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Functional and Molecular Expression of the Proton-Coupled Oligopeptide Transporters in Spleen and Macrophages from Mouse and Human

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Abstract

The aim of this study was to determine the expression and function of proton-coupled oligopeptide transporters (POTs) in spleen and macrophages, and their contribution to innate immune response induced by bacterial peptidomimetics γ -iE-DAP and MDP. Quantitative real-time PCR (qRT-PCR) and Western blot results revealed the mRNA and protein expression of PepT2, PhT1 and PhT2, but not PepT1, in the spleen of mice and human. In comparison to lymphocytes of the spleen, macrophages had higher transcript levels of PepT2 and PhT2. The cellular uptake of Ala-Lys-AMCA in mouse splenic macrophages was pH dependent with maximum uptake at pH 6.0, and the kinetic parameters were $K_m = 75.5 \pm 14.3 \mu\text{M}$ and $V_{max} = 25.4 \pm 2.1 \text{ pmol/min per mg protein}$. The uptake of Ala-Lys-AMCA by mouse splenic macrophages was not inhibited by histidine, but was significantly inhibited by Glycyl-Sarcosine (GlySar) and carnosine ($P < 0.01$), and by bacterial peptidomimetics γ -iE-DAP and MDP, ligands of nucleotide-binding oligomerization domain (NOD)-containing proteins. Carnosine and GlySar, but not histidine, attenuated the inflammatory response induced by γ -iE-DAP and MDP in mouse splenic macrophages. Functional expression of POTs was also demonstrated in THP-1 cells and dipeptides reduced the immune response induced by γ -iE-DAP. In conclusion, our findings are novel by providing important information on the molecular and functional expression of POTs in spleen. Moreover, it appears that the PepT2-mediated uptake of γ -iE-DAP and MDP in macrophages further contributes to the innate immune response.

Keywords

POTs; PepT2; Spleen; Expression; Function; NOD

1. Introduction

Proton-coupled oligopeptide transporters (POTs) are electrogenic membrane transporters that mediate the cellular uptake of di/tripeptides and many peptidomimetics utilizing an inwardly-directed proton gradient and negative membrane potential.^{1–3} In mammals, the

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POT family has four members which are encoded by SLC15 genes, including PepT1 (SLC15A1), PepT2 (SLC15A2), PhT1 (SLC15A4) and PhT2 (SLC15A3). All these members are predicted to contain 12 transmembrane domains, with the N- and C-termini in the cytosol. PepT1 and PepT2 have high amino acid identity, around 50%, and have broad substrate specificity in which they mediate the cellular uptake of small peptides and peptide-like drugs such as cefadroxil, valacyclovir and others. PhT1 and PhT2, in contrast, mediate the transport of histidine in addition to di/tripeptides. With respect to tissue distribution, PepT1 is abundantly expressed in the small intestine⁴ and PepT2 in the kidney,⁵ brain^{6,7} and lung.⁸ PhT1 is highly expressed in the brain and eye⁹ whereas PhT2 is found mainly in the lung, spleen and thymus.¹⁰

PepT1 has been shown to mediate the transport of bacterially produced peptidomimetics, such as fMet-Leu-Phe, Tri-DAP and MDP, in intestinal epithelial cells and Caco-2/BBE cell cultures.^{11,12} Tri-DAP and MDP can be recognized by nucleotide-binding oligomerization domain-containing 1 (NOD1) and 2 (NOD2) which are intracellular NOD-like receptors (NLRs).¹³ Intracellular accumulation of γ -iE-DAP (a constituent form of Tri-DAP) or MDP recognized by NLRs can activate NF- κ B, and then induce the production of proinflammatory cytokines, such as IL-6 and TNF α , which are part of NOD signaling pathways.¹³ However, whether all POT family members are relevant in the transport of γ -iE-DAP or MDP has not yet reached consensus. In fact, Marina-García *et al.*¹⁴ carried out studies regarding the internalization of MDP in bone-derived mouse macrophages and ruled out the involvement of PepT1.

