

The Radio-mitigative Effect of CpG-Oligodeoxynucleotides on Mice After Exposure to Carbon Ions Radiation.

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Research

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Abstract

Background: Heavy ion radiation constitutes a major health risk for astronaut in space flight, potential damage to healthy tissues surrounding the tumor target along its penetrating path should still be considered in hydrotherapy. Therefore, there is a demand for reliable countermeasure against heavy ions radiation. In this study, we will estimate the radiomitigative effect of CpG-ODN on immune tissues after carbon ions radiation (CIR).

Methods: Firstly, the 30 days' survival of mice was observed, peripheral blood cell was counted, the injury of three principal immune tissues (including bone marrow, thymus and spleen) was evaluated by histological examination, apoptosis and double strand breaks (DSB) were detected by TUNEL staining and γ -H₂AX immunohistochemistry respectively, and cytokine (G-CSF, IL-6 and TNF- α) was measured by ELISA assay.

Results: the 30 days' survival improved, the injury of three principal immune tissues were obviously ameliorated, the number of γ -H₂AX foci and TUNEL-positive nuclei decreased, and G-CSF, IL-6 and TNF- α expression increased by CpG-ODN treatment after CIR.

Conclusion: CpG-ODN could enhanced mice survival, and ameliorate immune tissues injury, the mechanism may be that CpG-ODN induced cytokines production and inhibited the double strand breaks (DSB) and apoptosis in order to stimulate the generation and mobilization of the immune cells and reestablish immune system to combat bacterial infections.

Introduction

Heavy ions radiation is densely ionizing, which have a maximum dose deposition upon entry in a medium, the depth-dose distribution is characterized by a low dose plateau upon entrance and pronounced maximum, the radiant energy is not only deposited by the primary interaction but also by secondary electrons, which may travel considerable distances from the core. Therefore, it produces more irreparable DNA break and death to cells in comparison with X rays or γ rays [1–4]. In space flight, heavy ion radiation constitutes a major health risk for astronaut, because unlike other radiation types in outer space, current shielding is unable to provide effective protection against heavy ions radiation [5, 6]. In hydrotherapy, although heavy ions radiation as an innovative modality of high precision tool for cancer therapy, potential damage to healthy tissues surrounding the tumor target along its penetrating path should still be considered [1]. Given space radiation protection and cancer therapy, there is a demand for reliable countermeasure against heavy ions radiation.

Recently, more and more attention has been focused on Toll-like receptor (TLR) ligands as radiation countermeasure [7]. TLRs are the key sensor elements of innate immunity and are evolutionary conserved receptors, which recognize highly conserved structural motifs known as pathogen-associated molecular patterns (PAMPs). Stimulation of TLRs by PAMPs, initiates signaling cascades which lead to the activation of transcription factors, such as NF- κ B. TLR signaling results in a variety of cellular response

including the production pro-inflammatory cytokines and effector cytokines which direct the adaptive immune response [8]. TLR ligands are characteristic of large group of pathogens, and cannot be easily mutated, which mediated specifically activation of TLRs. TLR ligands are appealing as potential radiation countermeasure since they have little effect besides activating TLRs [9].

Our previous research has shown that TLR9 ligand(CpG-oligodeoxynucleotides, CpG-ODN)could reduce bone marrow after γ -rays and protect RAW264.7 cell in vitro against heavy ions radiation [10]. However, the effect of CpG-ODN against injury induced by heavy ions radiation in vivo is still largely unknown. Immune tissue injury is of great important parameter of radiation injury as they are potentially life threatening. Carbon ions radiation (CIR) is a specific type of heavy ions radiation. In this regard based on ground experiments at accelerators, the present study has been undertaken to estimate the mitigative effect of CpG-ODN on immune tissue after exposure to CIR.

Materials And Methods

Reagent

CpG-ODN was synthesized at Shanghai Sangon Biological Engineering Technology Services Co Ltd (Sangon, Shanghai, China). CpG-ODN sequences were 5'-T ***CGT CG*** TTT T ***CG GC GCGC CG***-3' (regular letters represent phosphorothioate, bold and italic letters represent phosphodiester). The compound was diluted with phosphate buffer saline (PBS) to a final concentration 250 $\mu\text{g/ml}$ and stored at 4 °C.

Mice

Male C57BL/6 (20~22 g), 10~12 weeks' old were purchased from SLAC laboratory animals Co. Ltd (SLAC, Shanghai, China), and were kept under standard laboratory conditions (a temperature of 23 ± 2 °C with 24 h cycles of fresh air and 12 h light/dark cycle). Food and water were sterilized by ^{60}Co γ rays and high pressure, respectively.

Carbon ions radiation [CIR]

Mice was placed inside a specially designed and well-ventilated acrylic container with dimensions of 8.0 cm \times 3.5 cm \times 3.5 cm. the acrylic container was placed into the beam path at the entrance plateau throughout the exposure. Whole-body radiation of mice was performed using CIR at initial energy of 250 MeV/u and the average LET of 31.3 keV/ μm . Each mouse received 5 Gy of radiation at a dose rate of 0.8 Gy/min. CIR was supplied by Heavy Ion Research Facility at Institute of Modern physics, Chinese Academy of Sciences (HIRF, Lanzhou, China). The acquisition of data (preset beams converted by doses of radiation) was automatically calculated and controlled by a microcomputer.

