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ORIGINAL ARTICLE

Reactive oxygen species mediated T lymphocyte abnormalities in an iron-overloaded mouse model and iron-overloaded patients with myelodysplastic syndromes

Jie Chen[1] · Wen-yi Lu[1] · Ming-feng Zhao[1] · Xiao-li Cao[1] · Yan-yu Jiang[1] · Xin Jin[1] · Ping Xu[1] · Ting-ting Yuan[1] · Yu-chen Zhang[1] · Xiao Chai[1] · Juan-xia Meng[1] · Qing Li[1] · Xia Xiao[1] · Juan Mu[1] · De-guan Li[2] · Ai-ping Qi[3]

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Abstract The adverse effects of iron overload have raised more concerns as a growing number of studies reported its association with immune disorders. This study aimed to investigate alterations in the immune system by iron overload in patients with myelodysplastic syndrome (MDS) and an iron-overloaded mouse model. The peripheral blood from patients was harvested to test the effect of iron overload on the subsets of T lymphocytes, and the level of reactive oxygen species (ROS) was also evaluated. The data showed that iron-overloaded patients had a lower percentage of $CD3^+$ T cells and disrupted T cell subsets, concomitant with higher ROS level in lymphocytes. In order to explore the mechanism, male C57Bl/6 mice were intraperitoneally injected with iron dextran at a dose of 250 mg/kg every 3 days for 4 weeks to establish an iron-overloaded mouse model and the blood of each mouse was collected for the analysis of the T lymphocyte subsets and T cell apoptosis. The results showed that iron overload could reduce the percentage of $CD3^+$ T cells and the ratio of Th1/Th2 and Tc1/Tc2 but increase the percentage of regulatory T (Treg) cells and the ratio of CD4/CD8. We also found that iron overload induced the apoptosis of T lymphocytes and increased its ROS level. Furthermore, these effects could be partially recovered after treating with antioxidant *N*-acetyl-L-cysteine (NAC) or iron chelator deferasirox (DFX). Taken together, these observations indicated that iron overload could selectively affect peripheral T lymphocytes and induce an impaired cellular immunity by increasing ROS level.

Keywords Iron overload · T lymphocyte subsets · Reactive oxygen species · Apoptosis

Introduction

Iron is an important component of hemoglobin. It plays an important role in the body's oxygen transport, DNA synthesis, respiration, and cell metabolism [1, 2]. However, a variety of factors such as increased intestinal iron absorption or repeated blood transfusions can lead to an excessive amount of body iron deposits, causing parenchymal organ dysfunction [3–5]. Previous studies have shown that iron overload can directly damage hematopoietic stem/progenitor cells (HSPCs) and bone marrow microenvironment [6, 7]. Immunological abnormalities have also been observed in clinical and experimental iron overload, and disruption of proteins of adaptive immunological system could lead to or exacerbate iron overload [8, 9]. However, the effects of iron overload on the immune system have not been elucidated, and the exact mechanism is still uncertain.

Generally, T cell represents a key sentinel, acting in the innate immune and adaptive immune response. It can be divided into different subsets according to the different surface molecules, intracellular molecules expression,

Jie Chen and Wen-yi Lu share the first authorship.

✉ Ming-feng Zhao
mingfengzhao@sina.com

[1] Department of Hematology, Tianjin First Central Hospital, Tianjin 300192, China

[2] Institute of Radiation Medicine, Academy of Medical Science and Peking Union Medical College, Tianjin 300192, China

[3] Department of Rheumatology and Immunology, Tianjin First Central Hospital, Tianjin 300192, China

cytokine production, and state of immunity [10, 11]. Helper T (Th) cells and cytotoxic T (Tc) cells play a central role in regulating immune responses to the foreign insults, and they response by secreting cytokines that modulate the development and activity of immune effectors. The dominance of either of the Th or Tc subsets correlates directly with the outcome and severity of infection [12, 13].

Numerous studies have shown that the existence of an increase of the cytoplasmic reactive oxygen species (ROS) in iron overload conditions [14–18]. ROS are a group of highly reactive chemicals containing oxygen produced either exogenously or endogenously. Endogenous ROS are produced by the body's own aerobic metabolism, while exogenous ROS are produced by either ionizing radiation or chemicals. They are related to a wide variety of human disorders, such as chronic inflammation, age-related diseases, and cancers [19–21]. ROS are also essential for various biological functions, including cell survival, growth, proliferation, differentiation, and immune response [22, 23]. These molecules function as important participants in immune regulation in distinct suppressive cell population. Now, it is becoming clear that ROS play an important role in the immune system and are intimately involved in various aspects of the immune response [23–27].

In this study, we set out to prove our hypothesis that iron overload could affect the proliferation, development, and survival of peripheral T lymphocytes by increasing ROS production. We first established iron overload, iron chelation, and antioxidative mouse models and then analyzed components of immune cells in the peripheral blood. Our data showed that iron overload resulted in fewer leukocytes, abnormal T lymphocyte subsets, and increased cell apoptosis, which effects could be partially reversed by antioxidant *N*-acetyl-L-cysteine (NAC) or iron chelator deferasirox (DFX).

Materials and methods

Patients and controls

Twenty myelodysplastic syndrome (MDS) patients with iron overload were recruited from Tianjin First Central Hospital. Ten MDS patients without iron overload, matched for age and gender with the examined patients, were enrolled as controls. Their characteristics are listed in Table 1. The diagnosis and classification of MDS were performed according to the World Health Organization (WHO) recommendations. Iron overload was evaluated by serum ferritin and transferrin saturation [28, 29]. Patients with acute leukemia transformation or severe infection, undergoing chemotherapy or cytokine therapy, were excluded from this study. The blood routine and serum ferritin tests were performed before iron chelation therapy. This study was approved by the ethical committee of Tianjin First Central Hospital. Informed consent was obtained from all participants.

Sample collection

The peripheral blood from patients and donors was collected in ethylenediaminetetraacetic acid (K3EDTA) tubes.

