行政院國家科學委員會補助專題研究計畫成果報告

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- □赴國外出差或研習心得報告一份
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- □出席國際學術會議心得報告及發表之論文各一份
- ☑國際合作研究計畫國外研究報告書一份

執行單位：台灣大學公共衛生學院流行病學研究所

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ABSTRACT

Human monocytes/macrophages (MO/Mϕ) are prime target cells to be infected by dengue virus (DV). Tissue Mϕ are differentiated from circulating blood MO also contained DV antigens in infected individuals. To address the permissiveness and the response of differentiated MO/Mϕ to DV infection, we cultured peripheral blood MO for various days (1 d, 1, 2, 3, and >6 wks) and found that MO/Mϕ of different differentiation stages were equally and highly susceptible to productive DV infection. Furthermore, the DV infected MO/Mϕ released a diverse array of proinflammatory cytokines and chemokines, including TNF-α, IFN-α, IL-1β, IL-8, IL-12, MIP-1α and RANTES, but not IL-6, IL-15 and nitric oxide. Secretion of these mediators varied significantly in magnitude, onset, kinetics and duration. Small amount of initial virus input resulted in high levels of secretion of for IL-8, MIP-1α and RANTES, while higher initial virus inputwas required to induce IL-12 and IFN-α. However, large amount of virus inputonly marginally secreted TNF-α and IL-1β. More importantly, primary human MO/Mϕ cultured even for more than 45 days fully supported DV replication and release large quantities of cytokines and chemokines, indicating that these terminally differentiated MO/Mϕ were still functional competent in both productive infection and stimulated soluble factors playing roles in cell-medicated immune response. DV infection also enhanced lipopolysaccharide (LPS)-stimulated MO/Mϕ secretion of cytokines and chemokines. Interestingly, although LPS by itself did not induce IFN-α production by human MO/Mϕ, it markedly augmented the DV-induced IFN-α release as well as enhanced and sustained the infectious-virus production by these cells.. Taken together, our results might partly account for the host innate immunity and immunopathology induced by DV infection.

INTRODUCTION

Dengue virus (DV) infection affects 100,000,000 people and kills 25,000 every year worldwide (1, 2). More than hundreds of countries and billions of people are at risk (3). The pathogenesis of DV infection is not completely understood and no vaccine and specific therapy are yet available. Infection with DV results in clinical manifestations with a broad spectrum of severity, from self-limited dengue fever (DF) to potentially fatal dengue hemorrhagic fever-dengue shock syndrome (DHF-DSS) that represented the leading viral hemorrhagic fever in the world with 500,000 cases annually (1, 3). Virus-specific, serotype cross-reactive immune responses during secondary DV infection may give rise to more severe forms of DHF-DSS due to antibody-mediated enhancement of DV infection of monocyte(s)/macrophage(s) (MO/Mϕ) and/or activation of memory T lymphocytes (4-6). Both of these two mechanisms highlight the important role of antigen-specific immunity. However, only part of patients with secondary infection developed DHF-DSS and DHF-DSS could also occur in primary DV infection (7-11). It is likely that dysregulation of certain types of innate, bystander immune activation plays a third party in the immunopathogenesis leading to DHF-DSS and exacerbates the disease progression during either primary or secondary DV infection.

MO/Mϕ are the prime targets for DV in vivo (5). MO/Mϕ comprise heterogeneous subpopulations with distinct properties and functions. MO are the blood precursors of tissue Mϕ. Circulating MOs undergo differentiation and transmigrate through blood vessel walls to various tissues where they proceed the maturation process and become resident differentiated tissue Mϕ (12-15). Although the differentiation stage of MO/Mϕ is crucial in determining the susceptibility of these cells to productive infections by a number of viruses (16-26), the definitive influence of MO/Mϕ differentiation on DV infection has not been established. Because DV antigens were found primarily in, in addition to blood MO, a wide variety of tissue Mϕ, it is important to know whether or not the nonproliferating MO/Mϕ, after cell

differentiation, are able to support productive DV infection.

Cytokines play an important role in the pathogenesis of DV infection (27) and serum levels of certain cytokines were elevated during DV infection (28-30). Among these cytokines, IL-12 and IFN-α play a pivotal role in protection against intracellular pathogens and initiation of Th1-type immune response (31-36). Although MO/Mϕ were thought to be one of the major cell sources of these cytokines, evidence on IL-12 production directly induced by virus infection of human MO/Mϕ is not available. The levels of Th1 cytokines were promoted in DV-infected patients (30), thus suggesting a role of these two cytokines in immunity and pathogenesis of DV infection.

Chemokine superfamily can be structurally and functionally divided into C-X-C, C-C, and other subfamilies that recruit and activate different subsets of leukocytes and possess multipurpose functions in immunity and pathophysiology such as regulation of microbial infections, inflammation, hematopoiesis, and NK and endothelial cell biology (37-43). Given the fact that local inflammation and leukocyte extravasation were evident in various tissues and organs during DV infection, localized production of these mediators by resident tissue MO/Mϕs may be pivotal in initiation of the process.

It has been demonstrated that infection with certain viruses impairs the production of cytokines by MO/Mϕ in response to stimulation by bacterial components (44-52). This mechanism of virally induced, MO/Mϕ-mediated immunosuppression may partly account for the detrimental outcome of viral infection followed by a superinfection with bacterial pathogens. On the other side, bacterial endotoxin, lipopolysaccharide (LPS), was able to versatilely regulate virus replication and virally induced cytokine production by human MO/Mϕ (53-56). We have also previously documented a novel interaction among DV, LPS and CD14 at the surface of primary human MO/Mϕ (57). Hence, it deserves to investigate whether the regulatory effect of LPS and virally induced impairment of cytokine production could be observed in DV infection of human MO/Mϕ.