Treatment and Ethics approval

Mice were treated with either 50 μg CpG-ODN (250 $\mu\text{g/ml}$) or 0.2 ml PBS via intraperitoneal (i.p) injection. CpG-ODN was given 30 min, 24 h and 48 h after CIR. The effective dosage and the time points of CpG-

ODN treatment in this study was selected based on data from previous study [11] Mice were randomly divided into four groups as follows: a normal group (the non-irradiated mice with PBS treatment), a CpG-ODN group (the non-irradiated mice with CpG-ODN treatment), a CIR control group (the irradiated mice with PBS treatment) and a CIR plus CpG-ODN group (the irradiated mice with CpG-ODN treatment). All studies were performed under the guidelines and protocols of the Institutional Animals Care and Use Committee of the Lanzhou General Hospital of PLA (IACUC approval#15010936) according to the Guide for Care and Use of Laboratory Animals published by US NIH (publication No 96~101).

Survival

Irradiated mice were observed to monitor survival for 30 days after CIR. Moribund mice were euthanized. On 31th day, surviving mice were euthanized by cervical dislocation. Data were expressed as percentage survival. 28 mice per groups were used for the experiments. The Mean Survival Time (MST) was calculated by Kaplan-Meier method.

Blood analysis

The peripheral blood was obtained from halothane-anesthetized mice and collected in tubes containing heparin, then mice were killed humanely by cervical dislocation. Whole blood samples were evaluated by automated hematology system (CA-700NVET, STAC Group, China). White blood cell (WBC), lymphocytes, granulocyte, and monocytes counts were determined. T cells and B cells were examined by immunofluorescence [23]. The specific method is: 65 μ l of 10 % formaldehyde was added to surplus 100 μ l of blood and incubated for 10 min at room temperature, followed by the addition of 1ml Triton X-100 (diluted in PBS) to obtain 0.1 final concentrations. After 30 min of incubation at room temperature, 1ml cold wash buffer was added. Samples were centrifuged, suspended in 1ml 50 % methanol diluted in PBS (stock stored at -20 °C), and incubated at 4 °C for a minimum of 10 min. All cells were stained blue by 4', 6-diamidino-2-phenylindole (DAPI, Sigma, USA). Rabbit polyclonal antibodies of CD3 (cat: 100203) and CD19 (cat: 115507) from Bio Legend were used to identify T cells and B cells respectively. These samples were examined and quantified under fluorescent microscope (Nikon, Japan).

Bone marrow cell count and bone marrow histological examination

two femurs were removed at the times of euthanasia. The bone marrow cells within one femur were flushed with PBS, and the number of live bone marrow cells were determined by a cell counter analyzer system (CASY, Innovates, Germany). The other femur was fixed in 4 % paraformaldehyde for 24 h, treated with a formic acid sodium citrate decalcification solution for 5 days, embedded in paraffin and stained with Hematoxylin and Eosin (H&E).

Organ index

Mice were weighted and then euthanized by cervical dislocation. The whole thymus and spleen were rapidly excised, their masses were measured. Relative organ index was calculated: relative organ index= organ mass (mg)/body mass (g).

TUNEL assay and γ -H₂AX assay

As description in M. Leonor Fernández-Murga's article [27] whole thymus and spleen were fixed for 48 h in 10 % neutral buffered formalin, 5- μ m section was prepared and stained with H&E. Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining. 5- μ m section was deparaffinized, permeabilized with proteinase K (20 μ g/ml, Sigma) at 23 °C and rinsed 4 additional times with distilled water followed by incubation with a solution made up of TdT (1 μ g/200 μ l of mix solution), bovine serum albumin (1mg/ml), and biotin-16-dUTP (1 nmol/50 μ l of mix solution) in TdT buffer. Sections were then incubated in saline citrate for 15 min, rinsed and incubated with 2 % bovine serum albumin at 23 °C. Endogenous peroxidase activity was blocked by incubating the tissue sections with 3 % H₂O₂. The labeled DNA fragments were detected with peroxidase-conjugated antibody elicited against biotin. Slides were developed with DBA (diaminobenzidine tetrahydrochloride), and stained with hematoxylin. Double strand breaks (DSB) were investigated by γ -H₂AX immunohistochemistry. 5- μ m section was prepared. The primary antibodies used were mouse monoclonal anti- γ -H₂AX (1:100 Santa Cruz Santiago USA) at 4 °C overnight in humidified incubator. Unbound antibody was removed by several washing and goat anti-mouse antibody (1:100 Santa Cruz Santiago USA) was applied as a secondary labeled antibody for 30 min at room temperature. Slides were developed with DBA and stained with hematoxylin.

Statistical analysis

For the survival data, the difference in 30 days' survival between was analyzed by Kaplan-Meier plots. For other data, the difference between CIR control group and CIR plus CpG-ODN group was analyzed by the two-sided, non-paired Student's *t*-test. Data are expressed as *mean* \pm standard error mean (SEM). The software analysis program SPSS 13.0 (release 12.0K; SPSS Inc. Chicago, USA) was used. All differences were considered significant if *p* was less than 0.05.

Results

Effect of CpG-ODN on survival after CIR

Death is a typical endpoint in the study of radiation injury, which described as the most attributable consequence to immune system failure. Mice were irradiated with 5 Gy of CIR, it is important to note that in vivo C57BL/6 mice experiments where typically doses no more than 5 Gy of CIR are required to determine the sub-lethal dose [12]. Irradiated mice were monitored for 30 days, the number of mortalities in each group recorded daily. Exposure to 5 Gy of CIR caused mortality between 4 d and 14 d, the 30 days' survival only was 10.7 %. On the contrary, the mortality of irradiated mice treated by CpG-ODN occurred between 8 d and 12 d, the 30 days' survival was 57.1 %. This difference of survival was statistically significant (fig.1). To determine the radio mitigative effect of CpG-ODN, MST of mice was calculated. Compared with irradiated mice without CpG-ODN treatment, MST in irradiated mice with CpG-ODN treatment increased 11.1 days. these indicated that CpG-ODN enhanced mice survival after CIR.

