

Effect of Influenza virus A infection on human immuno-inflammatory genes expression and gene ontology analysis

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Abstract: Influenza virus A is important agent that causes respiratory infections with high rates of morbidity and mortality worldwide. The aim of the study is the detection of changes in mRNA expression of 84 human inflammatory genes and gene ontology analysis. From 90 patients only 8 gave positive IgM – IAV test. IAV infection caused over expression of 10 common (in all patients) prominent proinflammatory genes which affect functional pathways. After infection other 6 genes were downregulated, this suggests that the host-viral interaction is a cellular response against viral infection. In conclusion: Gene ontology analysis revealed that IAV stimulated 4 different key role immunological pathways represented by cytokine-cytokine receptor interaction, NOD-like receptor signaling, Toll-like receptor signaling, and asthma pathway. The patients are mostly infected with related subtypes of IAV. These results may help in further analysis of viral-host interaction.

Keywords: RT-PCR-arrays; Influenza A virus- white blood cell interaction; inflammatory gene expression; cytokines; cytokines receptors.

INTRODUCTION

Viral infections are often associated with significant morbidity and mortality causing various diseases that range from mild to severe respiratory tract infection, Hepatitis, encephalitis, haemorrhagic fever and other severe diseases. Therefore, rapid and accurate diagnosis is essential for therapy (Barenfanger, et al., 2000). Influenza virus A is a single stranded RNA virus cause viral respiratory tract infections. Signals lead to alterations in the gene expression in the cell (Green, 2000; Penelope, et al., 2004). The detection of these pathogens and the determination of their effects on immune-gene expression in white blood cells is important for ; gene ontology analysis, initiating antiviral therapy, avoiding unnecessary antimicrobial therapy, preventing nosocomial spread, decreasing the duration of hospital stays, and reducing management costs (Adams, et al., 2010; Henrickson, 2005). Several studies have been reporting on the up and down-regulation of a number of genes associated with inflammatory pathways (Andrejeva et al., 2004). Influenza viruses A (IAV) are responsible for 5 to 15% of viral respiratory infections (Heikkinen and Jarvinen, 2003). IAV infection in human stimulates a number of inflammatory genes such as interferons (IFNs), Tumor necrosis factor (TNF) and interleukins (IL) genes which lead to bronchiolitis and ends with asthma (Reemerset et al., 2010; Huang et al., 2009).

IAV infection induce cytokines production, these cytokines activate; migration of adhesion molecules, immune cells to infected sites; facilitates the recognition of IAV after infection, function as potent chemoattractants, stimulates hundreds of IFN stimulating genes, stimulates the upregulation of IFN which is the most important gene for viral response, production of many chemokines; increase the production of proinflammatory genes represented by IL1, IL6, and IFNs which initiate the cascades of antiviral responses in infected cells (Geiler et al., 2010; Matloubian et al., 2000; Guan et al., 2002; Kotenko et al., 2003; Huang et al., 2010; Dillon, 2010; Novoselova et al., 2010; Dalodet et al., 2002; Wang et al., 2009; Adams et al., 2010). Microarray analysis is a powerful method to determine gene expression in cells and tissues under a variety of complex biological conditions (Clewley, 2004). The aim of the present study is to analyze proinflammatory gene-expression profiles peripheral white blood cells after the infection with IAV. And to determine genes that influence the main functional immune-inflammatory pathways.

MATERIALS AND METHODS

Patients, blood samples and ELISA test

From May to July 2009, 90 patients were hospitalized at Ibn AL-Haytham Hospital in Amman/Jordan for showing respiratory tract infection symptoms. Blood samples were collected in EDTA tubes during the acute phase of the disease (within 48 hrs of infection). Serums were separated and forwarded for indirect ELISA-IgM assay (IBL- Hamburg Corporation) to confirm the viral type infection. After one month (after recovery) blood samples were taken from patients who showed positive results to analyze the gene expression changes in leukocytes in response to IAV infection.

