Lipopolysaccharide Activates Human Mast Cells To Induce Intestinal Epithelial Barrier Dysfunction
Y Ping-Chang, Z Peng-Yuan, W Chang-Sheng

Citation

Abstract
Background: To determine the pathway via which lipopolysaccharide (LPS) compromises the intestinal epithelial barrier function.

Materials and Methods: The human mast cell line HMC-1 cells were stimulated with LPS. The tryptase levels were evaluated in the supernatant. The human colonic epithelial cell line T84 cells were cultured with the supernatant from the culture of the HMC-1 cells that have been activated by LPS. The T84 monolayers' barrier function was evaluated by recording transepithelial electric resistance (TER) and horseradish peroxidase (HRP) flux.

Results: HMC-1 cells expressed the toll like receptor 4. LPS stimulation induced HMC-1 cells to release tryptase. The supernatant caused significantly decrease in TER and increase in HRP flux that could be inhibited by pretreatment with tryptase inhibitor BABIM.

Conclusion: LPS can induce human mast cells to release chemical mediators such as tryptase that can compromise the intestinal epithelial barrier function.

INTRODUCTION
The gastrointestinal mucosa is the largest body surface to interface with the external environment (7). The gastrointestinal tract is constantly in contact with an abundant commensal bacterial microbiota and is exposed perpetually to potentially harmful nutrients, microbes, and toxins. Accumulative evidence suggests that the intestinal bacterial flora plays crucial role in the pathogenesis of intestinal inflammatory diseases such as inflammatory bowel disease (IBD) (7, 8). Lipopolysaccharide (LPS), the outer envelope of Gram negative bacteria, is the major inducer of the inflammatory response in the intestinal tissue. LPS signaling is mainly mediated through the cell surface toll-like receptors (TLRs) (9). TLR4 is the major transducer of LPS and specifically binds the lipid A portion of LPS. The mucosal barrier plays important roles in preventing bacterial translocation and absorption of endotoxin from the lumen to the intestinal tissue. It has been reported that the bacterial LPS can compromise gastrointestinal function including intestinal motility, secretion and epithelial barrier functions (10, 11).

Mast cells originate in the bone marrow and are distributed in the mucosa and subcutaneous connective tissue where they serve as the first line of defense against attack by pathogens (12, 13) via releasing anti-pathogen mediators such as TNF-α. Mast cells are also the residential cellular components in the intestinal tissue. Its strategically distribution in the intestinal tissue makes them at the front line facing exogenous insults, such as microbita, microbial products, protein antigens and so on. Although it has been mentioned that mast cells play a certain role in the innate immunity, the majority reports about mast cells emphasize the pathological role in allergic or inflammatory diseases in the body based on their deleterious effects in the pathogenesis of type I hypersensitivity reactions and inflammation (10, 11). Tryptase is a constitutive protein in the granules of the mast cells. It consists of approximately 25% of the total protein in the cytosol of mast cells (12). A unique feature of tryptase is its cleaving protease activating peptide (PAR) 2 and modifying functions of the PAR2 bearing cells including epithelial cells of the intestine, skin, airway and urigenital tract (13). It is suggested that mast cell derived
trypase plays certain roles in several diseases, such as asthma, allergic dermatitis, inflammatory bowel disease (IBD), and so on (16).

The normal intestinal epithelial barrier can effectively to restrict the intact proteins, microbial toxins and some other noxious substances to be absorbed into the intestinal tissue (14). Mounting evidence shows that there are a bundle of factors may compromise the intestinal barrier functions, such as stress, infections and existing intestinal inflammation (15,16,17). Intestinal epithelial barrier permeability markedly increases in response to these factors. A plentiful resource of LPS is available in the intestinal lumen. The increase in absorption of LPS in the intestinal mucosa may be up regulated in the inflammatory situation (19). LPS is harmful to the intestinal tissue. It induces epithelial apoptosis, increases the production of active oxygen, damage blood vessel endothelium, increase epithelial permeability, inflammatory cell infiltration and tissue remodeling (18). It is reported that the intestinal tissue can be immunized by contacting LPS and becomes hyporesponsiveness to the subsequent stimuli of LPS (20). However, the intestinal tissue in some individuals can be still damaged by LPS having been contacting the luminal LPS since birth. There may have been some alternatives by which LPS compromise the intestinal tissue.