Effect of CpG-ODN on WBC and bone marrow after CIR

Peripheral blood WBC and bone marrow were not only the blood system, but also were immune principal components. It was generally agreed that radiation death in the sub-lethal dose range was due to reduction of WBC and impairment of bone marrow, which ultimately predisposed to infection, sepsis or even death [13]. Mice were irradiated with 5 Gy of CIR, WBC was detected at 1 d and 3 d. CIR lead to a rapidly decrease in number of WBC (including lymphocyte granulocyte and monocyte). Compared with irradiated mice without CpG-ODN treatment, WBC, lymphocyte, granulocyte and monocyte in irradiated mice with CpG-ODN treatment increased 1.38, 1.41, 1.30 and 1.40-fold at 1 d, and increased 3.00, 2.13, 3.83 and 2.00-fold at 3 d respectively (fig.2A, fig.2B, fig.2C and fig.2D). Bone marrow histological examination showed that there was a significant reduction in bone marrow cell accompanied by typical apoptotic changes such as karyopyknosis, meanwhile, there were larger empty space and extensive interstitial hemorrhage with sinusoidal filling in bone marrow cavity at 1 d after CIR. In comparison, pathological alterations of irradiated mice with CpG-ODN treatment were less severe as evidenced by less bone marrow cell reduction, higher hemorrhage, and decreased edema. At 3 d, pathological changes in bone marrow cavity appeared 'empty'. There was a marked reduction in cell number with collapsed sinusoidal structures that were almost entirely destroyed, and extensive hemorrhage within bone marrow cavity (fig.2E). a nearly 1.5-fold raised in the number of bone marrow cell for irradiated mice with CpG-ODN treatment compared with irradiated mice without CpG-ODN treatment (fig.2F). These indicated that CpG-ODN increased the number of peripheral blood WBC and ameliorated bone marrow injury after CIR.

Effect of CpG-ODN on thymus after CIR

The thymus is also an immune principal component and most sensitive to radiation [14]. CIR caused thymus wastage. The thymus index decreased from 2.19 to 1.25 at 1 d and 0.66 at 3 d after CIR. Compared with irradiated mice without CpG-ODN treatment, the thymus index in irradiated mice with CpG-ODN treatment raised 8 % at 1 d and 26 % at 3 d. This difference was statistically significant at 3 d (fig.3A). Histological examination showed that the thymus cortices structure had almost disappeared, and medulla has severely atrophied, the thymocytes were decreased in number and scattered in the reticulum of medulla after carbon ions radiation. the medullar of irradiated mice with CpG-ODN treatment was thicker, which contained much more thymocytes that of irradiated mice without CpG-ODN treatment (fig.3B). Apoptosis and DSB of thymocytes were detected by TUNEL staining and γ -H2AX immunohistochemistry after CIR. As fig.3C and fig.3D shown, compared with irradiated mice without CpG-ODN treatment, the number of γ -H2AX foci and TUNEL-positive nuclei were obviously decreases by CpG-ODN treatment. In addition, T cell in peripheral blood from thymus was detected by labeling CD3 (fig.3E). The number of T cell decreased rapidly and showed a reduction of 24.0 % and 79.0 % at 1 d and 3 d after CIR, CpG-ODN produced significant sparing effect on the irradiation-induced decrease, the number of T cell increased 1.21-fold at 1 d and 2.31-fold at 3 d after CIR (fig.3F). These indicated that CpG-ODN ameliorated thymus injury induced by CIR.

Effect of CpG-ODN on spleen after CIR

The spleen is known to play an important role in the immune system, which is one of the major injury sites after radiation. CIR caused spleen significantly atrophy, the spleen index of irradiated mice without CpG-ODN treatment decreased from 3.73 to 2.31 at 1th and 1.73 at 3 d. Compared with irradiated mice without CpG-ODN treatment, the spleen index in irradiated mice with CpG-ODN treatment raised 35 % at 1 d and 28 % at 3 d after CIR. This difference was statistically significant at 1 d and 3 d (fig.4A). Histological examination showed that the area of white pulp was obviously smaller, germinal centers shrunk, and lymphatic sinus were expanded and congested after CIR. Although there was reduction of white pulp in irradiated mice with CpG-ODN treatment, the reduction was less severe than in irradiated mice without CpG-ODN treatment (fig.4B). The area of white pulp was increased 10 % at 1 d and 8 % at 3 d by CpG-ODN treatment (fig.4C). Apoptosis and DSB of spleen were detected by TUNEL staining and γ -H₂AX immunohistochemistry. Compared with irradiated mice without CpG-ODN, the number of γ -H₂AX foci and TUNEL-positive nuclei in white pulp of spleen was decreased obviously in irradiated mice with CpG-ODN treatment (fig.4D and fig.4E). In addition, B cell in peripheral blood from spleen was detected by labeling CD19 (fig.4F). The number of B cell decreased also rapidly and showed a reduction of 24.0 % at 1 d and 79.0 % at 3 d after CIR. CpG-ODN boosted the number of B cells, the number of B cell increased 1.72-fold at 1 d and 9.65-fold at 3 d after CIR (fig.4G). These results suggested that CpG-ODN ameliorate spleen injury induced by CIR.

Effect of CpG-ODN on cytokines after CIR

Cytokine stimulated the generation and mobilization of the immune cells and reestablish immune system after radiation to combat bacterial infection. Some radiation countermeasures that prevent against radiation injury do so in large part by stimulating expression of cytokines [15]. CIR stimulated the transient elevation of G-CSF, IL-6 and TNF- α within 8 h that might be associated with stress reaction, and then these cytokines expression drastically decreased. Compared with irradiated mice without CpG-ODN treatment, the expression of G-CSF significantly increased in all time points, was doubled in maximal value, and maintained high level within 24 h in the irradiated mice with CpG-ODN treatment (fig.5A). As fig.5B showed that the expression of IL-6 also significantly increased by CpG-ODN treatment, maximal value of IL-6 level in the irradiated mice with CpG-ODN treatment was more twice as high as the irradiated mice without CpG-ODN treatment. TNF- α have similar expression trend with IL-6. Although the expression of TNF- α started declining at 2 h after CIR, CpG-ODN treatment showed much stronger ability in increasing TNF- α expression at all time points (fig.5C). There was significant difference between two groups. These indicated that CpG-ODN induces G-CSF, IL-6 and TNF- α expression after CIR.

