Expression profile of immune response genes in patients with Severe Acute Respiratory Syndrome

Renji Reghunathan\textsuperscript{†1}, Manikandan Jayapal\textsuperscript{†1}, Li-Yang Hsu\textsuperscript{2}, Hiok-Hee Chng\textsuperscript{3}, Dessmon Tai\textsuperscript{4}, Bernard P Leung\textsuperscript{1} and Alirio J Melendez*\textsuperscript{1}

Address: \textsuperscript{1}Department of Physiology, National University of Singapore, Singapore, \textsuperscript{2}Department of Infectious Disease, Tan Tock Seng Hospital, Singapore, \textsuperscript{3}Department of Rheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore and \textsuperscript{4}Department of General Medicine, Tan Tock Seng Hospital, Singapore

Email: Renji Reghunathan - phsrr@nus.edu.sg; Manikandan Jayapal - phsjm@nus.edu.sg; Li-Yang Hsu - liyang_hsu@yahoo.com; Hiok-Hee Chng - hiok_hee_chng@ttsh.com.sg; Dessmon Tai - dessmon_tai@ttsh.com.sg; Bernard P Leung - phslplb@nus.edu.sg; Alirio J Melendez* - phsmraj@nus.edu.sg

* Corresponding author    †Equal contributors

Abstract

Background: Severe acute respiratory syndrome (SARS) emerged in later February 2003, as a new epidemic form of life-threatening infection caused by a novel coronavirus. However, the immune-pathogenesis of SARS is poorly understood. To understand the host response to this pathogen, we investigated the gene expression profiles of peripheral blood mononuclear cells (PBMCs) derived from SARS patients, and compared with healthy controls.

Results: The number of differentially expressed genes was found to be 186 under stringent filtering criteria of microarray data analysis. Several genes were highly up-regulated in patients with SARS, such as, the genes coding for Lactoferrin, S100A9 and Lipocalin 2. The real-time PCR method verified the results of the gene array analysis and showed that those genes that were up-regulated as determined by microarray analysis were also found to be comparatively up-regulated by real-time PCR analysis.

Conclusions: This differential gene expression profiling of PBMCs from patients with SARS strongly suggests that the response of SARS affected patients seems to be mainly an innate inflammatory response, rather than a specific immune response against a viral infection, as we observed a complete lack of cytokine genes usually triggered during a viral infection. Our study shows for the first time how the immune system responds to the SARS infection, and opens new possibilities for designing new diagnostics and treatments for this new life-threatening disease.
of this disease are lymphopenia, transient thrombocytopenia, and normal neutrophil and monocyte counts [8]. It has been shown that SARS coronavirus infects and replicates in a wide variety of host cells, including PBMCs, in susceptible animals and human beings [9,10]. Hence, to understand the host response to this pathogen, we profiled the gene expression patterns of peripheral blood mononuclear cells (PBMC) from SARS patients, compared to healthy controls using oligo nucleotide microarrays. We found that in the PBMC from SARS patients a number of genes were differentially expressed, as compared to healthy controls, including immune-related genes and these genes are not the typical ones expected in a viral infection.

During a viral infection, most cell types in the body respond by secreting high levels of type 1 interferons (IFN-α and IFN-β) [11]. IFN-α/β can directly induce antiviral activities in neighboring cells, preventing viral spread by increasing the resistance of uninfected cells toward the virus. Moreover, these IFNs can activate Natural Killer (NK) cells mediated cytotoxicity toward virus-infected cells [11,12], and there is accumulating evidence that IFN-α/β contribute to driving the adaptive-immune response in the T helper cell type 1 (Th1) direction, via stimulation of IFN-γ expression [12]. NK cells can produce IFN-γ [13], which activates leukocytes, such as monocytes/macrophages, that, in turn, participate in the antiviral responses by producing free radicals and proinflammatory cytokines such as TNF-α [13]. During the response to viral infections, a key role is played by the expansion and activation of CD4+ and CD8+ T cells, which are central to the antiviral immunity, including their capability to inhibit replication and clear the infection. CD8+ cells have a direct effector role through cytotoxic T lymphocyte mediated lysis, and cytokine and chemokine production [14]. The role of CD4+ T cells in antiviral immunity is highly dependent on production of cytokines, notably IFN-γ [15], and the cytopathic activity exerted by a subset of CD4+ T cells [16]. Activation, coordination, and regulation of the above-described antiviral responses are mediated by complex mechanisms, where cytokines play important roles. However, to our surprise, we found that the patients’ response of SARS appears to be mainly an innate inflammatory response, rather than a specific immune response against a viral infection. There is no significant level of up-regulation of MHC-I genes, neither for major cytokines including IFNs, nor for genes involved in complement mediated cytosis, suggesting that the immune response against SARS-CoV may be different from other viral infections.

**Results and discussion**

To study the differential immune-gene expression patterns induced by SARS coronavirus, PBMCs from patients with SARS and normal subjects were examined using microarray technology. We chose to use PBMCs as these cells are more easily obtained from patients compared to other infected tissues, and the SARS-CoV has recently been shown to infect PBMCs [9,10]. The method of global gene expression analysis using oligonucleotide microarrays has proven to be a sensitive method to develop and refine the molecular determinants of several human disorders, including cancer and autoimmune diseases, and has provided us with signatures of the immune response [17]. Using this technology, complemented with powerful analytical methods, we compared the gene expression profiles of PBMC from a series of SARS patients with those of healthy donors. To ensure the reliability and reproducibility of the microarray analysis, Pearson Correlation factors using the signal from all the normal samples were calculated. All four control arrays have Pearson correlation coefficients (r) of >0.95, which suggests an excellent reproducibility among individual arrays in the same experiment and between normal control experiments.

