Role of toll-like receptors in naturally occurring influenza virus infection

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KEY MESSAGES

1. In naturally occurring influenza virus infection, there is differential increase in toll-like receptor (TLR) expression in antigen-presenting cells.
2. Increased TLR expression is associated with early innate suppression of virus, reducing influenza viral loads.
3. Concomitantly, TLR signalling induces pro-inflammatory and adaptive cytokine responses.
4. Targeting of TLRs may be a novel strategy to improve influenza control.

Hong Kong Med J 2014;20(Suppl 6):S11-5
RFCID project number: 09080102

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Introduction

Toll-like receptors (TLRs) are pattern recognition receptors expressed by antigen-presenting dendritic cells, monocytes/macrophages, and epithelial cells; when activated, they trigger the innate immune responses. Viral nucleic acids (dsRNA, ssRNA, CpG oligodeoxynucleotides) are detected by the endosomal TLRs 3,7,8, and 9, whereas bacterial components (peptidoglycans/lipoproteins, lipopolysaccharides) are detected by cell-surface TLRs 2 and 4. In influenza pathogenesis, TLRs induce expressions of type-I interferons and pro-inflammatory cytokines (eg IL-6, TNF-α), limiting viral replication/dissemination, mediating tissue inflammation, and linking to adaptive immunity development. Animal models suggested that TLR targeting may be clinically useful, as it can rapidly up-regulate innate immunity and provide broad-range virus strain non-specific protection against lethal influenza virus challenge. This study hypothesised that the 'virus-sensing' TLRs are up-regulated in patients with influenza virus infections, and TLR signalling pathway activation is associated with virus inhibition and pro-inflammatory cytokine expression. Adults hospitalised with influenza virus A infection were compared with healthy controls, and TLR expression in antigen-presenting cells, respiratory tract viral loads, plasma cytokines/chemokines, and signalling molecules were studied simultaneously. Patients' immune effector cells were stimulated ex vivo with TLR-specific ligands for response.

Methods

This prospective, case-control study was conducted from January 2010 to December 2011 during the influenza seasonal peaks in Hong Kong. Informed consent was obtained from each subject. Ethical approval was obtained from the Institutional Review Boards of the Chinese University of Hong Kong and the Hospital Authority of Hong Kong. Adults aged 18 years or older who were hospitalised for influenza A virus infection and presented within 48 hours of illness onset were recruited. Patients with antiviral treatment before enrolment, underlying immunocompromised conditions (eg autoimmune diseases, HIV/AIDS) or receiving immunosuppressant (including corticosteroids) were excluded. Age- and sex-matched controls were recruited outside the seasonal peak periods from the general outpatient clinics and the community for comparison. Those with any immunocompromised condition or a history of any febrile illness in the past 4 weeks were excluded.

Peripheral blood samples were taken. Expression profiles of TLRs 2, 3, 4, 7, 8, and 9 on blood monocytes (CD14+), 'total' dendritic cells (CD14-, CD16-, CD85k+), myeloid dendritic cells (CD16-, CD14-, CD85k+, CD123-) and plasmacytoid dendritic cells (CD16-, CD14-, CD85k+, CD123+) were analysed by flow cytometry using established methods. Intracellular signalling molecule expression on peripheral blood mononucleated cells (PBMCs), including activated MAPKs (phospho-p38 and phospho-ERK) and NF-κB (phospho-IκB) were similarly analysed by flow cytometry. Plasma concentrations of 14 'pro-inflammatory' or 'adaptive' immunity-related cytokines/chemokines, including IL-6, TNF-α, CXCL8/IL-8, CCL2/MCP-1, and IL-1β; and IFN-γ, CXCL10/IP-10, CXCL9/MIG,

CCL5/RANTES, and IL-12p70; and IFN-α2, CCL3/MIP-1α, IL-10, and sTNFR-1 (indicating TNF-α release) were measured using cytometric bead array with flow cytometry analysis or using enzyme-linked immunosorbent assay as previously described. PBMCs obtained from recruited participants were cultured and stimulated with TLR-specific ligands to assess their response for cytokine/chemokine production (compared with 'control medium'). These included peptidoglycan (TLR2-ligand), polyinosinic-polycytidylic acid (TLR3-ligand), ultra-purified lipopolysaccharide (TLR4-ligand), R837/Imiquimod (TLR7-ligand), ssRNA (TLR8-ligand), and CpG DNA (TLR9-ligand).