Reagents

Anti-mouse-CD3-percp, CD8-PE, CD8-percp, CD3-APC, CD3-percp, CD25-PE, FoxP3-APC, CD4-FITC, B220-APC, IFN-γ-FITC, and IL-4-APC were purchased from BioLegend Company (San Diego, CA, USA); anti-human-CD3-APC, CD8-PE, CD3-percp, CD8-percp, IFN-γ-FITC, IL-4-APC, and CD19-APC were purchased from BD Company (San Diego, CA, USA); iron dextran

Table 1 Patients characteristics at baseline

Characteristic	Patients without iron overload (n = 10)	Patients with iron overload (n = 20)	P value
Age (years), median (range)	65 (51–79)	66.5 (49–82)	0.582
Gender: male, n (%)	4 (40)	11 (55)	0.700
World Health Organization classification, n (%)			1.000
RA	4 (40)	7 (35)	
RAS	3 (30)	8 (40)	
RAEB	3 (30)	5 (25)	
C-reactive protein (mg/L), median (range)	4.35 (1.9–8.9)	4.9 (1.2–9.3)	0.163
Ferritin (ng/mL), median (range)	112.5 (46–463)	1932 (1007–11,159)	<0.001
Transferrin saturation (%), median (range)	37.6 (18.2–58.3)	88.2 (61.4–97.8)	<0.001
WBC ($\times 10^9$/L), median (range)	3.1 (1.3–8.1)	3.6 (1.1–7.6)	0.567
Hemoglobin (g/L), median (range)	80.5 (49–133)	75 (37–124)	0.878
Platelets ($\times 10^9$/L), median (range)	109.5 (18–470)	98 (11–365)	0.708

was purchased from Pharmacosmos A/S Company (Denmark); the ROS staining kit (S0033) and NAC were purchased from the Beyotime Institute of Biotechnology; calcein-acetoxymethyl ester (AM) fluorescent dye was purchased from Sigma-Aldrich Company (USA); DFX was purchased from Novartis; RPMI 1640 was purchased from Gibco Company (USA); annexin/propidium iodide (PI) kit was purchased from BD Biosciences Company; carboxyfluorescein succinimidyl ester (CFSE) was purchased from Molecular Probe Company (USA).