We analyzed the expression pattern of over ~8,700 genes from the PBMCs of 10 SARS patients, and compared the expression patterns with healthy control samples. Student’s unpaired T-test (P < 0.01) was performed and significant genes were selected. Cluster analysis was performed to find distinct groups of genes that were significantly changed. The criteria used for cluster analysis is different from filter criteria used for differentially expressed genes. 248 genes were selected for Hierarchical clustering (Figure 1) which includes all the 186 differentially expressed genes. The severity of the disease does not seem to change the genetic profile of the PBMCs: 5 of our patients were in intensive care, whereas the other 5 patients did not require intensive care, at the time the blood was taken. However, no substantial gene-expression differences were observed that could correlate to disease severity.

To our surprise, the expression of genes coding for cytokines was completely absent, and the immune-related genes which were overexpressed are usually associated with innate-immune response against bacterial infection and not against a viral infection (Table 1). Several genes were highly up-regulated in patients with SARS, such as, genes coding for Lactoferrin, S100A9 and Lipocalin 2 among others (Table 1). Lactoferrin is a 78– to 80-kDa glycoprotein synthesized by glandular epithelial cells and mature neutrophils, frequently used as a marker for neutrophil degranulation at sites of inflammation, having well recognized bacteriostatic and bactericidal properties [18]. It is now clear that the biological actions of lactoferrin are not restricted to its bacteriostatic and bactericidal properties. Indeed, a wide array of actions has been reported for lactoferrin, including enhancing NK cell
Figure 1
Clustering and distribution of differentially expressed genes. A. Hierarchical clustering of gene expression data from PBMCs from 10 SARS patients showing different classes of gene expression profiles. Each row represents a separate gene and each column a separate SARS patient. 248 genes have been selected for this analysis which is described in methods. The expression index for each gene (rows) in each sample (column) is indicated by a color code. The color scale ranges from saturated green for log ratios -3.0 and above to saturated red for log ratios 3.0 and above. Red indicates increased gene expression levels, whereas green indicates decreased levels compared with normal samples. B. Pie chart showing the percentage distribution of the differentially expressed genes from the PBMCs of 10 SARS patients.
Table 1: Immune-response related genes which were found to be significantly up-regulated in PBMCs of SARS patients. Level of expression is expressed in Fold change (average of fold changes of ten patients, S1-S10) as compared to that of control samples from normal human subjects (C1-C4).