The spleen is a unique lymphoid organ that combines innate and adaptive immune system responses, and contains B and T cells, macrophages, dendritic cells, and others.¹⁵ Anatomical features of the spleen, combined with its highly adaptive macrophages, make this organ a vital site of early exposure to bacteria. Therefore, as a major organ in the human body for antibacterial immune reactivity, it would seem important to characterize the role and relevance of POT members in splenic tissue. To our knowledge, studies on the expression of POTs in spleen are sparse and limited to mRNA detection.^{16,17} On the other hand, studies on POT protein expression and physiological function in spleen are completely lacking. With this in mind, the present study was designed: 1) to determine the mRNA and protein expression levels of PepT1, PepT2, PhT1 and PhT2 in spleen; 2) to characterize the transport properties of Ala-Lys-AMCA (a fluorescent dipeptide conjugate) and GlySar (a prototypical dipeptide) in mouse spleen macrophages; 3) to evaluate the effect of potential transport inhibitors on MDP- and γ -iE-DAP-induced inflammatory responses in macrophages; and 4) to delineate the relationship between POT mRNA expression, dipeptide uptake, and inflammatory response to bacterial products in THP-1 cells.

2. Materials and methods

2.1 Materials

Primers used in the qRT-PCR studies were synthesized by Sangon Corporation (Shanghai, China). Specific polyclonal antibodies for PepT1, PepT2, PhT1 and PhT2 were provided by Prof. Smith, University of Michigan (Ann Arbor, MI). The rabbit anti-mouse PepT1 antisera were raised against the COOH-terminal region (amino acids 690–709)⁴ as were the rabbit anti-mouse PepT2 antisera (amino acids 721–740).¹⁸ The rabbit anti-rat PhT1 antisera were raised against the COOH-terminal region, ARTDGGTASTRT, amino acids 561–572 (Lampire Biological Laboratories, Inc., Pipersville, PA) and the rabbit anti-rat PhT2 antisera were raised against the COOH-terminal region, PDSQNSTSRVRG, amino acids 571–582 (Lampire Biological Laboratories). The monoclonal antibody for glyceraldehyde phosphate dehydrogenase (GAPDH) was purchased from KangChen Corporation (Shanghai, China). Secondary anti-rabbit and anti-mouse antibodies were purchased from Multisciences

Biotech Corporation. The fluorescence dipeptide Ala-Lys-AMCA was purchased from Bio Trend Chemicals (Germany). Phorbol-12-myristate-13-acetate (PMA) and other chemicals were purchased from Sigma Aldrich (USA).

2.2 Animal and human spleen samples

Adult ICR mice weighing 20 ± 2 g were provided by the Experimental Animal Center of The Zhejiang Academy of Medicinal Sciences. All procedures were approved by the institutional animal care and use committee of Zhejiang University. Animals were allowed free access to standard laboratory chow and tap water. Human spleen samples from patients who underwent splenectomy were provided by the First Affiliated Hospital, College of Medicine, Zhejiang University, and were delivered to our laboratory in RNA stabilization (Qiagen, Germany) for mRNA quantitation study or in ice-cold HBSS buffer for cell isolation. All human spleen samples were obtained from surplus remnants with informed consent of the patients and protocols were approved by the institutional review board.

2.3 Cell culture

Cells from the human acute monocytic leukemia cell line (THP-1) were kindly provided by Prof. Jie Yan, School of Medicine, Zhejiang University. The THP-1 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, Invitrogen) at 37°C with 5% CO₂. Cells were differentiated into macrophages by PMA (100ng/mL) treatment before study for 48 hr.

