.0	5.5	0.0	SCF R	0.7
5.2	1,111.1	TGFa	0.0	0.0	0.0	0.0	0.0	TGFa	0.0
814.4	100,000.0	TGFb1	0.0	0.0	0.0	0.0	0.0	TGFb1	0.0
70.0	40,000.0	TGFb3	16.1	7.8	22.2	6.8	0.0	TGFb3	5.6
13.6	3,333.3	VEGF	0.2	0.6	0.0	0.0	0.0	VEGF	0.0
28.2	10,000.0	VEGF R2	4.8	3.9	0.0	0.0	0.0	VEGF R2	0.2
61.2	40,000.0	VEGF R3	6.5	0.0	4.3	0.0	0.0	VEGF R3	6.0
12.5	20,000.0	VEGF-D	0.0	0.0	0.0	0.0	0.0	VEGF-D	0.0

Heatmap

The biomarker values were standardized (centering and scaling) by subtracting the average and then dividing by the standard deviation. The standardized data were plotted in a heatmap with hierarchical clustering by Euclidean distance.

Principal Component analysis

The various expression levels of multiple biomarkers may come from a common underlying factor/mechanism. The principal component analysis (PCA) decompose the data set into different principal components (PCs) sorted by their contribution to total variance/variation in the dataset. These PCs are linear transformations/combinations of standardized biomarker values. By observing the location of a sample on the plot of the first 2 PCs explaining the most variation, we can tell the pattern of samples.

Software

All the analyses were conducted in the R programming language V3.6.0 (R Core Team 2017).

Results

Direct Streptococcus pyogenes' effects

Tables 2 to 6 summarize the effects of the bacteria on the cell culture of endothelial cells. There is a significant reduction in HCC1, IGFBP2, PDGFAA, and TIMP. Macrophage inhibiting factor and lymphocyte inhibition factors showed a decrease in the levels. There is a marked increase in E Cadherin, Angiostatin, DAN, Ep-CAM, CFG RIIBC, PDGF- AB, gp 130, TPO, Tie 2, and Angiogenin. ICAM-1, IL6, Endoglin, Trail 3, and PRE-CAM-1 showed an increasing trend.

Streptococcus pyogenes' Lysate Challenge

The results of the streptococcal lysate challenge on the endothelial cells are summarized in the heat map results (Figure 1). BLC, IL 17, BMP 7, PARC, Contactin2, IL 10 Rb, NAP 2 (CXCL 7), Eotaxin 2 were maximally increased. Other secreted markers that were induced – trappin2, SDF-1a, FGF7, CCL 28, 4-1BB, VCAM 1, VEGF R3, GITR, SIGLEC 5, IL13 R1, CD30, TGF B2 and GP 130. The markers which were unequivocally reduced were MIF, Fas, IL13R2, TIMP2, Follistatin,

Table 4. Increase in all the cytokines in the table listed (except Galactin7 and ST2)

