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World J Gastroenterol 2005;11(45):7091-7096
World Journal of Gastroenterology ISSN 1007-9327

• *BASIC RESEARCH* •

Effects of arginine supplementation on splenocyte cytokine mRNA expression in rats with gut-derived sepsis

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Supported by a research grant from the National Science Council, Taipei, Taiwan, China. No. NSC 92-2320-B-038-029
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Received: 2005-02-15 Accepted: 2005-06-09

Abstract

AIM: To investigate the effects of arginine (Arg)-enriched diets before sepsis and/or Arg-containing total parenteral nutrition (TPN) after sepsis or both on cytokine mRNA expression levels in splenocytes of rats with gut-derived sepsis.

METHODS: Rats were assigned to four experimental groups. Groups 1 and 2 were fed with a semipurified diet, while groups 3 and 4 had part of the casein replaced by Arg which provided 2% of the total calories. After the rats were fed with these diets for 10 d, sepsis was induced by cecal ligation and puncture (CLP), at the same time an internal jugular vein was cannulated. All rats were maintained on TPN for 3 d. Groups 1 and 3 were infused with conventional TPN, while groups 2 and 4 were supplemented with Arg which provided 2% of the total calories in the TPN solution. All rats were killed 3 d after CLP to examine their splenocyte subpopulation distribution and cytokine expression levels.

RESULTS: Plasma interleukin (IL)-2, IL-4, tumor necrosis factor-α (TNF-α) and interferon (IFN-γ) were not detectable 3 d after CLP. There were no differences in the distributions of CD45Ra+, CD3+, CD4+, and CD8+ cells in whole blood and splenocytes among the four groups. The splenocyte IL-2 mRNA expression in the Arg-supplemented groups was significantly higher than that in group 1. IL-4 mRNA expression in groups 3 and 4 was significantly higher than that in groups 1 and 2. The mRNA expression of IL-10 and IFN-γ was significantly higher in group 4 than in the other three groups. There was no difference in TNF-α mRNA expression among the four groups.

CONCLUSION: The influence of Arg on the whole blood and splenic lymphocyte subpopulation distribution is not obvious. However, Arg administration, especially before and after CLP, significantly enhances the mRNA expression levels of Th1 and Th2 cytokines in the spleen of rats with gut-derived sepsis.

Key words: Arginine; Sepsis; Cytokine mRNA expression

Shang HF, Hsu CS, Yeh CL, Pai MH, Yeh SL. Effects of arginine supplementation on splenocyte cytokine mRNA expression in rats with gut-derived sepsis. *World J Gastroenterol* 2005;11(45): 7091-7096
http://www.wjgnet.com/1007-9327/11/7091.asp

INTRODUCTION

Sepsis is a major cause of death in critically ill patients. Under a condition of sepsis, different endogenous mediators are oversecreted, which may result in an imbalance of the metabolic pathway[1,2]. These metabolic abnormalities that occur during sepsis mainly result from the secretion of cytokines[3]. Cytokines belong to a superfamily of low molecular weight glycoproteins that act as important regulatory proteins, and play a key role in inflammatory responses either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in numerous cell types[4-6]. Cytokines have beneficial properties in initiating immune responses and maintaining homeostasis in critically ill patients. However, exaggerated or prolonged secretion of cytokines may be detrimental for the host[4-6]. Modulation of cytokines is very important for patients under metabolic stress.

Arginine (Arg) is a semi-essential amino acid that possesses numerous useful physiologic properties[7]. Accumulating experimental and clinical evidence has suggested that Arg reduces protein catabolism and enhances immune function in severely injured animal models and critically ill patients[8–12]. Although a meta-analysis by Heyland *et al*[13]. suggested that immune-enhancing diets rich in Arg may be harmful in unstable critically ill patients, immunonutrition with Arg positively modulates postsurgical immunosuppressive and inflammatory responses[14]. Arg is considered to be an