Appreciable progress in understanding the pathogenesis of DV infection has been made by a number of recent studies on virus-host cell interaction. However, a majority of these studies has focused on DV infection of cancerous cells and it is therefore important to understand more details about the interaction of DV with its principal target cell, human MO/Mϕ. Based on the fact that DV infection only causes diseases in humans and no appropriate animal model is available, we established an in vitro-infection model using primary cultures of human MO/Mϕ undergoing self-differentiation. We found that DV was able to productively infect these cells of all differentiation states and triggered the secretion of multiple vasoactive proinflammatory cytokines and chemokines. Production of infectious progeny virions and these mediators varied remarkably in magnitude, onset, kinetics, duration and induction potential, and could be regulated by initial infectious doses and modified by LPS in a unique manner.

Materials and Methods

Virus. DV strain 16681 (serotype 2) which was initially isolated from a DHF patient was used for infection. The preparation and titers of virus stocks were described previously (57).

Primary culture of human MO/Mϕ. Human peripheral blood MO were isolated and cultured as previously described (57). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from heparinized whole blood by density gradient centrifugation on Histopaque (Sigma, St. Louis, Mo.) and then incubated in complete alpha minimal essential medium (a-MEM, Life Technology, Grand Island, N.Y.) containing 10% freshly prepared, heat-inactivated autologous human serum (ΔAHS) and 2 mM L-glutamine at 37°C with 7.5% CO_2 for 2.5 h to allow MO adhesion. Thereafter, the cultures were extensively washed with HBSS three times to remove nonadherent cells and adherent cells were detached by incubating the monolayers with an elution buffer (6mM EDTA in PBS supplemented with 5% FCS). The adherent cells were resuspended in complete α-MEM at a density of $4x10^5$ cells per ml and then cultured in 24-well (1 ml/well) or 48-well (0.5 ml/well) tissue culture plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere containing 7.5% CO_2. These purified adherent cells contained more than 95% MO by the criteria as described (57).

In vitro differentiation of MO into Mϕ. MO were cultured in complete α-MEM containing 10% ΔAHS without addition any stimulating agent to allow self-differentiation into Mϕ of various ages, from less than 1 d to more than 45 d of cultivation. Half of culture medium was replaced with fresh complete α-MEM every 5 to 6 days. The remainder of half of the old conditioned medium (CM) was very essential for promoting differentiation of MO/Mϕ and preventing these cells from death.

Morphological changes during MO/Mϕ differentiation was presented in Fig. 1. Briefly, freshly isolated MO were small in size and were evenly distributed. After 2 d of cultivation, these cells began to enlarge and contact with on another with frequent formation of cell clumps. Around 5 to 6 d of culture, the cell monolayers reached subconfluence and subpopulations of

cells began to fuse and form small multinucleated giant cells (MNGC) which contained about 2~3 nuclei per cell. After 6~7 days, the number of MNGC markedly increased and the cell monolayer reached fully confluence. Afterwards, the size of MNGC progressively enlarged and the number of nuclei in a single MNGC increased and ranged from 2 to more than 10, although there were still some MO/Mϕ possessing single nuclei. At about 10 days after culture, huge MNGC which possessed around 40~50 or more nuclei in a single cell formed. After 2 to 3 wk of culture, lipid droplets appeared in the cytoplasm of most of the cells, including MNGC and gradually increased in quantity with time. Accompanying with such lipid laden was a reduction in the number and size of MNGC and also the number of nuclei in a single MNGC. The cell population exhibit heterogeneous morphology throughout the course of differentiation. There were significant donor-to-donor and experiment-to-experiment variability in the speed and extent of cell differentiation and MNGC formation and the duration that the cultures could be maintained (from 30 d to > 100 d). The phagocytic activity slightly and NSE activity strongly increased after cell differentiation, while the NBT reduction capacity remained the same. Strikingly, the peroxidase activity could only be detected in cells cultured for less than two days. In addition, cell surface expression of CD14 decreased and CD68 increased after cell differentiation. High levels of CD11b and HLA-DR expression maintained throughout the course of cell differentiation.

Phagocytosis and cytochemistry. The phagocytic capacity of MO/Mϕ was assessed by incubating the cells with incomplete α-MEM containing yeasts (1×10^8/ml) or latex beads (1.1 μm in diameter; Sigma) at 37°C for 30 min. The cytoplasmic peroxidase and nonspecific esterase (NSE) activities were analyzed as described previously (••). The production of cytoplasmic superoxide was detected by a nitroblue tetrazolium (NBT) reduction test.

Infection of MO/Mϕ with DV. MO/Mϕ of different stages of differentiation were washed twice with HBSS and then once with incomplete α-MEM to remove ΔAHS in culture medium. The cells were then infected with DV at a multiplicity of infection (MOI) of 2~3 PFU per cell

or as indicated. The virus inoculum (about 40~60 μl per well of 48-well plate) was incubated with the cells in serum-free α-MEM with a final volume of 0.2~0.25 ml at 37°C for 2.5 h for viral adsorption. The culture plates were gently agitated every 15 to 20 min for optimal virus-cell contact. Thereafter, the unabsorbed viruses were removed by washing the cell monolayers once with HBSS and then with complete α-MEM containing 10% ΔAHS. The DV-infected and uninfected MO/Mϕ cultures were replenished with fresh complete α-MEM and further incubated for 40~48 h or time periods as indicated. At the end of incubation, the cell-free supernatants and adherent MO/Mϕ (in fresh α-MEM) were harvested seperately and stored in aliquots at -70°C until assayed for infectious-virus production and cytokine secretion.