LOD	MAX	(pg/ml)	0 hour treated	2 hour treated	6 hour treated	10 hour treated	14 hour treated	(pg/ml)	14 hour untreated
263.8	11,111.1	Activin A	346.3	251.0	343.9	281.7	472.9	Activin A	209.1
16.8	10,000.0	AgRP	30.3	16.5	45.4	18.9	184.3	AgRP	20.0
5.5	2,000.0	Angiogenin	37.9	29.9	43.4	29.8	144.1	Angiogenin	26.2
56.3	40,000.0	ANG-1	246.5	209.1	257.7	258.2	322.4	ANG-1	163.3
2,267.7	1,000,000.0	Angiostatin	4,942.9	3,576.9	8,953.9	3,369.3	40,517.2	Angiostatin	3,978.9
10.4	10,000.0	Cathepsin S	25.7	19.2	27.9	26.0	184.6	Cathepsin S	17.8
10.4	10,000.0	CD40	11.0	13.1	23.3	23.6	37.7	CD40	27.8
23.1	10,000.0	Cripto-1	5.2	0.0	20.4	0.0	35.2	Cripto-1	0.0
169.4	40,000.0	DAN	374.8	70.5	562.2	165.8	11,816.4	DAN	119.4
49.8	8,888.9	DKK-1	35.7	192.3	1,122.9	1,466.7	2,365.2	DKK-1	3,009.6
173.9	80,000.0	E-Cadherin	333.6	149.1	1,478.0	128.0	4,275.4	E-Cadherin	167.7
13.7	20,000.0	EpCAM	8.0	0.5	17.5	4.1	189.7	EpCAM	2.8
6.3	2,000.0	FAS L	20.3	4.6	19.0	5.0	40.8	FAS L	12.1
58.0	10,000.0	Fcg RIIBC	128.9	43.3	323.8	60.5	568.9	Fcg RIIBC	134.4
22.8	40,000.0	Follistatin	2.7	5.5	15.2	23.0	86.0	Follistatin	63.2
102.6	100,000.0	Galectin-7	25.0	48.2	58.6	21.9	56.5	Galectin-7	81.9
235.1	100,000.0	ICAM-2	433.2	203.9	3,766.3	3,949.3	9,610.2	ICAM-2	2,397.8
50.2	10,000.0	IL-13 R1	534.1	531.2	1,004.0	567.2	2,667.3	IL-13 R1	444.9
70.7	20,000.0	IL-13 R2	140.1	105.9	153.7	228.2	155.1	IL-13 R2	119.3
103.7	40,000.0	IL-17B	310.7	241.8	1,552.4	384.4	551.4	IL-17B	179.7
22.3	10,000.0	IL-2 Ra	98.5	57.8	108.3	88.1	207.3	IL-2 Ra	19.1
253.8	100,000.0	IL-2 Rb	199.8	134.6	170.4	265.7	191.3	IL-2 Rb	206.8
171.6	40,000.0	IL-23	0.0	4.7	122.3	26.1	59.0	IL-23	0.0
8.2	4,000.0	LAP(TGFb1)	34.5	66.5	188.4	175.7	267.7	LAP(TGFb1)	211.7
48.7	20,000.0	NrCAM	112.7	47.1	125.4	91.0	71.7	NrCAM	55.0
107.8	40,000.0	PAI-1	1,907.2	17,224.8	23,895.7	22,084.3	20,426.1	PAI-1	16,711.3
28.4	10,000.0	PDGF-AB	45.6	28.4	92.9	63.5	138.1	PDGF-AB	15.5
70.9	20,000.0	Resistin	49.0	4.1	56.6	26.7	49.8	Resistin	9.2
32.7	13,333.3	SDF-1b	14.9	6.3	52.0	5.0	10.3	SDF-1b	1.3
239.7	80,000.0	gp130	330.2	37.4	859.8	320.1	892.3	gp130	16.3
25.2	40,000.0	Shh-N	11.6	14.8	31.2	32.8	42.2	Shh-N	54.0
32.2	10,000.0	Siglec-5	91.6	10.7	344.8	45.1	95.8	Siglec-5	38.5
14.2	4,000.0	ST2	28.6	0.9	27.7	17.1	31.1	ST2	35.0
104.8	40,000.0	TGFb2	57.1	18.1	85.6	54.4	72.2	TGFb2	42.9
46.5	10,000.0	Tie-2	122.7	22.5	451.4	51.2	79.3	Tie-2	27.0
335.0	200,000.0	TPO	249.1	66.3	155.7	51.1	212.2	TPO	57.5
26.3	8,000.0	TRAIL R4	61.5	38.7	54.8	33.1	58.3	TRAIL R4	31.5
111.9	20,000.0	TREM-1	468.6	178.7	2,561.2	209.8	468.1	TREM-1	167.4
26.4	20,000.0	VEGF-C	57.8	1.9	37.9	35.5	37.3	VEGF-C	19.6
118.8	40,000.0	VEGF R1	106.8	593.6	2,745.2	3,514.9	4,425.7	VEGF R1	8,579.2

IGFBP-2, DKK1, TNF R2, Hb EGF. There are 18 biomarkers showing zero variance among all the samples, including BTC, IL-9, IL-29, MCP-4, CD40, DAN, E-Cadherin, b-NGF, EGF R, EG-VEGF, FGF-4, IGF-1, NT- 3, SCF, TGFb3, IL-5, BCMA, and E-Selectin. These biomarkers were excluded from the analysis. Details of the principal components and the results are shown in figure 1 and 2 (and supplement file). The results of the principal component analysis are significant.

Discussion

Direct Streptococcus pyogenes immune response – decreased biomarkers

Specific biomarkers like HCC1, IGFBP2, PDGF-AA, and TIMP decrease in the levels compared to controls. Hemofiltrate CC is a chemokine, which attracts and acts through CCR1 receptors.[16] It is widely secreted by various tissues. Insulin-like growth factor binding protein 2 reduces the risk of diabetes. IGFBP2 is implicated in the regulation of IGF in most tissues.[17] Blocking of IGF BP2 results in the reduction of tumor and metastasis.[18] Platelet-derived growth factor AA is involved in the migration of smooth muscle cells.[19-21] Reduction in the TIMP metallopeptidase inhibitor1 and also has anti-angiogenic activity is associated with a reduction in the adverse clinical events in acute kidney injuries.[22] MIF (macrophage migration inhibitory factor) and LIF (leukemia inhibitory factor) levels were reduced. MIF is a widely expressed pleiotropic cytokine, and it is involved in the stimulation of other inflammatory cytokines like TNF alpha, INF gamma, IL6, IL 12, CXCL8 etc.[23] LIF is involved in cell differentiation, and maturation and stimulation lead to JAK/STAT and MAPK cascades.[24]