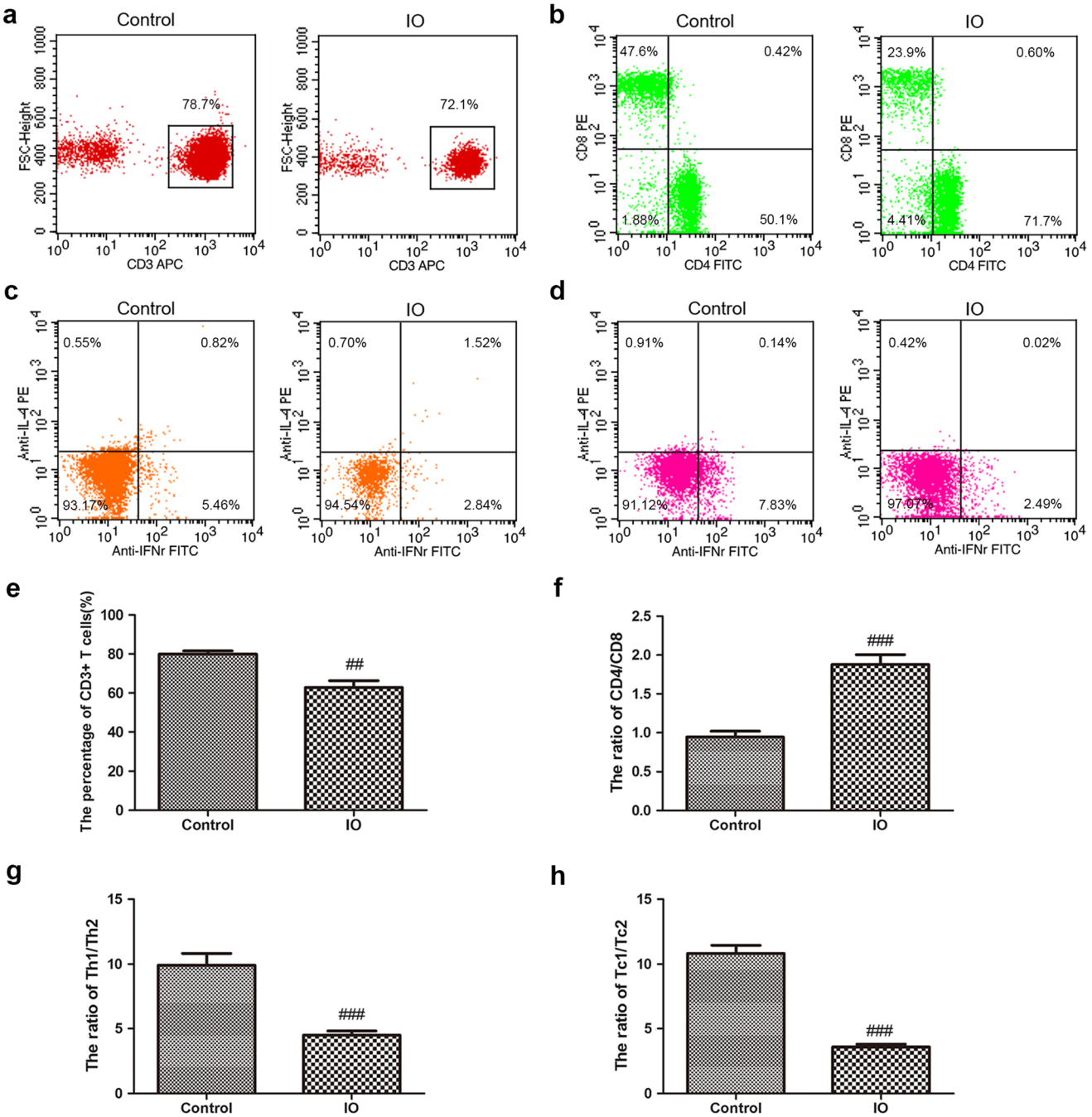

Fig. 1 Abnormal T cell subsets in peripheral blood cells from iron-overloaded patients. **a** A representative FACS analysis of $CD3^+$ T cells in lymphocyte. **b** A representative FACS analysis of $CD4^+$ and $CD8^+$ cells in $CD3^+$ T cells. **c** A representative FACS analysis of $CD4^+$IFN-γ^+Th1 cells and $CD4^+$IL-4^+Th2 cells from an IO patient and a control patient. **d** A representative FACS analysis of $CD8^+$IFN-γ^+Tc1 cells and $CD8^+$IL-4^+Tc2 cells from an IO patient and a control patient. **e** The percentage of $CD3^+$ T cells in lymphocytes was significantly decreased in IO patients compared with control. **f** The ratio of CD4/CD8 cells was increased in IO patients compared with control. The ratios of Th1/Th2 (**g**) and Tc1/Tc2 (**h**) were significantly decreased in IO patients compared with control (control, $n = 10$; IO, $n = 20$). Statistical analyses were performed with Student's t test or Mann–Whitney U test as appropriate. $^{\#\#}P < 0.01$, vs. control; $^{\#\#\#}P < 0.001$, vs. control

Animal and treatment

C57BL/6-Ly-5.1 (Ly5.1) male mice were obtained from the Institute of Peking University Health Science Center (Beijing, China). The mice were bred at the certified animal care facility in the Institute of Radiation Medicine of Peking Union Medical College (PUMC). All animal care and experimental procedures were conducted according to ethical standards of animal use and were approved by the Institutional Committee of Animal Care and Use of PUMC.

All mice were used at approximately 6–8 weeks of age, and the average weight was (20 ± 0.24 mg). Forty male mice were randomly divided into four groups: (a) a control group, (b) an iron-overloaded (IO) group (250 mg/kg), (c) an IO + NAC group, and (d) an IO + DFX group. The IO groups were injected with iron dextran intraperitoneally every 3 days for 4 weeks. The IO + NAC group mice were given NAC in drinking water (40 mM). The water bottles were changed twice per week with a freshly made NAC solution. The IO + DFX group mice received 2.5 mg DFX via gavage twice every 3 days for 4 weeks.

Identification of iron-overloaded mice model

Labile iron pool (LIP) level of lymphocytes was measured by calcein-AM fluorescent dye. The peripheral blood cells were washed twice in PBS and then incubated (5×10^5 cells/well) for 15 min at 37 °C with 0.125 μM calcein-AM and analyzed by a flow cytometer with the mean fluorescence intensity (MFI) calculated by the CellQuest software.

Hematological examination

Peripheral blood (150 μl/mouse) was harvested from the retro-orbital venous plexus and collected in RPMI 1640 containing 2% EDTA. Complete blood counts were obtained using a pocH-100i hematology analyzer (Sysmex, Japan). The cell counts included white blood cells (WBCs), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), the percentages of ANC (ANC%) and ALC (ALC%), red blood cells (RBCs), hemoglobin (HGB), and platelets (PLTs).

Subsets of lymphocytes

Surface staining and intracellular cytokine staining were performed as previously described [26, 27]. Briefly, surface staining was performed in 100 μl PBS with 3% (*v*/*v*) FBS and different antibody cocktails for surface markers (anti-CD3, anti-CD4, anti-CD8, and anti-B220) at room temperature for 15 min. Post-staining for surface markers, the intracellular cytokines were stained using intracellular staining kits from BD Biosciences. Following staining, the cells were washed and run through a BD Calibur flow cytometer (BD Biosciences) and 100,000 cells were sampled.

To detect the ratio of Th1/Th2 and Tc1/Tc2, peripheral blood mononuclear cells (PBMCs) were cultured with PMA (50 ng/mL), ionomycin (500 ng/mL), and GolgiStop (1.0 mL/mL) for 5 h. After surface staining, the cells were incubated with anti-IFN-γ and anti-IL-4

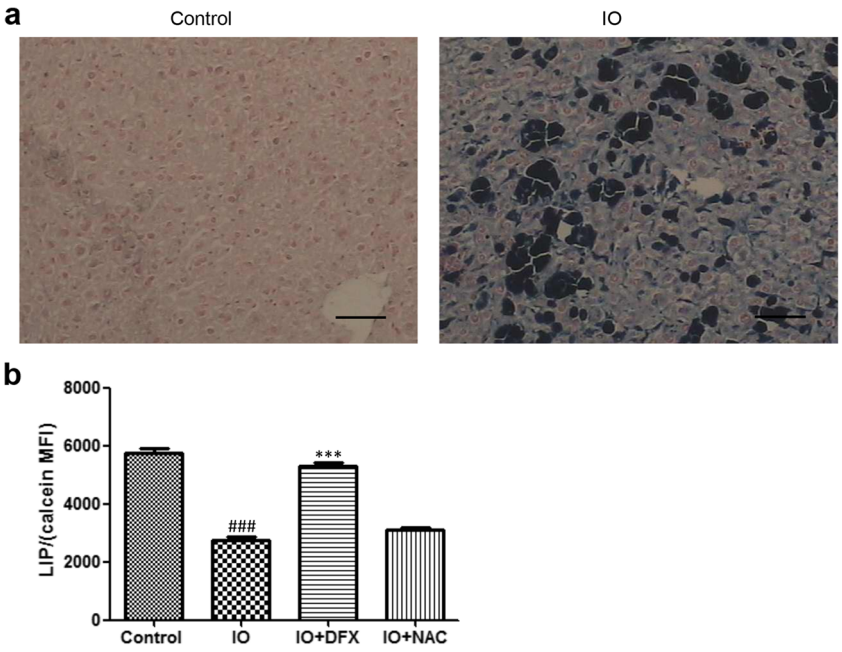

Fig. 2 The establishment of an iron-overloaded mouse model. **a** Iron deposition in the liver from the IO group mice and the controls (Perl's iron staining, *scale bar* = 50 μm). **b** The LIP level of lymphocytes in the IO group was increased compared with the control group, and concomitant treatment with DFX significantly attenuated this effects. Statistical analyses were performed with ANOVA test. The data represent the means ± SD; $n = 10$ per group. $^{###}P < 0.001$, vs. control; $^{***}P < 0.001$, vs. IO