Stimulation of DV-infected MO/Mϕ with LPS. After wash and replenishment with fresh culture medium, LPS (*Escherichia coli* serotype O55:B5 purchased from Sigma) at different concentrations (from 0 to 5 μg/ml) was added to the infected MO/Mϕ and maintained in the culture till the end of incubation.

DV titration. The titers of both extracellular and intracellular DV were determined by plaque assay with BHK-21 cells as previously described (57).

Assay for cytokines and chemokines. The levels of IL-8, IL-15, MIP-1α and RANTES in the culture supernatants were measured with an enzyme-linked immunosorbent assay (ELISA) using commercially available kits purchased from R&D (Minneapolis, Minn.). The ELISA kits for detection of IL-1β, IL-12 (both p40 subunit and p70 heterodimer), TNF-α and IFN-α were obtained from Biosource (Camarillo, Calif.), whereas the detection reagents for IL-6 were purchased from Genzyme ( ). The Assays were performed according to the instructions of the manufacturers. Nitric oxide production was assayed by measuring the levels of nitrite in the culture supernatants.

Results

Effects of cell differentiation on DV infection of human MO/Mϕ.. The effect of cell differentiation on the susceptibility of primary human MO/Mϕ to DV infection was explored by using infectious-virus production as an outcome measurement since it is the most convincing indicator for 'complete' viral replication. As shown in Fig. 2, the release of infectious virions from cells cultured for either 1 day, 1, 3 or even more than 6 weeks was identical, approximately 10^5 PFU per 4 x 10^5 cells at ~ 42 h post-infection. The individual-to-individual and experiment-to experiment variability was minimal (≤ 1 log PFU/4 x 10^5 cells). These results indicated that human MO/Mϕ are fully susceptible to and permissive for DV infection in vitro, regardless of the stage of their differentiation, and that infection of these primary differentiated MO/Mϕs could serve as a surrogate model to mimic DV interactions with differentiated tissue Mϕ.

Kinetics of DV replication in primary differentiated human MO/Mϕ. Time course of the production of infectious progeny DV was further investigated (57) and Fig 3). No intracellular and extracellular infectious viruses could be detected in the cultures before 12 h post-infection. However, infectious viruses were synthesized and released afterwards, followed by a log phase of growth, and the peak viral yields were reached at about 48 h post-infection (Fig. 3A). The infectious viral yields became progressively declined thereafter, but could still be detected in the culture medium of infected cells even after one week of infection (Fig. 3B). The growth kinetics of both intracellular and extracellular infectious viruses, albeit varied to some extent (about 1 log PFU/ml), appeared to be parallel (Fig 3). Interestingly, no noticeable cell death could be seen after DV infection as shown by trypan blue dye exclusion and MTT assay (not shown). Therefore, the decline in virus titers could not be explained by virus-induced cell killing. Instead, a virus-induced anti-viral state of the infected MO/Mϕ was likely to contribute to the control of virus multiplication.

Secretion of multiple cytokines and chemokines by primary differentiated MO/Mϕ after

DV infection. We next explored the profile of cytokines and chemokines generated by DV infection of the primary differentiated human MO/Mϕ. Cytokines and chemokines exert their activity by being secreted from the producing cells and binding to effector cells, we therefore measured their protein secretion instead of gene expression. As shown in Fig. 3 and Table 1, after DV infection of the 1-wk-old, primary differentiated MO/Mϕ, the proinflammatory cytokines, IL-1β and TNF-α, the Th1/NK-stimulatory cytokine, IL-12, and the type I IFN, IFN-α, were released and constitutive secretion of the CXC chemokine, IL-8, and the CC chemokines, MIP-1α and RANTES, was markedly enhanced. Although it has been reported that human MO/Mϕ could be stimulated to release IL-6, IL-15 and nitric oxide, we failed to detect these secretory products at any time after DV infection (data not shown). When the short-term and long-term kinetics of cytokin/chemokine secretion were examined (Fig. 3), it was found that the enhanced secretion of IL-8 took place by 18 h post-infection and sustained after one week of infection. Significant enhancement of RANTES secretion was observed about 2 d post-infection, peaked by 4 days after infection and subsided to the normal constitutive level by 8 d post-infection. The augmented production of MIP-1α occurred by 28 h post-infection, reached peak level at 2 d post-infection, and subsided to constitutive level by 6 d post-infection. Furthermore, no IL-12 induction was observed by 28 h after infection, whereas induction of IFN-α occurred by 18 h post-infection.