Extraction of mRNA from patient's leukocyte

The RNA is extracted within 24h after blood collection. RNA-extraction phase, treat the leukocytes concentrate (isolated from the blood Buffy coat) with Trizol reagent (Phenol-guanidin-isothiocyanate); Separation phase, using chloroform; RNA precipitation phase, using Isopropanol Alcohol; washing phase, using 70% ethanol, and dissolving phase; using Rnase-Dnase free water (Invitrogen) (Rinyet et al., 2005).

RNA quantification

Spectrophotometer (Bio-Rad) analysis was carried out using spectrophotometer (Bio-Rad) then gel-electrophoresis. In spectrophotometer the O.D_{260/280} ratio is checked. RNA is considered pure if the O.D ratio is between 1.8 and 2.0. RNA efficiency test Prior to RT PCR-Array, the RNA samples were tested for the efficiency of the reverse transcription to produce the cDNA (Promega USA) (Sebastian and Johanson, 2009).

Real time- PCR array

Real time-PCR array was performed for extract from IVA-infected samples in patient's leukocytes concentrate. cDNAs were synthesized using SABioscience kit. Each cDNA sample was added to RT-qPCR master Mix containing SYBR Green and reference dye (SABioscience). 25µL of cDNA-Master Mix mixture was added to each well across the PCR-arrays (two RT-PCR-array plates for each patient; one during PIV infection and the second for control, after recovery). Each PCR-array profiles the expression of 84 pathway-specific genes plus 12 internal controls (SABioscience). Thermocycling conditions as 95°C for 10 min and 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min) using Bio-Rad-iCycler real-time PCR detection system.

Statistical analysis

Non-parametric Wilcoxon signed ranks-test to calculate the Z-value and P-value for each gene (Joshua et al., 2006). Results From 90 patients subjected to ELISA-IgM test, only 8 patients were positive results for IAV. When subjected to electrophoresis can be used to produce the cDNA strand to be used for the RT-PCR Array technique.

Leukocyte gene expression analysis after IAV infection

The IAV infection upregulated for 10 pro- inflammatory genes in the 8 positive (Table 1). We found that 7 genes were downregulated in all infected patients (Table 2). The common up and downregulated genes are gathered in one heat map through the clustering of all 84 genes in all 8 infected patients to give a complete image for the inflammatory gene expression profile in the IAV infected leukocyte (Eisen et al., 1998) (Figure 1).

Table 1: The prominent common 10 up regulated genes and their frequencies in 8 patients after 48h of IAV infection

No.	Unigene	Gene name	Frequency/8 patients	Fold change	Total Frequency(%)
1	Hs.193717	IL 10	8	16.14	100
2	Hs.241570	TNF	8	18.22	100
3	Hs.303649	CCL 2	8	39.73	100

4	Hs.126256	IL1B	8	60.94	100
5	Hs.789	CXCL1	8	26.04	100
6	Hs.78913	C-X3-CR 1	8	21.16	100
7	Hs.624	IL8	8	53.02	100
8	Hs.514821	CCL 5	8	13.51	100
9	Hs.211575	IFNA 2	6	262.60	83.33
10	Hs.1722	IL1A	6	34.08	83.33

Table 2: The common 7 down regulated genes and their frequencies in 8 patients after 48h of IAV infection

No.	Unigene	Gene name	Frequency in 8 infected patients	Fold change	Total Frequency (%)
1	Hs.301921	CCR1	6	-250.1	66.66
2	Hs.407995	MIF	5	-19.30	58.33
3	Hs.247838	CCL24	4	-10.68	50
4	Hs.376208	LTB	4	-6.86	50
5	Hs.517106	CEBPB	7	-5.56	75
6	Hs.414629	CCL13	4	-5.35	50
7	Hs.591680	SCYE1	4	-4.68	50