Table 2 Counts of peripheral blood cells ($\bar{x} \pm s$, $n = 10$ in each group)

Groups	WBC ($\times 10^9$/L)	ANC ($\times 10^9$/L)	ALC ($\times 10^9$/L)	ANC%	ALC%	RBC ($\times 10^{12}$/L)	HGB (g/L)	PLT ($\times 10^9$/L)
Control	10.52 ± 0.64	0.99 ± 0.18	9.15 ± 0.40	10.14 ± 0.52	90.81 ± 1.63	8.64 ± 0.27	130.3 ± 9.31	562.7 ± 41.54
IO	9.36 ± 0.79	1.14 ± 0.06	8.55 ± 0.59#	11.87 ± 1.38#	86.48 ± 1.91#	7.73 ± 0.50#	109.6 ± 4.81#	458.2 ± 46.7#
IO + DFX	9.96 ± 0.46	1.10 ± 0.12	9.01 ± 0.38	11.01 ± 0.84	88.43 ± 1.26*	8.35 ± 0.23*	117.0 ± 4.85*	462.7 ± 23.71
IO + NAC	10.52 ± 0.67	1.07 ± 0.09	9.03 ± 0.22	10.82 ± 0.97	89.47 ± 0.61*	8.54 ± 0.42*	112.8 ± 6.01	456.6 ± 21.04

WBC white blood cell, *ANC* absolute neutrophil count, *ALC* absolute lymphocyte count, *RBC* red blood cell, *HGB* hemoglobin, *PLT* platelet

# $P < 0.05$, vs. control; * $P < 0.05$, vs. IO

for 40 min at room temperature. In order to detect the percentage of regulatory T (Treg) cells, after surface staining, PBMCs were labeled with FoxP3 for 30 min at room temperature. Finally, cells were washed and analyzed by a flow cytometer (BD Calibur).

Assess the apoptosis of T cells

Apoptosis were determined using an annexin V-FITC/PI apoptosis detection kit (BD Biosciences) according to the manufacturer's instruction. Briefly, the peripheral blood cells were washed twice with PBS and then washed once with annexin binding buffer. Samples were incubated in annexin V-FITC at routine temperature for 10 min in the dark and then labeled with PI at routine temperature for 5 min. Finally, cells were washed and analyzed by a flow cytometer (BD Calibur).

Flow cytometer measurements of ROS

ROS staining was performed using a ROS staining kit following the manufacturer's protocol. The peripheral blood cells were incubated with 10 μM DCFH-DA and anti-CD3 antibody in a humidified atmosphere of 5% CO_2 in air at 37 °C for 15 min. The levels of intracellular ROS were analyzed by measuring the MFI of 2′,7′-dichlorofluorescein (DCF) using a flow cytometer.

Statistical analyses

All experiments were performed at least three times. Analyses of flow cytometry data were conducted using CellQuest software. Continuous variables were presented as mean ± standard deviation (SD) or median. They were compared by a parametric (Student's t test, ANOVA) or non-parametric (Mann–Whitney U, Kruskal–Wallis) test as appropriate according to each variable distribution. Categorical variables were compared using Fisher's exact tests. Differences were considered to be statistically significant at $P < 0.05$. The statistical analyses were performed with the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, USA) or SPSS software (version 19.0).

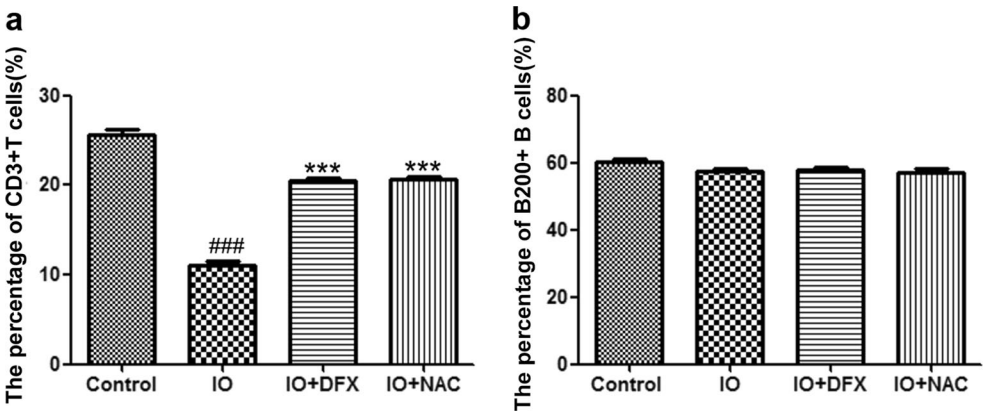

Fig. 3 Iron overload decreased the percentage of T cells. **a** The percentage of $CD3^+$ T cells in lymphocytes was decreased in IO group mice compared with control, and it was increased by DFX and NAC treatment. **b** Iron overload has no effect on the percentage of $B220^+$ B cells in mice peripheral blood lymphocytes. Statistical analyses were performed with ANOVA test as appropriate. The data represent the means ± SD; $n = 10$ per group. ### $P < 0.001$, vs. control; *** $P < 0.001$, vs. IO

Results

Lymphocyte immunophenotyping, T cell subsets, and ROS level in iron-overloaded patients

We found that LIP levels of lymphocytes from MDS patients with iron overload were higher than those of patients without iron overload (1068.83 ± 64.27 vs. 2047.32 ± 228.94, $P < 0.001$). The percentage of $CD3^+$ T cells and the ratio of Th1/Th2, Tc1/Tc2, and CD4/CD8 were evaluated in controls and 20 MDS patients with iron overload. Compared to the controls, the percentage of $CD3^+$ T cell and the ratio of Th1/Th2 and Tc1/Tc2 were significantly lower in iron-overloaded patients, whereas those of CD4/CD8 were higher (Fig. 1). We