Initial infectious dose is essential in determining MO/Mϕ secretion of cytokines and chemokines after DV infeciton. As shown in Table 1, infectious progeny viruses and each of the cytokines and chemokines were all released after DV infection in a dose-dependent manner according to initial doses of virus input. Interestingly, at any given input MOI equivalent, levels of the secretory products were greatly higher for the chemokines, IL-8, MIP-1α and RANTES, intermediate for the NK/Th1-stimulatory cytokines, IL-12 and IFN-α, and much lower for the proinflammatory cytokiens, TNF-α and IL-1β. More importantly, it was noted that the minimal infectious dose required for induction (or enhancement) of the secretion was remarkably varied

among these mediators. Enhancement of MO/Mϕ secretion of IL-8, MIP-1α and RANTES required only trace amount of input viruses (MOI ≤ 0.005 PFU per cell). Induction of IL-12 and IFN-α secretion required higher initial virus input (MOI ≤ 0.05 PFU per cell). However, much higher initial infectious doses were needed for induction of TNF-α and IL-1β secretion (MOI ≤ 0.5 and 5 PFU per cell for TNF-α and IL-1β, respectively). These observations also excluded the possibility that secretion of these cytokines and chemokines was caused by any contamination of endotoxin (LPS), because treatment of cells with very low concentrations of LPS triggered large amount of TNF-α, IL-1β and IL-6 release, but not IFN-α (data not shown). Our results clearly demonstrated that MO/Mϕ secretion of chemokines (IL-8, MIP-1α and RANTES) could be induced much more readily by DV infection compared to other cytokines tested here. Moreover, DV was also a potent inducer for IL-12 and IFN-α production. By contrast, DV infection could only poorly trigger the secretion of TNF-α and IL-1β by human MO/Mϕ.

Terminally differentiated human MO/Mϕ can support productive viral infection and secrete multiple cytokines and chemokines in response to infection. Although the phenotypes and enzymatic activities have been characterized (16, 58-64), little information is available on the functions of terminally differentiated human MO/Mϕ, particularly with respect to viral infection and cytokine secretion. The average life span of human MO/Mϕ in vivo ranges from one to two months, we therefore cultured the blood-derived MOs for 45 d and infected these self-differentiated MO/Mϕ with DV to investigate the susceptibility and cytokine responses of these fully matured MO/Mϕ to viral infection. These terminally differentiated cells revealed typical characteristics of "aged" MO/Mϕ and possessed condensed and lipid-vacuole containing cytoplasm, and their size became smaller than the MNGCs growing in vitro for 1 to 3 wk (Fig. 1). Surprisingly, these cells were fully susceptible to productive DV infection, releasing vast amount of infectious virions by 24 h post-infection. The viral yields peaked at 36 to 48 h after infection, with titers of 2×10^5 PFU and 3×10^3 PFU

per 4 x 10^5 cells for extracelular and intracellular viruses, respectively. In contrast to 1-wk-old MO/Mϕ, production of intracellular infectious viruses from these terminally matured MO/Mϕ was less prominent. Similarly, DV infection did not exert a cytopatheic effect on cell survival in that small differences in healthy cell numbers between uninfected and DV-infected cells could only be seen after 12 d of infection, long after the infectious-virus production had terminated.

Most importantly, after DV infection, these terminally differentiated MO/Mϕ secreted all of the cytokines and chemokines that were also released by DV-infected, 7-day-old MO/Mϕ. Basically, the hierarchy for the levels of secretion of these cytokines and chemokines by the 1.5-mo-old human MO/Mϕ was in consistence with that observed in 7-d-old primary MO/Mϕ (Table I). The peak titers could reach ~ 90 ng/ml, 25 ng/ml, and 2 ng/ml for IL-8, MIP-1α and RANTES, respectively. On the other hand, the peak titers of IL-12, IFN-α and TNF-α were between 100 and 200 pg/ml whereas the peak titer of IL-1β was only about 50 pg/ml. In addition, the time courses of secretion were distinct among these cytokines and chemokines, and to some extent, also differed from that observed in 7-d-old MO/Mϕ (Fig. 3). Induction (or enhancement) of IL-8, MIP-1α and *TNF-α* secretion commenced early after infection (≤ 24 h post-infection), followed by RNATES, IL-1β and IFN-α (≤ 36 h post-infection). Secretion of IL-12 was slightly delayed (≤ 48 h post-infection). Moreover, secretion of *MIP-1α and TNF-α peaked at ~ 2 d after* infection, consistent with the peak time of infectious-virus production. The level of IL-1β secretion reached peak at ~ 3 d post-infection, whereas the peak time of RANTES and IFN-α release occurred at ~ 4 d after infection. However, secretion of IL-8 and IL-12 did not attain the peak levels until 7 d post-infection, at that time the infectious-virus production had ceased. Furthermore, secretion of TNF-α and the CC chemokines, MIP-1α and RANTES, were transient, as TNF-α secretion terminated and release of MIP-1α and RANTES subsided to normal constitutive levels *between 8 – 10 d* after infection. Although secretion of IL-1β declined gradually after 3 d post-infection, the levels remained detectable at the end of

experiment (2 wks post-infection). Strikingly, the levels of secretion of IL-8, IL-12 and IFN-α sustained at their peak values even at 14 d after infection, a time more than one week after the cease of infectious-virus synthesis. It is interesting to note that there was a dramatic increase of IL-12 secretion by 7 d post-infection and multiple peaks of IL-12 and IFN-α production were observed during the late course of infection. The unique kinetics and long duration of secretion suggested that induction and/or release of these molecules was regulated by their own or other soluble mediators in an autocrine and/or paracrine manner and that lasting effects can be provoked by these cytokines/chemokines during the course of DV infection.