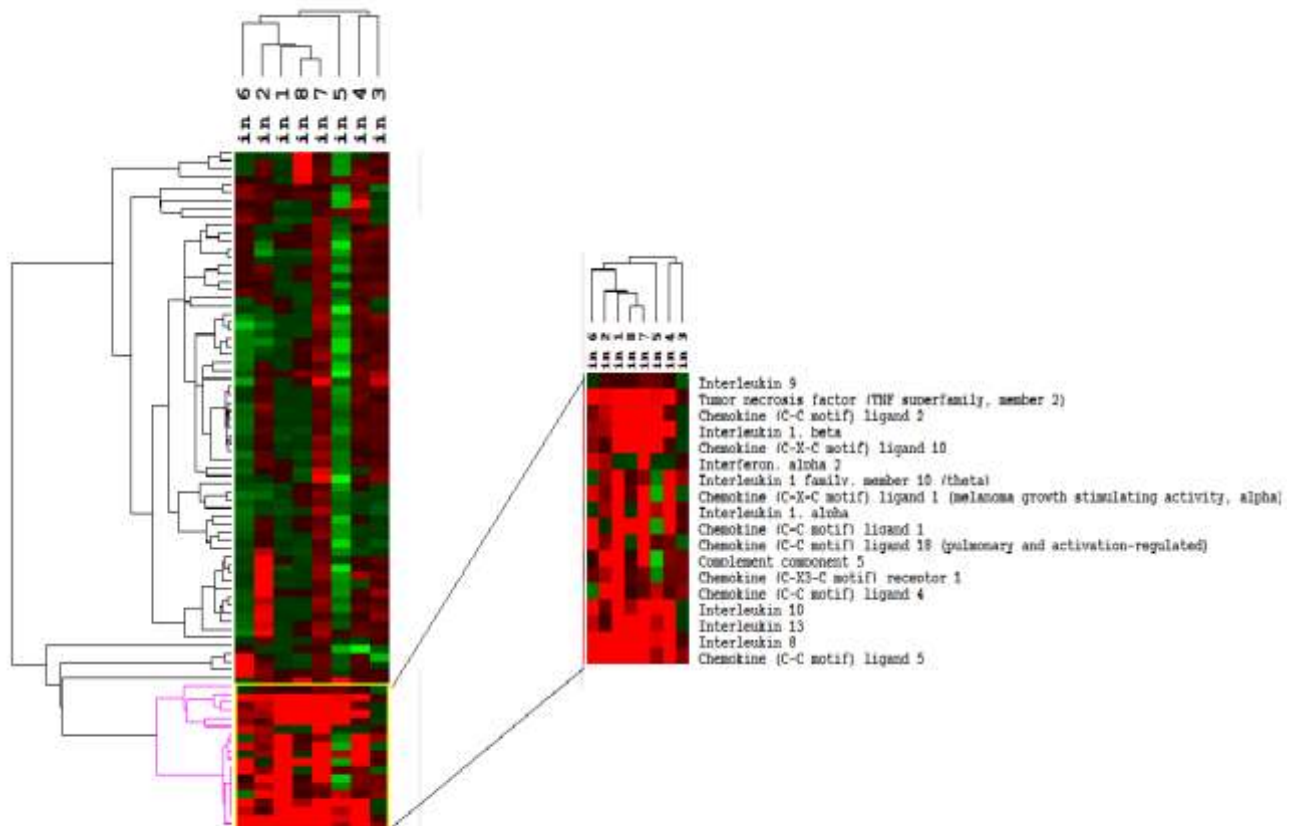


Figure 1: Cluster of 84 immune genes expression in 8 samples of the human leukocytes after IAV infections. The dendrogram depicts the 48 inflammatory-gene profile of leukocytes. Each panel represents one patient with 84 genes. The up regulated genes are with red color and the down regulated genes are with green color.