It is until recently revealed that mast cells express toll like receptor (TLR) 4 (21). TLR4 can recognize LPS with the aid of LPS binding protein (LBP) and CD14. LBP is an acute-phase protein, produced in the liver, which circulates in the bloodstream where it recognizes and forms a high-affinity complex with the lipid A moiety of LPS, as free molecules, fragments, or still bound to the outer membrane of intact bacteria. The role of LBP appears to be that of aiding LPS to dock at the LPS receptor complex by initially binding LPS and then forming a ternary complex with CD14, thus enabling LPS to be transferred to the LPS receptor complex composed of TLR4 and MD-2. Mast cells are reported to express various kinds of TLR including TLR4 that can be up regulated by incubation with IFN-γ. LPS can induce TNF-α release from mast cells (22).

Apart from the release of TNF-α when mast cells are stimulated by LPS, we hypothesized that mast cells may also release tryptase, the most abundant and important mediator in mast cells. The intestinal epithelial barrier will be damaged by the LPS-induced mast cell activation. In this study, we aimed at to characterize the LPS induced tryptase release from the human mast cell line HMC-1 cells; the subsequently induced intestinal epithelial barrier function. The results showed that LPS did induce HMC-1 cells to release tryptase in the exposure to LPS. Intestinal epithelial barrier function was markedly damaged.

**MATERIAL AND METHODS**

**CELL CULTURE**

A human mast cell line, HMC-1 cells (a gift from Dr Butterfield, Mayo Clinic, USA) was grown in Iscove’s media supplemented with 10% FCS and 2 mM glutamine. T84 epithelial cells, a human colonic epithelial cell line, were seeded (10⁶ cells) on tissue culture-treated Transwell filter supports (Costar, Cambridge, MA). Culture media consisted of a mixture of DMEM/F-12 (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated FCS (Cansera International, Rexdale, Ontario, Canada), 1.5% HEPES (GIBCO), and 2% penicillin-streptomycin (GIBCO). Cells were grown in 95% humidity at 37°C in 5% CO₂ for 7 days to attain polarized epithelial monolayers with daily replacement of media until transepithelial resistance (TER) measurements of >1,000 Omega /cm².

**EPITHELIAL BARRIER FUNCTION STUDY**

Two parameters, TER and the permeability to a macromolecular protein, horseradish peroxidase (HRP, MW = 44,000, type II, Sigma), were used in the present study to represent the T84 cell monolayers’ barrier function. TER is a measure of the ability of T84 cells to restrict passive movement of ions and was monitored during the experimental period using a Millicell-ERS system (Millipore, Bedford, MA). To measure the T84 monolayers’ permeability, 50 µg HRP was added to the 1 ml medium in the apical side of the monolayers. The monolayers were incubated for 2 h at 37°C. Media samples were obtained from the basal compartments and assayed for HRP concentration by kinetic enzymatic assay as previously described (22). Briefly, sample was added to phosphate buffer containing H₂O₂ and o-dianisidine (Sigma). Enzyme activity was determined from the rate of increase in optical density at 460 nm. HRP transport was calculated as recovery of added HRP.

**HMC-1 CELLS STIMULATION WITH LIPOPOLYSACCHARIDE (LPS)**

A series concentration, from 0.625 µg/ml to 10 µg/ml, of LPS was designed. Each dose was added to each culture well of 10⁶ HMC-1 cells in addition of LBP (10 ng/ml, R&D Systems, MN). One group consisted of 12 wells. The
HMC-1 cells were cultured at 37°C for 24 hours after the addition and the supernatants were harvested. Based on the preliminary results (data not shown), a dosage of 1 µg/ml LPS (this dose of LPS doesn’t influence the T84 monolayers’ barrier function) was added to the culture, supernatant samples were taken at the time points of 0.5, 8, 24 and 48 hours after the addition of LPS. The cells were washed and re-suspended with fresh culture medium after sampling at each time point in order to detect the amount of tryptase release in each period. The supernatants were centrifuged for 10 minutes at 14,000 rpm at 4°C. The aliquots were stored at -70°C for further assay. A control group consisted of 12 wells that were cultured with medium only.