LPS sustained DV replication and amplified DV-induced IFN-α production in primary differentiated MO/Mϕ. Viral infection of MO/Mϕ could be modulated by LPS (53-55). We have previously shown that treatment with LPS prior to or simultaneously during viral adsorption suppressed DV infection of human MO/Mϕ, while treatment after viral adsorption failed to do so (57). Since dual infection with bacteria is likely to occur during DV infection, we examined the consequences of MO/Mϕ infection by DV in the presence of LPS. Primary differentiated, 7-day-old MO/Mϕs were infected with DV and then treated with LPS which was remained in the culture throughout the course of infection. The virus titers of LPS-treated and untreated MO/Mϕ cultures were similar before 48 h post-infection, the log-phase of viral replication (57) and data not shown). However, after this time point, both intracellular and extracellular infectious-virus titers in untreated cultures dropped more rapidly than that in LPS-treated cultures, resulting in a significantly higher viral yields and prolonged duration of viral replication in the LPS-containing cultures (Fig. 5). The fact that LPS sustained DV multiplication late in the course of infection suggested that such treatment might alter the antiviral state, such as cytokine production, of the cells rather than directly activated virus replication.

It was known that LPS could potentiate certain cytokine expression by virally infected MO/Mϕ (56). On the other hand, viral infections could also impair MO/Mϕ cytokine

production in response to LPS stimulation (44-52). We therefore investigated the LPS-stimulated cytokine secretion by DV-infected human MO/Mϕ. We found that DV infection did not alter the cell responsiveness to LPS stimulation in secretion of cytokines tested here, except for IFN-α. Taking TNF-α as an example, dramatic difference in LPS-stimulated TNF-α production was not observed between DV-infected and unifected MO/Mϕ, regardless of the concentrations of LPS (from 10 ng/ml to 10 μg/ml) and the MOI equivalents (Figure 7 and not shown). Additive or synergistic increase in TNF-α secretion could be seen in some experiments when using low doses of LPS. Interestingly, although LPS *per se* was not able to induce MO/Mϕ secretion of IFN-α, treatment of this molecule markedly enhanced DV-induced secretion of this type I IFN from these primary differentiated cells (Fig. 6A). Such an augmentation occurred shortly after infection (< **24** h post-infection), a time at which the enhancing effect of LPS on DV replication was not evident (Fig. 6B). The data suggested that the enhancement of DV-induced IFN-α production by MO/Mϕ might not be an effect of enhanced virus replication.

Discussion

In the present study, we have extensively investigated the production of infectious viruses and secretion of cytoines and chemokines by primary differentiated human MO/Mϕ after DV infection and the modulatory effect of cell differentiation and bacterial LPS. To our knowledge, this report provides for the first time an evidence for direct induction of IL-12 production by human MO/Mϕ exposed to a viral pathogen and supports the idea that MO/Mϕ are one of the sources for IL-12 during viral infection of humans.

Human MO/Mϕ of various differentiation stages can support productive DV infection. Differentiated tissue Mϕ from the same or different tissues and organs contain heterogeneous subpopulations that differ in both phenotypes and functions (12-15) and are different in many respects from their less matured blood MO precursors, which also involve functionally distinct subsets (65). Considerable studies have shown that the cell susceptibility to viral infection altered with the days of in vitro-cultivation of blood MO/Mϕ (16-26). It has been reported that immature promonocytic U937 cells were not infected by DV unless they were provided with a differentiation signal and became Mϕ-like prior to DV infection (66). In the present study we demonstrated that DV could productively infect primary human MO/Mϕ regardless of the state of cell differentiation (Fig. 2, 3 and 4). Our observations may imply that DV can propagate in differentiated tissue Mϕ as well as in blood MO. In addition, a productive MO/Mϕ infection could be established in the absence of virus-specific Ab, suggesting a Fc receptor-independent pathway for DV infection (57). The noncytopathic, productive DV infection proceeded with MO/Mϕ differentiation, along with the production of intracellular infectious virions, suggested that blood MO may act as a Trojan horse for disseminating this blood-borne virus to different tissues where infectious DV are released and then infect resident tissue Mϕ in the vicinity. This proposed mechanism might partly account for systemic symptoms observed in dengue disease.

Differential magnitude and kinetics of DV-induced secretion of multiple cytokines and chemokines. At various times after DV infection of MO/Mϕ, multiple cytokines and

chemokines were released with distinct kinetics, quantities, duration and induction potential. For a given MOI equivalent, the magnitude of IL-8 secretion was highest, which was followed by MIP-1α and RANTES. The levels of IFN-α, IL-12 and TNF-α were intermediate, while secretion of IL-1β was only marginal. This hierarchy was the same for both 1-wk-old and 45-d-old MO/Mϕ (Fig. 3, Fig. 4, and Table 1). Similarly, the production kinetics between these two MO/Mϕ cultures was basically parallel. The onset of IL-8, MIP-1α and TNF-α secretion appeared earlier than that of RANTES, IL-1β and IFN-α, while the onset of IL-12 release appeared slightly later. Secretion of MIP-1α, RANTES, IL-1β and TNF-α peaked at early time after DV infection ($\leq 2 \sim 4$ d post-infection) and subsided thereafter. By contrast, secretion of IL-8, IL-12 and IFN-α proceeded till a plateau was reached and persisted for a long time. Secretion of these mediators by differentiated MO/Mϕ suggested that regional accumulation of these mediators might occur in various tissues to which DV has disseminated and thus lead to local inflammation. In consistence with this idea, it has been reported that DV infection of human Kupffer cells, albeit abortive, could result in significant cytokine production (67). The observations may also reflect the functional heterogeneity of MO/Mϕ with respect to their susceptibility to DV infection.

