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Suppression of cellular antibacterial effects by HIV

Key Messages

1. Immunopathogenesis of HIV and mycobacterial infections is discussed.
2. HIV transactivator protein (Tat) is the viral factor responsible for favouring mycobacterial growth. This can be reversed by immunoregulatory cytokines such as IFN-γ.
3. The use of small molecule inhibitors of Tat to abrogate its suppressive effects on the immune functions of macrophages/monocytes may have a role on the design of new therapeutic strategies against both pathogens.

Introduction

In 2007, over 33 million people were HIV positive, of whom a large percentage progressed to fulminant AIDS. People infected with HIV gradually lose their immune system and become infected with other opportunistic pathogens. HIV infects and integrates its viral genome into CD4+ T cells and macrophages. The viral proteins produced in infected cells dysregulate cellular responses, and in some cases lead to cell death. HIV transactivator protein (Tat) is one of the first viral proteins produced in infected cells. The primary function of Tat is to activate the HIV long terminal repeat (LTR) region, which contains the viral transcription activation sites, and to enhance the retroviral replication. With this Tat protein, HIV viral replication efficiency can be enhanced more than 100 fold. Tat can also dysregulate cytokine responses and affect AIDS pathogenesis.[1-3] It induces cytokines such as IL-10, IL-6, and TNF-α in monocytes/macrophages to activate HIV replication, induce T cell death, and promote HIV-associated B cell lymphomas.

As HIV can perturb and evade the host immune system, other pathogens take advantage of the immunocompromised state and invade the HIV-infected host. Co-infections with non-HIV pathogens including viruses, parasites, fungi, and bacteria are common in AIDS patients. These opportunistic infections change the course of disease progression, decrease host survival, and increase the risk of HIV transmission.

Mycobacterium tuberculosis (MTB) infection affects one third of the world's population and results in 1.5 to 2 million deaths annually. In Hong Kong, there are about 6000 new cases every year. According to the World Health Organization, countries with a high incidence of HIV infection also have higher incidence of tuberculosis. For hosts with normal immunity, only 10% of MTB-infected patients develop active disease. In the remaining 90%, the disease is latent. Latent MTB is reactivated when host immunity is perturbed such as in HIV infection, malnutrition, or after the use of immunosuppressive drugs.[4] The exact mechanism of tuberculosis reactivation remains unclear. Coinfection of HIV and MTB or *M avium* complex (MAC) is important in AIDS pathogenesis.

Gram-negative bacterial infections are also common in HIV-infected hosts. Lipopolysaccharide (LPS), the bacterial cell wall component of Gram-negative bacilli, is recognised by Toll-like receptor 4 and induces signalling cascades that trigger innate immune responses. Cytokines such as TNF-α, IL-6, and IFN-β are produced to combat invading pathogens. For example, in mouse models, IFN-β induces iNOS expression to enhance nitric oxide (NO) synthesis to help clear Gram-negative bacteria.[4] Furthermore, IFN-β synergises with TNF-α and IL-1β to induce indoleamine-2,3-dioxygenase expression that inhibits bacterial growth.[4] In HIV-1 infected patients, induced cytokines may become dysregulated and contribute to the septic shock syndrome. This study aimed to elucidate the perturbation of cellular signalling pathways by HIV, specifically the Tat protein, to aid design of novel immunotherapeutic regimens to abrogate or lessen the severity of cytokine dysregulation.

Methods

This study was conducted from February 2007 to January 2009. Recombinant

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HIV-1 Tat protein was purchased from Advanced BioScience Laboratories (Kensington, MD, USA). Endotoxin levels of Tat protein was <0.0025 EU/mg as measured by Pyrotell assay kit (Associates of Cape Cod, East Falmouth, MA, USA). The biological activities of the Tat protein were confirmed by HIV-LTR luciferase activities. Lipopolysaccharide extracted from *Escherichia Coli* serotype O26:B6 was purchased from Sigma-Aldrich (St Louis, MO, USA).

Primary blood monocytes (PBMo) were isolated from buffy-coat samples (donated by healthy volunteers), using the Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. CD14+ PBMo were purified from these cells using anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA, USA). Cell viability was over 99% as measured by the Trypan blue exclusion assay, and the purity of monocytes was 90 to 95% as verified by FACS using PE-conjugated anti-CD14 antibody (Beckman Coulter, Miami, FL, USA). Purified CD14+ PBMo were resuspended in RPMI containing 5% autologous plasma before culture. Primary human blood macrophages (PBMac) were obtained by an adherence method and cultured for 14 days in RPMI with autologous serum.