All nasopharyngeal samples collected at presentation were subjected to influenza virus detection using immunofluorescence or PCR assays for diagnosis; virus isolation was performed in parallel. Real-time reverse-transcriptase PCR assay (targeting M-gene) was performed on the original specimens to determine viral RNA concentration (copies/μL RNA) using established methods.

Results

A total of 42 patients hospitalised with influenza virus (A/H3N2, n=24; A/H1N1pdm09, n=18) and 20 controls were enrolled. Patients and controls were similar in terms of age (67.7±15.9 years vs 62.0±13.5 years, P>0.05) and sex distribution (male: 57% vs 55%, P>0.05). Among patients, 81% developed acute respiratory and/or cardiovascular complications, and 50% required supplemental oxygen therapy because of hypoxaemia. Four (9.5%) patients developed critical illness requiring ventilatory support, and one died.

Levels of expression of 'virus sensing' TLRs 3, 7, 8, and 9 and 'bacterial sensing' TLRs 2 and 4 in monocytes and dendritic cells were compared between patients and controls. Patients' blood samples were collected at a median of 2 (interquartile range, 1-2) days from symptom onset, prior to antiviral treatment. Expression of TLRs 8 and 9 increased significantly, but that of TLRs 2 and 4 was suppressed (Fig 1). There were trends for increased expression of TLRs 3 and 7 in the dendritic cells. Subgroup analyses of plasmacytoid and myeloid dendritic cells showed similar results. A/H3N2 and A/H1N1pdm09 virus infections did not differ significantly in terms of TLR expression profile and magnitude (monocytes and dendritic cells, all P≥0.1).