Differential requirement of the minimal initial infection doses for DV-induced secretion of cytokines and chemokines. It is important to note that, in addition to the levels of secretion, the minimal initial virus input required for inducing (or enhancing) the secretion varied significantly among these cytokines and chemokines of different categories (Table I). These observations provided several novel implications. First, because IL-8, MIP-1α and RANTES could readily be induced by very small amounts of viruses, these chemokines may play a more critical hierarchical role in the immunity and pathogenesis of DV infection. In addition, significant induction of IL-12 and IFN-α, which share overlapping functions, did not need high MOI equivalent, indicating a potent driving force for activation of Th1 type immune responses can be provided by DV-infected MO/Mϕ. Finally, it has been a prevailing thought that IL-1β

and TNF-α were the key mediators for development of DHF-DSS. Nevertheless, requirement of high doses of DV to induce small amounts of TNF-α and IL-1β may suggest that by the time when higher levels of these two cytokines were detected (28), the virus load and other cytokines and chemokines had reached extremely high and pathophysiological levels and resulted in detrimental consequences.

Terminally differentiated primary human MO/Mϕ are functionally competent in the context of viral infection. We showed that peripheral blood MO cultured in vitro for more than 45 d were fully permissive for DV infection and were able to support the production of multiple cytokines and chemokines for at least 2 wks after infection (2 mo of culture; Fig. 4). In an attempt to understand the physiological role of terminally differentiated MO/Mϕ, many methodologies were aimed at developing long-term human MO/Mϕ cultures. Long-term culture of MO/Mϕ, in which no other leukocytes could survive for that time, provided a means to know the definitive role of MO/Mϕ and avoid the confounding effects exerted by other types of cells. However, special culture conditions were required to achieve successful long-term growth and most of them might more or less lead to Mϕ activation (19, 23, 58-62, 64, 68, 69). Herein, we established a culture system devoid of cell activation by using autologous serum, without any exogenous growth factors, in the culture medium which was half-replaced every 5 ~ 6 days. Interestingly, we found that remaining of old CM was crucial for optimal MO/Mϕ differentiation. In fact, it has been shown that MO/Mϕ-CM was the best stimuli for differentiation of human MO-derived dendritic cells (DC) in vitro (70). It has been shown that latent human cytomegalovirus could be reactivated after long-term culture of allogenically stimulated MO/Mϕ (23). In their culture system, the terminally differentiated MO/Mϕ demonstrated characteristics of both MO/Mϕ and DC. Because during MO/Mϕ differentiation, these cells constitutively produce GM-CSF and other monokines and granulomonopoietic-enhancing activity that may orchestrate to determine the fate of MO differentiation (61-63, 70-73), it is intriguing in the future to further examine if these

self-differentiated MO/Mϕ also express DC characteristics and their role in antigen presentation after DV infection. Herein, we provided a new insight into Mϕ biology by demonstrating that terminally differentiated human MO/Mϕ were capable, both in supporting virus replication and in production of multiple cytokines and chemokines.

Significance of DV-induced production of cytokines and chemokine by differentiated MO/Mϕ. We demonstrated here that DV was pluripotent in inducing MO/Mϕ secretion of a diverse array of cytokines and chemokines. It is likely that these mediators, with distinct degree and kinetics of production, orchestrate in a sophisticated manner to determine the disease outcome of DV infection.

The clinical presentations of DF and DHF-DSS reveal many signs of local inflammation, which appear to result from extravasation of leukocytes to the site of infection. The onset and peak of chemokine production by differentiated MO/Mϕ occurred at early time after DV infection indicated that these molecules play a role in early recruitment of inflammatory leukocytes and take part in early response to viral infection as well as tissue injury (33, 37-42, 74, 75). Interestingly, endothelial cells were shown to produce chemokines after DV infection (76), suggesting a possible cooperative interaction of these two types of cells in regulating leukocyte trafficking by differential production of chemokines. Interestingly, in addition to its essential role in acute inflammation, IL-8 could stimulate virus replication by antagonizing the antiviral activity of IFN-α (77). The extremely high and durable levels of IL-8 secretion from DV-infected MO/Mϕ may give rise to overwhelming inflammation and less efficient in elimination of viruses during DV infection. Moreover, RANTES was shown to preferentially recruit and activate T lymphocytes with memory phenotypes (78) and, thus, might be important for rapid T cell-mediated effector functions during secondary DV infection.

It is important to note that secretion of IL-12 and IFN-α demonstrated multiple peaks and persisted long after the production of infectious viruses and cytokines had ceased or subsided. The observations suggested that their production could also be regulated by other cytokines in

an autocrine and/or paracrine manner. For example, TNF-α was shown to inhibit IL-12 production by human MO/Mϕ, (79) the decline of TNF-α might account for the burst secretion of IL-12 at ~ 7 d after infection (Fig. 4). Moreover, MIP-1α and RANTES signaling through their receptor CCR5, which is expressed by MO/Mϕ, was able to induce IL-12 production (•••). It is conceivable that sustained production of IFN-α and IL-12 by DV-infected differentiated MO/Mϕ may act to keep effector lymphocytes alive and activated and maintain and/or amplify Th1-type immune activation in vivo even after virus elimination (31-34, 80). Interestingly, replication efficiency of some viruses increased with MO/Mϕ differentiation due to reduced capacity of differentiated cells in producing IFN-α for viral restriction (20, 81). By contrast, we did not find any defect in IFN-α production by the primary differentiated MO/Mϕ in response to DV infection.