| Gen Bank ID | Description | Gene name | Fold change (S1-S10) |
|-------------|-------------|-----------|---------------------|
| NM_002343.1 | Lactotransferrin | LTF | 149.63 |
| M33326.1     | Carcinoembryonic antigen-related cell adhesion molecule 8 | CEACAM8 | 97.29 |
| NM_005564.1 | Lipocalin 2 | LCN2 | 80.40 |
| NM_004660.2 | S100 calcium binding protein A9 | S100A9 | 65.39 |
| NM_001725.1 | Bactericidal permeability-increasing protein | BPI | 49.94 |
| NM_005091.1 | Peptidoglycan recognition protein | PGLYRP | 47.86 |
| NM_001925.1 | Defensin alpha 4 | DEFA4 | 46.88 |
| U19970.1     | Antimicrobial LPS-binding protein CAP18 | CAMP | 43.36 |
| NM_005980.1 | S100 calcium-binding protein P | S100P | 39.52 |
| NM_00143.1 | Haptoglobin | HP | 34.78 |
| NM_004084.2 | Defensin alpha 1 | DEFA1 | 29.51 |
| NM_006865.1 | Leukocyte immunoglobulin-like receptor, subfamily A, member 3 | LILRA3 | 19.43 |
| NM_002870.1 | RAS oncogene family (RAB13) | RAB13 | 14.85 |
| NM_003064.1 | Secretory leukocyte protease inhibitor | SLPI | 11.35 |
| NM_00265.1 | Neutrophil cytosolic factor 1 | NCF1 | 11.30 |
| NM_00634.1  | Interleukin 8 receptor alpha | ILBRA | 10.85 |
| AL138717    | Rag D protein | RAGD | 7.84 |
| BG327863    | CD24 antigen | CD24 | 7.69 |
| NM_000419.2 | integrin, alpha 2b (antigen CD41B) | ITGAA2B | 5.50 |
| NM_001828.3 | Charcot-Leyden crystal protein | CLC | 5.47 |
| AI078167    | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | NFKBIA | 5.21 |
| NM_005621.1 | S100 calcium-binding protein P12 | S100A12 | 5.05 |
| NM_003255.2 | Tissue inhibitor of metalloproteinase 2 | TIMP2 | 4.93 |
| NM_003029.1 | Formyl peptide receptor 1 | FPR1 | 4.59 |
| NM_003384.1 | Nuclear factor, interleukin 3 regulated | NFIL3 | 4.50 |
| M25915.1    | Complement cytosis inhibitor (CLI) | CLU | 4.07 |
| NM_002965.2 | S100 calcium-binding protein A9 | S100A9 | 4.05 |
| NM_014214.1 | Inositol (myo)-1-(or 4)-monophosphatase 2 | IMPA2 | 3.95 |
| NM_003189.1 | T-cell acute lymphocytic leukemia 1 | TAL1 | 3.94 |
| U62027.1    | C3a receptor | C3AR | 3.83 |
| NM_001150   | Alanyl (membrane) aminopeptidase | ANPEP | 3.83 |
| BE789881    | RAS oncogene family | RAB3 | 3.75 |
| NM_001769.1 | CD9 antigen (p24) | CD9 | 3.73 |
| NM_014358.1 | C-type lectin, superfamily member 9 | CLEC9F | 3.53 |
| NM_006834.1 | RAS oncogene family member (RAB32) | RAB32 | 3.40 |
| J04162.1    | Leukocyte IgG receptor (Fc-gamma-R) | FCGR3A | 3.32 |
| NM_006866.1 | Lleukocyte immunoglobulin-like receptor, subfamily A2 | LILRA2 | 3.30 |
| U41070.1    | Leukotriene b4 receptor | LTBR | 3.23 |
| NM_006362.2 | Integrin, alpha M | ITGAM | 3.20 |
| BC003393.1 | Phosphoinositide-3-kinase, catalytic, beta polypeptide | PIK3CB | 3.13 |
| NM_002432.1 | Myeloid cell nuclear differentiation antigen | MND | 3.08 |
| NM_003264.1 | Toll-like receptor 2 | TLR2 | 3.05 |
| NM_004475.1 | Fibrinogen | FH | 3.04 |
| NM_00461.1  | Zyxin | ZYX | 2.96 |
| NM_003897.1 | Immediate early response 3 | IER3 | 2.91 |
| AF19873.1   | Serine (or cysteine) proteinase inhibitor alpha-1 | SERPINAI | 2.87 |
| NM_016205.1 | Platelet derived growth factor C | PDGF | 2.74 |
| NM_005479.1 | Frequently rearranged in advanced T-cell lymphomas | FRAT1 | 2.72 |
| AV756141.1  | Colony stimulating factor 2 receptor beta | CSF2RB | 2.71 |
| NM_006019.1 | T-cell, immune regulator 1 | TCR1 | 2.59 |
| J02959.1    | Leukotriene A-4 hydrolase | LAT4 | 2.59 |
| BC004188.1 | Tubulin, beta, 2 | TUBB2 | 2.57 |
| NM_001780.1 | CD63 antigen | CD63 | 2.53 |
| NM_003254.1 | Tissue inhibitor of metalloproteinase 1 | TIMP1 | 2.37 |
| AF070673.1  | Stannin | SNN | 2.35 |
| BC003570.1  | Similar to vesicle-associated membrane protein 3 | VAMP3 | 2.31 |
activity, as well as stimulating neutrophil aggregation and adhesion [18]. The myeloid-related proteins, S100A8, S100A9, and S100A12 have been shown to regulate lymphocyte, monocyte, and neutrophil migration and are found in the extracellular milieu during infections and inflammatory episodes [19,20]. The high levels of serum S100A8/A9 in chronic inflammatory pathologies such as rheumatoid arthritis and inflammatory bowel disease, as well as during bronchitis and tuberculosis [20], suggest that they might play a role in inflammatory reactions.

Lipocalins are a family of small, secreted proteins, which have little amino acid sequence homology (20–30%) but share a common three-dimensional structure [21,22]. Tissue distribution studies have revealed that a member of this family 24p3 lipocalin (24p3) is mainly expressed in the liver during an acute phase response [21]. It has also been detected in spleen, lung and the uterus. In the latter location, its expression has been found to be coincident with parturition, a time of major tissue remodeling and inflammation [22]. 24p3 has also been detected in the conditioned media of LPS stimulated murine PU5.1.8 macrophages and therefore it has been suggested to function in defense against infectious agents [22]. However, recent evidence proposed that the lipocalins may trigger apoptosis in immune cells via an unknown cell surface receptor [23]. SARS patients with respiratory distress fulfill criteria for Acute Respiratory Distress Syndrome (ARDS) and diffuse alveolar damage is seen in the lungs on histological examination of postmortem [24]. We speculate that upregulation of lipocalins are part of the host response in limiting unwanted tissue damage and in reducing inflammation and lung fibrosis. In addition, enhanced expression of lipocalins may play a role in the potential cause of lymphopenia observed in the majority of patients.

There is also up-regulation of expression of genes, such us, Bactericidal Permeability Increasing Protein (BPI) and Carcino Embryonic Antigen related Cell adhesion Molecule 8 (CEACAM 8 or CD66b). BPI is released from activated neutrophils and is an endogenous antibiotic, which rapidly kills Gram negative bacteria by high affinity binding to the LPS component of the cell wall [25]. CEACAM 8 is expressed by activated monocytes and granulocytes, and significant up-regulation of this gene indicates the involvement of innate immune cells in SARS. Other genes which encode proteins like Leukotrien-B4 receptor (LTB4R), Leukotrien-A4 hydrolase, IL-8 receptor (IL-8RA), anaphylatoxin C3a receptor-1 (C3aR1), Neutrophil Cytosolic Factor 1 (NCF 1), S100 calcium binding protein A9, Defensin, (DEFA 1/4), LPS binding protein CAP18 (CAMP), and Peptidoglycan Recognition Protein (PGLYRP) are involved in chemotaxis, inflammatory reac-