Discussion

Exposure to heavy ions radiation, immune organs atrophy and immune cells fall within a few days, which was likely to lead to considerable more serious immunological problems, with additional exposure to a bacterial challenge, leads to a very high level of morbidity which is equal to mortality. The bacterial challenge utilized bacterial known to be associated with infection, which are part of the normal bacterial flora of the mouth, skin and intestines [16]. In the mouse studies, these bacterial challenges are non-toxic

to normal mice. But under the conditions of heavy ions radiation, mice were morbidity or mortality [17–19]. Therefore, morbidity or mortality could be prevented by relieving immune tissues injury. Our previously study have documented that CpG-ODN relieved RAW264.7 macrophage cell damage induced by CIR. the present study was to estimate the mitigative effect of CpG-ODN on immune tissues after CIR by using C57BL/6 mice model.

Mice were exposed to 5 Gy of CIR, it is important to note that in vivo C57BL/6 mice experiments where typically doses no more than 5 Gy of CIR are required to determine the sub-lethal dose. Radiation death in the sub-lethal dose range was due to immune tissues destruction, which ultimately predisposed to infection and sepsis [12]. In this study, the radiomitigative effect of CpG-ODN on immune tissue was manifested indirectly by significantly enhancing mice survival. Bone marrow, thymus and spleen are the immune principal components and the major sites of radiation injury. Compared with irradiated mice without CpG-ODN treatment, the numbers of WBC (including lymphocyte granulocyte and monocyte) in irradiated mice with CpG-ODN treatment significantly increased. the direct evidence from histological examination showed that the injuries of bone marrow, thymus and spleen were ameliorated by CpG-ODN treatment after CIR. DSB have a close correlation to DNA injury induced by heavy ions radiation, which are thought to be more difficult to repair and is probably the most lethal attack, with as little as one unrepaired DSB being capable of triggering apoptosis. Thus, DSB have been considered as highly significant biological endpoint [21, 22]. The phosphorylation of the histone protein H₂AX (γ -H₂AX) has been used as a beacon of DSB and TUNEL staining was used to detect cell apoptosis. Many γ -H₂AX foci and TUNEL-positive nuclei were observed in thymus and spleen of irradiated mice, but CpG-ODN provided a significant reduction in the elevation of γ -H₂AX foci and TUNEL-positive nuclei. These results indicated that CpG-ODN ameliorated bone marrow, thymus and spleen injuries after CIR by inhibiting cell DSB and apoptosis, which were basically in agreement with our previous vitro experiment (Data not shown).

Although the effect of CpG-ODN against immune tissues injury induced CIR is not fully understood, the primary mechanism may be that CpG-ODN stimulates immune cell to secrete a group of cytokines. Published data suggested that TLR5 agonist (flagellin) could trigger immune tissues possibly via pro-inflammation cytokines, including IL-1, IL-6, IL-11, IL-12, G-CSF, IFN- α and TNF- α , which stimulated the generation and mobilization of the immune cells and reestablish immune system after radiation to combat bacterial infections [22]. The biological responses trigger immune cells by CpG-ODN display remarkable parallels to flagellin. The result showed that CpG-ODN induced G-CSF, IL-6 and TNF- α production after CIR. We anticipate that CpG-ODN and flagellin share operationally similar characteristics. Other possible mechanism of action is that NF- κ B activation plays a pivotal role. The important radiomitigative strategy is to activate the NF- κ B pathway TLRs are the key sensor elements of innate immunity, which are important for the defense against microbial infection. Stimulation of TLRs by special agonist initiates signaling cascades, and lead to the activation of NF- κ B. The role of NF- κ B regulates anti-apoptotic genes coding for proteins especially the TNF receptor-associated factor (TRAF), checks the activates of the caspase enzyme family, inhibit DNA damage, and block major apoptotic pathways. The idea of using NF- κ B pathway function to reduce radiation injury was initially tested using flagellin, which

activates NF- κ B through TLR5. TLR9 is one of the members of TLR family, and expressed in peripheral blood leukocyte, thymus, spleen, and other lymphoid organs. Our previous study has demonstrated CpG-ODN enhance cell survival after radiation by activation NF- κ B and inhibited Caspase pathway.

Overall, the present study demonstrated that the radiomitigative effect of CpG-ODN on immune tissues after CIR. The results showed that CpG-ODN could enhanced mice survival, and ameliorate bone marrow, thymus and spleen injuries by inhibiting DSB and apoptosis. The mechanism may be that CpG-ODN stimulates a group of cytokines production and NF- κ B activation. A better understanding of mechanism will require further studies.

Conclusion

CpG-ODN could enhanced mice survival, and ameliorate immune tissues injury, the mechanism may be that CpG-ODN induced cytokines production and inhibited the double strand breaks (DSB) and apoptosis in order to stimulate the generation and mobilization of the immune cells and reestablish immune system to combat bacterial infections.

Declarations

Ethical Approval and Consent to participate

All animal experiments conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 85-23, National Academy Press, Washington, DC, revised 1996), with the approval of the Laboratory Animal Center of the Second Military Medical University, Shanghai. The approval ID for this study was SMMU-20161762

Consent for publication

All authors reached an agreement to publish the study in this journal.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Chao Zhang and Hao Hu designed the study. Hua Fu, Chao Zhang, Xiang-hui Zhu performed the experiments. Sha Li analyzed the data. Chao Zhang and Hu Liu wrote the paper, Jianming Cai supported fund assistance. All authors read and approved the final manuscript.