It was known that MIP-1α, RANTES, IFN-α and IL-12 can induce NK cell activation, chemotaxis, adhesion and transendothelial migration (31-33, 38, 42, 43), and MIP-1α was shown to possess dual roles in viral infection by conferring NK cell-mediated protection and tissue inflammation (74, 82). Therefore, regulation of NK cell activity by these mediators may give rise to resolution of DV infection and/or tissue pathology (27). Furthermore, some of the secreted cytokines and chemokines possess suppressive activity on hematopoiesis (40, 43, 83) and may contribute to bone marrow suppression observed in dengue illness (84).

Effect of LPS on the production of infectious virions, cytokines and chemokines by DV-infected primary differentiated human MO/Mϕ. Dual infection with bacteria during the course of viral infection usually aggravates disease progression (85). Bacterial LPS was able to modulate virus infection of human MO/Mϕ, by either inhibiting or amplifying virus replication (53, 55). We have previously shown that pretreatment of human MO/Mϕ with LPS markedly inhibited DV infection via blockade of the access for virus entry (57). In the present study, we found that infectious-virus production was indeed increased and prolonged in the presence of LPS, further supporting the notion that LPS was able to prevent DV entry but unable to elicit an

intracellular antiviral state of human MO/Mϕ for DV clearance.

It has been reported that secretion of IL-12 and some other cytokines after stimulation with bacterial components was impaired in MO/Mϕ infected with certain viruses (44-52). Such a virally mediated impairment of cytokine production may predispose a host already acquired a viral infection more susceptible and vulnerable to bacterial superinfection. However, in our experimental system, stimulation of MO/Mϕ with LPS after DV infection could still potently trigger the secretion of multiple cytokines and chemokines, indicating that DV infection did not suppress MO/Mϕ to produce these mediators. It is noteworthy that although LPS by itself was unable to induce IFN-α production, it potently augmented the DV-induced secretion of this type I IFN at early time after infection and throughout the course of infection. This was consistent with the observations that human MO/Mϕ needed to be primed for LPS-stimulated IFN-α production (86). Interestingly, early augmentation of IFN-α did not result in reduction of virus replication in the LPS-treated cultures (Fig. 5), suggesting that IFN-α-mediated antiviral activity might not be the sole mechanism underlying virus clearance in our experimental system. Alternatively, MO/Mϕ activation by LPS may trigger an undefined activity counteracting the antiviral activity of IFN. Together with the lack of IL-12 suppression, enhanced IFN-α production by DV-infected MO/Mϕ treated with LPS provided a novel potential pathogenic mechanism by which bacterial coinfection may exaggerate the Th1 type immune response in DV infection (87-89). The LPS-mediated enhancement of virus replication and cytokine production may partly account for the pathogenesis of severe dengue illness in dengue epidemic areas where bacterial infection is also prevalent.

Immunopathogenesis of DV infection. Based on the observations, a cytokine-induced inflammation and pathogenic cascade was proposed. At first, secretion of chemokines would form a local gradient to specifically recruit circulating neutrophils, lymphocytes NK cells and MO to the sites of infection. Meanwhile, these chemokines and IL-12, TNF-α and IL-1β act locally on endothelial cells to increase vascular permeability and adhesion molecule expression,

thereby facilitating blood leukocyte adherence to and diapedesis through vascular endothelium. As a result, increased numbers of inflammatory cells were brought to the site where antigen-bearing tissue Mϕ are located. Thereafter, the cellular infiltrates are further influenced by the synergistic interactions of cytokines and chemokines released from infected tissue Mϕ and undergo functional maturation. More importatnly, the long-lasting cytokines, such as IL-12, IFN-α and IL-1β may contribute to the differentiation, maintenance and activation of CTL, Th1 and NK cells in secondary lymphoid or other organs. These effector cells exert cytotoxic actions and secrete IFN-γ, IL-2, and other mediators, which may essentially participate in the orchestrations of the complex cytokine network with host immune system.

Since DV infection could induce human MO/Mϕ secretion of a diverse array of cytokines and chemokines, it is worthy to investigate the molecular mechanisms underlying the induction. Is virus replication necessary? Are these mediators induced directly by DV or some of them are triggered by other endogenous cytokines? What is the mutual regulation among these molecules? What are the cell surface and intracellular requirements? We have shown that a CD14-related pathway was involved in DV entry (57). Interestingly, CD14 and toll-like receptor were shown to mediate respiratory syncytia virus-induced cell activation (•••). It is fascinating to explore the role of these innate pattern recognition receptors in DV-induced MO/Mϕ activation

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Table 1.

Release of infectious viruses, cytokines and chemokines by mock- and DV-infected MO/Mϕ inoculated with different MOI equivalents

Input MOI (PFU/cell)	Viral yields (PFU/ml)	pyrogenic cytokines		Th1/NK-stimulatory cytokines		C-X-C and C-C chemokines		
		TNF-α	IL-1β	IL-12	IFN-α	IL-8	MIP-α	RANTES
mock	-	UD	UD	UD	UD	1975.03±109.35	112.09±1.33	34.52±3.22
5	$4.83x10^5$	134.99±12.07	18.63±1.31	339.18±25.97	248.17±11.98	74224.81±1136.30	19165.06±135.12	813.82±67.90
0.5	$1.54x10^5$	15.46±4.61	UD	97.81±3.00	68.19±4.06	58406.18±439.15	2095.56±488.74	381.30±26.72
0.05	$3.28x10^4$	UD	UD	15.29±2.65	9.10±0.49	27045.24±463.80	293.02±4.96	218.95±9.42
0.005	$1.68x10^3$	UD	UD	UD	UD	9370.08±339.44	134.23±0.70	200.65±17.79