2.4 Quantitative real-time PCR (qRT-PCR) analysis

The mRNA expression of POTs, IL-6 and TNF α was determined by qRT-PCR as described previously.¹⁹ Briefly, mouse spleen was obtained after immediate sacrifice of the animal and the total RNA was isolated with RNeasy mini kit (Qiagen, Germany). The cDNA was synthesized using 500 ng RNA and Prime Script RT reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. Expression of target genes was determined using 2 μ L cDNA with SYBR Premix Ex Taq (TaKaRa, Japan) and primers described previously¹⁹ or in Table 1 on the StepOne plus Real-Time PCR system (Applied Biosystems, Life Technologies). For each sample, cDNA was measured three times, and the averaged cycle threshold (Ct) was used. Relative mRNA levels of PepT1, PepT2, PhT1 and PhT2 were calculated using the delta Ct method and described as $2^{-\Delta Ct}$, $\Delta Ct = \text{Avg. CT (Target gene)} - \text{Avg. CT (GAPDH)}$. To determine the fold-change, results were calculated using the $\Delta\Delta Ct$ (comparative threshold cycle) method and described as $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (\text{Avg. CT (Target gene)} - \text{Avg. CT (GAPDH)})_{\text{sample}} - (\text{Avg. CT (Target gene)} - \text{Avg. CT (GAPDH)})_{\text{control}}$

2.5 Western blot analysis

Protein level expression of POTs in mouse spleen tissues and cells was evaluated by Western blot analysis as described previously.¹⁹ In general, protein samples of mouse spleen tissue or cells were isolated by RIPA buffer and protein concentration was determined with BCA Protein Assay Kit (Beyotime, China). Protein samples were stored at -80°C until use and without repeated freeze-thaw cycles. Equal amounts of total protein sample (about 30 μ g) were separated by 7.5% SDS-PAGE. Protein in the gel was electroblotted onto Polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, USA) at 4°C and blocked with 7.5% not fat milk overnight in Tris-buffered saline/Tween 20 at 4°C. The membrane was then incubated with polyclonal antibody (1:2000 for PepT1, 1:5000 for PepT2, and 1:1000 for PhT1 and PhT2 in the blocking buffer) or monoclonal antibody (GAPDH, 1:10000 in the blocking buffer) at room temperature for 1 hr with gently shaking. After washing with TBST for 5 min, 5 times, the membrane was incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000 in the blocking

buffer) for 1 hr and then washed 5 times again. PepT1, PepT2, PhT1, PhT2 and GAPDH proteins were detected on X-ray film by an enhanced chemiluminescence system (ECL, LumiGLO, USA).

2.6 Isolation of mouse spleen macrophages

Spleen macrophages were isolated from mouse according to the method of Xu *et al.*²⁰ In brief, the mice were immersed in ethanol for 3 min immediately after being sacrificed by ether inhalation. Once the abdominal cavity was opened, the spleen was isolated and transferred to PBS. Splenic cells were collected by gently pressing of a frosted glass pillar in RPMI1640 medium (Gibco)/10% fetal calf serum (FCS) and filtering through a stainless steel screen (74 μ m mesh). The red blood cells were removed by red blood cell lysate treatment and then centrifuged. After rinsing with PBS for 3 times by centrifugation at 200 g for 5 min, the cell pellet was suspended with RPMI1640 medium/10% FCS and transferred to cell plates. Macrophages of spleen were separated after 2 hr cultures at 37°C by gently removing the nonadherent cells.