Discussion

IAV infection upregulated 10 human proinflammatory genes (IFNA2, IL1B, IL8, CCL2, IL1 A, CXCL1, CX3CR1, TNF, IL10, and CCL 5) (Table 1). Upregulation of mRNA of interferon, alpha 2 (IFNA2) with high significance induces several antiviral proteins which lead to general inhibition of protein synthesis and replication of the invader virus (Bone et al., 2005). Another study showed that infection with IAV stimulates the upregulation of IFN which give a protective role against viral infections (Huang et al., 2010). IFN production also increased significantly in most vertebrates after the infection of IAV (Dillon, and Runstadler, 2010). Previous study revealed that the infection of mouse with IAV cause upregulation of 46-interferon induced genes in addition to other cytokines (Novoselova et al., 2010). In the present study the mRNA transcription of IFNA2 upregulated with 262.6 fold after 48 h of IAV infection.

Similar results were revealed that IFNA2 is highly upregulated in viral infections and stimulates gene expression in NK cells and induces hundreds of IFN stimulating genes (ISGs) which activate antiviral state in the infected cells (Matikainen et al., 2001; Dalodet et al., 2002; Sato et al., 2000). Interleukin 1-B cytokine is an important mediator of inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. It activates IL-1R type 2 which in turn stimulates IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity (Stuart and Nancy, 2001). The high significant mRNA expression (60.94 fold) of IL1B gene in all infected patient with high frequency (100%) in this study suggests its immune role against the IAV. The effect of IAV on IL1B level, also seen in previous study which showed that IL1B and IL6 cytokine levels were upregulated during the IAV infection (Adams et al., 2010).

Interleukin 8 is upregulated with 53.02 fold. The activation of IL8 was found to cause bronchiolitis and ends with asthma (Huang et al., 2009). Another study showed that IL8 is upregulated after IAV and then stimulates the innate immune system response and may cause asthma complication (Utgaard et al., 1998). CCL 2 is upregulated with high significance (39.73 fold). CCL2 has direct and indirect effect on immune responses toward viral infections through its functions as a chemotactic factor for monocytes and basophiles (Joshua et al., 2006; Zlotnik et al., 2006). It activates CCR2 which in turn acts as a receptor for the monocyte chemoattractant proteins (Joshua et al., 2006). CCL2 transcription is also upregulated in human monocyte culture after IAV infection (Bubfeld et al., 1998). Another study showed that CCL2 is a potent inducer of dendritic cell maturation (Bernt et al., 2005). In this study the interleukin-1A is upregulated with high significance (34.08 fold). Interleukin-1A is a member of the IL-1 family which seems to participate in the pulmonary immune response against pathogens (Rosseau et al., 2007; Higginbotham et al., 2002) (Table 2). IL1A pulmonary concentration was also upregulated and elevated in IAV (H1N1 A/Puerto Rico/8/34) after mice infection (Aleksandret et al., 2005). Another study showed that the IL1A is significantly upregulated and increased in the bronchoalveolar fluid of IAV asthmatic patients (Montes et al., 2006). CXCL1 chemokine upregulated in this study with high significance (26.04 fold). The function of CXCL1 chemokine gene (the melanoma growth stimulatory activity/growth-regulated protein) is to regulate the cell trafficking of leukocytes (Huang et al., 2009 (Table 1).