**EVALUATION OF TRYPTASE**

For tryptase activity assay, triplicate aliquots (10 µL) of the supernatants were added to 200 µL of buffer (50 mmol/L Tris/HCl, pH 7.6, 120 mmol/L NaCl, 20 µg/mL heparin) containing 0.5 mmol/L substrate (tosyl-Glycine-Proline-Arginine-pNitroanilide, New England, USA) and incubated at room temperature for 17 hours (± inhibitors as indicated). Cleavage of the substrate was measured using a microtiter plate reader (absorbance 405 nm).

**IMMUNOCYTOCHEMISTRY**

The HMC-1 cells were fixed in 4% formaldehyde for 30 minutes. The cells were then washed with 0.05 M Tris buffer (TBS; pH 7.6), and were incubated for 2 hours with rat anti-human TLR4 antibody diluted 1:200 in 1% BSA/TBS as described. Cells were incubated 45 minutes with FITC-conjugated rabbit anti-rat antibody (Vector Laboratories, Burlington, CA) diluted 1:200 in 1% BSA/TBS as well as 5µg/ml propidium iodide. After they were washed, the cells were smeared on glass slides and covered in antifade medium (Vectashield; Vector Laboratories), and observed with a confocal microscope (LSM 510).

**FLOW CYTOMETRY**

The HMC-1 cells were processed as described above. After staining, the positive stained cells were counted with a FACScan.

**WESTERN BLOT**

Total HMC-1 cell lysates were obtained by lysing the naïve HMC-1 cells with lyse buffer, and proteins were quantified spectrophotometrically using Bio-Rad DC Protein Assay kit (Bio-Rad) per manufacturer's instructions. Equivalent amounts (40 µg) of total cellular protein were fractionated by SDS-PAGE and blotted onto nitrocellulose membranes (Amersham Biosciences). Membranes were sequentially incubated with primary antibodies to TLR4 (rat anti-human, Biocompare, diluted 1/1000). HRP-conjugated rabbit anti-rat secondary antibody (Sigma-Aldrich) was diluted 1/5000. Detection of chemiluminescence was obtained by using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

**RT-PCR**

The TLR4 gene sequence was obtained from the PubMed (U88880 ). Primers were designed with the software Primer3. The pair of primers was: forward: AGA TGG GGC TCA GAC GAT GGA C and Reverse: CCA GAA CCA AAC GAT GGA C. Total RNA was isolated from 1 × 10^6 cells using TRI reagent (Sigma-Aldrich). Contaminating DNA was removed using DNase 1 treatment. For RT-PCR, 1 µg of total RNA was reverse transcribed into cDNA with an oligo(dT)15 primer using first strand cDNA synthesis kit (Roche). The integrity of RNA extraction and cDNA synthesis was verified by PCR by measuring the amounts of GAPDH cDNA in each sample using GAPDH-specific primers to generate a 211-bp product. PCR mixtures contained 2.5 mM MgCl2, 1.25 U of Taq polymerase, 0.2 mM dNTPs (Promega), and 50 pmol of TLR4 gene-specific primers. Thermocycling conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension step of 72°C for 10 min was performed. Products were resolved on 1.5% Tris-borate-EDTA (TBE)-agarose gels containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich), and images were captured using the GeneGenius Gel Documentation and Analysis System (Syngene).

**ELECTRON MICROSCOPY**

At the end of each experiment, the HMC-1 cells were collected for electron microscopy. The HMC-1 cells were fixed with 2% glutaraldehyde for 2 hours and postfixed with 1% osmium tetroxide for 1 hour. After dehydration with a series of graded ethanol, the cells were embedded in EPOF. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate and observed with an electron microscope (JEC 1200). The sections were coded to avoid bias of the observer. Thirty mast cells were randomly selected from each group. The granules of intact, PMD and AND in each selected HMC-1 cell were counted.
RESPONSE TO LPS INDUCED MAST CELL TRYPTASE RELEASE