2.7 Uptake studies

Macrophages isolated from mouse spleen or THP-1 cells (differentiated by PMA) were washed twice with MES buffer (g in 1 liter ddH₂O: 8.00 NaCl, 0.40 KCl, 0.14 CaCl₂, 0.20 MgSO₄·7H₂O, 0.06 Na₂HPO₄·12H₂O, 0.06 KH₂PO₄, 0.35 NaHCO₃, 1.00 glucose, 1.95 MES, pH 6.0, 37°C). Uptake was initiated by adding a specific concentration of Ala-Lys-AMCA or GlySar in MES buffer (pH 6.0), and then terminated at specific times by removing the buffer and rapidly washing 3x with ice-cold MES buffer. Inhibition studies were conducted by preincubating the cells with competitive compounds for 30 min. For pH-dependent analyses, uptake was performed in buffers at different pH (e.g., 5.4, 6.0, 7.4 and 8.4) and in buffers at pH 6.0 for other experiments which are terminated at 120min. The cells were then disrupted in 0.1% SDS/0.05% Triton X-100 and the concentration of Ala-Lys-AMCA in cell lysate was determined by SPECTRA max M2 spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California, United States) with excitation at 355 nm and emission at 435nm, while GlySar was assayed by HILIC-MS/MS. The method was validated for selectivity, linearity, accuracy, precision, extraction recovery and stability. For each experiment, a calibration curve was constructed. The presence of competitive compounds was demonstrated not to affect the fluorescence of Ala-Lys-AMCA. Protein content was determined with BCA kit (Beyotime, China), and fluorescence data were calculated as pmol/min per mg protein.

2.8 HILIC-MS/MS conditions for GlySar analysis

The concentration of GlySar was quantified by a UPLC ACQUITY instrument (Waters, USA) equipped with a TQD detector. LC-MS analysis was performed on an Atlantis HILIC Silica (2.1 $\times$ 50 mm, 3.0 μ m, Waters Corp. Milford, MA, USA) under positive ion mode, and isoniazid was used as internal standard. A gradient elution program was conducted with mobile phase A (10mM ammonium acetate) and mobile phase B (acetonitrile) as follows: 0 (20% A), 0.5min (20% A), 0.8min 40% A, 1.8min (40% A), 1.9min (20% A) and finished at 2 min. The flow rate of mobile phase was 0.35mL/min, injection volume was 7 μ L and column temperature was controlled at was 25°C. Capillary: 4.4 kV, Cone: 25 V, Source Temp: 140°C, Desolvation temperature: 400°C were set for MS analysis. MRM was employed for quantitation based on the transitions of protonated molecular ions of GlySar at m/z 146.82-90.12 and isoniazid at m/z 137.8-120.91.

2.9 Statistical analysis

Graphpad Prism version 5.01 for windows was used for statistical analysis of the data. All data are given as mean $\pm$ SD. Unpaired two-tailed Students's *t* test was used to compare difference between groups. A difference was considered statistically significant when the *P* value was less than 0.05.

3. Results

3.1 mRNA and protein expression of POTs in spleen

To clarify the expression of POTs in spleen, the total RNA isolated from mouse and human spleen was transcribed to cDNA, quantitated by qRT-PCR and then normalized by GAPDH (Figure 1A). Protein expression of PepT2, PhT1 and PhT2 in mouse spleen was demonstrated by specific antibodies (Figure 1B). The results showed that among these four POT members, PepT1 was not detected at either the transcript or protein level, while PhT1/2 exhibited higher mRNA expression than PepT2 in both the mouse and human spleen.

3.2 mRNA and protein expression of POTs in lymphocytes and macrophages

To elucidate the cell type of POT expression, macrophages and lymphocytes of spleen were isolated, and mRNA and protein levels were evaluated by qRT-PCR and Western blot, respectively. The results revealed that significantly higher mRNA expression of PepT2 and PhT2 was found in macrophages compared with lymphocytes of both mouse and human (Figures 2A and 2B). Greater expression of PepT2 protein was observed in mouse macrophages than lymphocytes, as determined by Western blot (Figure 2C). These results demonstrated that PepT2 and PhT1/2 were expressed in spleen, especially in splenic macrophages.