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Figures

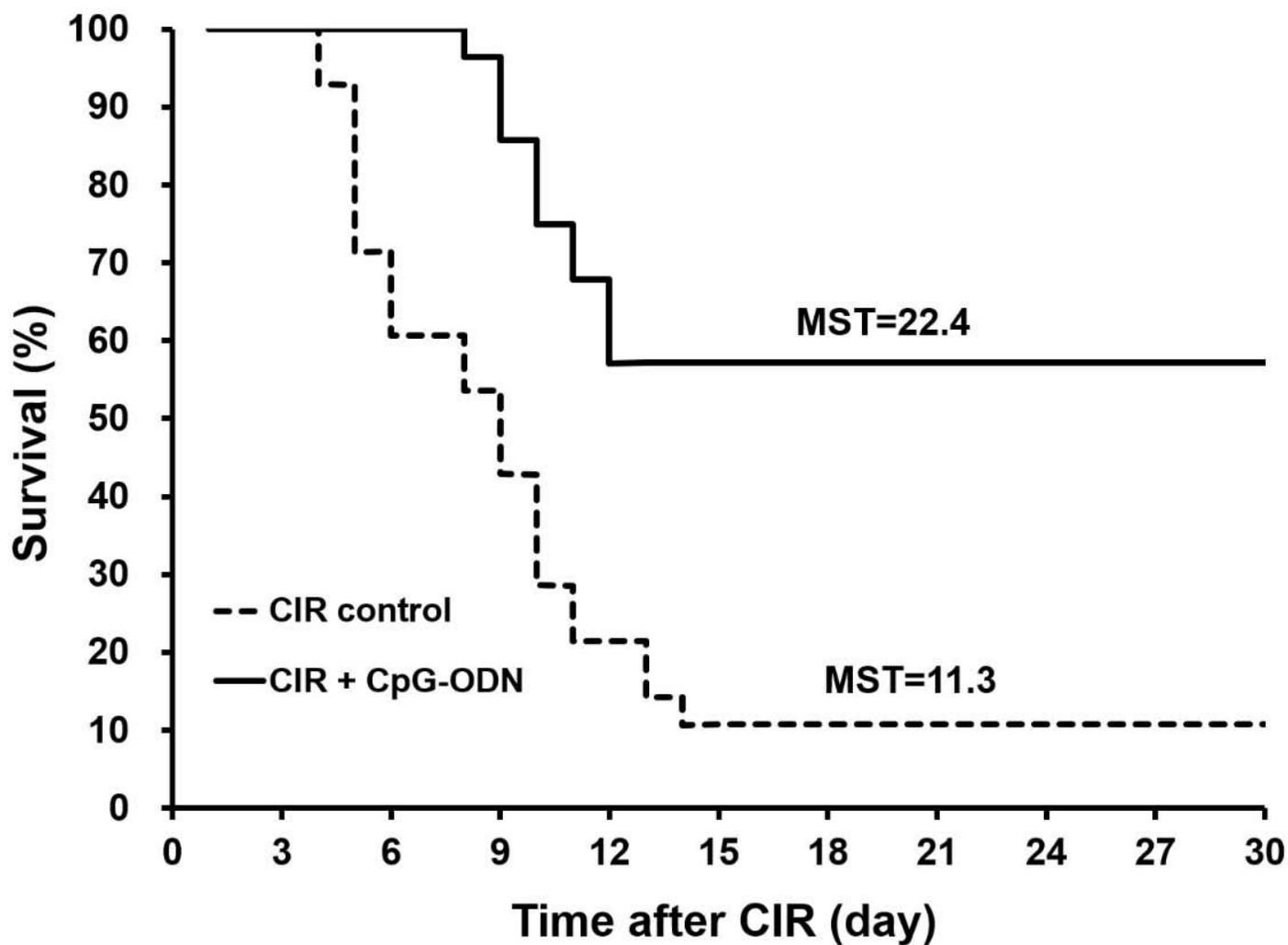


Figure 1

Effect of CpG-ODN on survival after CIR. Survival was monitored for 30 days after 5 Gy of CIR. (n=18 mice/group in each of three separate experiments. the difference in 30-day survival between CIR control group and CIR plus CpG-ODN group was analyzed by Kaplan-Meier plots).