---

**Table 2: Immune-response related genes which were found to be significantly down-regulated in PBMCs of SARS patients. Level of expression is expressed in Fold change (average of fold changes of ten patients, S1-S10) as compared to that of control samples from normal human subjects (C1-C4).**

| Genbank ID   | Description                                           | Gene name              | Fold change (S1-S10) |
|--------------|-------------------------------------------------------|------------------------|---------------------|
| D13720.1     | IL2-inducible T-cell kinase                           | ITK                    | 3.64                |
| AF288571.1   | Lymphoid enhancer factor-1                            | LEF1                   | 3.63                |
| NM_005356.1  | Lymphocyte-specific protein tyrosine kinase           | LCK                    | 3.20                |
| NM_003151.1  | Signal transducer and activator of transcription 4    | STAT4                  | 3.14                |
| BG435404     | ADP-ribosylation factor-like 7                        | ARL7                   | 2.99                |
| J04132.1     | T cell receptor zeta-chain                            | CD3Z                   | 2.96                |
| BG532690     | Alpha 4 subunit of VLA-4 receptor                     | ITGA4                  | 2.91                |
| NM_001838.1  | Chemokine (C-C motif) receptor 7                      | CCR7                   | 2.83                |
| A1743792     | Sialyltransferase 1                                   | SIAT1                  | 2.71                |
| NM_001558.1  | Interleukin 10 receptor, alpha                         | IL10RA                 | 2.71                |
| BF669455     | Sialomucin                                            | CD164                  | 2.58                |
| NM_000733.1  | Epsilon polypeptide of CD3                            | CD3E                   | 2.58                |
| AI073984     | Interferon consensus sequence binding protein 1       | IC3BP1                 | 2.48                |
| NM_005475.1  | Lymphocyte adaptor protein                            | LNK                    | 2.38                |
| AB014560.1   | Ras-GTPase activating protein SH3 domain-binding protein 2 | G3BP2     | 2.31                |
| NM_004757.1  | Small inducible cytokine subfamily E, member 1        | SCYE1                  | 2.19                |
and superoxide metabolism [26]. Similarly, Formyl Peptidase Receptor (FPR) genes are expressed by activated neutrophils. Regulation of expression of FPR on neutrophils plays a key role in neutrophil polarization and chemotaxis. Chemotaxis is effected by a neutrophil membrane mesh, via remodeling of the actin component of the membrane [27]. Up-regulation of CD24 and FcγR3A indicates some degree of neutrophil, B cell and NK cell activity. FcγR3a, a low affinity receptor for IgG, expressed in activated macrophages and NK cells, fa-

Table 3: Genes involved in homeostasis and cell growth, which were found to be significantly up-regulated in PBMCs of SARS patients. Level of expression is expressed in Fold change (average of fold changes of ten patients, S1-S10) as compared to that of control samples from normal human subjects (C1-C4).