3.3 Uptake of Ala-Lys-AMCA in macrophages of mouse spleen

To study the functional activity of POTs in macrophages of mouse spleen and determine their roles in uptake of POT substrates, the specific fluorescence substrate Ala-Lys-AMCA was studied in the absence and presence of other POT substrates/inhibitors. The uptake of Ala-Lys-AMCA was time dependent (Figure 3A), and also pH dependent (Figure 3B). Moreover, the uptake was significantly diminished by competing compounds such as carnosine, GlySar and cephalixin (5 mM) but not histidine (5 mM), which is a substrate of PhT1/2 (Figure 4A). The uptake of Ala-Lys-AMCA was concentration dependent, saturable and followed Michaelis-Menten kinetics ($V_{\max} = 25.4 \pm 2.1$ pmol/min per mg protein, $K_m = 75.5 \pm 14.3 \mu\text{M}$) (Figure 3C). In addition, uptake of GlySar was decreased by carnosine and cephalixin (5 mM) but not histidine (Figure 4B). These observations demonstrated that the proton-coupled peptide transporter PepT2 but not PhT1/2 participated in the transport of fluorescence dipeptide Ala-Lys-AMCA and GlySar in mouse splenic macrophages.

3.4 Inhibitory effect of γ -iE-DAP and MDP on uptake of Ala-Lys-AMCA mediated by PepT2

To determine whether PepT2 might mediate the intracellular uptake of γ -iE-DAP and MDP in splenic macrophages, the inhibitory effect of γ -iE-DAP (5 mM) and MDP (5 mM) on Ala-Lys-AMCA uptake was evaluated. The results revealed that γ -iE-DAP ($P < 0.001$) and MDP ($P < 0.05$) significantly inhibited the macrophage uptake of Ala-Lys-AMCA. Moreover, the inhibitory effect of γ -iE-DAP was stronger than that of carnosine at the same concentration (5 mM) (Figure 5).

3.5 PepT2 contributes to IL-6 and TNF α expression induced by MDP and γ -iE-DAP

To further evaluate the role of PepT2 in intracellular uptake of γ -iE-DAP and MDP, and role in immune activation, the effect of potential inhibitors on gene expression of

inflammatory cytokines induced by intracellular accumulation of γ -iE-DAP and MDP was determined. Macrophages of mouse spleen were stimulated by γ -iE-DAP (10 μ g/mL) or MDP (10 μ g/mL) in the absence and presence of carnosine, GlySar and histidine, and the mRNA levels of IL-6 and TNF α were determined. Figure 6A showed that IL-6 and TNF α mRNA expression induced by γ -iE-DAP (10 μ g/mL) was substantially decreased by the presence of carnosine (5 mM, $P<0.001$) or GlySar (5 mM, $P<0.01$) but not by histidine (5 mM). Carnosine or GlySar also attenuated the induction of IL-6 and TNF α expression by MDP (10 μ g/mL), but to a lesser degree. In contrast, immune activation by LPS (10 ng/mL) stimulation was not affected by carnosine (5 mM) (Figure 6B). Our observations suggested that PepT2 played an important role in γ -iE-DAP and MDP stimulated NOD signaling pathways in mouse splenic macrophages.

3.6 POT mRNA expression, dipeptide uptake, and immune activation of cytokines by γ -iE-DAP in THP-1 macrophages

To further evaluate the role of POTs in the intracellular uptake of NOD ligands, the expression of POT transcripts was determined in THP-1 macrophages. Figure 7A shows the mRNA expression of PepT2, PhT1 and PhT2, but not PepT1 in THP-1 cells before or after PMA treatment. Transport activity of POTs in THP-1 cells was evaluated by uptake of Ala-Lys-AMCA and GlySar, which were both significantly reduced by competing compounds (5 mM) such as carnosine and cephalixin but not histidine (Figure 7B and 7C, respectively). When the NOD1 ligand γ -iE-DAP was applied, THP-1 macrophages were immunity activated as indicated by the significant increase in mRNA expression of IL-6 and TNF α . On the other hand, the NOD2 ligand MDP had no effect (Figure 8A). In a manner similar to that in splenic macrophages, carnosine decreased the induction of IL-6 and TNF α in THP-1 cells by γ -iE-DAP but was without effect during LPS stimulation (Figure 8B and 8C, respectively). Histidine also had an inhibitory effect on the immune activation by γ -iE-DAP in THP-1 cells, the mechanism of which is unclear at present.