Total cellular RNA extraction and cDNA synthesis were performed as described in our previous reports.[1-3] The cDNA produced was then subjected to quantitative real-time polymerase chain reaction (QRT-PCR) assay using Applied Biosystems TaqMan probes including IFN-β, GAPDH, and 18S rRNA (reference gene). Results of QRT-PCR were analysed with reference to the comparative Cτ (cycle number to threshold) according to the manufacturer's instructions. All samples were run in triplicate along with template controls.

The U133 Plus 2.0 Microarray is composed of >54 000 probe sets that can be used to analyse over 47 000 human transcripts and variants. Microarray procedures, including RNA quality control, sample labelling, gene chip hybridisation, and data acquisition, were undertaken together with the Genome Research Centre, University of Hong Kong. The quality of total RNA was checked by the Agilent 2100 Bioanalyzer. The RNA was then amplified and labelled with MessageAmp II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion, USA). Briefly, total RNA (1 µg) was reverse transcribed to double-stranded cDNA by using an oligo(dT) primer bearing a T7 promoter. The double strand cDNA was then used as a template for in vitro transcription to generate biotin-labelled cRNA. After fragmentation, 15 µg of cRNA was hybridised to the microarray gene chips for 16 hours. The gene chips were washed and stained using the GeneChip Fluidics Station 450 (Affymetrix, USA), and then scanned with the GeneChip Scanner 7G (Affymetrix, USA).

The IFN-β bioassay was performed as previously described,[3] but with minor modifications. Supernatants from the PBMac treated with different stimulants were collected. The collected samples or 400 pg/mL recombinant IFN-β (PBL, Piscataway, NJ, USA) were mixed with control rabbit immunoglobulin fraction (Dako, Glostrup, Denmark) or rabbit anti-IFN-β neutralising antibodies (Abcam, Cambridge, UK) and incubated for 30 minutes before being added onto T98G cells. After 24-hour incubation, the adherent T98G cells were washed with PBS and infected with encephalomyocarditis virus (EMCV) [10^8 titre/mL] and cultured in 2% foetal bovine serum medium. After being infected for 24 hours, the virus-induced cytopathic effects were visualised using light microscopy after staining the cells with 0.1% crystal violet dissolved in 5% ethanol.

PBMac were fixed and stained with anti-NFκB p65 antibodies (Santa Cruz Biotechnology) followed by rhodamine-conjugated secondary antibodies (Millipore). Cell nuclei were stained with 4,6-diamidine-2-phenylindole dihydrochloride. Immunofluorescence of the stained cells was quantified by Cellomics ArrayScan HCS VTI Reader (Thermo Fisher Scientific, Waltham, MA, USA). The nuclear translocation of NFκB p65 was determined by Nuclear Translocation BioApplications from Cellomics and expressed as nucleus/cytoplasm intensity ratio. A higher intensity ratio indicated a higher level of nuclear localisation of NFκB p65. Representative images were captured using a 40x objective lens by immunofluorescence microscopy.

The BCG vaccine, Danish strain 1331, was purchased from Statens Serum Institut. The concentration of BCG (range, 0.5-5 cfu per cell) used in the experiments was optimised in our previous projects. The vaccine was free from virulent mycobacteria. Multiple stains of MAC including *M avium* and *M intracellulare* were provided by the Department of Microbiology, University of Hong Kong. The mycobacteria were cultured in Middlebrook 7H9 broth and quantified on 7H10 agar plates (BD Difco). For the colony forming unit (CFU) measurement, cells were lysed and the lysate were cultured for the presence MAC in Middlebrook 7H9 broth and quantified on 7H10 agar plates. The colonies formed by MAC were counted.

Fluorescein-labelled *E coli* was purchased from Molecular Probes Primary human macrophages. Different treatments were incubated with MAC or fluorescein-labelled *E coli* for 2 hours. The surface unbounded bacteria were washed with PBS. The cells were fixed and analysed by flow cytometry.

PBMac incubated with fluorescein-labelled microspheres and FITC-labelled *E coli* were fixed with 1% paraformaldehyde and analysed by a flow cytometer (Elite, Beckman Coulter, Fullerton, CA, USA).

