Doxorubicin-Induced Systemic Inflammation Is Driven by Upregulation of Toll-Like Receptor TLR4 and Endotoxin Leakage

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Abstract

Doxorubicin is one of the most effective chemotherapeutic agents used for cancer treatment, but it causes systemic inflammation and serious multiorgan side effects in many patients. In this study, we report that upregulation of the proinflammatory Toll-like receptor TLR4 in macrophages by doxorubicin is an important step in generating its toxic side effects. In patient serum, doxorubicin treatment resulted in leakage of endotoxin and inflammatory cytokines into circulation. In mice, doxorubicin damaged the intestinal epithelium, which also resulted in leakage of endotoxin from the gut flora into circulation. Concurrently, doxorubicin increased TLR4 expression in macrophages both in vitro and in vivo, which further enhanced the sensitivity of these cells to endotoxin. Either depletion of gut microorganisms or blockage of TLR4 signaling effectively decreased doxorubicin-induced toxicity. Taken together, our findings suggest that doxorubicin-triggered leakage of endotoxin into the circulation, in tandem with enhanced TLR4 signaling, is a candidate mechanism underlying doxorubicin-induced systemic inflammation. Our study provides new insights for devising relevant strategies to minimize the adverse effects of chemotherapeutic agents such as doxorubicin, which may extend its clinical uses to eradicate cancer cells. Cancer Res; 76(22): 1–12. ©2016 AACR.

Introduction

Increasing clinical evidence suggests that cancer therapeutic agents (CTA) can trigger systemic inflammation that leads to poor prognosis. For example, doxorubicin (DOX), one of the most effective CTA to date, induces severe inflammatory responses in various organs including the liver, kidney, intestine, and blood vessels (1–5). In addition to its known major adverse effect of cardiac toxicity (6). The levels of proinflammatory cytokines, such as IL1 and TNFα, were significantly increased following doxorubicin administration; meanwhile, blocking their functions alleviated the tissue damage caused by doxorubicin (7–10). Further investigations uncovered the roles of the toll-like receptor (TLR) family in this process—in particular TLR4, as heart failure was completely unobserved in TLR4-knockout mice treated with doxorubicin (11). However, TLR4 is a receptor mainly responsible for innate immune response against bacterial infection; it remains unclear how it could be involved in these CTA-induced tissue damages.

We postulated that the entry of endotoxin into the circulation triggers TLR4-mediated systemic inflammation. Endotoxin is a major component of Gram-negative bacteria and a typical ligand of TLR4 (12). Under normal conditions, a large amount of bacteria presenting endotoxin reside in the intestine and are strictly confined by the barrier of the intestinal mucosa. But this barrier could be broken by doxorubicin, which is known to be capable of disrupting the epithelium to induce oral ulcers, intestinal inflammation, and hemorrhagic cystitis in cancer patients (13, 14). If doxorubicin caused damage in the intestinal mucosa, endotoxin could enter the circulation and stimulate systemic inflammation. Furthermore, doxorubicin was found to upregulate the expression of TLR2 and 4 in cardiomyocytes (15). It is possible that this molecule can directly elevate the level of TLR4 in macrophages, resulting in stronger inflammatory responses to endotoxin and more severe damages to various organs.

Therefore, we hypothesized that the disruption of the intestinal mucosa barrier by doxorubicin, leading to the entry of endotoxin into the circulation that elicits TLR4-mediated inflammation in multiple organs, could serve as a new mechanism for the multiorgan toxicity of doxorubicin in the body. To validate this, we compared the levels of endotoxin and inflammatory cytokines in serum samples of patients with or without doxorubicin treatment. On the basis of the result, we tested the toxicity of doxorubicin in animal models in which gut microorganisms were depleted, and studied the correlation between TLR4 expression, doxorubicin administration and global inflammatory responses both in vitro and in vivo.