Increased biomarkers with direct bacterial infection

Increased activity was found in E Cadherin is involved in cell-to-cell interactions, and they have tumor suppressor effects.[25] Angiostatin, DAN, is an inhibitor of BMP and TNF. Angiostatin is engaged in the reduction of angiogenesis.[26,27] There is a marked increase in EPCAM, which are complex proteins that promote transcription factor-mediated pluripotency reprogramming,[28] CFG RIIC and PDGF AB.[29] The Platelet-derived growth factor has active angiogenic potential and mitogenesis and

Table 5. Mild increase in GMCSF, IL6, IL13, TNFb and TNF RI and mild reduction in TIMP 2

LOD	MAX	(pg/ml)	0 hour treated	2 hour treated	6 hour treated	10 hour treated	14 hour treated	(pg/ml)	14 hour untreated
2.3	666.7	BLC	0.0	0.0	0.0	0.0	0.0	BLC	0.0
15.4	4,000.0	Eotaxin	0.0	0.0	0.0	0.0	0.0	Eotaxin	0.0
11.9	1,000.0	Eotaxin-2	0.0	0.0	0.0	0.0	0.3	Eotaxin-2	0.0
35.0	20,000.0	G-CSF	0.0	0.0	0.0	0.0	0.1	G-CSF	2.0
4.8	1,000.0	GM-CSF	0.0	0.0	0.0	3.4	6.1	GM-CSF	0.9
14.8	4,000.0	I-309	0.0	0.0	0.0	1.1	1.6	I-309	0.0
56.5	33,333.3	ICAM-1	23.2	54.7	69.6	76.2	159.3	ICAM-1	52.0
14.2	2,000.0	IFNg	0.0	0.0	0.0	0.0	7.0	IFNg	0.0
5.4	2,000.0	IL-1a	0.0	0.0	2.8	6.0	15.0	IL-1a	11.4
2.3	1,000.0	IL-1b	0.0	0.0	0.0	0.0	0.0	IL-1b	0.0
5.7	222.2	IL-1ra	0.0	0.0	0.0	0.0	0.0	IL-1ra	7.2
6.8	2,000.0	IL-2	0.8	0.0	0.0	0.0	0.0	IL-2	4.5
5.2	2,000.0	IL-4	0.0	0.0	0.0	0.0	0.0	IL-4	0.0
17.3	4,000.0	IL-5	0.0	2.7	0.0	0.0	0.0	IL-5	8.7
8.1	2,000.0	IL-6	0.0	9.2	22.7	77.4	136.3	IL-6	53.8
19.5	10,000.0	IL-6R	0.0	0.0	0.0	0.0	0.0	IL-6R	0.0
14.0	4,000.0	IL-7	0.0	3.3	0.0	0.0	0.0	IL-7	9.8
2.1	500.0	IL-8	4.6	16.5	7.0	4.9	17.2	IL-8	128.7
8.8	4,000.0	IL-10	0.0	0.8	0.0	0.0	0.1	IL-10	2.4
44.0	20,000.0	IL-11	0.0	0.0	0.0	0.0	3.8	IL-11	0.0
17.9	10,000.0	IL-12p40	0.0	0.0	0.0	0.0	0.0	IL-12p40	0.0
1.2	500.0	IL-12p70	0.3	0.5	0.2	0.0	1.0	IL-12p70	0.6
17.2	1,000.0	IL-13	4.2	9.1	23.0	23.5	69.3	IL-13	97.3
41.0	4,000.0	IL-15	0.0	0.0	0.0	4.5	27.9	IL-15	32.1
14.8	5,000.0	IL-16	0.0	0.0	0.0	0.0	0.0	IL-16	1.0
11.1	4,000.0	IL-17	0.0	0.0	0.0	0.0	0.6	IL-17	0.0
15.2	2,000.0	MCP-1	7.2	24.0	100.7	276.7	266.4	MCP-1	219.0
3.0	4,000.0	MCSF	0.0	0.0	0.0	0.0	0.0	MCSF	0.0
11.2	5,000.0	MIG	0.0	0.0	0.0	0.0	0.6	MIG	0.2
17.1	3,333.3	MIP-1a	0.0	0.0	0.0	0.0	0.0	MIP-1a	0.0
2.8	1,000.0	MIP-1b	0.0	0.0	0.0	0.0	0.0	MIP-1b	0.0
6.7	3,333.3	MIP-1d	0.0	0.0	0.0	0.0	0.0	MIP-1d	0.0
4.0	2,000.0	PDGF-BB	0.0	0.0	0.9	3.9	9.6	PDGF-BB	5.7
29.9	6,666.7	RANTES	0.0	0.0	0.0	0.0	0.0	RANTES	0.0
89.8	13,333.3	TIMP-1	0.0	1,286.7	2,866.2	2,781.3	3,291.9	TIMP-1	3,752.9
24.5	40,000.0	TIMP-2	0.0	53.1	359.7	444.6	566.4	TIMP-2	1,439.1
37.2	2,000.0	TNFa	0.0	44.1	18.3	49.4	41.8	TNFa	55.4
66.1	20,000.0	TNFb	0.0	6.2	0.0	13.5	19.9	TNFb	2.3
36.6	40,000.0	TNF RI	0.0	0.0	7.5	1.2	10.8	TNF RI	7.0
118.3	40,000.0	TNF RII	0.0	0.0	0.0	0.0	9.1	TNF RII	0.0