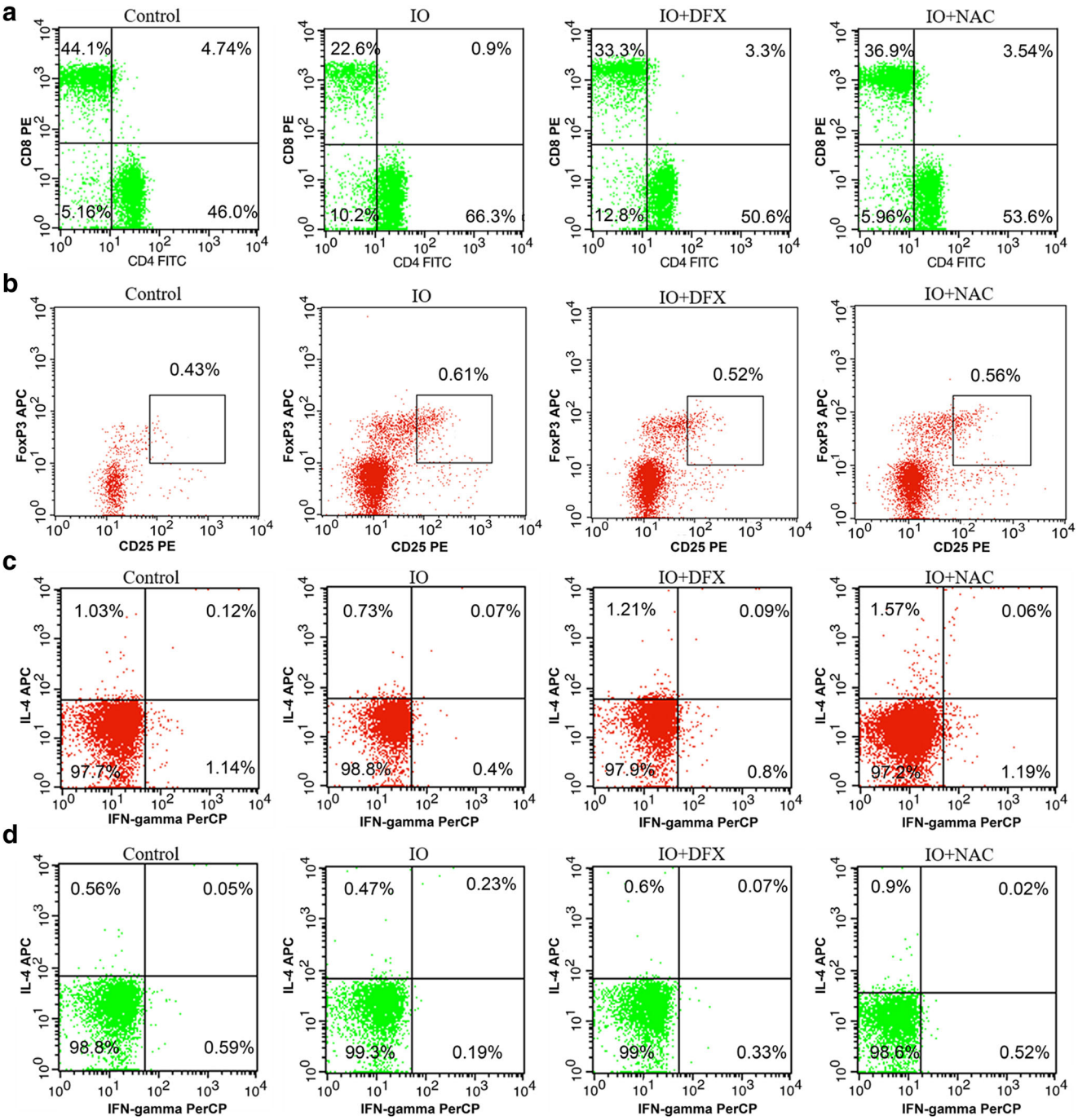

Fig. 4 Flow cytometry analysis of the subsets of T cells from different groups mice. **a** A representative FACS analysis of $CD4^+$ and $CD8^+$ cells in $CD3^+$ T cells. **b** A representative FACS analysis of CD25highFoxp3$^+$ Treg cells in $CD4^+$ cells from different group mice. **c** A representative FACS analysis of $CD4^+$IFN-γ^+Th1 cells and $CD4^+$IL-4^+Th2 cells from different group mice. **d** A representative FACS analysis of $CD8^+$IFN-γ^+Tc1 cells and $CD8^+$IL-4^+Tc2 cells from different group mice

also found that ROS levels of $CD3^+$ T lymphocytes from iron-overloaded patients were higher than control (107.49 ± 10.58 vs. 29.09 ± 3.04, $P < 0.001$), which indicated that ROS might participate in the pathogenesis of iron overload.

The establishment of an iron-overloaded mouse model

According to our previous studies, we injected mice intraperitoneally with iron dextran for 4 weeks to establish an iron-overloaded mouse model, and iron deposits in the liver were easily observed [6, 7] (Fig. 2a). To confirm the iron accumulation in the immune system, LIP levels of lymphocytes were evaluated (Fig. 2b). Our results demonstrated that this experimental murine model reflected an iron-overloaded pathogenic condition.

Iron overload affects peripheral T lymphocytes

Compared to the control, significantly lower numbers of lymphocytes were observed in the peripheral blood of experimental mice after receiving iron dextran for 4 weeks ($P < 0.05$). The platelet, RBC counts, and HGB levels were also decreased after iron overload. Although the frequency of ANC was increased after iron overload, the absolute number of ANC did not show a significant difference between different groups. After treating with NAC, the percentage of lymphocytes in the IO group mice was significantly increased, while the absolute number of lymphocytes did not show a significant difference (Table 2).