Materials and Methods

Reagents

Doxorubicin was purchased from Fenghua Biotech (purity>99%) and dissolved in saline. TAK-242 was provided by
MedChem Express (purity > 98%) and dissolved in a fat emulsion (16). LPS (Lipopolysaccharides from Escherichia coli 0111:B4) was purchased from Sigma-Aldrich. Rapamycin was purchased from Sangon Biotech (purity > 98%) and dissolved in 0.9% sodium chloride solution containing 1% DMSO at a concentration of 1 mg/mL. Other chemical reagents were purchased from Sangon Biotech.

Commercial kits to determine the levels of creatine kinase (CK), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), aspartate transaminase (AST), and alanineaminotransferase (ALT) were provided by Jiancheng Biotech. ELISA kits for TNFα, IL1β, and IL6 were purchased from Sizhengbai Biotech. Cell culture medium RPMI-1640 and FBS were purchased from Gibco BRL and Hyclone, respectively.

Human serum samples
Serum samples were collected from healthy donors and cancer patients with or without doxorubicin treatment. The study was approved by the Nanjing University Human Research Ethics Committee. All human samples were collected at Jinling Hospital (Nanjing, China) after the informed consent was obtained. We studied 10 healthy donors (Healthy donor; mean age 53 years, SD = 6), 10 cancer patients without doxorubicin treatment (DOX− patient; mean age 58 years, SD = 7) and 14 cancer patients with doxorubicin treatment (DOX+ patient; mean age 57 years, SD = 10). Detailed information about the clinical sample donors can be found in Supporting Information (Supplementary Table S1).

Mouse peritoneal macrophages
Primary mouse peritoneal macrophages were used in all the experiments. They were harvested and cultured according to a published method (17). Purified macrophages (purity > 90%) were incubated in 12-well plates (density of 1 × 10^6 per well) in RPMI-1640 medium with 10% (v/v) FBS at 37°C in a humidified atmosphere of 5% CO2.

To study the effect of gut microorganisms on proinflammatory cytokine levels following doxorubicin administration, we treated the mice with 20 mg/kg, i.p. doxorubicin. Macrophages were harvested and cultured at days 0, 2, and 4 after doxorubicin treatment. Serum-free medium was added for 12 hours before it was collected for subsequent ELISA analyses.

To study the effect of doxorubicin on TLR4 expression in peritoneal macrophages, the mice were treated with doxorubicin (20 mg/kg, i.p.) or an equal volume of PBS for 2 days, before their hearts, kidneys, livers and intestines were harvested for subsequent Western blotting analyses.

To assess whether doxorubicin administration increased cellular sensitivity to LPS, we treated the cells with doxorubicin (100 ng/mL) or and LPS (100 ng/mL) for 24 hours before the cells were harvested for subsequent ELISA analyses.

Animal treatments
C57BL/6N mice, ages 6 to 8 weeks, were provided by Vital River Laboratories (Beijing, China). For the accuracy of experiments, all of the control mice were littermates (18). The TLR4<sup>−/−</sup> mice (C57BL/10ScN background, The Jackson Laboratory Number: 003752), ages 6 to 8 weeks and carrying a spontaneous deletion of TLR4, and littermates were purchased from the experimental animal center of Nanjing University (Nanjing, China; refs. 19–22).

Animal protocols were reviewed and approved by the Animal Care and Use Committee of Nanjing University, and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. They were fed in a specific pathogen-free (SPF) animal facility with controlled light (12 hours light/dark), temperature and humidity, with food and water available.

We established a depletion-of-gut-microorganisms (DGM) mice model to study the source of endotoxin and its correlation with doxorubicin’s toxicity. Mice were initially given a treatment of 1 g/L ampicillin, 0.5 g/L vancomycin, 1 g/L metronidazole, 1 g/L neomycin and 100 g/L glucose for 4 weeks. Mice were then switched to a different antibiotic cocktail of 2 g/L streptomycin, 0.17 g/L gentamycin, 0.125 g/L ciprofloxacin, 1 g/L bacitracin, and 100 g/L glucose throughout the experiment. We confirmed intestinal depletion by collecting feces, homogenizing them in PBS, and serially diluting and plating the samples on trypticase soy agar with 10% sheep blood (Thermo Fisher Scientific) in an anaerobic chamber (37°C; 48 hours; refs. 23, 24).