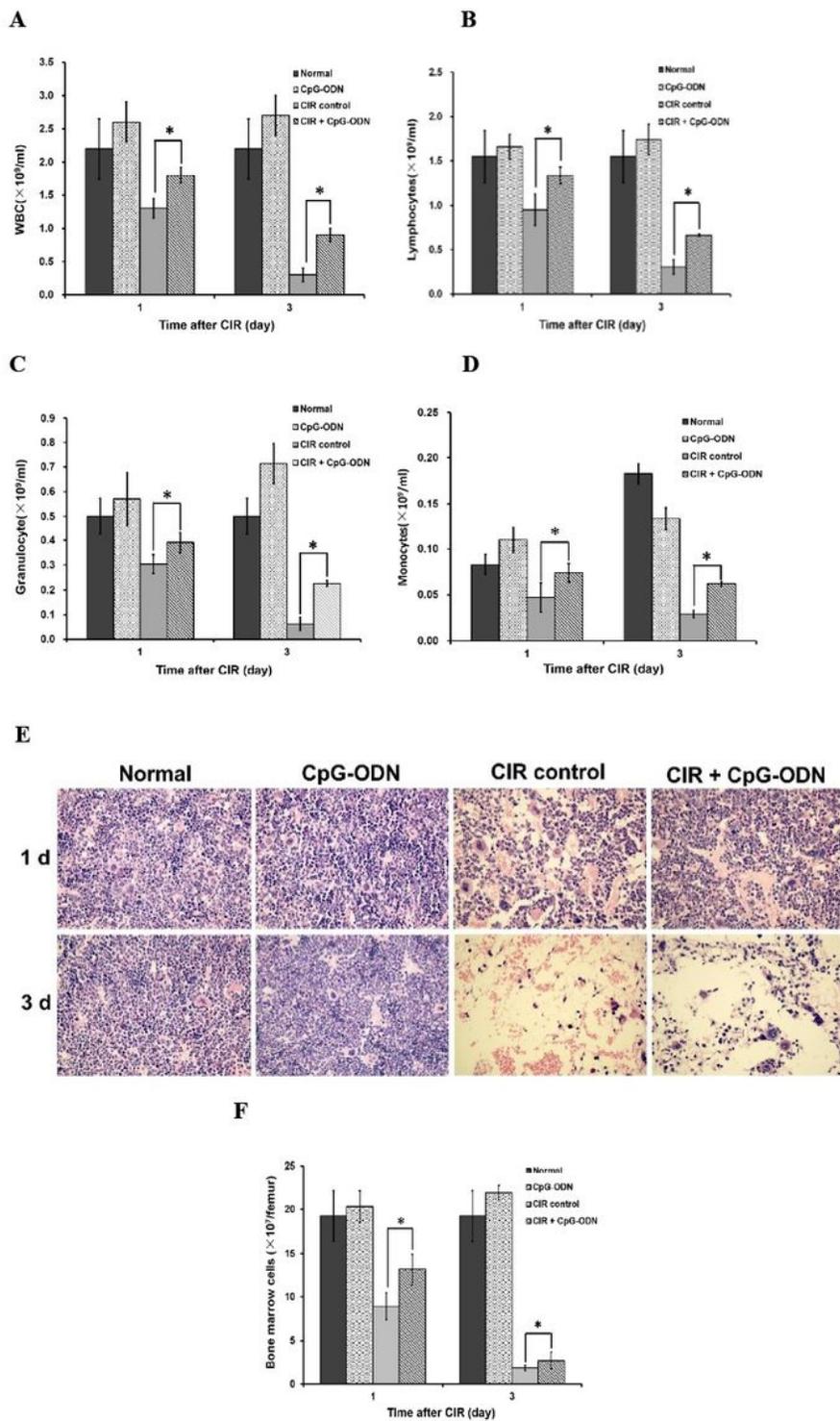


Figure 2

Effect of CpG-ODN on WBC in peripheral blood and bone marrow after CIR. (A) The number of WBC was assayed at 1 d and 3 d after CIR. (B) The number of lymphocyte was assayed at 1 d and 3 d after CIR. (C) The number of granulocytes was assayed at 1 d and 3 d after CIR. (D) The number of monocytes was assayed at 1 d and 3 d after CIR. (E) Representative histological specimens of the bone marrow at 1 d and 3 d after CIR are showed. (F) The number of bone marrow cell was determined at 1 d and 3 d after

CIR. (n=6 mice per group in each of three separate experiments. Data are represented mean \pm SEM *P<0.05 between CIR control group and CIR plus CpG-ODN group by Student's t test).