acts on various tissues. Angiogenin may maintain blood homeostasis and participates in anti-inflammatory activity and has antibacterial and antiviral properties.[30]

Streptococcus pyogenes' lysate effects and Heat map analysis

When the cells were treated with *S. pyogenes*' lysate, the levels of BLC -the B lymphocyte chemoattractant protein (CXCL13) was increased.[31] Contactin 2 is a neuronal membrane protein, and it acts as an active cell adhesion molecule.[32-34] IL 17 is an inflammatory protein, and it was induced after lysate treatment. IL17 induces the production of GCSF and chemokines like CXCL1 and 2.[35] IL17 is strongly associated with chronic inflammation associated with autoimmune disorders. PARC (parkin like ubiquitin ligase) is a cytoplasmic anchor protein to p53-associated protein complexes.[36-38]

CXCL7 is involved in neutrophil chemotaxis, adhesion to the endothelial cells, and trans-endothelial migration of the cells.[39-41] Chemokine CXCL 7 is engaged in neutrophil-platelet crosstalk, and also it is actively involved in the growth of renal cell carcinoma.[42] IL10 Rb is the receptor for IL10, and it actively participates in inflammatory signaling.[43, 44] IL 10 regulates memory T cell development in response to viral infections.[45] Eotaxin 2 is an eosinophilic chemoattractant protein, and it acts through CCR3, and it is actively involved in the recruitment of other inflammatory cells also.[46] BMP 7 acts on the BMP receptor, which is actively involved in the process of inflammation and atherogenesis. Exogenous administration of BMP7 improves left ventricular remodeling and function in acute myocardial infarction.[47]

Trappin 2 is a serine protease inhibitor, and it has anti-inflammatory actions on the mucosal surfaces.[48] It also has anti-retrovirus activities on the mucosal surfaces. SDF1 alpha and its chemokine receptor play a significant role in hematopoietic cell mobilization, cancer metastasis, and ischemic injury repair in myocardial infarction tissues.[49] FGF 7 (fibroblastic growth factor) has an active role in tissue repair.[50] CCL 28 is a mucosa-associated epithelial chemokine, and it is associated with the recruitment of the cells, and it helps in T and B cell accumulation in mucosal surfaces.[51] 4-1BB (CD137) signalosome promotes T cell prolifer-