To study the role of gut microorganisms in doxorubicin induced multiorgan damages, mice were divided into four groups: (i) Mice received drinking glucose solution without the addition of microbiotic (WT group); (ii) DGM mice (DGM group); (iii) WT group mice received 20 mg/kg doxorubicin, intraperitoneal (i.p.) injection (WT+DOX group); and (iv) DGM mice received 20 mg/kg doxorubicin, intraperitoneal injection (DGM+DOX group). The animals were examined for their body weights, mortality, and damages in different organs.

To verify whether endotoxin contributed to the toxicity of doxorubicin, mice were divided into three groups: (i) DGM mice received 20 mg/kg, i.p. doxorubicin administration (DGM+DOX group); (ii) DGM mice received doxorubicin (20 mg/kg) and 2 mg/kg, i.p. LPS administration (DGM+DOX+2mg/kg LPS group); and (iii) DGM mice received doxorubicin (20 mg/kg) and LPS (5 mg/kg) administration (DGM+DOX+5mg/kg LPS group). The animals were examined for their body weights, mortality, and damages in different organs.

To study the influence of doxorubicin treatment on TLR4 expression in peritoneal macrophages, the mice were treated with doxorubicin (20 mg/kg, i.p.) or an equal volume of PBS for 2 days, before their hearts, kidneys, livers and intestines were harvested for TLR4 analysis.

To assess whether doxorubicin treatment would increase the toxicity of LPS in vivo, mice were divided into four groups: (i) Mice treated with saline at the equal volume as used in other experimental groups (Sham group); (ii) WT mice received 20 mg/kg, i.p. doxorubicin administration (DOX group); (iii) WT mice received 5 mg/kg, i.p. LPS administration (LPS group); (iv) WT mice received doxorubicin (20 mg/kg) and LPS (5 mg/kg) administration (DOX+LPS group). The animals were examined for their body weights, mortality and damages in different organs.

To study the role of TLR4 in the process of doxorubicin-caused intestinal impairment and systemic inflammation, TAK-242 (10 mg/kg body weight, i.v. injection) was used to inhibit the signal transduction of TLR4. The TLR4<sup>−/−</sup> mice were further used to confirm the function of TLR4 in the generation of doxorubicin’s toxicity. Having been challenged by doxorubicin or doxorubicin combined with LPS, the animals were examined for damage in their tissues and the cytokines in the serum.

To study the role of mTOR signaling in the side effect of doxorubicin, mice were divided into two groups: (i) Mice treated...
with 2 mg/kg, i.p. Rapamycin per day resolved in 0.9% sodium chloride solution containing 1% DMSO for 30 days before they were injected with doxorubicin (20 mg/kg; Rapamycin+DOX group). (ii) Mice treated with 0.9% sodium chloride solution containing 1% DMSO (i.p.) per day at the equal volume as the Rapamycin+DOX group for 30 days before receiving doxorubicin (20 mg/kg, DOX group). The animals were examined for their body weights, mortality, and damage in different organs.

**Serum marker of multiple organ damages and cytokine analysis**

The serum and cell supernatant were collected and stored at −80°C until analysis, in which the levels of CK, LDH, BUN, AST, and ALT were assessed with corresponding kits. The levels of the inflammatory cytokines (TNFα, II1β, and IL6) were determined by ELISA kits.

**Histological and immunofluorescence analysis**

The cardiac, renal, hepatic, and intestinal tissue samples were fixed in Bouin’s buffer, embedded in paraffin and sectioned. For histologic analyses, the slices were counterstained with hematoxylin and eosin (H&E). For the histologic quantitative analyses of gut tissues, inflammatory foci, crypt depth, and altered villi were scored for each section. For immunofluorescence (IF) analysis, the slices were washed with PBS and blocked with 5% BSA for 1 hour, followed by incubation in primary (4°C; overnight) and secondary antibodies (room temperature; 1 hour), consequently, with proper rinse. The bands were visualized with Lumin-GLO Reagent (CST).

**Statistical analysis**

Data are presented as mean ± SEM. The differences between groups were analyzed using the Student t test when only two groups were compared or one-way ANOVA when more than two groups were compared. A P value of ≤0.05 was considered significant.