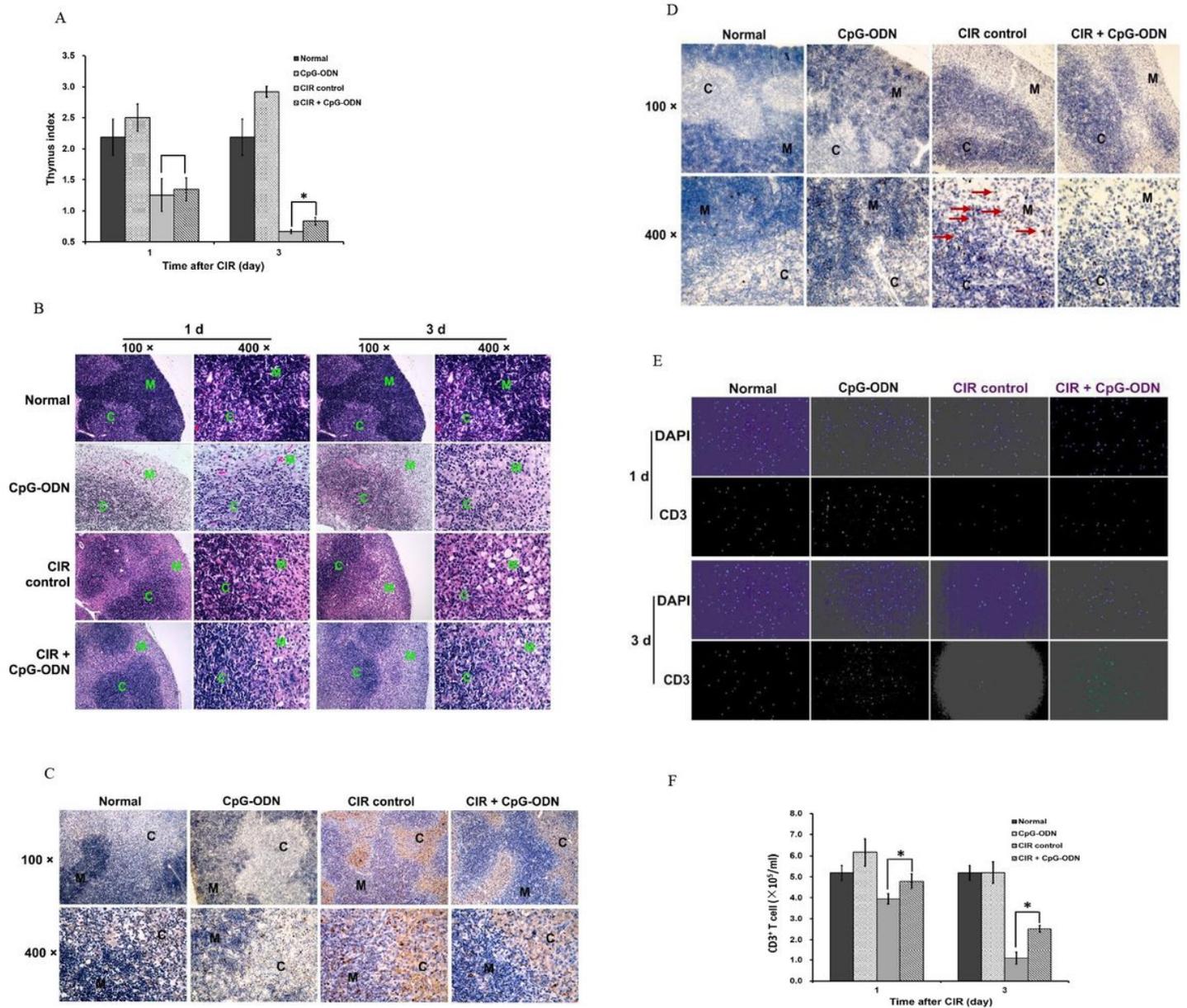


Figure 3

Effect of CpG-ODN on thymus after CIR. (A) The index of thymus was calculated by the ratio of thymus mass (mg) and body mass (g) at 1 d and 3 d after CIR. (B) Representative histological specimens of thymus (100 \times and 400 \times) at 1 d and 3 d after CIR are showed. (C) Apoptosis of thymus cells was detected by TUNEL assay at 1 d after CIR. (D) DSB of thymus cell was detected by anti- γ -H2AX labeling at 1 d after CIR; (E) CD3 labeling T cell was observed in peripheral blood, (F) the number of CD3 labeling T cell was quantified at 1 d and 3 d after CIR. ('C' represents cortex and 'M' represents medulla. n=6 mice per group in each of three separate experiment. Data are represented mean \pm SEM *P<0.05 between CIR

control group and CIR plus CpG-ODN group by Student's t test). Red arrows represent γ -H2AX positive cells.