CX3CR1 (chemokine fractalkine) is upregulated with high significance (21.16 fold) and acts as a mediator for both adhesive and migratory leukocytes and also acts as a co-receptor for viral envelope protein (Huang et al., 2009). In another study, the CXCL1 showed high levels of expression (30.5-fold) during IAV infection (Crompton, et al., 1988). The results of these studies are consistent with the findings of these studies especially in the highly upregulation of CXCL1 after IAV infection and the presence of asthma complication signs and symptoms in some of the patients. IAV infection stimulates the mRNA expression of this gene which may interact with IAV invasion and mediates different immune processes and facilitate the recognition and binding with the IAV after invading the host cell (Matloubian et al., 2000). TNF was upregulated in this study with high significance (18.22 fold). This gene encodes a multifunctional proinflammatory cytokines through activation of more than 60 proinflammatory genes (Mehta et al., 2003) (Table 1). It was recorded previously that TNF mRNA was significantly increased by infection of influenza virus and Sendai virus (Zhu et al., 2010; Veckman et al., 2006). Another study also showed that IAV infection to human cells cause upregulation of TNF and IL6 at the first 24 h of infection (NGET et al., 2010).

Interleukin-10 cytokine upregulated with 16.14 fold (Table 1). The upregulation of this potent anti-inflammatory cytokine may be induced by the invading virus to make use of its anti-inflammatory function and to provide a safe environment for viral replication. IL10 represses the expression of TNF- α , IL6 and IL1 by activating macrophages. Another study showed a significant increase in IL10 mRNA expression in virus infection accompanied with acute asthma (Grissellet et al., 2005). Another study revealed a wide involvement of interleukin 10 to prevent viral infections and produce regulatory cells that are involved in protection against allergic diseases (Mege et al., 2006).

Previous study reported that IL10 appears to play a detrimental role during host response to acute influenza virus infection (Keeret et al., 2008). Another study showed a significant increase in IL10 mRNA expression in virus infection accompanied with acute asthma, but not observed in virus infection without asthma (Grissellet et al., 2005). CCL 5 is upregulated after infection with IAV with high significant (13.51 fold) (Table 1). Previous studies showed that CCL5 is upregulated and accumulated in respiratory secretion after 24 h of IAV infection (Brydon et al., 2003; Cully et al.,

2006). Recent study also showed that the infection of A549 cells with IAV stimulates the upregulation of CCL5 and other cytokines (Geileret al., 2010).

Gene ontology for upregulated genes after IAV infection

The function of gene ontology (GO) is to classify functional categories based on co-occurrence with sets of genes in a gene list and rapidly unraveling new biological processes associated with cellular functions and key pathways. GO also provides investigators with much more power to analyze their genes using many different biological aspects in a single space (Huang et al., 2009). IAV infection upregulated 10 proinflammatory genes in human leukocytes which classified in to four groups participate in important inflammatory pathways and represented by; 10(100%) genes activate the cytokine-cytokine receptor interaction pathway, 8 (80 %) genes involved in the NOD-like receptor signaling pathway, 7 (70%) genes involved in Toll-like receptor signaling pathway, and 4 (40%) genes involved in asthma pathway (Table 3).

Table 3: Gene ontology for 10 common upregulated genes after infection with IAV

Gene pathway	Gene number and Gene percentage (%)	Gene name
Cytokine-cytokine receptor interaction pathway	10 (100)	CCL2,CCL5, CXCL1, CX3CR1, IFNA2, IL1A, IL1B, IL8, IL10, TNF
NOD-like receptor signaling pathway	8 (80)	CCL2, CCL5, CCL11,CC17, CXCL1, IL1b, IL8, TNF
Toll-like receptor signaling pathway.	7 (70)	CCL4,CCL5,CXCL10, IFNA2, IL1B, IL8, TNF
Asthma pathway	4 (40)	CCL11, IL10, IL13, TNF

Conclusions

RT-PCR-Array allows comparison of viruses with respect to their impact on cellular pro-inflammatory gene expression. Human WBC gene expression profiles, induced by IAV demonstrate different alterations of inflammatory genes which are involved in complex interaction between these viruses and human WBCs. Gene ontology showed that IAV viruses stimulate four different inflammatory pathways with specific genes. The infection with IAV stimulates asthma pathway which leads to asthma complication. Further studies at the protein level and protein-protein interactions within the IVA infected cells will be required to explore the underlying mechanism of the effects of IAV infection during the course of infection.

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