| Gen Bank ID | Description | Gene name | Fold change (S1-S10) |
|-------------|-------------|-----------|---------------------|
| BC005248.1  | Eukaryotic translation initiation factor 1A | EIF1AY | 44.79 |
| NM_001008.1 | Ribosomal protein S4, Y-linked | RPS4Y | 20.75 |
| NM_001062.1 | transcobalamin I (vitamin B12 binding protein, R binder family) | TCNI | 16.88 |
| NM_002357.1 | MAX dimerization protein | MAD | 11.88 |
| NM_012387.1 | Peptidyl arginine deiminase, type V | PADI4 | 9.12 |
| NM_000184.1 | Hemoglobin, gamma A | HBG1 | 8.1 |
| NM_004653.1 | SMC (mouse) homolog, Y chromosome | SMCY | 7.07 |
| NM_029394.1 | Ribonuclease, RNase A family, 2 | RNASE2 | 5.99 |
| M60721.1   | Homeo box 1 | Hlx1 | 5.85 |
| NM_005178.1 | B-cell CLL/lymphoma 3 | BCL3 | 5.11 |
| NM_001964.1 | Early growth response 1 | EGR1 | 4.9 |
| NM_006931.1 | Solute carrier family 2, member 3 | SLC2A3 | 4.89 |
| NM_02863.1 | Phosphofructokinase, liver | PYGL | 4.89 |
| NM_021122.2 | Fatty-acid-Coenzyme A ligase, long-chain 2 | FACL2 | 4.87 |
| L13974.1   | Nuclear factor (erythroid-derived) 2 | NFE2 | 4.77 |
| AI313232.1 | H2A histone family, member O | HIST2H2AA | 4.48 |
| BC002649.1 | H1 histone family, member 2 | HIST1H1C | 4.44 |
| AF073310.1 | Insulin receptor substrate-2 | IRS2 | 4.43 |
| NM_055303.2 | Hemoglobin, epsilon 1 | HBE1 | 3.97 |
| AF073890.1 | Cathepsin X precursor | CTSZ | 3.69 |
| AB002539.1 | Syntaxin binding protein 2 | STXB2 | 3.69 |
| NM_017445.1 | H2B histone family, member S | HIST1H2BK | 3.64 |
| NM_003118.1 | Secreted protein, acidic, cysteine-rich (osteonectin) | SPARC | 3.62 |
| AL353759.1 | Two genes for novel histone I | HIST1H2AC | 3.54 |
| NM_003681.1 | Pyridoxal (pyridoxine, vitamin B6) kinase | PDKX | 3.54 |
| BC001886.1 | Ribonucleotide reductase M2 polypeptide | RRME | 3.53 |
| AI082078.1 | Actin, alpha 1 | ACTN1 | 3.42 |
| AF087942.1 | Glycogenin-IL | GYG | 3.39 |
| AL538601.1 | Sjogren syndrome antigen A2 | SSA2 | 3.36 |
| NM_001805.1 | CCAAT enhancer binding protein | CEBP | 3.26 |
| NM_003528.1 | H2B histone family, member Q | HIST2H2BE | 3.13 |
| BC001906.1 | Similar to metaxin | MTX1 | 3.11 |
| NM_006755.1 | Transaldolase 1 | TALDO1 | 3.07 |
| BC000893.1 | Histone family, member A | HIST1H2BK | 3.06 |
| AL161952.1 | Glutamate-ammonia ligase | GLUL | 3.05 |
| BC002842.1 | H2B histone family, member B | HIST1H2BD | 2.78 |
| NM_004219.2 | Pituitary tumor-transforming 1 | PTG1 | 2.75 |
| NM_012228.1 | Pinil-like transcription factor | PILB | 2.73 |
| LI2711.1   | Transketolase | TKT | 2.68 |
| BE561479.1 | Glyceraldehyde-3-phosphate dehydrogenase | GAPD | 2.61 |
| NM_012331.2 | Methionine sulfoxide reductase A | MSRA | 2.6 |
| M33197.1   | Glyceraldehyde-3-phosphate dehydrogenase | GAPD | 2.59 |
| NM_005195.1 | CCAAT enhancer binding protein | CEBP | 2.57 |
| NM_018840.1 | Putative Rab5-interacting protein | C20or24 | 2.45 |
| AA314406.1 | Thyroid Hormone Receptor Associated protein | TRAP95 | 2.44 |
| NM_004832.1 | Glutathione-S-transferase like, glutathione transferase omega | GSTLP | 2.42 |
| NM_012372.2 | Solute carrier family 21 (organic anion transporter) | SLC21A1 | 2.42 |
Antibody Dependent Cell mediated Cytotoxicity (ADCC) by NK cells [13]. There is moderate up-regulation of the kappa light chain of the Nuclear Factor (NFκB 1A) and the B-cell lymphoma 3-encoded protein (BCL3) (Table 1). The major form of NFκB is a heterodimer of p65/p50 subunits, which regulates expression of many proteins in activated immune cells by binding to DNA. It interacts with NFκB through its ankyrin repeats and it favors p50 dimerization by recruiting p50 monomers from the cytoplasmic pool of p105/p50 dimers, and thereby enhancing nuclear translocation and DNA binding of p50 dimers [28]. BLC3 is expressed by activated B cells and T cells on mitotic stimuli, playing an important role in transcription activation [29].

In SARS patients there is down-regulation of genes (Table 2) which regulate proliferation and differentiation of T-cells, such as the Lymphocyte-specific protein tyrosine kinase (LCK), which is required for phosphorylation of CD3. There is down-regulation of genes coding for the epsilon polypeptide of the TCR (CD3E), IL-2 induced T-cell kinase (ITK), the Zeta chain of the TCR (CD3Z), the Alpha 4 subunit of VLA-4 receptor (ITGA4), the Chemokine (C-C motif) Receptor 7 (CCR7), and the Interleukin 10 receptor alpha (IL10 RA). All these point to a potential state of unresponsiveness towards the SARS-CoV antigens. It has been described that T cell function in patients with peritonitis had decreased Th1 function, without increased Th2 response, and furthermore T cell proliferation and cytokine secretion correlated with mortality [30]. In our study, there is up-regulation of genes involved in homeos-
tasis and cell growth (Table 3); for example, genes involved in DNA synthesis, nucleosome assembly and protein synthesis, such as genes encoding Translation Initiation Factor 1A (EIF1AY), and histone proteins. There is down-regulation of genes coding for negative regulators of the cell cycle, such as, Retinoblastoma-like 2 protein (RBL2), Cyclin-Dependent Kinase Inhibitor 1B (CDKN1B) and anti-apoptotic protein TNF induced protein GG2-1 etc (data not shown). These findings indicate a high degree of proliferation of immune cells; however, there is no significant level of increase in the expression of cytokines like IL-2 or IL-3 and their receptors, which are required for the proliferation and the differentiation of T cells and effector functions of B cells and NK cells. So the increased cell proliferation may be of a granulocyte lineage rather than a monocytic lineage. We confirmed our microarrays findings by Real time PCR on selected upregulated genes such as Lactoferrin Lipocalin, S100P, FCGR3A, and TLR2 (Figure 2). The average expression of all the five genes by real-time RT-PCR was compared to the average expression levels found by microarray analysis (Table 4). The real-time PCR method verified the results of the gene array analysis and showed that those genes that were up-regulated as determined by microarray analysis were found to be up-regulated by real-time PCR analysis. Moreover, since we did not find any upregulation of cytokine-genes usually associated with a viral infection, we selected a few cytokines and compared the SARS samples, with samples collected from 5 influenza virus-infected patients. The observed results, in figure 2, showed that in the influenza patients the mRNA for type I interferons (IFNα and IFNβ), TNFα and IL-12-p40 were upregulated, whereas genes upregulated in the SARS patients (LTF, Lipocalin, S100P, FCGR3A and TLR2) were not upregulated in the influenza patients. Therefore, firstly, this confirms that cytokines usually triggered during a viral infection were not detected in the SARS samples (but were triggered in the influenza patients); and secondly, genes triggered in the SARS patients were not triggered in influenza patients.