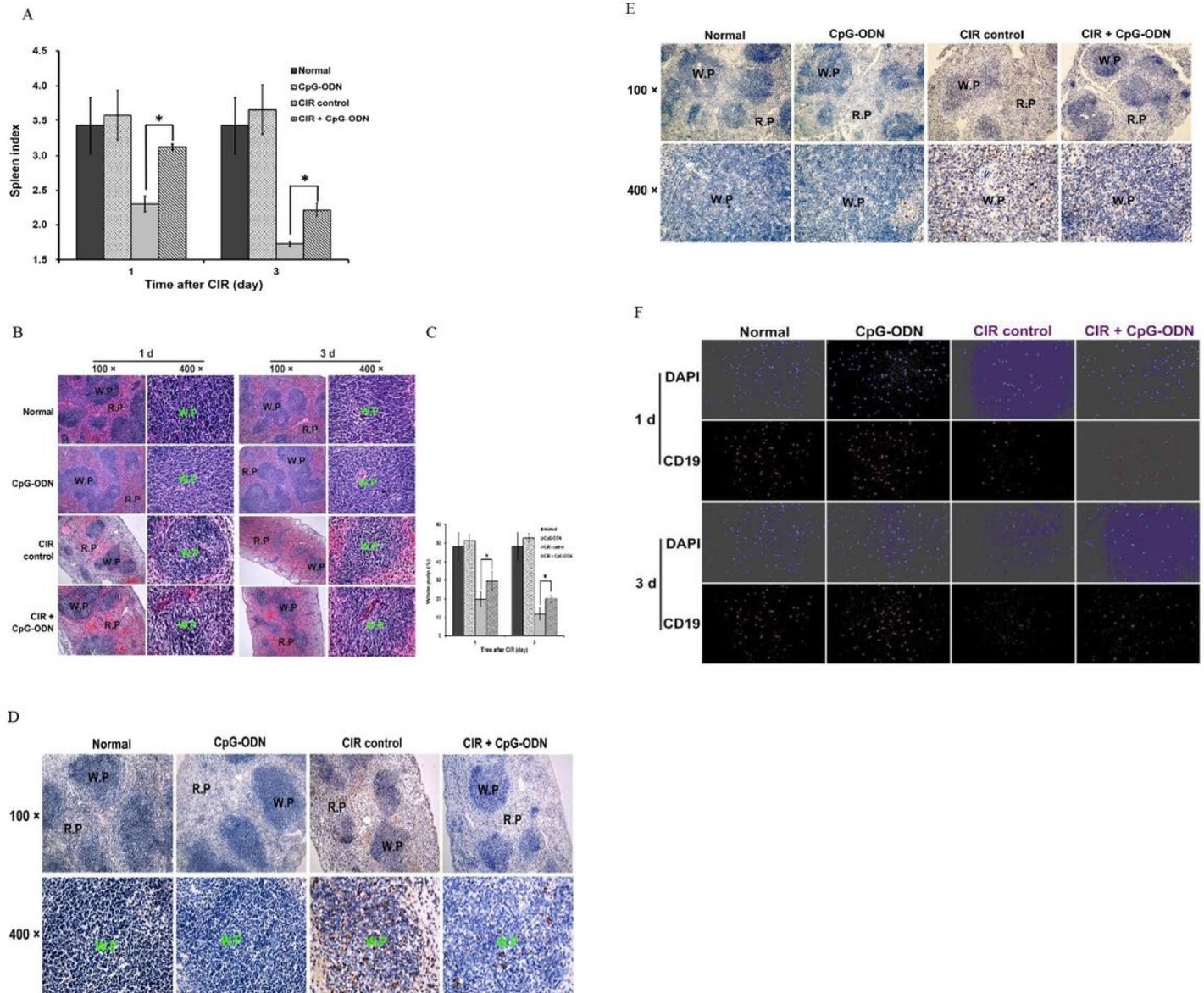


Figure 4

Effect of CpG-ODN on spleen after CIR. (A) The index of spleen was calculated by the ratio of spleen mass (mg) and body mass (g) at 1 d and 3 d after CIR. (B) Representative histological specimens of spleen (100 \times and 400 \times) at 1 d and 3 d after CIR are showed. (C) The area of white pulp was measure at 1 d and 3 d after CIR. (D) Apoptosis of spleen cell in white pulp was detected by TUNEL assay at 1 d after CIR. (E) DSB of spleen cell in white pulp was detected by anti- γ -H2AX labeling at 1 d after CIR. (F) CD19 labeling B cell was observed after CIR. (G) the number of CD19 labeling B cell was quantified at 1 d and 3 d after CIR. ('R.P.' represents red pulp and 'W.P.' represents white pulp, n=6 mice per group in each of three separate experiment. Data are represented mean \pm SEM *P<0.05 between CIR control group and CIR plus CpG-ODN group by Student's t test).

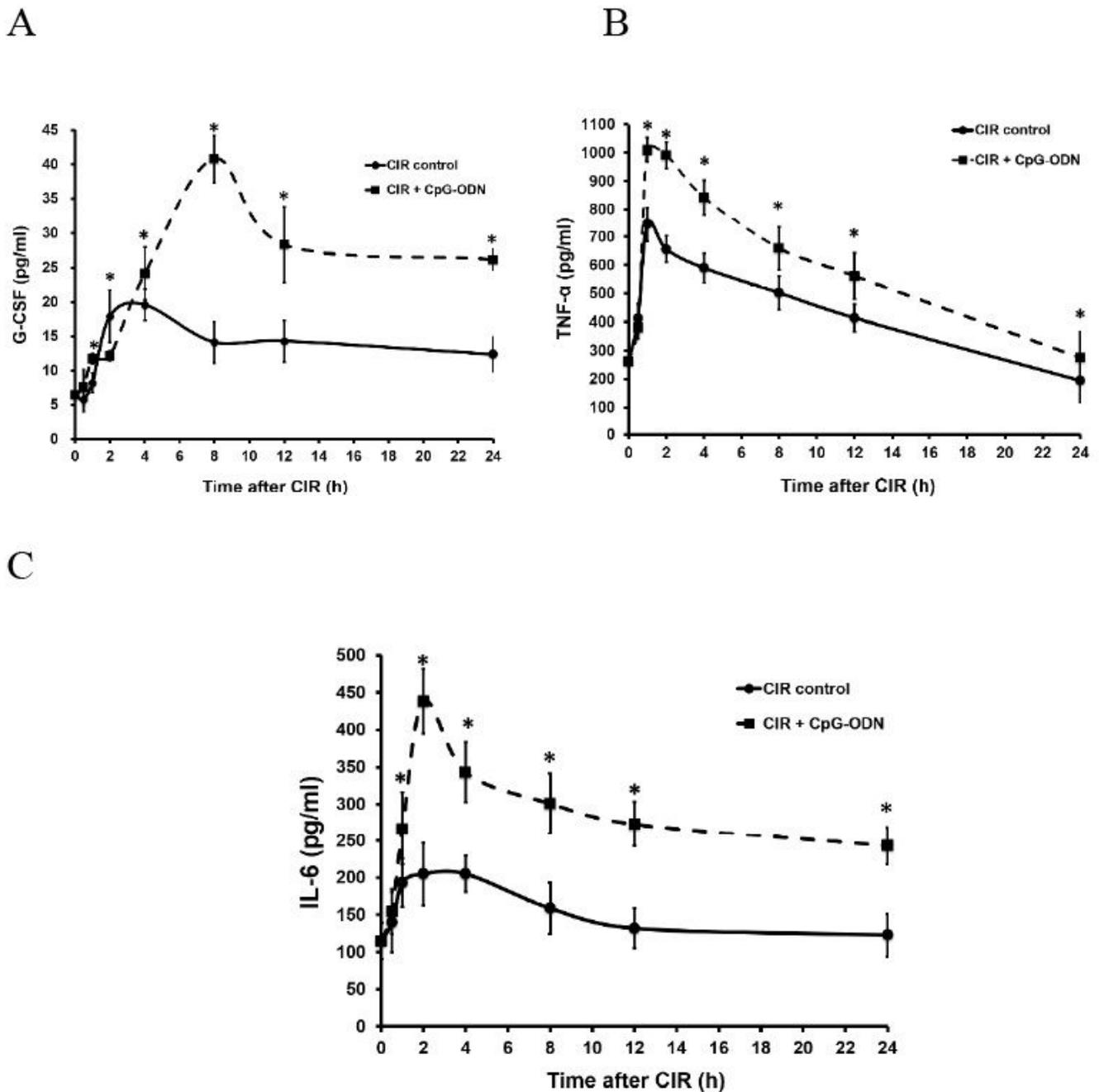


Figure 5

Effect of CpG-ODN of cytokines after CIR. (A) the level of G-CSF was measured within 24 h after CIR; (B) the levels of IL-6 were measured within 24 h after CIR; (C) the level of TNF- α was measured within 24 h after CIR (n=6 mice per group in each of three separate experiments. Data are represented mean \pm SEM *P<0.05 between CIR control group and CIR plus CpG-ODN group by Student's t test).