T lymphocytes play a pivotal role in the host immune defense against infection and tumor. A flow cytometer was used to examine the effects of iron overload exposure on T cells. Our results showed that after 4 weeks of iron dextran treatment, the frequency of T cells in the peripheral blood was significantly decreased (Fig. 3a). Compared to the IO group, the frequency of T cells in the peripheral blood from IO + NAC and IO + DFX groups was increased (Fig. 3a), but there was no significant difference in the frequency of B cells in the peripheral blood between different groups at these time points (Fig. 3b).

Effects of iron overload on the T lymphocyte subsets

We examined the subsets of T lymphocyte in different groups, and our results showed that the ratio of CD4/CD8 and the percentage of Treg cells were substantially increased after iron overload (Figs. 4 and 5a, b). However, the ratio of Th1/Th2 and Tc1/Tc2 was decreased, which suggested the balance between type 1 and type 2 cytokine-producing cells was disrupted after excessive iron deposition (Figs. 4 and 5c, d). Furthermore, these effects were partly reversed after treating with NAC and DFX.

Iron overload induces the apoptosis of $CD4^+$ and $CD8^+$ T cells

We further tested the effect of iron overload on the apoptosis of $CD4^+$ T and $CD8^+$ T cells. As shown in Fig. 6, the apoptosis

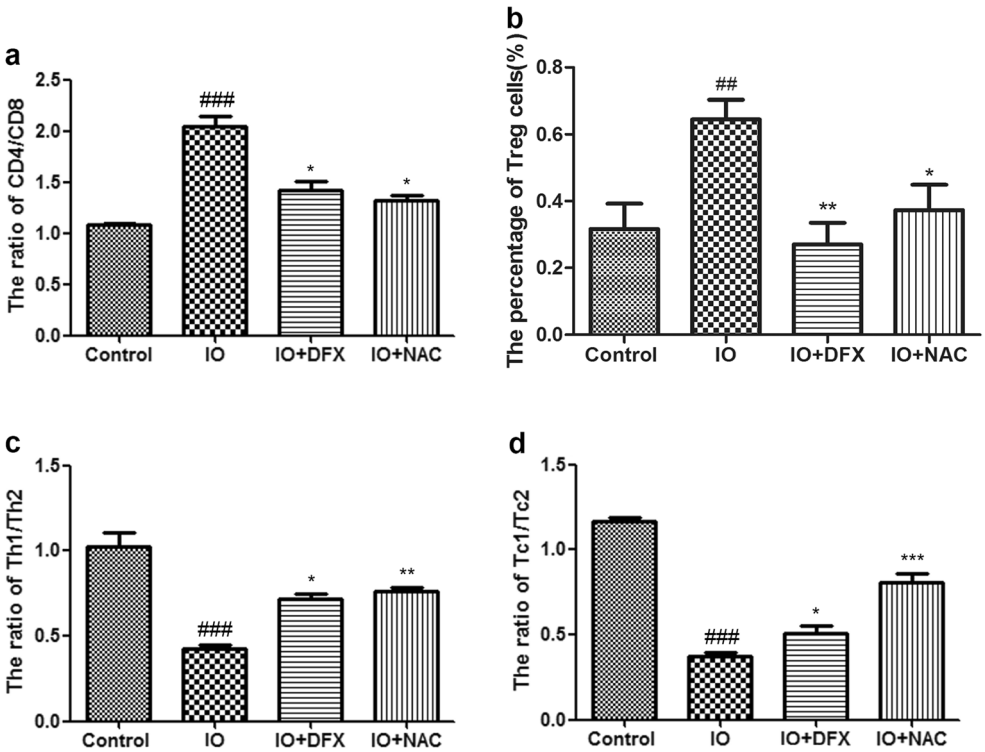

Fig. 5 Iron overload affects the subsets of T cells. Compared to the controls, iron overload increased the ratio of CD4/CD8 (**a**) and the frequency of Treg cells (**b**), which effect could be reversed by treating with NAC or DFX. The ratios of Th1/Th2 (**c**) and Tc1/Tc2 (**d**) in the peripheral blood of IO group mice were significantly decreased. Statistical analyses were performed with Kruskal–Wallis or ANOVA test as appropriate. $n = 10$ per group. $^{\#\#}P < 0.01$, vs. control group; $^{\#\#\#}P < 0.001$, vs. control group; $^{*}P < 0.05$,vs. IO group; $^{**}P < 0.01$, vs. IO group; $^{***}P < 0.001$, vs. IO group