essential amino acid for patients with catabolic diseases[7,15]. Previous reports by our laboratory showed that preventive use of a Arg-supplemented enteral diet or Arg administered both before and after cecal ligation and puncture (CLP) enhances peritoneal macrophage phagocytic activity and bacterial clearance[16]. Also, total lymphocyte yields in Peyer's patches and intestinal immunoglobin (Ig) A secretion are improved after Arg administration both before and after CLP[17]. In order to understand the protective mechanisms of Arg, the cytokine mRNA expression and protein secretion in septic hosts need to be investigated. However, cytokine protein detection is usually limited, due to lack of sensitive commercial ELISA kits and the short half-life of most cytokines in plasma. Wu *et al*[18]. have investigated the relation between cytokine mRNA expression and organ damage after sepsis. Cui *et al*[19]. reported that an Arg-supplemented diet decreases the expression of inflammatory cytokines in burn rats. As we know, there is no study investigating the effects of Arg on Th1 and Th2 cytokine mRNA expressions of splenocytes in septic rats. Therefore, the aim of this investigation was to study the effect of Arg-supplemented diets before sepsis and Arg-enriched total parenteral nutrition (TPN) after sepsis or both on plasma cytokine levels and splenic cytokine mRNA expression in gut-derived sepsis. Also, the lymphocyte subpopulations of whole blood and splenocytes were analyzed to understand the effects of Arg on the phenotype of lymphocytes in a septic condition.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-230 g were housed in stainless steel cages in a temperature- and humidity-controlled room with a 12:12 h light-dark cycle. Animals were allowed free access to standard rat chow for 3 d prior to the experiment. All procedures conducted in this study were approved by the Taipei Medical University Animal Care Committee.

Surgical procedure and grouping

All rats were divided into four groups. Groups 1 and 2 were fed a semipurified diet. Rats in groups 3 and 4 were fed an identical diet except that part of the casein was replaced by Arg, which provided 2% of the total energy intake (Table 1). After the rats were fed the experimental diets for 10 d, sepsis was induced by CLP according to the method of Wichterman *et al*[20]. Briefly, the rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the abdomen was opened through a midline incision. The cecum was isolated, and a 3-0 silk ligature was placed around it, ligating the cecum just below the ileocecal valve. The cecum was then punctured twice with an 18-gauge needle and placed back into the abdomen. The abdominal wound was closed in two layers.

Immediately after CLP, all rats underwent placement of a catheter for TPN infusion. A silicon catheter (Dow Corning, Midland, MI, USA) was inserted into the right internal jugular vein. The distal end of the catheter was tunneled subcutaneously to the back of the neck, and exited through a coiled spring which was attached to a swivel, allowing free mobility of animals inside individual metabolic cages. TPN at 2 mL/h was administered on the first day. Full-strength TPN (56-64 mL/d, according to body weight) was given thereafter for 3 d. The infusion speed was controlled by a Terufusion pump (Model STC-503, Terumo, Tokyo, Japan). The TPN solution without fat was prepared in a laminar flow hood. Sterilized fat emulsions were added to the TPN solution daily just before use. The TPN solution was infused for the entire day at room temperature. No enteral nutrition was administered during the period of TPN. Groups 1 and 3 were infused with conventional TPN. Groups 2 and 4 were supplemented with Arg, which replaced 10% of the total amino acids, and provided 2% of the total energy of the TPN solution. TPN provided 280 kcal/kg body weight, and the energy (kcal):nitrogen (g) ratio was 119:1. The calorie density was almost 1 kcal/mL. The TPN solutions were isonitrogenous and identical in nutrient composition except for the difference in the amino acid content (Table 2). There were four groups in this study: group 1, no Arg supplementation before or after CLP (–/–); group 2, a semipurified diet given before and Arg-enriched TPN after CLP (–/+); group 3, an Arg-supplemented diet before and conventional TPN after CLP (+/–); and group 4, Arg supplementation both before and after CLP (+/+). There were 10 rats in each group.