In this study we have performed extensive analysis of gene expression of PBMCs of SARS patients using a microarray platform that includes more than 8000 gene sequences. However, one potential drawback in our current experimental design is that the gene expression levels were compared between patient samples and normal controls using PBMCs, rather than purified individual cell types. While it would be of interest to determine the exact proportions of monocytes, lymphocytes and contaminating granulocytes in our PBMC preparations, unfortunately, given the highly infective nature of patient samples and lack of suitable flowcytometry facility with appropriate bio-safety control, FACS analysis was, however, not possible.

Our results suggest that the response of SARS affected patients seems to be mainly an innate inflammatory response, rather than a specific immune response against a viral infection. There is no significant level of up-regulation of MHC-I genes or major cytokines including IFNs (α, β, and γ), or genes involved in complement mediated cytolysis, suggesting that the immune response against the SARS-CoV may be different from other viral infections, or that the virus may be using a unusual strategy to evade the host immune system and cause the pathogenesis and mortality.

**Conclusions**

This differential gene expression profiling of PBMCs from patients with SARS, strongly suggests that the response of SARS affected patients seems to be mainly an innate inflammatory response, rather than a specific immune response against a viral infection. There is no significant level of up-regulation of MHC-I genes or major cytokines, including IFNs, or complement mediated cytolysis, suggesting that the immune response against the SARS-CoV may be different from other viral infections or that the virus may be using a different strategy to evade the host immune system and cause the pathogenesis and mortality. The severity of the disease does not seem to change the genetic profile of the PBMCs: 5 of our patients were in intensive care, whereas the other 5 patients did not

| Gene name | Microarray Mean expression | Microarray Fold increase | Realtime RT-PCR mRNA conc.(ng) | Realtime RT-PCR Fold increase |
|-----------|---------------------------|--------------------------|-------------------------------|-------------------------------|
| LTF       | 12403.95/83.78            | 148.05                   | 1204/13                       | 92.6                          |
| Lipocalin | 9788/135.94               | 72                       | 1455/25                       | 58.2                          |
| FCGR3A    | 6202.81/1885.35           | 3.29                     | 650/205                       | 3.17                          |
| S100P     | 7496.57/292.49            | 25.63                    | 475/45                        | 10.5                          |
| TLR2      | 3459.33/1141.69           | 3.03                     | 563/200                       | 2.8                           |

*Calculated as the average of 10 SARS samples over (/) the average of the 4 normal controls, and expressed as fold increase.
Microarray hybridization and scanning
Fragmented cRNA (10 to 11 µg/probe array) was used to hybridize to human focus array (HG-Focus Array) at 45°C for 16 hr with constant rotation of 60 rpm in a Gene chip hybridization oven 640 (Affymetrix). The chips were washed and stained using Gene chip fluidsics Station 400 (Affymetrix). Staining was performed using streptavidine phycoerythrin conjugate (SAPE, Molecular Probes, Eugene, OR), followed by the addition of biotinylated antibody to streptavidine (Vector Laboratories, CA), and finally with streptavidine phycoerythrin conjugate. Probe arrays were scanned using Agilent Gene Array Scanner Series US 74900593 (Agilent technologies, USA).

Data filtering and analysis
An absolute expression analysis was performed using Microarray Suite Software 5.0 (Affymetrix) and relative mRNA expression levels were expressed as plus or minus fold changes compared to normal controls. Each chip was scaled to an overall intensity of 500 to correct for minor differences in overall chip hybridization intensity, and to allow comparison between chips. The data from ~8700 genes was import in to MicroDB 3.0 and Data Mining Tool 3.0 (Affymetrix) for further analysis. Pearson Correlation coefficient (r) was used to ensure the reproducibility of the data using signal from normal samples. T-test was performed to identify genes that were differentially expressed in SARS patients over normal samples. The statistical significance of the differential expression of any gene was assessed by computing P value for each gene. Any gene for which this P value was < 0.01 was considered to be differentially expressed. We selected 186 genes for further analysis that met the following criteria:

(i) Changes in expression of at least 2 fold higher or lower comparing the normal

(ii) Signal >500

(iii) Detection P < 0.01 and

(iv) Genes which met the above criteria in at least 30% of samples.

Finally, data representing 10 SARS patients fold changes over control samples were averaged.

Genes were annotated according to biological process using the Gene ontology: tool for the unification of biology from the Gene Ontology Consortium [31]. The complete set of raw data was deposited into the NCBIs' Gene Expression Omnibus (GEO) and it can be accessed through the GEO accession ‘GSE1739’ http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1739.


Hierarchical clustering

Unweighted average linkage Hierarchical clustering was applied for samples using the 'Genes' software [32]. Genes with 'Present' call (P < 0.01) and significantly changes in expression of at least 2 fold higher or lower comparing the normal were selected. Finally 248 genes which are passing this filter criteria in at least 15% of samples and above were selected for clustering.