Table 6. Mild increase in ALCAM, Endoglin, MICB, PECAM1, µPAR, VCAM 1 and Xedar

LOD	MAX	(pg/ml)	0 hour treated	2 hour treated	6 hour treated	10 hour treated	14 hour treated	(pg/ml)	14 hour untreated
16.2	10,000.0	4-1BB	0.0	0.0	0.0	0.0	2.1	4-1BB	0.0
14.9	10,000.0	ALCAM	0.0	0.0	3.5	1.3	16.8	ALCAM	6.9
19.7	10,000.0	B7-1	0.0	0.0	0.0	0.0	8.8	B7-1	0.0
37.0	20,000.0	BCMA	0.0	0.0	0.0	0.0	0.0	BCMA	0.0
17.3	10,000.0	CD14	0.0	0.0	0.0	0.0	2.8	CD14	0.0
27.2	10,000.0	CD30	0.0	0.0	0.0	0.0	0.0	CD30	0.0
26.3	10,000.0	CD40L	0.6	0.0	0.0	0.0	1.2	CD40L	0.0
24.1	10,000.0	CEACAM-1	0.0	0.0	0.0	0.0	3.1	CEACAM-1	1.5
5.1	4,000.0	DR6	0.4	0.0	0.9	1.9	2.5	DR6	5.1
23.1	20,000.0	Dtk	0.0	0.0	2.5	0.4	12.9	Dtk	0.0
10.5	4,000.0	Endoglin	0.3	8.1	17.9	36.4	51.4	Endoglin	9.6
57.8	20,000.0	ErbB3	0.0	0.0	0.0	0.0	0.0	ErbB3	6.0
39.3	13,333.3	E-Selectin	0.0	0.0	0.0	0.0	2.9	E-Selectin	0.0
5.0	2,000.0	Fas	0.8	0.5	0.2	0.7	1.1	Fas	2.4
3.0	2,000.0	Flt-3L	0.0	0.0	0.0	1.1	0.0	Flt-3L	0.5
23.1	10,000.0	GITR	0.0	0.0	0.0	1.6	0.0	GITR	7.5
34.6	40,000.0	HVEM	0.0	2.9	0.0	0.0	0.0	HVEM	0.7
80.3	100,000.0	ICAM-3	0.0	3.4	0.0	0.0	6.8	ICAM-3	3.5
192.9	100,000.0	Contactin-2	0.0	0.0	8.9	0.0	7.1	Contactin-2	38.5
12.1	4,000.0	IL-1 RI	0.0	2.3	0.0	1.2	0.0	IL-1 RI	1.4
38.8	10,000.0	IL-2 Rg	0.0	0.0	0.0	0.0	1.8	IL-2 Rg	0.0
9.8	4,000.0	IL-10 Rb	0.0	0.7	0.0	0.0	0.7	IL-10 Rb	0.0
23.6	10,000.0	IL-17R	7.9	26.3	9.6	3.9	0.6	IL-17R	8.2
55.4	20,000.0	IL-21R	0.0	3.7	9.7	0.0	1.7	IL-21R	0.0
8.9	4,000.0	LIMPII	0.0	0.0	1.2	0.0	4.9	LIMPII	3.4
1.5	1,000.0	Lipocalin-2	3.6	1.7	1.7	0.7	1.5	Lipocalin-2	2.1
162.9	100,000.0	L-Selectin	8.9	29.3	0.0	17.0	32.6	L-Selectin	53.5
5.2	2,000.0	LYVE-1	0.0	0.6	2.7	0.0	3.4	LYVE-1	0.0
15.2	10,000.0	MICA	15.0	12.2	5.6	1.9	13.9	MICA	12.9
40.0	15,000.0	MICB	33.5	59.2	1.8	1.7	51.9	MICB	22.9
9.3	5,000.0	NRG1-b1	0.0	0.0	0.0	0.0	0.7	NRG1-b1	0.0
293.9	100,000.0	PDGF Rb	0.0	10.7	0.0	0.0	65.0	PDGF Rb	5.1
39.7	20,000.0	PECAM-1	19.5	103.2	185.0	207.1	326.8	PECAM-1	127.5
8.3	3,333.3	RAGE	0.2	1.9	0.0	0.0	0.0	RAGE	0.0
24.7	10,000.0	TIM-1	0.0	11.1	0.0	3.2	0.0	TIM-1	0.0
7.9	5,000.0	TRAIL R3	0.0	2.2	13.8	35.0	74.4	TRAIL R3	17.6
9.0	10,000.0	Trappin-2	148.1	4.6	1.2	3.4	0.0	Trappin-2	0.1
21.1	40,000.0	uPAR	0.0	20.7	51.2	82.8	111.1	uPAR	75.0
320.9	200,000.0	VCAM-1	0.0	34.0	15.9	23.7	77.0	VCAM-1	0.0
34.6	10,000.0	XEDAR	0.0	0.0	15.8	12.4	22.7	XEDAR	0.0

ation and survival and results in increased T cell effector functions.[52,53] VCAM1 is an inflammatory protein involved in cell to cell adhesions, and it also effectively induces angiogenesis.[54,55]

Glucosteroid induced TNFR related protein (GITR) is expressed by T cells and its ligands, and it boosts T cell activity. GITR agonistic stimulation is emerging as a promising therapeutic concept.[56] SIGLEC 5 is a leucocyte receptor that recognizes sialic acid structures and helps in leucocyte recruitment.[57] VEGFR3 is a receptor for vascular endothelial growth factors C and D and it is involved in lymph-angiogenesis and to some extent in VEGF A induced angiogenesis as well.[58] IL13 overcomes insulin resistance by promoting anti-inflammatory macrophage differentiation in adipose tissue.[59] CD30 is expressed on the surfaces of the endothelial cells though it is primarily expressed by lymphoid tissues. They are expressed in non-lymphomatous tumors. CD30 signaling is involved in proliferation, differentiation and survival (anti-apoptosis).[60] TGF B2 is expressed in the endothelium, and it plays an essential role in angiogenesis.[61] GP130 is a glycoprotein that participates in IL6 mediated inflammation and vascular pathologies, and it also has a negative feedback control.[62]