**Results**

**Endotoxin from gut microflora causes inflammation and tissue damage following doxorubicin administration**

To study the role of endotoxin in doxorubicin-induced inflammation and tissue damages, we measured the levels of both endotoxin and TNFα in different human serum samples. Their levels in patients who received doxorubicin treatment (endotoxin, 342.39 U/L; TNFα, 73.44 pg/mL) were significantly higher than those in patients without doxorubicin treatment (endotoxin, 127.5 U/L; TNFα, 9.7 pg/mL) and healthy donors (endotoxin, 200.10 U/L; TNFα, 13.54 pg/mL; Fig 1A). We assumed that the increased endotoxin came from gut microbiota, a main reservoir of bacteria in the body. To validate this, we examined whether doxorubicin would trigger less severe inflammation in DGM mice, in which gut microorganisms were experimentally removed. Indeed, we detected remarkably lower levels of endotoxin and TNFα in the serum of DGM+DOX mice than in the WT+DOX group (endotoxin: 56.26 vs. 121.53 U/L; TNFα: 65.34 vs. 189.65 pg/mL; Fig 1B). Also, lower levels of inflammatory cytokines were detected in the supernatant of the macrophages isolated from the abdominal cavity of DGM+DOX mice than those from WT+DOX mice (TNFα: 7.68 vs. 57.26 pg/mL; II1β: 83.03 vs. 177 pg/mL; IL6: 162.67 vs. 752.58 pg/mL; Fig 1C). Further inspection of the gut epithelium sections indicated that doxorubicin treatment caused focal accumulation of inflammatory cells in the lamina propria and interstitial edema, as well as shortening of small intestinal villi in WT mice; however, these impairments were much less severe in the DGM group (Fig 1D and E).

The above findings suggested that endotoxin from gut microflora mediated doxorubicin-triggered inflammation—but were these endotoxin responsible for doxorubicin’s toxicity to various tissues? To answer this question, we examined whether removal of gut microbiota—the source of endotoxin—could abolish the multiorgan damages caused by doxorubicin. We found no difference between the DGM+DOX mice and the untreated controls. All animals in the DGM+DOX group survived through the 8-day period without significant body loss, in sharp contrast with the WT+DOX mice that suffered a consistent body weight loss until all of them died between days 6 and 8 (Fig 2A). Similarly, the DGM+DOX mice had significantly lower levels of CK (691.84 vs. 1,677.14 U/L), LDH (1,481.63 vs. 2,058.5 U/L), BUN (16.56 vs. 23.89 mmol/L) and ALT (29.57 vs. 69.56 U/L) than WT+DOX mice, indicating that removal of gut microorganisms could effectively prevent the toxicity of doxorubicin to the cardiac (CK and LDH), renal (BUN) and hepatic (ALT) tissues, respectively (Fig 2B). Furthermore, we observed that, compared with the DGM+DOX group, the WT+DOX group showed serious disorganization of myofibrillar arrays and intense infiltration with neutrophil granulocytes in cardiac tissue samples, and cytoplasmic vacuolization in the renal and hepatic tissue samples (Fig 2C). To verify whether endotoxin is a direct cause of those side effects of doxorubicin, the DGM mice were treated with different levels of LPS (2 and 5 mg/kg) following doxorubicin administration. Following doxorubicin and LPS administration, endotoxin in the serum of the DGM+DOX+2 mg/kg LPS and DGM+DOX+5 mg/kg LPS groups showed higher level than in the DGM+DOX group (Supplementary Fig S1). We detected remarkably higher levels of proinflammatory factors (TNFα and II1β) in the serum of DGM+DOX+2mg/kg LPS and DGM+DOX+5mg/kg LPS groups than in the DGM+DOX group (NFα: 77.76 vs. 38.19 pg/mL; II1β: 215.71 and 309.92 vs. 113.49 pg/mL; Fig 2D and Supplementary Fig S2). We also observed lower survival rates and body weights in DGM+DOX+2 mg/kg LPS and DGM+DOX+5 mg/kg LPS groups than in the DGM+DOX group (Fig 2D and Supplementary Fig S3). The DGM+DOX+2 mg/kg LPS mice and DGM+DOX+5 mg/kg LPS mice had significantly higher levels of CK (2,303.16 and 3,399.11 vs. 691.84 U/L), LDH (2,257.68 and 3,037.83 vs. 1,481.63 U/L), BUN (19.62 and 26.02 vs. 15.89 mmol/L), and ALT (58.43 and 89.59 vs. 29.57 U/L) than DGM+DOX mice. These findings indicated that endotoxin in circulation could be a direct cause of doxorubicin to the cardiac (CK and LDH), renal (BUN), and hepatic (ALT) tissues, respectively (Supplementary Fig S4A). Moreover, compared with the DGM+DOX group, damages in heart, kidney, liver, and intestine were more severe in the DGM+DOX+2 mg/kg LPS mice and DGM+DOX+5 mg/kg LPS mice (Supplementary Fig S4B). These
data confirmed that removal of gut microbiota was effective in abolishing the damage of doxorubicin to multiple tissues/organisms. Collectively, we demonstrated that the entry of endotoxin from the gut to the circulation, as a consequence of doxorubicin-caused intestinal damage, could be an important mechanism for doxorubicin-induced systemic inflammation and multiorgan toxicity.