A batch of HMC-1 cells was stimulated by the addition of LPS (1 µg/10⁶ HMC-1 cells) for 24 hours at 37°C. The culture was centrifuged for 5 minutes at 500 rpm. The supernatants were collected using to stimulate the T84 monolayers. Confluent T84 monolayers were stimulated by adding 1.5 ml the supernatant to the basal compartment of the Transwells. The TER of the T84 monolayers was recorded at 0 hour and 24 hour after the addition of the supernatant. The influence of the supernatant on the T84 monolayer TER was calculated with the TER at 24-hour divided by the TER at 0-hour and expressed as the percentage of the baseline TER. The T84 monolayer's permeability was assessed with HRP flux from the 24th hour to the 26th hour after the addition of the supernatant. Control groups included a group of the Transwells that the T84 cells were treated with fresh medium; another group T84 cells treated with fresh medium plus 1 µg/ml LPS; the third control group was pretreated with 100 µMol/ml for 30 minutes, then added the supernatant to the basal side of the Transwells.

STATISTICS

Data were presented as mean ± SD. Differences of means between groups were analyzed with student t test or F test. P<0.05 was set as significant criteria.

RESULTS

TRYPTASE RELEASE FROM THE MAST CELLS IN RESPONSE TO LPS STIMULATION

The lowest dose (0.625 µg/ml) of LPS used in the experiments apparently induced tryptase release from the HMC-1 cells. The release of tryptase increased following the increase of LPS in the culture in a dose-dependant manner (Fig 1-A). Tryptase inhibitor efficiently blocked the degradation of the substrate by tryptase (data not shown). We also detected a low level of tryptase in the culture of naïve HMC-1 cells.
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Figure 2
Figure 2: Human mast cell line HMC-1 cell degranulation in response to LPS stimulation. A, the average ratio of anaphylactic degranulation in the 30 randomly selected HMC-1 cells. *, p<0.05, compared with the 0-hour group. B, the average ratio of piecemeal degranulation in the 30 randomly selected HMC-1 cells. *, p<0.05, compared with the 0-hour group. C-F, representative electron microphotographs of HMC-1 cells from naïve group (C), 0.5-hour group (D), 24-hour group (E) and 48-hour group (F).

Figure 3
THE HMC-1 CELLS EXPRESS TLR4 RECEPTORS
The TLR4 receptors were identified in the HMC-1 cells. Immunocytochemistry results showed TLR4 receptor protein expression on the surface of the HMC-1 cells (Fig 3A). Flow cytometry demonstrated more than 50% HMC-1 cells were positively stained by anti-TLR4 antibodies (Fig 3B). Western blot data specifically confirmed the TLR4 protein existence in the HMC-1 cells (Fig 3C). RT-PCR showed TLR4 gene expression on the HMC-1 cells increased significantly in the exposure to LPS stimulation at 30 minute time point (Fig 3D, lane 1), and kept higher levels at 24- and 48-hour (Fig 3D, lane 2) time points.

Figure 5
THE HMC-1 CELLS RELEASED TRYPTASE COMPROMISED THE INTESTINAL EPITHELIAL BARRIER FUNCTION
The supernatant used in assessing the T84 cell barrier function was from those HMC-1 cells treated with 1 µg/ml LPS for 24 hours. The tryptase level in the supernatant was determined as 7.56 pg/ml. As shown in Fig 4, the TER decreased significantly in the T84 monolayers after incubated with the supernatant derived from the LPS treated HMC-1 cells whereas HRP flux showed a great elevation of the permeability to HRP in the T84 monolayers. Pretreated with tryptase inhibitor BABIM efficiently but partially blocked the influence of the supernatant on TER and HRP flux. Those control wells of T84 cells with 1 µg/ml LPS didn't show statistical difference from the naïve controls.

In the anti-TLR4 antibody treated HMC-1 cells, the results showed the cells didn't increase tryptase release levels. The ratio of degranulation was approximately the same as the controls.
Figure 6

Figure 4: Human colonic epithelial cell line T84 cell monolayer barrier function was down regulated by the supernatant from the HMC-1 cells that have been activated by LPS stimulation. A, transepithelial electric resistance (TER) was down regulated by LPS-induced mast cell activation. The bars stand for the percentage of TER as compared with the baseline before the exposure to the supernatant. *, p<0.05, compared with the supernatant from the naïve HMC-1 cells; #, p<0.05, compared with the group treated with the supernatant from the LPS treated HMC-1 cells. B, bars represent the recovery of HRP in the basal side of the Transwells. *, p<0.05, compared with the group treated with the supernatant from the LPS treated HMC-1 cells. B, bars represent the recovery of HRP in the basal side of the Transwells. C-F, representative electron photomicrographs from the T84 cell monolayers. C, naïve T84 cell monolayers. A small HRP endosome was located in the cytoplasm (arrow). D-F, these photomicrographs were taken from the T84 cells that were treated with the supernatant from LPS-treated HMC-1 cells. More HRP endosomes were located in the up region (D) and low region (E) of the cytoplasm (arrows). HRP products also were located in the paracellular spaces (empty arrows). ×3,000.