Reduced biomarkers with lysate

MIF is a widely expressed pleiotropic cytokine, and it is involved in the stimulation of other inflammatory cytokines like TNF alpha, INF gamma, IL6, IL 12, CXCL8 etc.[23,63] MIF is elevated in type 1 and 2 diabetes. Fas activation is associated with autoimmune disorders, which can be modulated by downregulation.[64] TIMP 2 are matrix metalloproteinases which are involved in inflammation in the cancer cells.[65] IGFBP2 are inhibitory and stimulatory to some of the tumours.[66] IL13 R2 is involved in mediating inflammation leading to myocarditis. Hence a reduction in these receptors could reduce inflammatory changes.[67,68] Dickkopf 1 family proteins are active modulators of Wnt pathways, and mostly their effects are inhibitory.[69] TNF R2 has proinflammatory and some anti-inflammatory aspects as well. The stimulatory and inhibitory effects had attracted considerable interest in the treatment of autoimmune diseases and cancer.[70] Follistatin is actively involved in activin A-follistatin regulation of cardiac inflammation

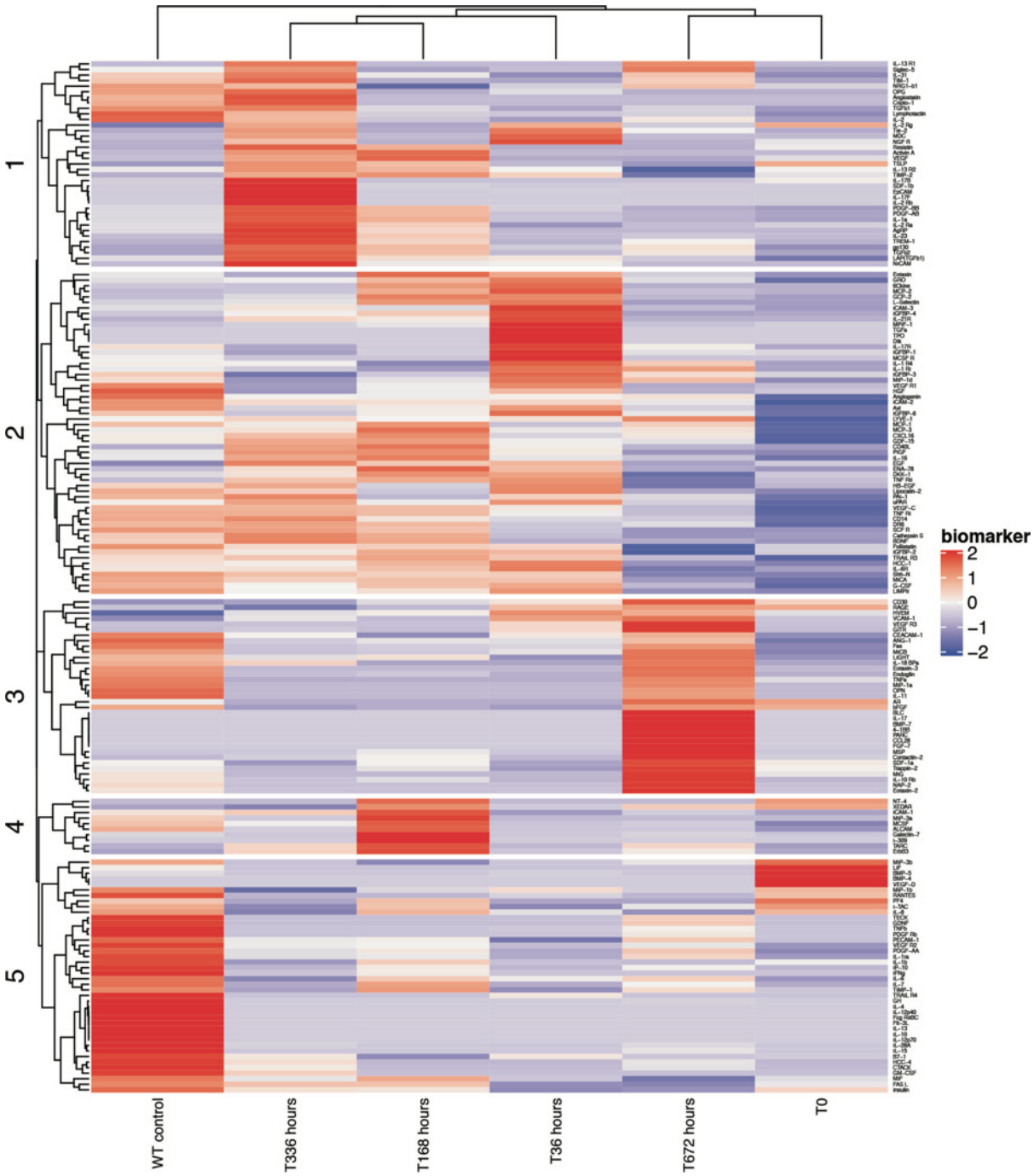

Fig. 1A. Heat map and the classification of the principal components (1-5) for analysis of the biomarkers after S. pyogenes lysate treatment on the endothelial cells.