Real-time quantitative PCR

Real-time PCR was performed for ten genes, namely: Lactoferrin (Primers: 5’ tgc ttc tgt tcc tcc tgg 3’ and 5’ tcc agc ggt ctc cgg aac gcc 3’); Lipocalin (Primers: 5’ aag ccc ctg ctc tgt gcc atc agc 3’ and 5’ cag cct gat gct gta tgc cag ggt 3’); S100P (Primers: 5’ cat gat cat aga cgt ctt ttc 3’ and 5’ aca cga tga act cag cag 3’); TLR2 (Primers: 5’ gta tct gca aag gga gct gat gag gta tgc cag ggt 3’); FCGRA3A (Primers: 5’ ctc cgg ata ttc tgt ggt act 3’ and 5’ tgg aga gtc gtt ttc cag 3’); IFNA (Primers: 5’ atg gcc tgt acc ttt gtt 3’ and 5’agg aag att tcc tca tca c 3’); IFNB (Primers: 5’ atg acc aag aag tgt ctc cca a 3’ and 5’ ttc ttc ctc gag cag ggt tct ca 3’); IL-12 p40 (Primers: 5’ atg tgt cag cag tgt gtc atc 3’ and 5’ ctt gat aat cct gta ctct 3’); TNFA (Primers: 5’ ipg tga cca gcc tgt agc cta tgt ggt 3’ and 5’ gca atc ccc a a aag tag acc tgc cca gac 3’); GAPDH (Primers: 5’ acc aca gtc cat gcc atc ac 3’ and 5’ tcc acc acc ctg tct gcg ta 3’). For amplicon detection, the Light Cycler RNA Master SYBR Green Kit (Roche) was used as described by the manufacturer. PCRs were performed in a LightCycler® instrument (Roche) as follows: reverse transcription at 61 °C for 20 min, initial denaturation at 95°C for 2 min; amplification for 45–65 cycles of denaturation (95°C, 5s, ramp rate 2°C/ s), annealing (optimal temperature, 5s, ramp rate 2°C/ s) and extension (72°C, product length [bp]/25 s, ramp rate 2°C/ s). A single online fluorescence reading for each sample was taken at the end of extension step. Quantitative results were expressed by identification of the second derivative maximum points, which marked the cycles where the second derivatives of the fluorescence signal curves are at maximum. These points were expressed as fractional cycle numbers. Then, these cycle numbers were plotted against the logarithm of the concentrations of serially 2-fold diluted standard samples to obtain a standard curve. The concentrations of unknown samples were calculated by extrapolation from this standard curve. Positive sample specificity was confirmed by determining the melting curve (95°C, 5s, ramp rate 20°C /s; 68°C, 15s, ramp rate 20°C /s; 95 °C, 0s, ramp rate 0.1°C /s, continuous measurement).

Authors’ contributions

RR carried out the sample preparation, hybridization and drafted the manuscript. MJ carried out statistical analysis, data filtration, and data deposition and helped with manuscript preparation. LYH, HHC and DT provided the blood samples and the clinical comments for the study. BPL advised on study design and development. AIM devised and coordinated the study, revised and finalized the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Biomedical Research Council-Young Investigator Award grant R-185-000-044-305. We thank A-K Fraser-Andrews for preproofreading the manuscript.

References

1. Peiris JS, Chu CM, Cheng VC, Chan KS, Hung IF, Poon LL, Law KL, Tsang BS, Hon TY, Chan CS, Chan KH, Ng JS, Zheng BJ, Ng WL, Lai WR, Guan Y, Yuen KY. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet. 2003, 361:1767-1772.
2. Lee N, Hui D, Wu A, Chan P, Cameron P, Joyce GM, Ahuja A, Yung MW, Leung CB, To KF, Li SF, Sputo CC, Corder R, Sun TL. A Major Outbreak of Severe Acute Respiratory Syndrome in Hong Kong. N Engl J Med 2003, 348:1986-1994.
3. Peiris JS, Lai ST, Poon LL, Guan Y, Lam YL, Lim W, Nicholls J, Yee WK, Yan WW, Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 2003, 361:1319-1325.
4. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnichenko T, Fouchier RA, Berger A, Bur- guiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Viele S, Klein HD, Osterhaus AD, Schmitz H, Doer K. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003, 348:1967-1976.
5. Kaizake TG, Erdman D, Goldsmith CS, Zaki SR, Perret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarnier J, Paddock CD, Rota P, Fields B, DeRisi J, Tang JY, Cox N, Hughes JM, LeDuc J, Bellini WJ, Anderson LJ. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003, 348:1953-1966.
6. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle GA, Tyler S, Vogrig R, Ward D, Watson B, Brunham RC, Krajden M, Ar- tsoh A, Majic S, Zaki SR, Schmaljohn C, Leibfritz D, Hulske J, Lui SF, Szeto CC, Chung S, Sung JJ. A community outbreak of coronavirus-associated SARS pneumonia in Hong Kong. N Engl J Med 2003, 348:1967-1976.
7. Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YS, Khattra J, Asano JK, Barber SA, Chan SY, Cloutier A, Coughlin SM, Freeman D, Ginn N, Griffith OL, Leach SR, Mayo M, McDonald H, Montgomery SB, Pandoth PK, Petruscu AS, Robertson AG, Schein JE, Sididique A, Smaliles DE, Scott JM, Yang GS, Plummer F, Andoanov A, Artsoh B, Bastien N, Bernard K, Booth TF, Bowness D, Czub M, Dredett M, Fernandez L, Del Rio R, Dervos A, Fouchier RA, Gobert A, Bur- guiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Viele S, Klein HD, Osterhaus AD, Schmitz H, Doer K. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003, 348:1953-1966.
8. Ruan Y, Wei CL, Ye AL, Vega VB, Thoreau H, Su ST, Chia JM, Ng P, Chiu KP, Lim L, Zhang M, Peng XX, Lin EO, Lee NY, Yee SL, Ng LF, Chee RE, Stanton LW, Long PM, Liu ET. Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. Lancet 2003, 361:1779-1785.
9. Gillim-Ross L, Taylor L, Schhoffman J, Redour J, Paul S, Masters PS, Wentworth DE. Discovery of Novel Human and Animal Cells Infected by the Severe Acute Respiratory Syndrome Coronavirus by Replication-Specific Multiplex Reverse Transcription-PCR. JCM 2004, 42:3196-3206.
10. Li L, Wu J, Shao J, Zhu H, Wu N, Li M, Yao H, Hu M, Dennin RH: SARS-coronavirus replicates in mononuclear cells of peripheral blood (PBMCs) from SARS patients. J Clin Virol 2003, 28:239-44.