Table 1 Composition of the semipurified diet (g/kg)

Ingredients	Arg-supplemented	Non-supplemented
Casein	180	220
Arg	20	–
Total nitrogen	35.2	35.2
Corn starch	677	657
Soybean oi[1]	44	44
Vitamin[1]	10	10
Salt mixture[2]	35	35
Methyl-cellulose	30	30
Choline chloride	1	1
DL-methionine	3	3

[1]The vitamin mix contained 0.6 mg/g thiamine hydrochloride, 06 mg/g riboflavin, 0.7 mg/g pyridoxine hydrochloride, 3 mg/g nicotinic acid, 1.6 mg/g calcium pantothenate, 0.02 mg/g D-biotin, 0.001 mg/g cyanocobalamin, 1.6 mg/g retinyl palmitate, 20 mg/g DL-a-tocopherol acetate, 0.25 mg/g cholecalciferol, and 0.005 mg/g menaquinone. [2]The salt mixture contained 500 mg/g calcium phosphate diabasic, 74 mg/g sodium chloride, 52 mg/g potassium sulfate, 220 mg/g potassium citrate monohydrate, 24 mg/g magnesium oxide, 3.5 mg/g manganese carbonate, 6 mg/g ferric citrate, 1.6 mg/g zinc carbonate, 0.3 mg/g cupric carbonate, 0.01 mg/g potassium iodate, 0.01 mg/g sodium selenite, and 0.55 mg/g chromium potassium sulfate.

Lymphocyte subpopulation distribution in whole blood and spleen

On d 3 after CLP, all surviving rats were weighed and anesthetized with pentobarbital. The survival rates in groups 1-4 were 10/17, 10/19, 10/18, 10/15,

Table 2 Composition of the TPN solution (mL/L)

	Arg-supplemented	Non-supplemented
50% Glucose	412	400
20% Fat emulsion	50	50
10% Moriamin-SN[1]	450	556
Arg (g)	5	–
Infuvita[2]	8	8
3% NaCl	35	35
7% KCl	10	10
8.7%K_3PO_4	10	10
Ca-gluconate	10	10
$MgSO_4$	4	4
$ZnSO_4$	2	2
Choline chloride (g)	1	1

[1]From Chinese Pharmaceuticals, Taipei, Taiwan. Contents per deciliter: 1 250 mg Leu, 560 mg Ile, 1 240 mg Lys acetate, 350 mg Met, 935 mg Phe, 650mg Thr, 130 mg Trp, 450 mg Val, 620 mg Ala, 790 mg Arg, 380 mg Asp, 100 mg Cys, 650 mg Glu, 600 mg His, 330 mg Pro, 220 mg Ser, 35 mg Tyr, and 1 570 mg aminoacetic acid (Gly). [2]From Yu-Liang Pharmaceuticals, Taoyuan, Taiwan. Contents per milliliter: 20 mg ascorbic acid, 660 IU vitamin A, 40 IU ergocalciferol, 0.6 mg thiamine HCl, 0.72 mg riboflavin, 8 mg niacinamide, 0.8 mg pyridoxine HCl, 3 mg D-panthenol, 2 mg dl-a-tocopheryl acetate, 12 μg biotin, 80 μg folic acid, and 1 μg cyanocobalamin.

respectively. There were no differences in the survival rates among the groups as described previously[16]. A middle abdominal incision was made, and the spleen was aseptically removed and teased on a stainless mesh immersed in chilled RPMI-1640 (Gibco, BRL, Grand Island, NY, USA). After filtration through a sterile nylon mesh, cell suspensions were washed thrice in HBSS and resuspended in RPMI-1640. Flow cytometry was used to determine the proportions of CD45Ra, CD3, CD4, and CD8 from the whole blood or splenocytes. One hundred microliters of heparinized whole blood or 10^5 splenocytes suspended in 100 μL HBSS were stained with fluorescein-conjugated mouse anti-rat CD3 (Serotec, Oxford, UK) and phycoerythrin-conjugated mouse anti-rat CD45Ra (Serotec) to distinguish T cells from B cells, respectively. Fluorescein-conjugated mouse anti-rat CD8 and phycoerythrin-conjugated mouse anti-rat CD4 (Serotec) were used to identify T helper cells and cytotoxic T lymphocyte cells, respectively. After staining for 15 min, 1 mL red blood cell (RBC) lysing buffer (Serotec) was added to lyse the RBCs and to fix the stained lymphocytes. Fluorescence data were collected on 5×10^4 viable cells and analyzed by flow cytometry (Coulter, Miami, FL, USA).

Plasma cytokine immunoassay

Plasma interleukin (IL)-2, IL-4, IL-10, interferon (IFN-γ), and tumor necrosis factor (TNF-α) concentrations were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Primers of cytokines

The primers of IL-2, IL-4, IL-10, TNF-α, and IFN-γ, and the housekeeping gene (18S rRNA) of rats were purchased from PE Applied Biosystems (Foster City, CA, USA).