4. Discussion

Using a combination of cellular, molecular and biochemical approaches, several new findings were elucidated regarding the role and relevance of POT family members in spleen. In particular, we found that: 1) PepT2, PhT1 and PhT2 transcripts were observed in mouse and human spleen along with protein expression of these same transporters in mouse spleen; no PepT1 mRNA or protein was detected, 2) PepT2, PhT1 and PhT2 transcripts were observed in splenic lymphocytes and macrophages from mouse and human spleen; PepT2 protein expression was much greater in mouse macrophages than in lymphocytes, 3) transport characteristics of Ala-Lys-AMCA and GlySar were consistent with PepT2 functional activity in mouse splenic macrophages, 4) MDP and γ -iE-DAP significantly inhibited the uptake of Ala-Lys-AMCA in mouse splenic macrophages suggesting these peptides were also substrates of PepT2, 5) MDP and γ -iE-DAP induction of pro-inflammatory cytokines in mouse splenic macrophages were inhibited by PepT2 but not PhT1/2 substrates, 6) PepT2, PhT1 and PhT2 transcripts were observed in THP-1 cells along with dipeptide transport consistent with the functional activity of PepT2 but not PhT1/2, and 7) pro-inflammatory cytokines were induced by γ -iE-DAP in THP-1 cells. Collectively, these results demonstrate that PepT2 has a significant role in the spleen, especially macrophages, in mediating the innate immune response to bacterially-produced chemotactic peptidomimetics.

Transport activity of POTs in mouse macrophages was studied using the fluorescence dipeptide substrate Ala-Lys-AMCA.²¹ The stability of Ala-Lys-AMCA was verified (data not shown), and its intracellular uptake in macrophages of mouse spleen was saturable and of high affinity ($K_m=75.5$ μ M). The uptake of Ala-Lys-AMCA in macrophages was also

sensitive to pH and could be inhibited by specific substrates of POTs such as carnosine, GlySar and cephalixin but not by histidine, which is a substrate of PhT1/2. Thus, the above observations indicated the involvement of PepT2 but not PhT1/2 in the transport of Ala-Lys-AMCA. Since the uptake of GlySar was also decreased by carnosine and cephalixin but not histidine, these observations may be explained by the intracellular localization of PhT1/2 in lysosomes, which has been suggested by others using immunohistochemical staining methods.^{10,22} Furthermore, the expression of PhT1 and PhT2 was detected in human macrophages²³ and, as a result, the presence of these peptide/histidine transporters in mouse spleen, especially macrophages, may be involved in the transport of di/tri-peptides out of lysosomes after the digested proteins are hydrolyzed into small peptide fragments.

Our results showed that NOD ligands (i.e., MDP and γ -iE-DAP) significantly inhibited the intracellular uptake of Ala-Lys-AMCA, which is a specific substrate of POTs. It is known that NOD1 and NOD2 are members of the NOD protein family, which is one group of intracellular pattern recognition receptors (PRRs) that mediates the recognition of specific bacterial components and triggers signaling cascades, thereby, activating the host immune response.²⁴ Ligands recognized by NOD proteins need to be transported into the cell cytoplasm in order to access NOD proteins localized in the intracellular milieu.¹³ But it remains unclear just how these ligands get into the cell and whether POT transporters contribute to cell entry.^{11,12,14} MDP is the minimal bioactive peptidoglycan motif common to all bacteria, which can be recognized by NOD2 and has been previously reported as a substrate of PepT1 in Caco-2/BBE cells ($K_m=4.3$ mM).²⁵ However, whether PepT2 is involved in the uptake of MDP is still controversial. Another NOD ligand is γ -iE-DAP, which is found in PGN of all Gram-negative and certain Gram-positive bacteria and recognized by NOD1. Tri-DAP, comprised of γ -iE-DAP and an L-Ala residue, is also recognized by NOD1. Involvement of PepT1 in the intracellular uptake of Tri-DAP in Caco-2/BBE cells and PepT2 in the uptake of γ -iE-DAP in lung epithelium has been previously suggested.^{12,26} However, Marina-García *et al.*¹⁴ ruled out PepT1 involvement in the internalization of MDP in bone-derived mouse macrophages and, instead, demonstrated the importance of clathrin- and dynamin-dependent endocytic pathways.