Elevated level of TLR4 increases endotoxin toxicity following doxorubicin administration

As TLR4 is involved in doxorubicin-induced cardiopathy (11) and an important receptor of endotoxin (25), we assessed whether its expression was altered in response to doxorubicin administration. We observed that the doxorubicin treatment elevated...
the level of TLR4 expression in the ex vivo cultured peritoneal macrophages, and that doxorubicin in a higher dose (100 vs. 50 ng/mL) exerted a stronger augmentation effect (Fig. 3A). A similar effect of doxorubicin was found in mice: The administration of this drug resulted in upregulated expression of TLR4 in the cardiac, renal, hepatic, and intestinal tissues, as evidenced by both Western blotting (Fig. 3B) and immunofluorescent staining (Fig. 3C). Interestingly, treatment of macrophages with a combination of...
doxorubicin and LPS stimulated remarkably higher levels of inflammatory cytokines (TNFα, IL1β, and IL6), compared with using LPS alone (Fig. 3D). This finding not only further indicated that doxorubicin could directly upregulate the expression of TLR4 and thereby increase the cellular sensitivity to LPS, but also inspired us to assess whether doxorubicin treatment could amplify the toxicity of endotoxin/LPS at a global level. Indeed, we detected significantly higher serum levels of TNFα and IL1β in mice receiving combined treatment of doxorubicin and LPS than those treated with LPS alone (TNFα: 188.6 vs. 58.64 pg/mL; IL1β: 309.64 vs. 207.18 pg/mL; Fig. 4A), and observed lower body weights and survival rates in the former group (Fig. 4B). In

Figure 3.
TLR4 expression level is upregulated following DOX administration. A, representative Western blotting and quantitative results showing the protein levels of TLR4 in peritoneal macrophages in response to PBS or DOX. *, P < 0.05 versus DOX/PBS and 50 ng/mL DOX/100 ng/mL DOX. Left, representative Western blotting. Right, bar graphs illustrating quantitative results. B, representative Western blotting and quantitative results showing the protein levels of TLR4 in the cardiac, renal, hepatic, and intestinal tissues of the mice following PBS or DOX administration. *, P < 0.05 versus DOX/PBS. Left, representative Western blotting. Right, bar graphs illustrating quantitative results (n = 10). C, immunofluorescent staining for TLR4 on heart, kidney, liver, and intestine slices of wild-type mice treated with PBS/DOX. Red, TLR4; blue, DAPI. D, levels of TNFα, IL1β, and IL6 in macrophage supernatant in the PBS, DOX, LPS, and DOX+LPS groups (n = 10). *, P < 0.05 versus LPS/DOX+LPS.
addition, the animals receiving combined treatment had remarkably elevated levels of CK (4,143.26 vs. 1,957.75 U/L), LDH (3,051.66 vs. 1,721.16 U/L), BUN (20.86 vs. 16.00 mmol/L), AST (178.41 vs. 33.07 U/L), and ALT (98.4 vs. 44.35 U/L) than the animals treated with LPS alone (Fig. 4C). The maximum number of cytoplasmic vacuolizations and intense infiltrations with neutrophil granulocytes was also found in various tissue samples from the DOX+LPS group (Fig. 4D).
TLR4 is involved in doxorubicin-induced intestinal impairment and multiorgan damages