Real-time reverse-transcription polymerase chain reaction (RT-PCR) method

Total RNA from rat spleen was isolated using the TRIzol reagent according to the manufacturer's protocol. RNA was reverse-transcribed using the Reverse Transcript system (Frementas, Vilnins, Lithuania). Briefly, 20 μL water containing 2 μg RNA was mixed with 1 μL oligo (dT) primer (0.5 μg/μL) and incubated for 5 min at 70 ℃. To the mixture, 22 μL $MgCl_2$ (25 mmol/L), 10 μL 10× RT-buffer, 20 μL dNTP (10 mmol/L), 2 μL RNase inhibitor, and 2.5 μL MultiScribe-RT (50 U/μL) were added and incubated at 25 ℃ for 10 min, then at 42 ℃ for 30 min. The reaction was stopped by heating the samples for 5 min to 95 ℃. cDNA was used for the real-time PCR assay performed with an ABI 7700 Sequence Detection System (PE Applied Biosystems) according to the supplied guidelines. The PCR reaction for IL-2, IL-4, IL-10, IFN-γ and TNF-α was carried out using a TaqMan PCR kit (PE Applied Biosystems).

Statistical analysis

Data were expressed as mean±SD. Differences among the groups were analyzed by ANOVA using Duncan's test. $P<0.05$ was considered statistically significant.

RESULTS

There were no differences in initial body weights and body weights after feeding the experimental diets for 10 d and after TPN administration for 3 d (data not shown). In this study, we were unable to detect plasma IL-2, IL-4, TNF-α and IFN-γ 3 d after CLP. Plasma IL-10 levels could be detected, but there was no difference in IL-10 levels among the groups (data not shown).

No differences in the distribution of CD45Ra+, CD3+, CD4+, and CD8+ cells in blood and splenocytes were observed among the four groups (Figures 1A and 1B). The expression of IL-2 mRNA in splenocytes was significantly higher in the Arg-supplemented groups (groups 2, 3, and 4) than in group 1 (–/–) (Figure 2A). The mRNA expressions of IFN-γ and IL-10 in group 4 (+/+) were significantly higher than in the other three groups (Figures 2B and 2D). IL-4 mRNA expression in groups 3 and 4 was higher than that in groups 1 and 2 (Figure 2C). There were no differences in splenocyte TNF-α mRNA expression among the four groups (Figure 2E).

DISCUSSION

In this study, 2% of total energy was supplied in the diet by Arg. This amount of Arg has been proved to enhance immune functions in rodents with gut-derived sepsis[21]. We administered TPN for 3 d after CLP, because the severity of infection and mortality are the highest at this time point in an established septic animal model[20]. In this

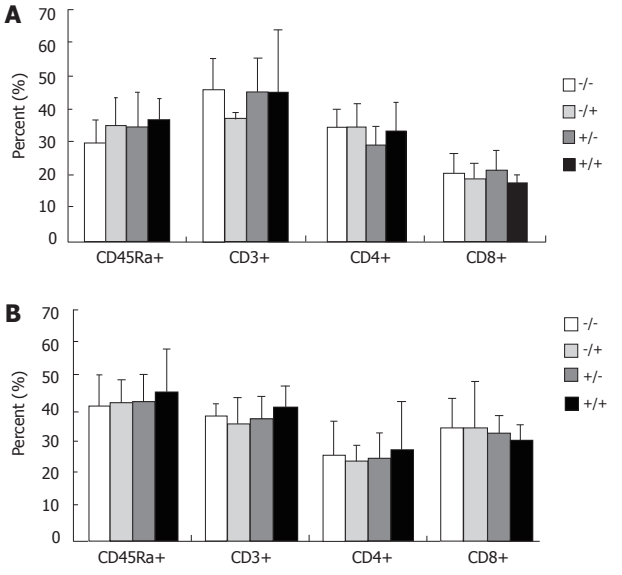

Figure 1 Distribution of CD45Ra+, CD3+, CD4+, and CD8+ lymphocytes in whole blood (**A**) and splenocytes (**B**) among the four groups 3 d after CLP (*n* = 10 in each group).

study, we did not include a sham-operation control (TPN without CLP) group; therefore, this study was not able to assess whether Arg supplementation could restore immune functions comparable to the control rats.