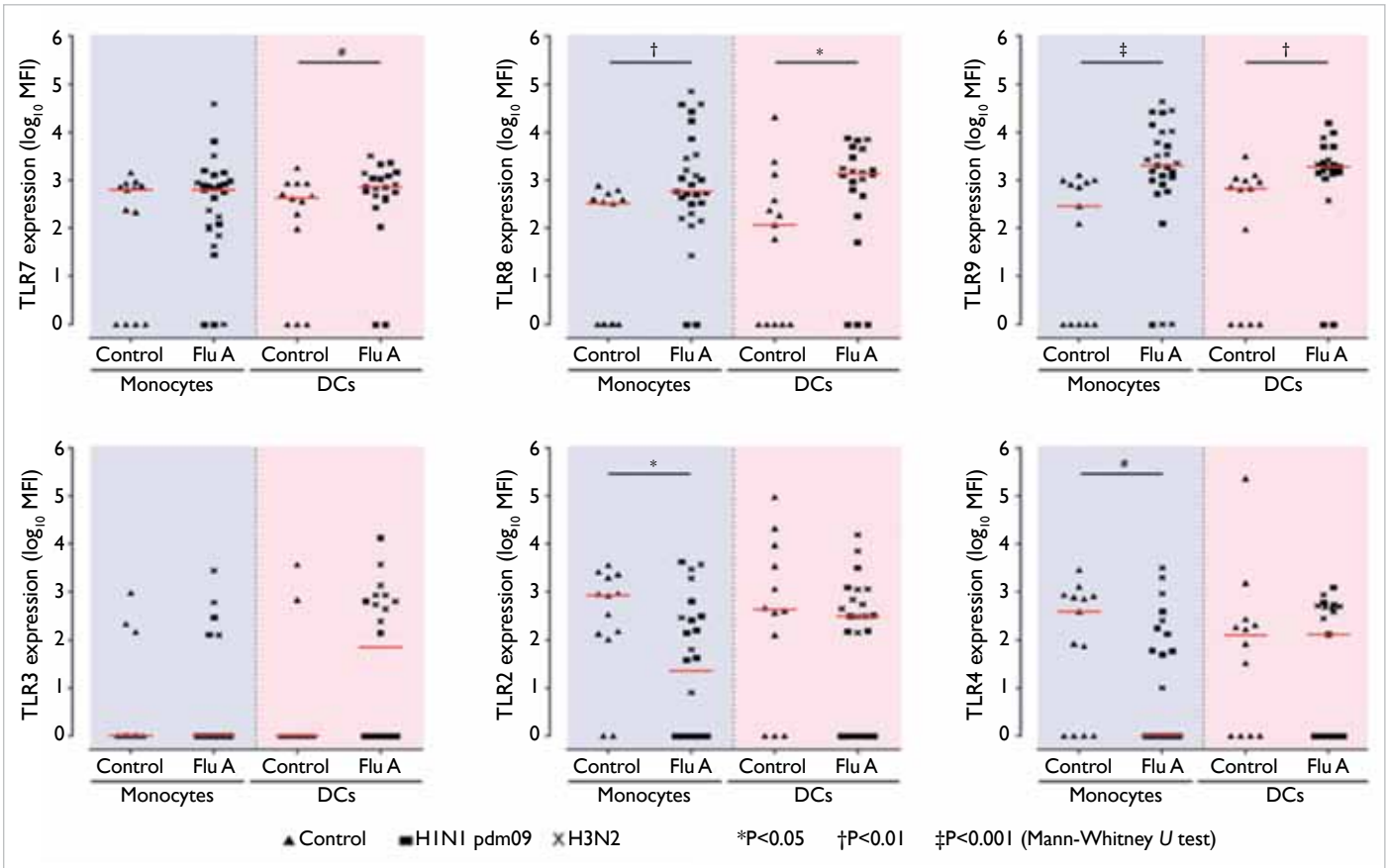

FIG 1. Expressions of toll-like receptors (TLRs) in terms of mean fluorescence intensity (MFI) in monocytes and dendritic cells (DCs) in influenza patients and controls: values are represented in logarithmic scale; horizontal bars represent median values

The relationship between nasopharyngeal viral RNA concentration ('viral load') at time of presentation and TLR expression level in influenza patients was examined (Fig 2). There were significant negative correlations between TLR 3, 8, and 9 expression levels and viral load (ie a lower level of TLR expression was associated with a higher viral load), particularly for the dendritic cells. Similar trends were shown for TLR7. Multivariate linear regression analysis showed that a higher viral load was independently associated with a more severe illness as indicated by pneumonia and hypoxaemia (β=+0.80; standard error [SE], 0.37; 95% confidence interval [CI], 0.05 to 1.55; P=0.037), adjusted for time elapsed from onset (β=-0.37; SE, 0.16; 95% CI, -0.70 to -0.04; P=0.029) and virus strain (β=+0.60; SE, 0.33; 95% CI, -0.07 to +1.26; P=0.076). Although TLRs 2 and 4 were generally suppressed, in several patients with culture-confirmed bacterial superinfection, these were up-regulated; TLRs 3, 7, 8, and 9 were unaffected.

Relationships between levels of expressions of TLR, signalling molecules, and plasma cytokines were examined. Increased cellular expression of TLRs 7, 8, and 9 correlated with increased plasma levels of proinflammatory cytokines, including IL-6, soluble TNF receptor-1 (indicating TNF-α release), CXCL8/IL-8, CCL2/MCP-1, and the adaptive cytokines IFN-γ, CXCL10/IP-10, and CXCL9/MIG (Spearman's *rho*, +0.30 to +0.49; all P<0.05). Increased TLR expression was also shown to correlate with increased intracellular signalling molecules phospho(p)-IκB, pp38-MAPK, and pERK (Spearman's *rho*; +0.49 to +0.63; all P<0.05).

These in vivo findings were supported by the ex vivo experiment results that showed a significant difference between patients and controls in their PBMCs' cytokine responses towards TLR-specific ligand activation (Table 1). For instance, stimulation of the TLR9 signalling pathway resulted in smaller increases in IL-6, TNF-α, CXCL10/IP-10, and IFN-α from baseline in patients than in controls, but