Electron microscopy showed HRP endosomes in the T84 monolayers. A few small HRP endosomes could be seen in the naïve T84 cells. Most of them were located on the apical region (above the nucleus). After treatment with the supernatant, the HRP endosome number markedly increased in the T84 cells. The HRP endosomes were distributed in all regions of the cells including apical, middle and the basal regions. The size of the endosomes was also increased. Image analysis data showed HRP endosome area was significantly increased in the HMC-1 cells treated with the supernatant. We also identified HRP products in the paracellular spaces in some wells.

DISCUSSION

We have shown that human mast cell line, HMC-1 cells can release tryptase in response to LPS stimulation. The intestinal epithelial barrier function can be compromised in the exposure to the milieu of co-culture with mast cells that have been activated in the presence of LPS.

Mast cells are residential cells in the intestinal tissue. Accumulated evidence demonstrates that mast cells play important roles in both innate and acquired immune functions (7, 8). However, what we have understood most about mast cells is the inflammatory effector activity. The present data have added one more piece of information to the pathological role of mast cells. Publications have demonstrated that significantly increased mast cells in the intestinal mucosa of the patients with intestinal chronic inflammation such as IBD (25, 26). The intestinal epithelial barrier dysfunction in the patients with IBD has been noticed. Some IBD patients show that the intestinal epithelial hyperpermeability precedes the relapse of IBD clinical phenomena (27). It has not been certain how the mast cells in the intestinal mucosa of IBD get activated. The causes can be multiple. Psychological stress activates mast cells in the skin (28), in the intestine (29) and other organs (30). Clinical investigation demonstrates that the increase in mast cell number in the intestinal mucosa of the patients with IBD and food allergy (25, 31). Studies revealed intestinal epithelial hyperpermeability in the patients with IBD (32). Some patients showed a phenomenon that their intestinal epithelial hyperpermeability preceded the clinical IBD symptoms (33). It is certain that mast cell activation in the intestinal mucosa can damage intestinal epithelial barrier function via release of the chemical mediators (34). But the causes of mast cell activation have not been fully understood. The present data demonstrate that the bacterial LPS can activate the human mast cell line, HMC-1 cells directly. There is a plenty source
of LPS in the intestinal lumen. The LPS can be absorbed into the intestinal tissue upon the intestinal epithelial barrier dysfunction occurs. The absorbed LPS then may act on the residential mast cells in the intestinal mucosa to cause the mast cells to release chemical mediators, such as histamine, tryptase, etc.

Tryptase consists of about 25% of the total protein in the cytosol of mast cell. Many publications suggest that mast cell derived tryptase plays a crucial role in the some of the intestinal diseases (30). The unique feature of tryptase is that it can cleave PAR2 receptors on the basolateral membrane of the enterocytes (31). We also have identified that the intestinal epithelial cells express PAR2 receptors on both apical and basal side of the human colonic cell membrane (data not shown). Other investigators also reported PAR2 expression on the rat intestinal mucosa (32). The subsequent experiments confirmed our hypothesis that mast cell derived tryptase can cause the intestinal epithelial barrier dysfunction. The data showed that the TER decreased significantly, that is an indicator of the paracellular permeability increase. Intact protein antigens, microbial products and other noxious substances may be absorbed into the mucosa in such an environment. HRP is a macromolecule (MW = 44,000 Dalton). We noticed in the experiments that HRP was transported across the T84 monolayers via transcytosis and paracellular pathway. The HRP found in the cytoplasm of the T84 cells appears as endosomes. The quantification of these HRP endosomes demonstrated that much more amount of HRP was transported across the T84 monolayers via transcytosis in the LPS group than that in the naïve groups. The data from the experiments with tryptase inhibitor BABIM further confirmed the decrease in TER and increase in HRP flux in the experiments was originated from tryptase that was derived from the HMC-1 cells, at least partially if not all, because there are an array of mediators may have been released from the HMC-1 cells.