and fibrosis.[71] Heparin-Binding Epidermal Growth Factor-Like Growth Factor inhibits cytokine-induced NF-κB Activation.[72] The heat map analysis shows a significant change in more proteins, and thereby it is possible to infer that *S. pyogenes* has a role in immune regulation.

The above observations indicate that the immune system undergoes various modifications by the *S. pyogenes*' direct challenge. Certain cytokines/parameters are increased, and some are decreased. In the long term, the immune memory and its regulation are complex, and it is also subjected to many positive and negative feedback regulations. Also, when the lysate is added, modulations are seen. Hence, *S. pyogenes* is associated with changes in the immune system, which can influence potential regulations in the immune homeostasis of the individuals. The negative impact causing rheumatic heart diseases by Streptococcus pyogenes can have a positive influence in modifying the immune-related and metabolic functions.

Chaos signaling and decoding

It is indeed difficult to predict the immune response in the future to a certain extent. It can be inferred that the

Ord	Biomarker	Ord	Biomarker	Ord	Biomarker	Ord	Biomarker	Ord	Biomarker
Cluster1									
1	IL-13 R1	9	TGFb1	17	Activin A	25	IL-17F	33	TREM-1
2	Siglec-5	10	Lymphotactin	18	VEGF	26	IL-2 Rb	34	gp130
3	IL-31	11	IL-2	19	TSLP	27	PDGF-BB	35	TGFb2
4	TIM-1	12	IL-2 Rg	20	IL-13 R2	28	PDGF-AB	36	LAP(TGFb1)
5	NRG1-b1	13	Tie-2	21	TIMP-2	29	IL-1a	37	NrCAM
6	OPG	14	MDC	22	IL-17B	30	IL-2 Ra		
7	Angiostatin	15	NGF R	23	SDF-1b	31	AgRP		
8	Cripto-1	16	Resistin	24	EpCAM	32	IL-23		
Cluster2									
1	Eotaxin	13	Dtk	25	Axl	37	DKK-1	49	BDNF
2	GRO	14	IL-17R	26	IGFBP-6	38	TNF RII	50	Follistatin
3	6Ckine	15	IGFBP-1	27	LYVE-1	39	HB-EGF	51	IGFBP-2
4	MCP-2	16	MCSF R	28	MCP-1	40	Lipocalin-2	52	TRAIL R3
5	GCP-2	17	IL-1 R4	29	MCP-3	41	PAI-1	53	HCC-1
6	L-Selectin	18	IL-1 RI	30	CXCL16	42	uPAR	54	IL-6R
7	ICAM-3	19	IGFBP-3	31	GDF-15	43	VEGF-C	55	Shh-N
8	IGFBP-4	20	MIP-1d	32	CD40L	44	TNF RI	56	MICA
9	IL-21R	21	VEGF R1	33	PIGF	45	CD14	57	G-CSF
10	MPIF-1	22	HGF	34	IL-16	46	DR6	58	LIMPII
11	TGFa	23	Angiogenin	35	EGF	47	SCF R		
12	TPO	24	ICAM-2	36	ENA-78	48	Cathepsin S		
Cluster3									
1	CD30	8	ANG-1	15	TNFa	22	IL-17	29	Contactin-2
2	RAGE	9	Fas	16	MIP-1a	23	BMP-7	30	SDF-1a
3	HVEM	10	MICB	17	OPN	24	4-1BB	31	Trappin-2
4	VCAM-1	11	LIGHT	18	IL-11	25	PARC	32	MIG
5	VEGF R3	12	IL-18 BPa	19	AR	26	CCL28	33	IL-10 Rb
6	GITR	13	Eotaxin-3	20	bFGF	27	FGF-7	34	NAP-2
7	CEACAM-1	14	Endoglin	21	BLC	28	MSP	35	Eotaxin-2
Cluster4									
1	NT-4	3	ICAM-1	5	MCSF	7	Galectin-7	9	TARC
2	XEDAR	4	MIP-3a	6	ALCAM	8	I-309	10	ErbB3
Cluster5									
1	MIP-3b	10	IL-8	19	IL-1b	28	IL-12p40	37	HCC-4
2	LIF	11	TECK	20	IP-10	29	Fcg RIIBC	38	CTACK
3	BMP-5	12	GDNF	21	IFNg	30	Flt-3L	39	GM-CSF
4	BMP-4	13	TNFb	22	IL-6	31	IL-13	40	MIF
5	VEGF-D	14	PDGF Rb	23	IL-7	32	IL-10	41	FAS L
6	MIP-1b	15	PECAM-1	24	TIMP-1	33	IL-12p70	42	Insulin
7	RANTES	16	VEGF R2	25	TRAIL R4	34	IL-28A		
8	PF4	17	PDGF-AA	26	GH	35	IL-15		
9	I-TAC	18	IL-1ra	27	IL-4	36	B7-1		