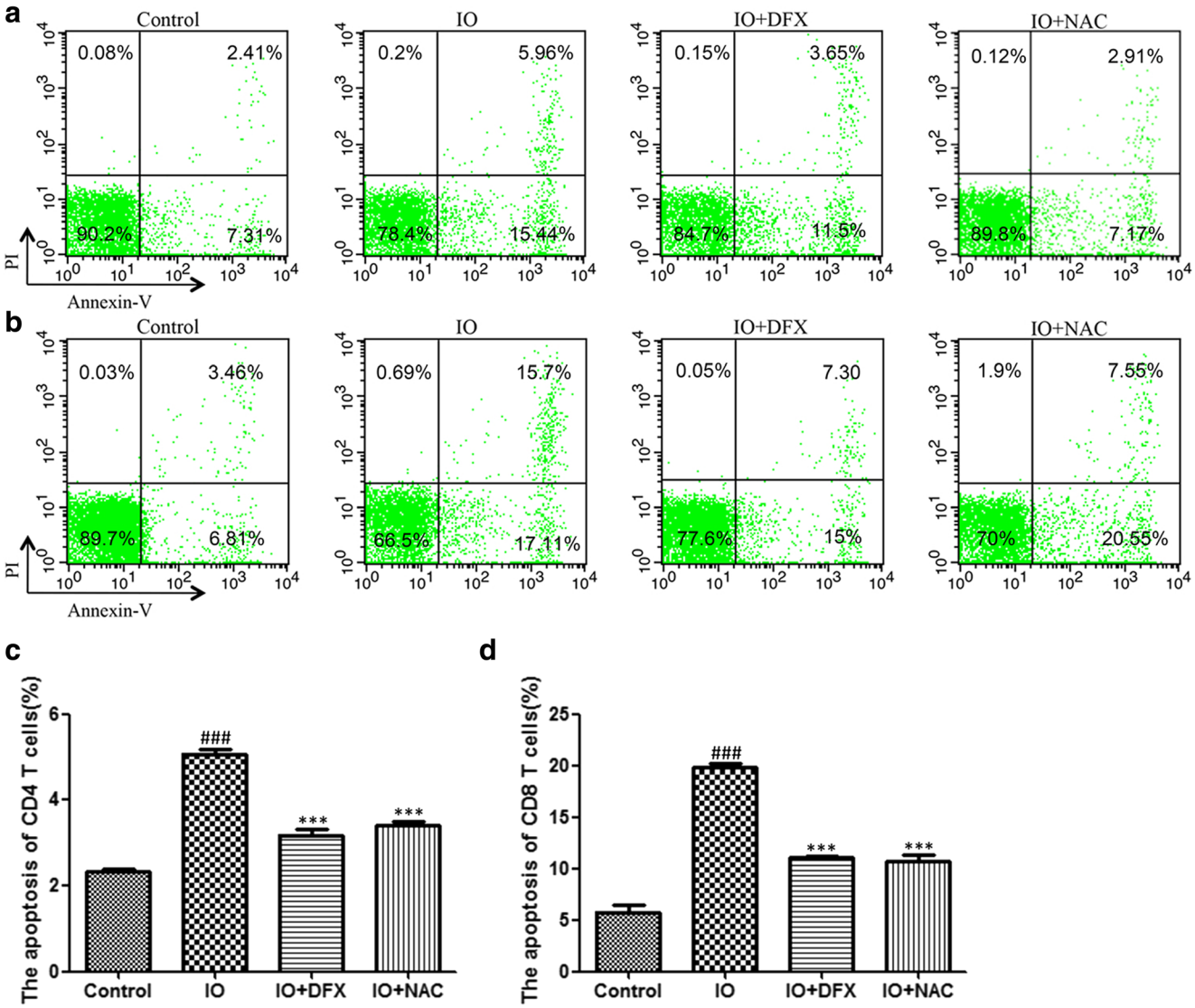

Fig. 6 Iron overload induces the apoptosis of $CD4^+$ and $CD8^+$ T cells. A representative FACS analysis of the apoptosis of $CD4^+$ T cells (**a**) and $CD8^+$ T cells (**b**) from different group mice. Compared to the controls, iron overload increased the apoptosis of $CD4^+$ T cells (**c**) and $CD8^+$ T cells (**d**), and the ratios were decreased by treating with NAC or DFX. Statistical analyses were performed with Kruskal–Wallis or ANOVA test as appropriate. $n = 10$ per group. $^{###}P < 0.001$, vs. control group; $^{*}P < 0.05$, vs. IO group; $^{**}P < 0.01$, vs. IO group; $^{***}P < 0.001$, vs. IO group

of $CD8^+$ T cells and $CD4^+$ T cells (annexin V^+PI^+) in iron-overloaded mice was significantly higher than that in control group mice, and DFX or NAC treatment could reverse this effect.

Iron overload increases ROS production in T cells

It is well known that iron is the main catalyst of ROS in an organism, and a growing body of evidence demonstrates that there is a positive correlation between ROS and LIP levels. In this study, we investigated the levels of ROS in $CD3^+$ T cells in iron-overloaded mice. There was a significant increase in ROS levels in the IO group compared to the controls, which could be reversed by DFX and NAC treatment (Fig. 7).

Discussions

It is well known that a link exists between cell-mediated immunity and iron metabolism [13]. Indeed, both iron deficiency and iron overload can exert subtle effects on the immune status [30, 31]. Our data showed that iron overload could reduce the percentage of T cells, especially $CD8^+$ T cells. Moreover, the ratios of Th1/Th2 and Tc1/Tc2 were dramatically reduced among iron-overloaded patients and iron-overloaded mice, while the percentage of Treg cells was increased. In addition, it also resulted in ROS accumulation in T cells and induced T cell apoptosis. Importantly, these effects could be partly reversed after treating with an antioxidant agent or iron chelator.