The culture supernatants were collected for NO measurement by Griess reagent system (Sigma). The sample supernatants were mixed with Griess reagent and measured by a microplate reader. The viability of the cells

was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the results from the Griess experiment were normalised.

Results

Tat inhibits phagocytosis of E coli but not MAC in human macrophages

HIV-1 Tat protein activates HIV LTR promoter, enhances HIV replication, and dysregulates the cytokine response.[1–3] We hypothesised that HIV-1 Tat could modulate macrophage functions to provide a favourable milieu for opportunistic microbial infections. Gram-negative bacteria and MAC infections are commonly encountered in HIV-1-infected and AIDS patients. In normal host immune response, macrophages engulf the bacteria and degrade them in a process called phagocytosis. We investigated whether HIV-1 Tat could affect the phagocytosis of the bacteria. The first step was to investigate whether HIV-1 Tat could mediate the cellular uptake of the fluorescein-labelled microspheres into macrophages. Fluorescein-labelled microspheres were incubated with primary peripheral human blood macrophages (PBMac) for 30 minutes after HIV-1 Tat pretreatment for 24 hours. HIV-1 Tat inhibited endocytosis of the fluorescein-labelled microspheres (Fig a).

We then measured the effects of HIV-1 Tat on phagocytosis of microbes in macrophages. PBMac were pretreated with HIV-1 Tat protein for 24 hours, followed by MAC infection for 2 hours. The cells were then washed by phosphate buffer saline and lysed. The lysate were plated on the agar plate for 14 days and the colonies formed were counted. The colonies formed in HIV-1 treated or mock-treated samples were similar, and that HIV-1 Tat did not affect the phagocytosis of MAC (Fig b).

We also examined whether HIV-1 Tat could affect the phagocytosis of *E coli* by PBMac. The treatment was similar to the study of phagocytosis of MAC, in which PBMac were treated by two different doses of HIV-1 Tat for 24 hours prior to the addition of FITC-labelled *E coli* for 1 hour. The cells were fixed and the FITC signals were measured by flow cytometry. HIV-1 Tat suppressed the FITC-labelled *E coli* phagocytosis by about 30% in PBMac (Fig c). In summary, HIV-1 Tat suppressed the endocytosis of the microspheres and phagocytosis of *E coli*, but had no effects on MAC.

Tat and MAC did not affect PI3Kinase pathways

To examine the effects of HIV on PI3K pathways, PBMac were pretreated with or without Tat (100 or 200 ng/ml) for 24 hours before the addition of MAC (MOI=4 CFU/cell) for various time points. For shorter time points, MAC was added for 0.5, 1, and 2 hours. Protein samples were collected by lysing the cells using a relevant lysis buffer. For longer time points, protein samples were collected on day 1, 4, and 6 after MAC was taken up. This was followed by trypsinsation and washing with PBS twice. The levels
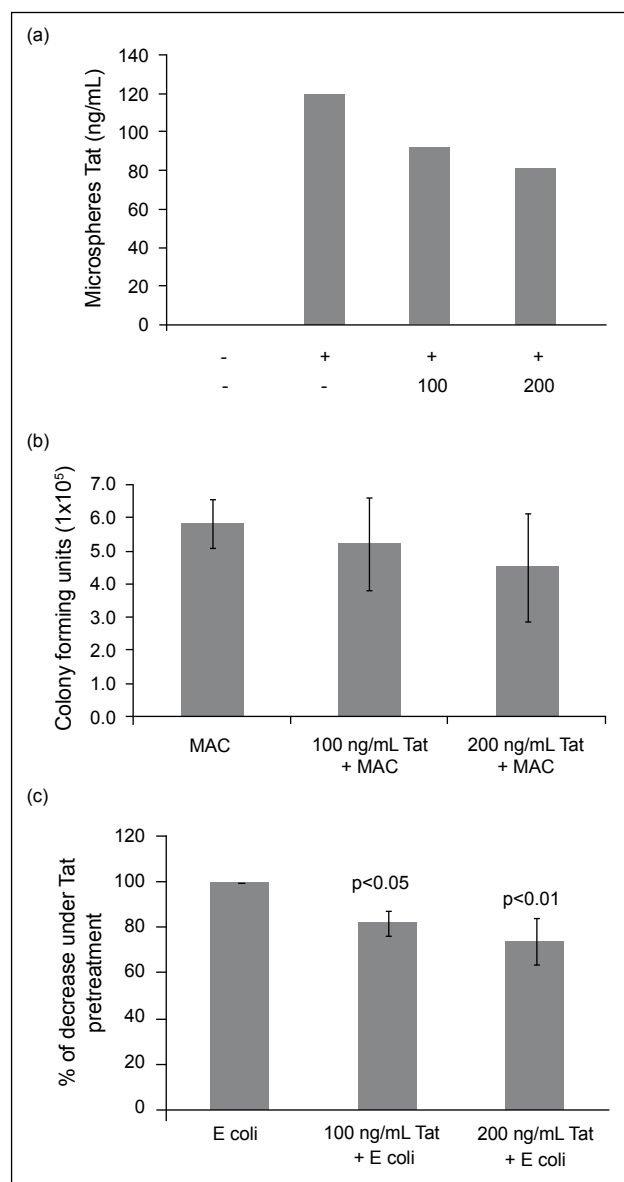