To validate the essential role of TLR4 in the whole process of doxorubicin-caused intestinal impairment, systemic inflammation and multiorgan damages, we suppressed or completely abolished its function in the animal models, by injecting TAK-242 or using TLR4<sup>−/−</sup> mice, respectively. Histologic observation revealed that the damages to the intestinal tissues were clearly reduced in TAK-242-treated and TLR4<sup>−/−</sup> mice administrated with doxorubicin, in comparison with those in the Sham+DOX group (Fig. 5A). Meanwhile, the serum levels of endotoxin and inflammatory cytokines were significantly lower in TAK-242-treated and TLR4<sup>−/−</sup> mice receiving doxorubicin treatment than in the Sham+DOX animals (endotoxin: 97.99 and 88.90 vs. 121.53 U/L; TNFα: 79.74 and 38.77 vs. 254.57 pg/mL; IL1β: 127.51 and 115.45 vs. 222.05 pg/mL; Fig. 5B and C), suggesting that suppressing or abolishing TLR4 functions could relieve the intestinal damages and endotoxin leakage into the circulation caused by doxorubicin administration.

The role of TLR4 in doxorubicin-induced multiorgan toxicity was subsequently confirmed. We observed higher body weights and lower death rates in TAK-242+DOX+LPS and TLR4<sup>−/−</sup>+DOX+LPS groups than in the Sham+DOX+LPS group (Fig. 6A). In addition, TAK-242+DOX+LPS and TLR4<sup>−/−</sup>+DOX+LPS groups had remarkably decreased levels of CK (1.604.23 and 1.405.33 vs. 3.579.97 U/L), LDH (1.186.47 and 930.33 vs. 2.284.7 U/L), BUN (11.99 and 7.88 vs. 19.26 mmol/L), and ALT (66.43 and 39.82 vs. 89.24 U/L) than the animals in the Sham+DOX+LPS group (Fig. 6B). Furthermore, compared with the Sham+DOX+LPS group, the TAK-242+DOX+LPS and TLR4<sup>−/−</sup>+DOX+LPS groups showed slight disorganizations of myofibrillar arrays and intense infiltrations with neutrophil granulocytes in cardiac tissue samples, and the lower number of cytoplasmic vacuolizations in the renal and hepatic tissue samples (Fig. 6C).

Discussion

The severe adverse effects of doxorubicin on multiple organs, notably including its cardiac toxicity leading to life-threatening heart failure, have substantially restricted its extended clinical use (6). However, their underlying mechanism remains elusive. Here, we report for the first time that the entry of endotoxins (ET) to the circulation from gut microbiota, due to the disruption of gut epithelium by doxorubicin, substantially contributes to doxorubicin-caused systemic inflammation and multiorgan damages.

This finding provides a new answer to why causes systemic inflammation in the cancer patients receiving doxorubicin treatment. In fact, evidences developed both in clinically and in laboratory have long demonstrated that doxorubicin could cause devastating systemic inflammation in vivo, including hepatitis, nephritis, phlebitis, and mucositis throughout the digestive tract, and increase inflammatory cytokines levels in the circulation (1–5). Anti-inflammation treatments not only suppressed doxorubicin-caused myocardial inflammation but also prevented cardioamypothy (7–9). Previous studies suggested that the systemic inflammation was stimulated by tissue damage or cell apoptosis due to the toxicity of this drug (26). However, inflammatory responses triggered by tissue damage should be transient and self-restricted. Instead, doxorubicin caused chronic and much more destructive inflammation that usually includes the activation of TNF signaling pathway, generation of massive ROS and persistent expression of multiple proinflammatory cytokines—all of which belong to typical characteristics of inflammatory responses against endotoxin (27, 28). In addition, the central role of TLR4 in mediating this pathologic process—as demonstrated by both our and other studies—further suggests that endotoxin is the most likely antigen to trigger tissue inflammation, because the principal function of this receptor is to recognize the component of invaded bacteria, notably including endotoxin (17, 29).