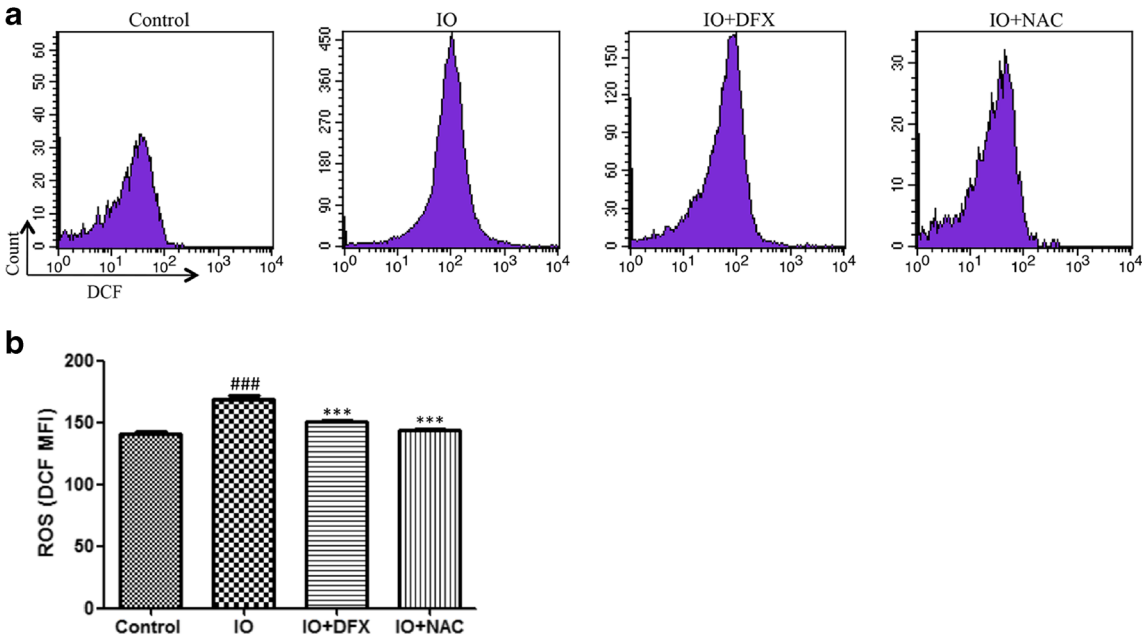

Fig. 7 Iron overload increases ROS production of T cells. **a** A representative FACS analysis of the level of ROS in $CD3^+$ T cells from different group mice. **b** Compared to the controls, IO increased the levels of ROS in $CD3^+$ T cells, and the elevated ROS level could be suppressed by NAC and DFX. Statistical analyses were performed with ANOVA test. The data represent the means ± SD; $n = 10$ per group. $^{###}P < 0.001$, vs. control group; $^{***}P < 0.001$, vs. IO group

Recent studies suggest that iron overload can lead to immune disorders and increase the risk of bacterial and fungal infections, but the specific effects and mechanism of action remain unclear [32, 33]. In our preliminary studies, we successfully established the iron-overloaded mouse model and showed the inhibitory effects on peripheral blood cell counts [7]. On the basis of the iron-overloaded model, we assessed whether iron overload injured the immune system. We first counted the peripheral blood cells, and our results showed that iron overload resulted in reduced lymphocytes. Furthermore, iron overload significantly decreased the frequency of $CD3^+$ T cells compared with the control group, while the frequency of $B220^+$ cells was not altered.

We further examined the effects of iron overload on T cell subsets and T cell apoptosis in our iron-overloaded mouse model. It reduced the percentage of T cells, especially $CD8^+$ T cells, but increased the percentage of Treg cells and the ratio of CD4/CD8. These results were consistent with previously published results, which showed decreased $CD8^+$ numbers and increased CD4/CD8 ratios in hereditary hemochromatosis patients [34, 35]. Furthermore, the lower number of $CD8^+$ T lymphocyte in hereditary hemochromatosis is associated with decreased $CD8^+$ effector memory, which indicated an inability of $CD8^+$ lymphocyte to differentiate into mature phenotype [35]. Thus, iron overload might affect the ability of the host to mount an effective response of antigen-specific $CD8^+$ T cells to infection. This also supports the hypothesis that iron overload increased the risk of bacterial and fungal infections. However, other studies have shown that iron overload decreased $CD4^+$ T cells and increased $CD8^+$ T cells, which resulted in a reduced CD4/CD8 T cell ratio [31, 36]. These apparently inconsistent observations may be related to different conditions used in different studies, including iron dosage, model of administration, and treatment duration time.

$CD4^+$ Th cells are divided into Th1 and Th2 cells based on their cytokine secretion patterns. $CD8^+$ T cells are vital for the antiviral cellular immune response which directly kill the target cells infected by viruses, bacteria, and other pathogens. Similar to $CD4^+$ Th cells, $CD8^+$ T cells can be divided into Tc1 and Tc2 subsets. Tc1 and Th1 secrete IFN-γ, IL-2, IL-12, and several other cytokines which mediate cellular immunity. Similarly, Tc2 and Th2 secrete IL-4, IL-5, IL-6, IL-10, and several other cytokines which mediate humoral immunity. Our data showed that iron-overloaded mice had a decreased ratio of Th1/Th2 and Tc1/Tc2, which indicated a defective type 1 immune response [37]. Type 1 immune response, which is involved in cellular immunity, is the main immune response following infection. Thus, our results may explain the increased susceptibility of iron overload patients to infection.

As the need for repeated RBC transfusion, MDS patients often have secondary iron overload. To confirm our findings in clinical samples, we examined the lymphocyte subsets of MDS patients with or without iron overload.

We found that patients with iron overload had a lower percentage of $CD3^+$ T cells and disrupted T cell subsets, which was consistent with the results of animal experiment. However, our study has some limitations. Several studies revealed that the lymphocytes of MDS patients tend to exhibit numerical and qualitative abnormalities. Thus, it is difficult to separate the MDS-related factor and iron overload. Although we confirmed our conclusion in animal experiment, further study including non-MDS patients is still needed.

On the basis of the previous data, we tried to explain the mechanism of iron overload in regulating the immune cells. It was reported that T lymphocytes can take up the toxic non-transferrin-bound iron (NTBI) in the plasma through some specific transporter. Increased intracellular NTBI can catalyze the production of ROS through Fenton's reaction and subsequently stimulate a series of cell signaling pathways to induce cell apoptosis [38–42]. Our data also show that ROS level in $CD3^+$ T cells was significantly increased. It is known that ROS play an important regulatory role in the activation and differentiation of T cells [43–45]. Therefore, we speculated that iron overload may induce T lymphocyte abnormality by increasing the ROS production. We used a ROS scavenger NAC to evaluate whether the effects of excessive iron on T lymphocytes could be reversed. Our data showed that disrupted T cell subsets and apoptosis could be partially reversed after treating iron-overloaded mice with NAC. These results indicate that iron overload may regulate T lymphocytes by increasing ROS production.

Taken together, the present study showed that iron overload could induce T lymphocyte subset abnormalities and increase T cell apoptosis, suggesting that the aberrant T cell responses may increase the probability of infection after iron overload. Furthermore, our results also revealed that ROS was an important mediator in this process, which provided a new strategy to treat iron overload-induced immune abnormalities. Further studies are needed to investigate the mechanisms by which iron overload increases the ROS level.

Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All procedures performed in studies involving animals were in accordance with the guidelines for the care and use of animals and the ethical standards of the Institutional Committee of Animal Care and Use of PUMC. Informed consent was obtained from all participants for being included in this study.

Conflict of interest The authors declare that they have no conflict of interest.

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