11. Guidotti LG, Chisari FV: Cytokine-mediated control of viral infections. Viralology 2000, 273:221-227.

12. Biron CA: Role of early cytokines, including α and β interferon (IFN-α/β), in innate and adaptive immune responses to viral infections. Semin Immunol 1999, 10:383-390.

13. Biron CA, Nguyen KB, Pien GC, Couzens LP, Salazar-Mather TP: Natural killer cells in antiviral defence: function and regulation by innate cytokines. Annu Rev Immunol 1999, 17:189-220.

14. Harty JT, Tewinkel A, White DW: CD8+ T cell effector mechanisms in resistance to infection. Annu Rev Immunol 2000, 18:275-308.

15. Guidotti LG, Chisari FV: Noncytolytic control of viral infections by the innate and adaptive immune response. Annu Rev Immunol 2001, 19:65-91.

16. Price DA, Klenerman P, Booth BL, Phillips RE, Sewel AKL: Cytotoxic T lymphocytes, chemokines and antiviral immunity. Immunol Today 1999, 20:212-216.

17. Shaffer AL, Rosenwald A, Hurt EM, Giltnane JM, Lam LT, Pickeral O, Staudt LM: Signatures of the Immune Response. Immunity 2001, 15:375-385.

18. Baveye S, Blass E, Mazurier J, Spik G, Legrand D: Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. Clin Chem Lab Med 1999, 37:281-286.

19. Yang Z, Yao T, Rafferty MJ, Youssef P, Di Girolamo N, Geczy CL: Proinflammatory properties of the human S100 protein S100A12. J Leukoc Biol 2001, 69:986-994.

20. Frosch M, Streu A, Vogl T, Wulffraat NM, Kuis W, Sunderkotter C, Harms E, Sorg C, Roth j: Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis Rheum 2000, 43:628-637.

21. Flower DR, North ACT, Astwood TK: Mouse oncogene protein 24p3 is a member of the lipocalin protein family. Biochem Biophys Res Commun 1991, 180:69-74.

22. Liu QS, Nilsen-Hamilton M: Identification of a new acute phase protein. J Biol Chem 1995, 270:22565-22570.

23. Denny RN, Teodorov JG, Richard FA, Green MR: Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. Science 2001, 293:829-834.

24. Lew TW, Kwek TK, Tai D, Earnest A, Loo SK, Lee Y, Leang LM, Tai D, Earnest A, Liu SK: Acute respiratory distress syndrome in critically ill patients with severe acute respiratory syndrome. J A M A 2003, 290:374-380.

25. Maren MN, Wilde CG, Collins MS, Sable L, Thornton MB, Scott RW: The role of BPI protein a natural inhibitor of bacterial endotoxin. J Immunol 1992, 148:532-537.

26. Melchjorsen J, Sorensen LN, Paludan SR: Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. J Leukoc Biol 2003, 74:331-343.

27. Jematis A, Kloxin KN: Cytoskeletal regulation of chemotactic receptors: Molecular complexity of N-formyl peptide receptors with G proteins and actin. Eur J Haematol 1993, 51:288-293.

28. May MJ, Ghosh S: Signal transduction through NF-κB. Immunol Today 1998, 19:80-88.

29. Na SY, Choi JE, Kim HJ, Jhung BH, Lee YC, Lee JW: Bcl3, an IkappaB protein, stimulates activating protein-1 transactivation and cellular proliferation. J Biol Chem 1999, 274:28491-28496.

30. Heidecke CD, Hensler T, Weighardt H, Zant N, Wagner H, Siewert JR, Holzmann B: Selective defects of T lymphocyte function in patients with lethal intra-abdominal infection. Am J Surg 1999, 178:288-292.

31. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G: Gene ontology: tool for the unification of biology from the Gene Ontology Consortium. Nat Genet 2000, 25:25-29.

32. [http://genome.tugraz.at/].