The main source of endotoxin in the body is the Gram-negative bacteria residing in the intestinal tract (30, 31). Healthy epithelium confines the endotoxin in the intestine and prevents them from entering the circulation. The epithelial cells are constantly under self-renewal, as a mechanism to resist the disturbance from food intake and digestion process, which nevertheless makes themselves more vulnerable to the chemotherapeutic agents than other normal cells. This is why patients receiving doxorubicin treatment always suffer from peptic ulcer and even gastrointestinal hemorrhage (32). Damages in the intestinal epithelium will inevitably lead to the leakage of endotoxin into the circulation. In our study, we observed severe damages in the intestinal epithelium of doxorubicin-treated mice, including the loss of epithelial cells, the shrink of the villus and large areas of inflammatory infiltration. Before causing systemic inflammation, the leakage of endotoxin first triggers inflammatory responses in the intestine, which in turn exaggerates the tissue damages. This dynamic pathologic process was evidenced by the result that removal of gut microibota prevented the damage caused by doxorubicin administration. It is very likely that both the initial damage to the epithelium and the subsequent inflammation collaboratively promoted the leakage of endotoxin to the circulation.

Circulating endotoxin is closely related to the heart failure. Clinical investigations discovered that the circulating endotoxin was elevated in HF patients and the monocytes from the patients were hypersensitive to LPS (33, 34). Recent studies suggested that the circulating endotoxin originated from gut microflora, and that the systemic inflammatory responses stimulated by the circulating endotoxin substantially contributed to the pathology of heart failure (35). These findings were consistent with our study that elimination of intestinal microflora dramatically alleviated the inflammation response in multiple organs and decreased the mortality rate in the doxorubicin-treated animals. Besides endotoxin, other microbial products, such as nucleic acids or polysaccharides, may also contribute to the inflammation via TLR2/9-mediated pathways, which were previously reported to be involved in the generation of doxorubicin’s toxicity (36, 37).

Because the mTOR signaling pathway is involved in LPS-mediated inflammation and tissue damages (38, 39), we wondered whether systemic inflammation would be lenient if mTOR signaling was suppressed. The mice were treated with doxorubicin and Rapamycin. We detected higher body weights and survival rates in the DOX+Rapamycin group than in the doxorubicin group (Supplementary Fig. S5). The levels of CK, LDH, BUN, and ALT were lower in the DOX+Rapamycin group than in the doxorubicin group (Supplementary Fig. S6A). Moreover, lenient damages in cardiac, renal and hepatic tissue samples were observed in the DOX+Rapamycin group than the doxorubicin group (Supplementary Fig. S6B). These findings indicated that mTOR signaling pathway could play a role in mediating the side effects of doxorubicin. It has been widely accepted that the
unwanted toxicity of doxorubicin largely comes from its stimulation of intracellular production of ROS that damages cell membrane and induces drastic apoptosis (40, 41). Nevertheless, ROS is also likely to contribute to the systemic inflammation during doxorubicin treatment, as there were studies that demonstrated that ROS could significantly increase the level of TLRs, such as TLR9 and TLR2 (42). Besides ROS, other inflammatory mediators, such as IL6, GM-CSF and IFNγ were also found to upregulate the expression of TLR-4 in different published studies (43). Intriguingly, the expression of IL6, GM-CSF, and IFNγ could all be enhanced by doxorubicin treatments in vivo or in vitro (44–46). Moreover, the upregulation of TLRs (including TLR4) seems a common phenomenon in the inflammation process as there is evidence that LPS, oxidative LDL and CpG could all increase the expression of some TLRs. Taken together, the upregulation of TLR4 might be due to the inflammation reactions triggered by doxorubicin and the release of some key inflammatory mediators (ROS, IL6, GM-CSF).