Fig. (a) HIV transactivator protein (Tat) inhibited the endocytosis of fluorecein-labelled microspheres. (b) Tat did not affect the phagocytosis of *Mycobacterium avium* complex (MAC). (c) Tat inhibited the phagocytosis of *Escherichia coli*.

of phospho-Akt (which is downstream of PI3Kinase) were measured by Western analysis. MAC did not activate the phosphorylation of Akt over the period of time points tested. Also, Tat did not affect MAC-induced phosphorylation of Akt at all time points tested.

Effects of Tat on iNOS pathways

In previous reports, PBMac did not produce nitrite upon mycobacteria infection. Therefore, we used a mouse macrophage cell line RAW264.7, which is known to produce nitrite in vitro. Cells were pretreated with or without Tat (100 or 200 ng/ml) for 24 hours before the addition of BCG (MOI=1 CFU/cell) for 24 hours. Cell-free supernatants were collected and formation of nitrite was measured by the Griess assay. BCG induced nitrite formation in RAW cells

within 24 hours of treatment. However, Tat did not have any modulating effects on BCG-induced nitrite formation.

Microarray analysis

To delineate the mechanisms underlying the effects of Tat on modulating immune responses during mycobacteria infection, we performed whole genome microarray experiments. Monocytes from five different individuals were treated with Tat. Total RNA from each sample was pooled together before performing the reverse transcription step. This was to contain the cost of genechip experiments and yet to provide a roadmap for QRT-PCR in each sample and also by Western blots for protein levels. Analyses of gene expression changes in Tat-treated cells (100 ng/ml for 3 hours) were compared to those cells with mock treatment alone. For analysis by the software GeneSpring GX, gene induction of greater than two fold was listed for confirmation by QRT-PCR. Tat upregulated the expression of phagocytosis-related genes including CD40 and CD44. These genechip analysis results formed the basis of our multiple projects.

Effects of mycobacteria on induction of phagocytosis-related genes

As CD40 and CD44 could be upregulated by Tat, we further examined whether Tat could affect the CD40 and CD44 expression during mycobacteria infection. Human macrophages were infected with BCG (MOI=1 CFU/cell) for 0.5, 1, 3, and 6 hours. RNA samples were harvested and PCR of a panel of phagocytosis-related genes were performed. BCG did not increase any phagocytosis-related genes including CD14, CD32, CD35, and CD40 in human macrophages. The results also demonstrated Tat did not have additional effects on the expression of phagocytosis-related genes during mycobacterial infection.

Tat inhibits LPS-induced IFN-β

As Tat could inhibit the phagocytosis of $E\ coli$, we further examined the cytokine expression profile of the blood monocytes/macrophages during Gram-negative bacterial infection in the presence of HIV-1 Tat. Lipopolysaccharide (from the outer membrane of the Gram-negative bacteria) contributes to the pathophysiological changes associated with sepsis. Its effects act through the dysregulation of cytokine (IL-6, TNF-α, and IFN-γ) production. We postulated that HIV-1 Tat may dysregulate the cytokine response to LPS and further cripple the immune system. PBMo were pre-incubated with HIV-1 Tat for 4 hours prior to LPS treatment for another 1 hour. As in previous studies, LPS induced IFN-γ mRNA expression within 1-hour incubation. The IFN-γ mRNA induction was inhibited in the presence of HIV-1 Tat protein in a dose-dependent manner (data not shown).