Circulating cytokine levels are usually used as a marker of injury or infection[3]. In order to understand the possible effects of Arg on the systemic cytokine expression in sepsis, IL-2, IL-4, IL-10, IFN-γ, and a pro-inflammatory cytokine TNF-α were measured. IL-2 and IFN-γ were produced by Th1 lymphocytes. Th1 cytokines enhance cell-mediated immunity. A predominant Th1 effect results in activation, growth, and differentiation of T and B lymphocytes as well as macrophages. Th2 cytokines, including IL-4 and IL-10, enhance humoral immunity. A predominant Th2 effect results in the activation of B lymphocytes, upregulation of antibody production, and mucosal immunity[22]. The effects of Th1 or Th2 lymphocytes were counter-regulatory.

In this study, we were unable to detect plasma IL-2, IL-4, TNF-α, and IFN-γ 3 d after CLP. Previous reports by our laboratory showed that IL-1β, IL-2, and IFN-γ were undetectable 24 h after CLP[23,24]. Cruickshank *et al*[25]. also reported that plasma IL-1, TNF, and IFN-γ are rarely detected in the plasma of injured patients. The inability to measure circulating cytokines may be due to a lack of highly sensitive assay kits and late assay times, or due to the fact that the quantities of cytokines do not enter the

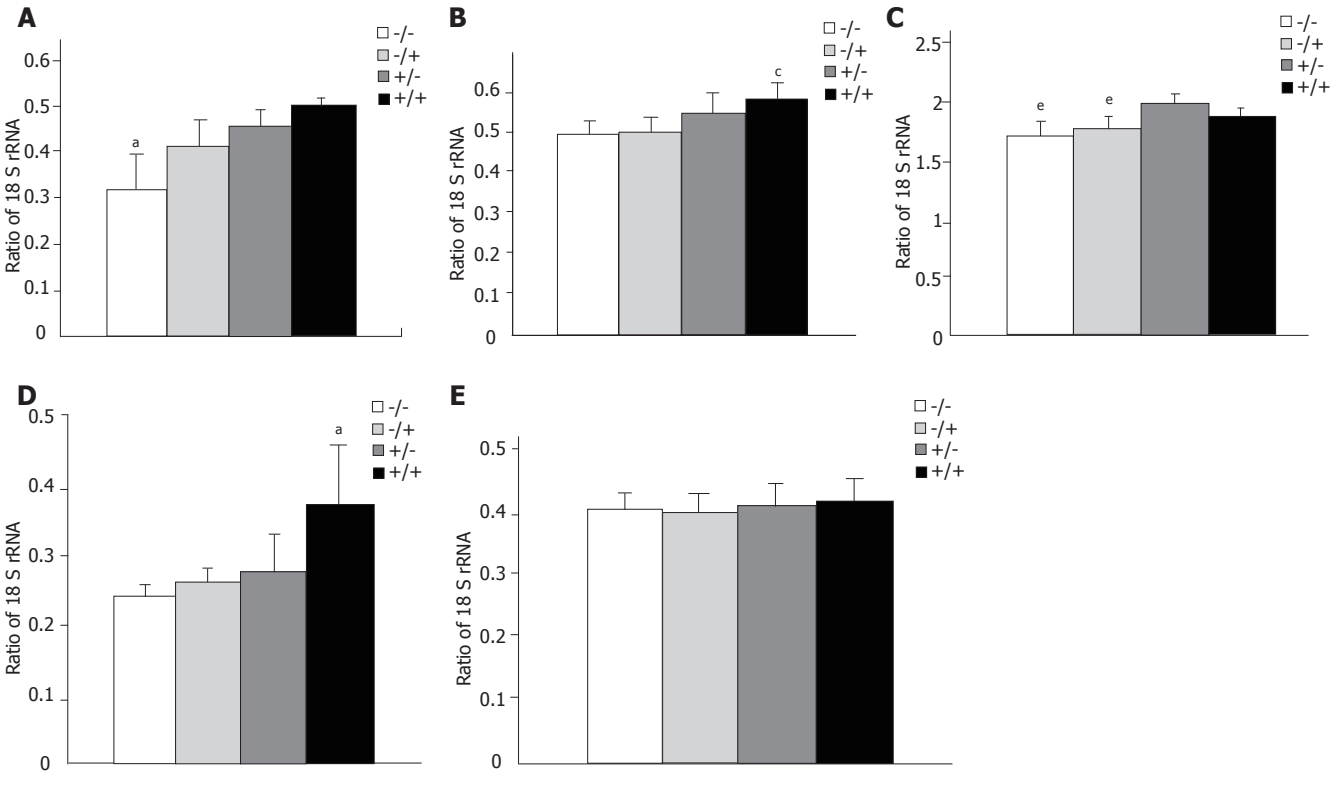

Figure 2 Expression of interleukin (IL)-2 (**A**), interferon (IFN-γ) (**B**), IL-4 (**C**), IL-10 (**D**) and tumor necrosis factor (TNF-**α**) (**E**) messenger RNA in the spleen as determined by real-time reverse-transcription polymerase chain reaction (*n* = 10 in each group). [a]*P*<0.05 *vs* other groups; [c]*P*<0.05 *vs* group 1 (–/–) and group 2 (–/+); [e]*P*<0.05 *vs* group 3 (+/–) and group 4 (+/+).

systemic circulation, even though the levels of cytokines in the tissues have increased[4]. Therefore, we used real-time RT-PCR to quantify splenic cytokine mRNA expression, this is a highly sensitive and reproducible tool to investigate the cytokine profiles at the mRNA level[26,27]. Our findings showed that Arg administration enhanced both Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cytokine mRNA expression, and Arg given both before and after had a synergistic effect on enhancing cytokine mRNA expression in a septic condition. These results are consistent with our previous report that Arg supplementation promotes intestinal IgA secretion and enhances peritoneal macrophage phagocytic activity in septic rats[16,17]. Although Th1 type cytokines promote the differentiation of cytotoxic T cells, these