1. The results were obtained from one representative experiment for stringent comparison among these cytokines and chemokines.
2. MO/Mϕ were cultured for 7 days, at which time the cell monolayers were washed and then either mock-infected with C6/36 cell CM or infected with DV at an MOI of 5, 0.5, 0.05 or 0.005. After 2.5 hours for viral adsorption, the cultures were washed twice to remove unabsorbed viruses and then replenished with fresh complete α-MEM. Culture supernatants were collected at 44 h post-infection and analyzed for the titers of infectious viruses, cytokines and chemokines.
3. The concentrations of each cytokines and chemokines were presented as pg/ml. Each experimental point is expressed as the mean±S.E of results obtained from three independent wells with cells from the same donor.
4. UD: undetectable; concentration < 7 pg/ml..

Figure Legends

Fig. 1 Morphological alteration during the in vitro differentiation of primary human MO/Mϕ. (*to be completed*)

Figure 2. Production of infectious DV by primary human MO/Mϕ at various differentiation stages. Peripheral blood monocytes were cultured with ΔAHS for 1 day, 1, 3, or > 6 weeks, at which time the cells were washed and then infected with DV at an MOI of 2 ~ 3 PFU per cell in the absence of serum. After 2.5 h of viral adsorption, the cells were washed and the cultures were further incubated for 40 ~ 48 h at that time the culture supernatant was harvested and assayed for infectious-virus production. The results are expressed as the mean ± S.E. of data n separate wells from different experiments.

Figure 3. Early (A) and long-term (B) kinetics of the production of various cytokines and chemokines by 7-day-old human MO/Mϕ infected with DV. The cells were cultured and treated as described in the legend to Fig. 2. At indicated time, the culture supernatant was harvested and assayed for infectious-virus production and cytokine/chemokine secretion. The cryolysates of MO/Mϕ were analyzed for intracellular infectious-virus titers. Results in (A) and (B) were separately derived from one of the representative experiments. Each time point represents the mean ± S.E. of results from three independent wells. The levels of these cytokines and chemokines in the supernatant of mock-infected control cultures remained constant before and after infection.

Figure 4. Kinetics of DV replication and virally induced secretion of cytokines and chemokines in cultures of terminally differentiated human MO/Mϕ. Human peripheral blood monocytes were cultured with 10% ΔAHS and replaced half of the culture medium with fresh complete α-MEM to allow self-differentiation to MO/Mϕ. After 45 days of culture, the cells were washed and infected with DV at an MOI of 3 PFU per cell. Culture supernatants were harvested at the time points as indicated and assayed for infectious-virus production and cytokine/chemokine secretion. Cell cryolysates were analyzed to determine the intracellular

infectious-virus titer. For each time point, three separate wells were prepared and analyzed, and the results are expressed as mean ± S.E.. Some error bars are too small to be seen. Representative data out of four independent experiments were shown.

Figure 5. LPS enhanced and sustained DV replication in differentiated human MO/Mϕ. 7-d-old MO/Mϕ were washed and then infected with DV at an MOI of 3 PFU per cell. After 2.5 h of viral adsorption, cell monolayers were washed and replenished with fresh complete α-MEM with (DV-LPS: hatched bar) or without (DV: dotted bar) LPS (5 μg/ml). Culture supernatants were harvested at different time points and were analyzed for extracellular and intracellular infectious-virus titers. For each time point, three separate wells were prepared and analyzed, and the results are expressed as mean ± S.E.. Representative data out of four independent experiments were shown.

Figure 6. Enhancing effect of LPS on DV-induced IFN-α secretion. 7-d-old MO/Mϕ were washed and then infected or left uninfected with DV at an MOI of 3 PFU per cell. After 2.5 h of viral adsorption, cell monolayers were washed and replenished with fresh complete medium. LPS (5 μg/ml) was added to some of the infected or uninfected cultures at 6 h post-infection (DV: hatched bar; LPS: dotted bar; DV-LPS: black bar). Culture supernatants were harvested at different time points and assayed for infectious-virus and IFN-α titers. For each time point, three separate wells were prepared and analyzed, and the results are expressed as mean ± S.E.. Representative data out of four independent experiments were shown.

Figure 7. DV infection did not suppress LPS-stimulated TNF-α production. (A) Experimental protocol. 7-d-old MO/Mϕ were treated or left untreated for 20 h and then, without wash, the cells were then mock-infected or infected with DV at an MOI of 3 PFU/cell. After 6 h of infection, some of the cultures were treated with LPS at a concentration of either 1 μglml or 10 ng/ml. After 48 h post-infection, the culture supernatants were harvested and analyzed for TNF-α production by ELISA. (B) LPS (hi) +/- DV: cells were pretreatred with LPS (1 μglml) and then infected or not infected with DV; Mock +/ LPS(hi or lo) and DV +/ LPS (hi or lo):

mock-infected cells or DV infected cells were stimulated with LPS (hi: 1 μglml; lo: 10 nglml) after 6 h of infection. The results are expressed as the mean ± S.E. of data from 3 seperate wells. Representative data out of eight independent experiments were shown. In some experiments, the additive or synergistic production was less pronounced as shown here when using high doses of LPS. Production of other cytokines demonstrated similar results. Measurements with the TNF-α ELISA kits purcahsed from Biosource and Genzyme obtained the same results.