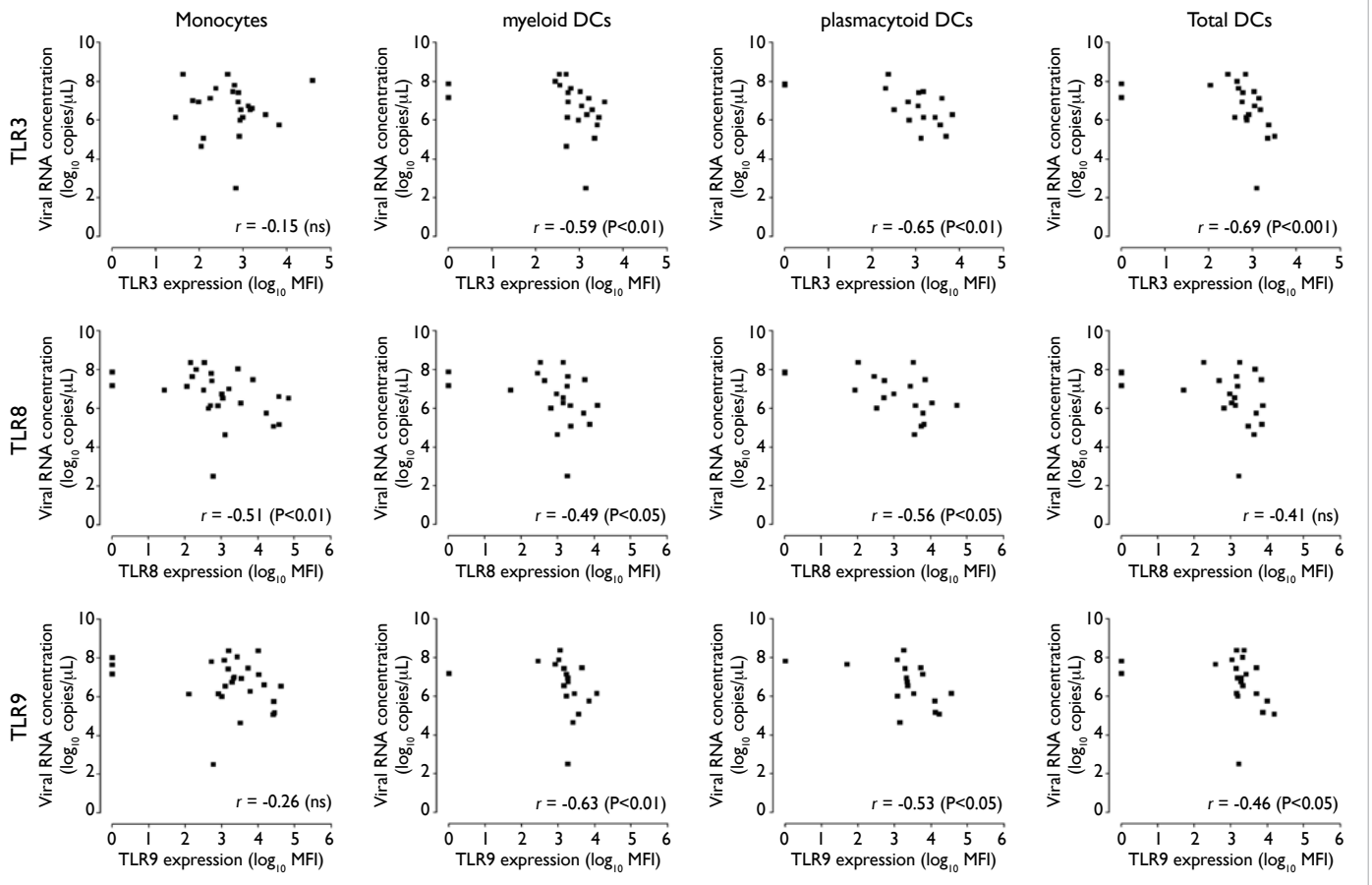

FIG 2. Negative correlations between expressions of toll-like receptors (TLRs) and influenza viral load in the respiratory tract: values are represented in logarithmic scale; trends of negative correlation between viral RNA concentration and TLR7 expression are also observed: 'total' dendritic cells [DC] (*r*= -0.34, P=0.12), plasmacytoid DCs (*r*= -0.31, P=0.21), and myeloid DCs (*r*= -0.34, P=0.18)

TABLE. Ex vivo cytokine/chemokine response of peripheral blood mononucleated cells (PBMCs) to toll-like receptor (TLR)-specific ligands in influenza patients and controls: in convalescent-phase samples from six influenza patients, median (interquartile range) fold-change for TLR9 ligand: IL-6 (4.8 [1.2-7.1]), TNF-α (2.4 [1.0-4.2]), CXCL10/IP10 (18.6 [0.8-34.5]), IL-10 (1.8[1.1-2.7]), IFN-α (2.1[1.2-39.4]), for TLR7 ligand: IL-6 (2.1 [0.7-42.7]), TNF-α (1.4 [1.0-3.9]), CXCL10/IP-10 (1.0 [1.0-5.3]), IL-10 (1.0 [0.9-1.7]), time interval from acute-phase samples (9 [7-14] days)