The present study showed transport activity of PepT2 in spleen macrophages that highly expressed NOD1 and NOD2 proteins.²⁴ Intracellular accumulation of MDP and γ -iE-DAP can induce NF- κ B activation, followed by the secretion of pro-inflammatory cytokines. Our results demonstrated a significant inhibition by PepT2 substrates, including carnosine and GlySar but not histidine (PhT1/2 substrate), on the induction of inflammatory cytokine mRNA expression by MDP and γ -iE-DAP. These results indicated that PepT2 is involved in the intracellular uptake of MDP and γ -iE-DAP in macrophages of spleen. Interestingly, γ -iE-DAP decreased the uptake of Ala-Lys-AMCA to a lower level than MDP, at equivalent concentrations, suggesting a higher affinity of PepT2 for γ -iE-DAP. Thus, it was not surprising to find that a much greater inhibitory effect by γ -iE-DAP than MDP on the induction of pro-inflammatory cytokine expression. Moreover, the expression and function of POTs in THP-1 macrophages were similar to that in spleen macrophages except that MDP did not induce an immune response, a result that might be due to the lower expression of NOD2 protein in THP-1 macrophages.

In conclusion, the present study provides the first documented evidence of POT expression in spleen and, in particular, the expression of PepT2 and PhT1/2 in macrophages of spleen. Moreover, the study demonstrated that PepT2 but not PhT1/2 mediated the transport of di/tripeptides and peptidomimetics in macrophages, and played an important role in the innate immune response induced by bacterial peptidomimetics γ -iE-DAP and MDP.

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Abbreviations

POTs	proton-coupled oligopeptide transporters
qRT-PCR	quantitative real-time PCR
Ala-Lys-AMCA	β -alanyl-L-lysyl-Ne-7-amino-4-methyl-coumarin-3-acetic acid
NOD	nucleotide-binding oligomerization domain
γ-iE-DAP	γ -D-glutamyl- <i>meso</i> -diaminopimelic acid
MDP	muramyl dipeptide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
IL-6	interleukin-6
TNFα	tumor necrosis factor alpha
fMet-Leu-Phe	N-formyl-methionyl-leucyl-phenylalanine
Tri-DAP	L-alanyl- γ -D-glutamyl- <i>meso</i> -diaminopimelic acid

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Figure 1. mRNA and protein expression of POTs in spleen. A: mRNA expression of PepT1, PepT2, PhT1 and PhT2 in the mouse (■) and human (□) spleen. B: protein expression of PepT1, PepT2, and PhT2 in mouse spleen. Immunoreactive bands of PepT2, PhT1 and PhT2 but not PepT1 were detected by specific antisera. Jejunum, lung and brain were used as positive controls for PepT1, PepT2 and PhT2, and PhT1, respectively. Data are expressed as mean $\pm$ SD, n=3.

Figure 2.

Expression of POTs in macrophages and lymphocytes of spleen. A and B: mRNA expression of POTs in lymphocytes (BT) and macrophages (Mφ) isolated from the spleen of mouse and human, respectively. C: protein expression of PepT2 in lymphocytes (BT) and macrophages (Mφ) isolated from mouse spleen. Data are expressed as mean $\pm$ SD, n=3. Differences between lymphocytes and macrophages are denoted as: **P<0.01 and ***P<0.001.

Figure 3.