Fig. 1B. Legends for Figure 1

endothelial response could be significant and probably chaotic, which determine transcription and gene regulation.[73] Chaos in the immune system could be a natural method of selection to strengthen immune functions. Optical laser chaos signals, which are high speed when studied are found to synchronize and it has many features.[74,75] Similarly, decoding these chaotic signals in immune system would potentially lead to our better understanding of immune system.

Streptococcus and viral infections – both together forever

In the recent times coronavirus infections (Covid-19) pandemic is rampant, and the infection selectively affects various countries, and the mortality statistics were varied in different countries. The Southeast Asian countries, India and neighboring countries, Africa and eastern European countries are relatively less affected so far, at his time of writing. The *S. pyogenes*', tropical bacterial infections and other viral are common in these areas, and they could provide cross-immunity to the Covid19 infections.[76] Bacteria can synthesize restriction enzymes like nucleases and inhibit viruses.[77] It has been shown that bacterial presence can reduce the intensity of viral infections.[78] Our study also reflects the immune regulation changes due to *S. pyogenes* as well as by its lysate.

Environmental modifications

In the theory of natural selection, the environment or the Nature in various forms could offer protection by its selective mechanisms in various geographic locations.[79] These could not necessarily be simple mechanisms but also as chaotic or cross-immunity methods. The commonly available Streptococcus bacteria and the common viruses could be the mechanism of choice in the form of natural selection by 'unhygienic' means.[80]

Metabolic protection and autoimmunity regulation

The rarity of diabetes and rheumatic heart disease was observed by legendary physicians like Joslin and

Steinchron in the early 1920s and 1930's respectively.[81,82] Our study also suggests various metabolic modulators being stimulated and some inhibited. Also, Joseph Barach made similar observations in that period and attributed the views to immune regulation changes. Our observations, such as increased IL13, decreasing Fas, and Dickkopf proteins, also indicate possible metabolic protection. Antibodies seen in rheumatic fever are also found in antiphospholipid antibody syndromes.[83] However, the incidence of autoimmune diseases in rheumatic heart disorders is very rare or possibly mutually exclusive by negative feedback mechanisms, at least in the clinical experience of the author.

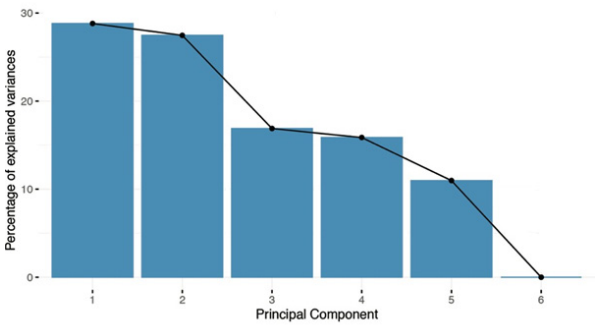

Fig. 2. Shows the variance explained by each principal component

Cancer prevention by S. pyogenes

Though certain viral infections can predispose patients for malignancies, infections can help to prevent malignancies.[84] This has been evaluated in the past as early as 1700's. William Coley in the 19th century has shown that *S. pyogenes*' vaccination can reduce the cancer progression, and improve the cure rates.[85] Hence, *S. pyogenes* vaccinations can be evaluated for this purpose. BCG vaccines are known to have anti-tumour activity in urinary bladder malignancies.[86]

Limitations and future perspectives

Further studies need to be performed to observe the immune changes in animals after direct *S. pyogenes* challenge and after the lysate administration. Specifically, the immune changes regulating the autoimmune disorders, cancer regulation, atherosclerotic processes, and host defense activities to viruses need to be studied in-depth in animal models.

Conclusion

Streptococcus pyogenes and its lysate has immunomodulation actions when tested with endothelial cells, which have pleiotropic functions. Further studies need to be performed to identify its potential benefits.

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