In order to delineate the mechanism of HIV-1 Tat on the suppression of LPS-induced IFN-γ expression, we measured the activation status of transcription factor NF-kappa B, which plays a crucial role in IFN-γ transcription.

Activation of transcription involves the translocation of an activated NF-kappa B p65 subunit from the cytoplasm to the nucleus for binding to the promoter of IFN-γ. We measured the LPS-induced translocation of the NF-kappa B p65 subunit with or without the presence of HIV-1 Tat using an automated fluorescence screening system (Cellomics) and analysed the results using the computer programme (BioApplication). HIV-1 Tat abrogated the translocation of the LPS-induced NF-kappa B p65 submit from the cytoplasm to the nucleus (data not shown).

As we delineated that HIV-1 Tat suppressed LPS-induced NF-kappa B activation and further inhibited IFN-γ activation, we next investigated whether HIV-1 Tat could inhibit intracellular antiviral functions of macrophages. The antiviral activity of IFN-γ was measured by its effects to protect the indicator cells (T98G, a human glioblastoma line) from EMCV-induced cytotoxicity. The presence of IFN-γ inhibits EMCV from replicating in susceptible T98G cells. Supernatants from the PBMac treated with or without HIV-1 Tat and LPS were collected and transferred to the T98G cells. After 24-hour incubation of the T98G cells with the supernatants, EMCV were added to the cells for infection. LPS-treated supernatants protected the T98G cells from EMCV-induced cytotoxicity. With the HIV-1 Tat pretreatment of PBMac prior to LPS challenge, the protective effects were abrogated (data not shown).

In conclusion, HIV-1 Tat inhibits the LPS-induced cytokine response and further suppresses cellular antiviral activities. Such deficiency in antimicrobial responses may provide a favourable environment for HIV replication as well as for invasion by opportunistic pathogens, leading to progression of disease.

Discussion

Both HIV-1 and mycobacteria have efficient immune evasion mechanisms to subvert immunity. IL-10 is induced by both infections through the double-stranded RNA dependent kinase and mitogen-activated kinases.[1] IL-10 is an anti-inflammatory cytokine capable of downregulating IL-2 synthesis and T-cell functions, and suppression of proinflammatory cytokine expression. Thus, both microbes and their encoded proteins are likely to act in concert to cripple cellular antimicrobial responses and enhance each other's survival and replication.

We have embarked a series of projects to explore the interactions between the two microbes at the cellular and molecular levels.[1-3] Results from this project are part of a larger effort to investigate the cross-talks and mechanisms. In the current study, HIV-1 Tat suppressed the endocytosis of the microspheres and phagocytosis of $E\ coli$, but had no effects on MAC. Both HIV Tat and MAC did not have effects on the activation of PI3K and the Akt system as reflected by respective phospho-protein kinase studies.

As Tat could inhibit the phagocytosis of *E coli*, we further examined the cytokine expression profile of the blood monocytes/macrophages during Gram-negative bacterial infection in the presence of HIV-1 Tat. HIV-1 Tat inhibits LPS-induced cytokine responses and further suppresses the cellular antiviral activities.[3] In other words, Tat cripples Toll-like Receptor-4 recognition of bacterial endotoxin, causing a deficiency in antimicrobial responses favourable to HIV replication.

High doses of Tat can induce the cytokine signalling suppressor gene SOCS-2 (one of a group of suppressors) to intervene with IFN-γ-activated Jak-Stat1 signalling. This results in deficient MHC antigen II expression on the cell surface of antigen-presenting cells including fully differentiated primary human macrophages.[2] Thus, there is a tug of war between IFN-regulated activities against mycobacterial infection and the HIV-1-encoded Tat to subvert such cellular antimicrobial effects.

In conclusion, IFN-γ may have a role in suppressing the action of HIV-1 in its enhancement of mycobacterial growth in human macrophages. This may provide a scientific rationale for the use of IFN-γ and related cytokines in AIDS patients with aggressive mycobacterial infections. Alternatively, the use small molecule inhibitors of Tat to abrogate its suppressive effects on the immune functions of macrophages/monocytes may have a role on the design of new therapeutic strategies against both pathogens.

Acknowledgements

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