The present data show two secreting peaks of tryptase secretion that occurred at 0.5- and 8-hour time points respectively during the 48-hour observation period. The mediators in mast cells are categorized into two groups, the preformed mediators such as histamine, tryptase, etc. and the newly generated mediators, such as prostaglandins, a series of cytokines and so on (33). The present data showed that the so called preformed mediator tryptase also could be released sometime after the acute secretion phase. We detected the second secretion peak of tryptase 24 hours after the stimulation of LPS. The tryptase released at the second peak is for sure not the remains of the first secretion since we took all the media out of the culture, the cells were washed with saline and we didn't detected high levels of tryptase at the 8-hour time point. The possible explanation on this point is that the release of tryptase from mast cells acts as a feedback signal to initiate the synthesis of tryptase in mast cells. The new formed tryptase releases in encountering the existence of the stimulating signals such as that in the LPS activated TLR4-MD2 signal pathway. This point is needed to be approved by further investigation. The second phase of mast cell mediator release responds for the late phase allergic reactions. The occurrence time of the late phase reaction varies from case to case, from several hours to a few days according to the clinical observation in the patients with allergic diseases (34). Different remedies have been tried in the treatment of allergic disorders, but not many have been approved to be satisfactory. We only have begun to appreciate the action of tryptase in the disorders of inflammation and allergy recently. Some inhibitors of tryptase have been tried clinically (35). The two release phases of tryptase from mast cells can be a useful reference in therapeutic strategy design of the patients with allergic disorders or chronic inflammation.

It is only in recent years the types of mast cell degranulation attracted attention from the scientists in this area. PMD is defined as a type of releasing mediators chronically, whereas AND is referred to the phenomenon that mediators release acutely such as in the pathogenesis of anaphylactic shock. In the present study, we observed both AND and PMD types of degranulation in the HMC-1 cells after the exposure to the stimulation of LPS. More AND type degranulation in the HMC-1 cell degranulation in acute allergic reactions. The event that tryptase levels increased significantly in the culture at the 0.5-hour time point demonstrates that a relatively large amount of tryptase (and others) can be released at the acute phase of mast cell degranulation. Morphological study showed more AND type degranulation in the HMC-1 cells that confirmed the point. It is noteworthy that more PMD HMC-1 cells were observed at 48-hour time point in this study, which suggests that the slowly mediator release in the HMC-1 cells lasts for a relatively long time after the exposure to LPS.

Here we report that the human mast cell line, HMC-1 cells constitutively express TLR4 mRNA and proteins. The receptors are involved in the stimulation of human mast cells by the agonist LPS. These results are in agreement with
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those previously obtained on mouse bone marrow-derived mast cell from TLR4-deficient mice (a). It appears that LBP is necessary in the reactions between LPS and TLR4 since LPS didn't show any effects on the tryptase release from the HMC-1 cells without the presence of LBP in this study.

CONCLUSIONS

In conclusion, we have reported that the human mast cell line, the HMC-1 cells express TLR4 receptors. LPS activates the HMC-1 cells to release tryptase that has two secretive peaks at 0.5 and 24 hours respectively after the exposure to LPS stimulation. Using the supernatant from the culture of the HMC-1 cells that have been activated by exposing to LPS, we observed the co-cultured human colonic epithelial cell line T84 cells' barrier function was significantly compromised.

CORRESPONDENCE TO

Dr Ping-Chang Yang HSC, Room 3N5C McMaster University 1200 Main St. West Hamilton, ON, Canada L8N 3ZS Tel: (905) 525-9140 Ext. 22585 Fax: (905) 522-3454 Email: yangp@mcmaster.ca

References

Author Information

Yang Ping-Chang, MD, PhD
Assistant Professor, Department of Pathology and Molecular Medicine, McMaster University

Zheng Peng-Yuan, MD, PhD
Professor, Department of Gastroenterology, Zhengzhou University, the Second Hospital

Wang Chang-Sheng, MD
Professor, Allergy Unit, Shanxi Medical University, the First Hospital