cytokines usually respond to viral infection and intracellular pathogens. The secretion of IFN-γ may activate macrophages, promote microbicidal activity and stimulate expression of the secretory component for intestinal sIgA by epithelial cells[28]. IL-4 and IL-10 are anti-inflammatory cytokines released by Th2 cells. Furthermore, IL-10 induces the synthesis and secretion of sIgA[22,29,30].

In this study, Arg seemed to have no effect on splenocyte TNF-α mRNA expression. This result is inconsistent with a report by Cui *et al*[19]. who showed that dietary Arg supplementation decreases the mRNA expression of inflammatory cytokines in the spleen after thermal injury. Since the insults of scald burns and gut-derived sepsis differ, the inflammatory cytokine response may vary. Burn injury results in generalized inflammation in organs remote from the region of thermal injury due to activated neutrophil and reactive oxygen metabolites[31,32], whereas CLP causes peritoneal infection with mixed intestinal bacterial flora, which may result in a direct damage to the tissue and organs by circulating bacterial toxins.

In order to understand the effect of Arg on the distribution of lymphocyte subpopulations, the total B lymphocytes (CD45Ra+), total T cells (CD3+), helper T cells (CD4+), and cytotoxic T cells (CD8+) within the blood and splenocytes were evaluated. The results showed that there were no differences in the ratio of B cell and T cell subpopulations among the groups. This finding may indicate that the differences in cytokine mRNA expression after Arg supplementation do not influence the distribution of systemic and splenic lymphocyte subpopulations. Although the effects of Th1 and Th2 lymphocytes are counter-regulatory, cytokine mRNA expression and protein secretion may be regulated by different mechanisms in various tissues and organs, and the final performance of the immune response to specific tissues or organs may vary. Whether there are intracellular factors which regulate the post-transcriptional expression of these cytokines requires further investigation.

In conclusion, the influence of Arg supplementation on the distribution of whole blood and splenic lymphocyte subpopulations is not obvious. However, Arg supplementation, especially before and after CLP, significantly enhances the splenic mRNA expression of Th1 and Th2 cytokines in rats with gut-derived sepsis.

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Science Editor Wang XL and Guo SY **Language Editor** Elsevier HK