Uptake of Ala-Lys-AMCA in macrophages isolated from mouse spleen. (A) Time-dependent uptake of Ala-Lys-AMCA (25 μ M) in mouse spleen macrophages. (B) pH-dependent uptake of Ala-Lys-AMCA (25 μ M) in mouse spleen macrophages. (C) Kinetics of Ala-Lys-AMCA uptake in mouse spleen macrophages. The line represents the best fit of data to a single Michaelis–Menten term ($R^2=0.954$).

Figure 4.

Uptake of Ala-Lys-AMCA in macrophages isolated from mouse spleen (A) Effect of histidine, dipeptides and cephalixin on the uptake of Ala-Lys-AMCA in macrophages isolated from mouse spleen. Control, control group was incubated without inhibitors (5mM), +Car: carnosine, +Cef: cephalixin, +His: histidine. (B) Effect of histidine, dipeptides and cephalixin on the uptake of GlySar (100 μ M) in macrophages isolated from mouse spleen. Data are expressed as mean $\pm$ SD, n=3. Compared with the control group: **P<0.01 and *** P<0.001.

Figure 5. Inhibitory effect of MDP (5 mM), γ -iE-DAP (5 mM) and carnosine (Car, 5 mM, as positive control) on the uptake of Ala-Lys-AMCA (50 μ M) in macrophages isolated from mouse spleen. Data are expressed as mean $\pm$ SD, n=3. Compared with the control group: *P<0.05, **P<0.01 and ***P<0.001.

Figure 6. Effect of peptides and histidine on the inflammatory response induced by MDP and γ -iE-DAP. A: Carnosine (Car) and GlySar but not histidine (His) inhibited the induction of IL-6 and TNF α mRNA expression by MDP (10 μ g/mL) and γ -iE-DAP (10 μ g/mL) in macrophages isolated from mouse spleen. B: Carnosine had no effect on the induction of IL-6 and TNF α mRNA expression by LPS (10 ng/mL) in mouse spleen macrophages. UNT, untreated cell group. Data are expressed as mean $\pm$ SD, n=3. Compared with indicated group: *P<0.05, **P<0.01 and ***P<0.001.

Figure 7.

Expression and transport activity of POTs in THP-1 cells. A: mRNA expression of POTs in THP-1 cells before (■) and after treatment of PMA (100 μM, 2 hr) treatment (□). B: Effect of carnosine, cephalixin and histidine on the uptake of Ala-Lys-AMCA in THP-1 cells. C: Effect of carnosine, cephalixin and histidine on the uptake of GlySar (100 μM) in THP-1 cells. Control, control incubated without inhibitors (5 mM), +Car: carnosine, +Cef: cephalixin, +His: histidine. Data are expressed as mean ± SD, n=3. Compared with control group **P<0.01 and ***P<0.001.

Figure 8.

Effect of peptides and histidine on the inflammatory response induced by MDP and γ -iE-DAP in THP-1 cells. A: Expression of IL-6 and TNFα was significantly increased by γ -iE-DAP (10 μ g/mL) but not by MDP (10 μ g/mL). B: Carnosine (Car, 5 mM) and histidine (His, 5 mM) significantly decreased IL-6 and TNFα mRNA expression induced by γ -iE-DAP (10 μ g/mL). Compared with the +Car and +His groups: * $P < 0.05$ and ** $P < 0.01$. C: Carnosine (5 mM) and histidine (5 mM) had no effect on IL-6 and TNFα mRNA expression induced by LPS (10 ng/mL). Data are expressed as mean $\pm$ SD, $n = 3$.

Table 1

Primers of IL-6 and TNFα of mouse

Target gene	Forward primer	Reverse primer	Product (bp)
IL-6	TCCCTCTCTGCAAGAGACTTCCATCC	GGGAAGGCCCGTGGTTGTCACC	69
TNFα	CCACGTCGTAGCAAAACCACCAAG	ATCCATGCCCGTTGGCCAGGAG	84