TLR-specific ligand activation	IL-6	TNF-α	CCL2 (MCP-1)	CXCL10 (IP-10)	IFN-γ	IL-10	IFN-α
TLR3 (PolyIC)							
Patients	99.5 (26.4-390.6)	101.0 (24.3-374.0)	4.0 (1.0-17.6)	1.0 (1.0-1.0)	1.5 (1.0-2.1)	39.5 (8.4-61.1)	1.0 (0.9-1.5)
Controls	84.5 (41.7-243.3)	84.6 (29.2-241.3)	1.2 (1.0-16.9)	1.0 (1.0-1.0)	1.8 (1.2-3.6)	53.2 (21.7-85.5)	1.3 (1.0-1.7)
TLR7 (Imiquimod)							
Patients	7.1 (2.6-41.6)†	1.3 (1.0-3.8)†	3.7 (1.0-16.9)	2.6 (1.0-12.4)‡	1.0 (0.8-1.5)	1.3 (1.0-2.1)*	1.2 (0.9-2.3)
Controls	4.2 (0.7-9.6)	1.0 (0.8-1.9)	1.5 (1.0-20.9)	1.0 (1.0-1.2)	1.4 (1.0-2.0)	1.0 (1.0-1.2)	1.2 (0.9-4.1)
TLR8 (ssRNA)							
Patients	2.8 (1.1-15.3)	1.5 (1.0-6.7)	1.5 (1.0-5.6)	1.0 (1.0-1.0)	1.1 (0.9-1.8)	1.0 (1.0-1.7)	1.0 (0.9-1.1)
Controls	2.9 (1.5-5.8)	2.2 (1.0-6.9)	1.0 (1.0-2.3)	1.0 (1.0-1.0)	1.0 (0.8-1.5)	1.0 (1.0-1.1)	1.0 (0.8-1.3)
TLR9 (CpG DNA)							
Patients	1.5 (0.9-3.3)*	1.0 (1.0-1.0)†	2.2 (1.0-8.2)	1.0 (1.0-3.9)‡	1.2 (1.0-1.6)	1.0 (1.0-1.0)‡	1.2 (1.0-1.3)‡
Controls	2.6 (1.6-7.0)	1.6 (1.0-2.9)	1.0 (1.0-16.9)	9.2 (1.0-27.8)	1.2 (1.0-2.2)	1.5 (1.3-2.0)	2.1 (1.3-10.5)
TLR2 (PGN)							
Patients	122.8 (25.9-390.6)	314.6 (84.5-1129.5)	3.8 (1.0-16.9)	1.0 (1.0-1.7)	1.3 (1.0-2.0)	47.7 (12.4-84.0)	1.1 (0.9-1.4)
Controls	89.8 (62.7-310.2)	278.3 (65.4-434.7)	1.3 (1.0-9.6)	1.0 (1.0-1.0)	1.4 (1.0-2.2)	32.5 (15.1-89.1)	1.5 (0.9-2.6)
TLR4 (LPS)							
Patients	118.7 (26.3-394.0)	125.4 (29.3-719.4)	3.7 (1.0-14.8)	1.0 (1.0-1.0)	1.3 (1.0-2.5)	37.3 (8.6-65.8)	1.3 (1.0-1.5)
Controls	84.5 (41.7-243.3)	96.3 (21.6-453.7)	1.2 (1.0-16.9)	1.0 (1.0-1.8)	1.5 (1.1-2.9)	49.4 (31.7-97.9)	1.1 (0.9-2.2)

* P≤0.1 using Mann-Whitney *U* test
† P≤0.05 using Mann-Whitney *U* test
‡ P≤0.01 using Mann-Whitney *U* test

such responsiveness for cytokine production was higher with TLR7 ligand binding in patients than in controls. In both cases, the responses normalised when patients' illness subsided, indicating that the TLR-signalling pathways were active during influenza virus infection. No difference was found between patients and controls for TLRs 2 or 4 ligand activation. Results were similar between influenza A/H3N2 and A/H1N1pdm09 virus infections.

Discussion

Our results on natural infections are consistent with earlier in vitro and animal studies, which showed that the 'viral sensing' TLRs 3, 7, 8, and 9 are up-regulated in antigen-presenting cells, and signal the innate virus inhibitory and inflammatory responses in influenza. TLR9 activation in dendritic cells strongly induces the release of type-1 IFNs and pro-inflammatory cytokines, and up-regulates co-stimulatory molecules (eg CD80/86); TLR7/8 activation induces IFNs, pro-inflammatory IL-6, TNF-α, CCL2/MCP-1, and CXCL8/IL-8, and promotes dendritic cell maturation; TLR3 in epithelial cells causes tissue inflammation through IL-6, TNF-α, and CXCL8/IL-8 induction and effector cell recruitment. Data in humans also showed significant increase in TLRs 8 and 9 expression, and increasing trends in TLRs 3 and 7 expression, together with highly increased inflammatory cytokines in severe influenza virus A/H1N1pdm09 infections.[1] Notably, the 'bacterial sensing' of TLRs 2 and 4 were suppressed. Their down-regulation may impair phagocyte recruitment and bacterial elimination, thus explaining the increased risk of secondary infections post-influenza. There was no significant difference in TLR expression (pattern/magnitude) between influenza virus A/H3N2 and A/H1N1pdm09 infections, unlike their adaptive immune responses. This may reflect the 'less specific' nature of innate immunity, which can be advantageous when considering TLR targeting for prophylaxis.