Our findings may provide insights for understanding the immunotoxicology of other cancer chemotherapeutic agents, because systemic inflammation is commonly found in the cancer patients undertaking chemotherapy, not only limited to anthracyclines (47). The side effects of chemotherapeutics were mostly attributed to their toxicity to cells in normal organs and tissues (48, 49). The inflammatory reactions following the chemotherapy were always considered as a consequence, but not the reason, of such cytotoxicity (50). Nevertheless, our present finding that the product of gut microflora leaking from the intestinal tract

![Figure 5](image_url)

**Figure 5.**
DOX-caused intestinal damage relieves when TLR4 was suppressed or abolished. A, representative histologic images of H&E staining of the intestine of Sham, TAK-242, TLR4−/−, Sham+DOX, TAK-242+DOX, and TLR4−/−+DOX. B, serum concentrations of endotoxin in Sham, TAK-242, TLR4−/−, Sham+DOX, TAK-242+DOX, and TLR4−/−+DOX groups (n = 10). *P < 0.05 versus Sham+DOX/TAK-242+DOX or TLR4−/−+DOX. C, serum concentrations of TNFα and IL1β in Sham, TAK-242, TLR4−/−, Sham+DOX, TAK-242+DOX, and TLR4−/−+DOX groups (n = 10). *P < 0.05 versus Sham+DOX/TAK-242+DOX or TLR4−/−+DOX.
promoted systemic inflammation during the treatment with chemotherapeutics suggested that inflammation could directly derive from chemotherapy and might play a significant role in inducing systemic toxicity. As such, we could develop new strategies aimed at controlling inflammation for the treatment of chemotherapy-related adverse effects. As our study indicated that the gut microflora was the source of some key inflammatory mediators, such as endotoxin, the application of antibiotics to temporarily eliminate the bacteria in gut may be a convenient and effective way to reduce the chemotherapy related physiological

Figure 6.
Toxicity of multiple organs after combined administration of DOX and LPS is lenient when TLR4 was suppressed or abolished. A, body weights and survival rates in the Sham, TAK-242, TLR4^{Δ-Δ}, Sham + DOX + LPS, TAK-242 + DOX + LPS, and TLR4^{Δ-Δ} + DOX + LPS groups (n = 10). B, serum levels of CK, LDH, BUN, and ALT in Sham, TAK-242, TLR4^{Δ-Δ}, Sham + DOX + LPS, TAK-242 + DOX + LPS, and TLR4^{Δ-Δ} + DOX + LPS groups (n = 10). *P < 0.05 versus Sham + DOX + LPS/TAK-242 + DOX + LPS or TLR4^{Δ-Δ} + DOX + LPS. C, Representative histologic images of H&E staining of the heart, kidney, and liver of Sham, TAK-242, TLR4^{Δ-Δ}, Sham + DOX + LPS, TAK-242 + DOX + LPS, and TLR4^{Δ-Δ} + DOX + LPS groups.
toxicity. Accordingly, several other strategies, inhibition of TLR signal pathway and suppression of the mTOR signaling pathway and restoration of intestine integrity, can be investigated as potential approaches to prevent or alleviate adverse effects of doxorubicin and other chemotherapy agents.

In conclusion, our present study demonstrates that doxorubicin-caused disruption of the intestinal epithelium, which enables the leakage of endotoxin from gut microflora into circulation, is an important cause for the common adverse effects of doxorubicin, including systemic inflammation and multiorgan damages. The administration of doxorubicin not only impaired the epithelial barrier, but also directly upregulated the expression of TLR4 in both the intestine and other tissues, which further amplified the global immunotoxicity of endotoxin. Either depletion of gut microflora or inhibition of TLR signaling proved effective in abolishing or alleviating DOX’s toxicity in the animal models. This can be a general mechanism for further understanding and control of systemic toxicity caused by a broader class of cancer chemotherapeutic agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Wang, C. Wang, J. Zhang, L. Dong
Development of methodology: L. Wang, L. Dong

References
2. Antoniak S, Pawlinski R, Mackman N. Doxorubicin-induced cardiac and hepatic toxicity is mediated by protease-activated receptor 1 (PAR-1). Circulation 2010;122:A1144.

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