This study suggested that TLRs play an important role in virus control in the early phase of naturally occurring influenza. Higher expression levels of TLRs 3, 8, and 9 (and trends for TLR7) in the innate immune cells, particularly the dendritic cells, were associated with lower level of virus

replication ('viral load') in the respiratory tract for both virus strains. Reduced viral load was associated with milder illness. As all patients were studied within the first 48 hours of illness onset, these observations were unlikely to be the sole result of adaptive immunity. These results are in line with recent mice experiments in which TLR activation rapidly produced virus inhibitory responses (predominantly through type-I IFNs and IFN-stimulated mechanisms), conferring protection against influenza challenge. Aerosolised TLR9 and TLR2/6 agonists given 3 days before or shortly after lethal challenge with influenza virus A/H3N2 or A/H1N1pdm09 reduced mice lung viral titres and enhanced survival.[2] Starting intranasal TLR3 agonist pretreatment 6 hours before lethal influenza challenges reduced lung viral titres and mortality; the protection was 'broad-range' (A/H1N1, A/H3N2, A/H1N1pdm09, A/H5N1, A/H9N2).[3] Pretreatment with TLR9 and TLR3 agonists up-regulated the TLRs within hours, and protected mice against lethal influenza virus (A/H1N1, A/H3N2, A/H5N1) infections for 7 to 14 days.[4] Prophylactic TLR7/8 or TLR7 agonist administration also resulted in virus inhibition and improved mice survival. Thus, potentiating the innate antiviral responses through TLR targeting/activation may be useful in enhancing initial influenza virus control in humans.

In patients with influenza virus infection, TLR-signalling led to the inflammatory response, and linked to the adaptive response. Increased TLRs 7, 8, and 9 expression correlated with the key intracellular signalling molecules (MAPKs, NF-κB/IκB), and higher levels of pro-inflammatory cytokines (IL-6, TNF-α). TLR9 and TLR7/8 (which signal through the MyD88 pathway) were associated with the 'adaptive' cytokines (Th1-related IFN-γ, CXCL10/IP-10, CXCL9/MIG). TLR's active role in cytokine induction was supported by our ex vivo experiments, in which significant differences in response towards TLR ligands between influenza patients and controls (in IL-6, TNF-α, CXCL10/IP-10, IFN-α release), and changes in the 'responsiveness' during/after the illness (which was governed by the ligand used, cell type, disease stage, or immune exhaustion). A direct association between TLR hyperexpression and disease severity was not observed, but this was shown for the 'downstream' pro-inflammatory cytokines. Perpetuating, uncontrolled pro-inflammatory cytokine responses can lead to immunopathological damage in severe influenza, and further stimulation of TLRs at a more advanced disease stage may exacerbate tissue inflammation. It is uncertain whether TLR blockade alone can reduce inflammation as compensatory mechanisms might exist. Nevertheless, our data in natural influenza provided evidence that TLR's role in regulating the adaptive responses could be harnessed to boost immunogenicity of influenza vaccines (eg TLR9 or TLR7 ligands as adjuvants), which are already in clinical trials. This approach may be useful for vaccines for elderly and immunologically-naive people. Further study on the innate responses against influenza virus in infected patients is warranted.

Conclusion

Low TLR expression level is associated with a high viral load in vivo; TLR targeting and activation, using specific TLR agonists, can lead to rapid, broad-range protection against influenza challenges. Our data provide a basis for clinical studies on TLR targeting/activation, and assist their future planning. This novel strategy is useful for preventive intervention, such as pre-/post-exposure prophylaxis, which may contribute to influenza disease control.

Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#09080102). Results of this study have been published in: Lee N, Wong CK, Hui DS, et al. Role of human Toll-like receptors in naturally occurring influenza A infections. Influenza Other Respir Viruses 2013;7:666-75.

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