J Mol Med (2007) 85:1263–1270
DOI 10.1007/s00109-007-0235-9

ORIGINAL ARTICLE

The different effects of indirubin on effector and $CD4^+CD25^+$ regulatory T cells in mice: potential implication for the treatment of autoimmune diseases

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Received: 27 March 2007 / Revised: 15 May 2007 / Accepted: 29 May 2007 / Published online: 17 July 2007

Abstract $CD4^+CD25^+$ regulatory T (Treg) cells play an essential role in the induction and maintenance of peripheral self-tolerance. Indirubin, a traditional Chinese medicine, was clinically used in the treatment of chronic myelocytic leukemia as well as some autoimmune diseases, including Alzheimer's disease, diabetes, and so on. The effects of indirubin on $CD4^+CD25^+$Treg cells, which play a critical role in controlling autoimmunity, have not been addressed. In the present study, we observed the cell levels, phenotypes, and immunoregulatory function of $CD4^+CD25^+$Treg cells in indirubin-treated mice. Treatment with indirubin significantly enhanced the ratios of $CD4^+CD25^+$Treg cells or $CD4^+CD25^+Foxp3^+$Treg cells to $CD4^+$T cells in peripheral blood, lymph nodes, and spleens ($P<0.01$ compared with control mice). $CD4^+CD25^+Foxp3^+$Treg cells to CD4 single positive cells in the thymi of indirubin-treated mice were significantly higher than those in control mice. Furthermore, splenic $CD4^+CD25^+$Treg cells in indirubin-treated mice showed immunosuppressive ability on the immune response of T effector cells to alloantigens or mitogen as efficiently as the control $CD4^+CD25^+$Treg cells in vitro. The present studies indicate that $CD4^+CD25^+$Treg cells are more resistant to indirubin than effector T cells in vivo. The selectively enhanced $CD4^+CD25^+$Treg cell levels by indirubin made host to be more favorable for immune tolerance induction, which opened one possibility for indirubin to treat autoimmune diseases.

Keywords $CD4^+CD25^+$Treg cells · Foxp3 · Indirubin · Autoimmune diseases

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Abbreviations

Treg	regulatory T cells
AIDS	acquired immune deficiency syndrome
DTH	delayed-type hypersensitivity reaction
CTLA4	cytotoxic T-lymphocyte-associated protein 4
FCM	flow cytometry
FITC	fluorescein isothiocyanate
Foxp3	forkhead box protein 3
GITR	glucocorticoid-induced tumor necrosis factor receptor
LNs	lymph nodes
MFI	median fluorescence intensity
MLR	mixed lymphocytes reaction
PBMCs	peripheral blood mononuclear cells
PE	phycoerythrin
PI	propidium iodide

Introduction

$CD4^+CD25^+$ regulatory T (Treg) cells are crucial in maintaining the homeostasis of the immune system and immunologic self-tolerance. It was shown that $CD4^+CD25^+$Treg cells could regulate effector T cells, antigen-presenting cells (APCs), B cells, and natural killer (NK) cells directly or indirectly via cell-to-cell contact or soluble factors in a dose-dependent manner [1, 2]. Impairing $CD4^+CD25^+$Treg cell number or activity is closely related to the occurrence of autoimmune diseases, such as insulin-dependent diabetes mellitus, autoimmune hemolytic anemia, systemic lupus erythematosus, collagen-induced arthritis, multiple sclerosis, and experimental allergic encephalomyelitis [3–6]. Foxp3 is a recently identified transcription factor which is specifically expressed in $CD4^+CD25^+$Treg cells [7–9]. The expression of Foxp3 is essential for the development and endowing immunosuppressive function of $CD4^+CD25^+$Treg cells [10].

Indirubin is a traditional Chinese medicine which is clinically used in the treatment of chronic myelocytic leukemia [11–15]. It has been demonstrated that indirubin derivatives have the potent antiproliferative and inducing apoptosis activity in various human cancer cells [16–18]. Indirubin has the ability to inhibit numerous important protein kinases including cyclin-dependent protein kinases (CDKs) [19], glycogen synthetase kinase 3 [19–21], c-Src kinase, and c-Jun NH2-terminal kinase [16, 22].

Indirubin inhibits inflammatory reactions in delayed-type hypersensitivity in a mouse model [23]. Indirubin and its derivatives may have an anti-inflammatory effect by suppressing the release of cytokines, including IFN-γ and IL-6 by mouse splenocytes [23]. The effects of indirubin on effector T cells and $CD4^+CD25^+$Treg cells have not been addressed so far. In the present study, we tried to investigate the effects of indirubin on the cell numbers, phenotype, and function of $CD4^+CD25^+$Treg cells in mice. Interestingly, the treatment with indirubin significantly enhanced the percentages of functional $CD4^+CD25^+Foxp3^+$Treg cells in the periphery, indicating the different sensitivity to indirubin of $CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$Treg cells in mice. This study supports that indirubin may be a potential reagent for immune tolerance induction.

Materials and methods

Mice Six- to eight-weeks-old female BALB/c and C57BL/6 mice were obtained from Institute of Genetics and Development, Chinese Academy of Sciences (Beijing, China). All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Reagents Indirubin was obtained from Cal Biochem. A 10 mM solution of indirubin was prepared in the vehicle containing Sodium CMC(C-5013 highviscosity, Sigma-Aldrich) and polysorbate 80 dimethyl sulfoxide, stored as small aliquots at 4°C. Indirubin was administered intraperitoneally (IP) at a dose of 50 mg/kg/day for 4 weeks. Solution including 0.2% CMC and 0.25% polysorbate 80 were administered to the control mice. At least three independent experiments were performed for each assay.

Monoclonal antibodies (mAbs) and reagents The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb (RM4-5; rat IgG2a), FITC-labeled anti-mouse CD8 mAb (53-6.7; rat IgG2a), FITC-labeled rat anti-mouse CD25 mAb (7D4; IgM), FITC-conjugated anti-mouse GITR mAb (DTA-1, rat IgG2b), phycoerythrin (PE)-labeled rat anti-mouse CD4 mAb, PE-labeled anti-mouse CD8α mAb (53-6.7; rat IgG2a), Cy5-labeled anti-mouse CD25 mAb, Cychrome-labeled anti-mouse CD4 mAb.

In addition, PE-labeled anti-mouse Foxp3 mAb (FJK-16s) and its staining kit were obtained from eBiosciences (San Diego, CA). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, MD) in our laboratory.

Mitomycin C ($C_{15}H_{18}N_4O_5$) was obtained from Kyowa Hakko (Tokyo, Japan). The culture medium was RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM 2-ME (Sigma, St. Louis, MO). [^{3}H] thymidine was purchased from China Institute of Atomic Energy (Beijing, China).

Cell preparation Mouse peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient (Sigma, St. Louis, MO) described before [24]. After indirubin treatment for 1 month, thymus, spleen, and lymph nodes (LNs including cervical, inguinal, and axillary LNs) were harvested. Single-cell suspensions were prepared by grinding the tissues with the plunger of a 5-ml disposable syringe and were then suspended in RPMI1640 medium. Splenocytes were treated with a hemolysis buffer (17 mM Tris–HCl and 140 mM NH_4Cl, pH 7.2) to remove red blood cells as described before [25].

Purification of mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ splenocytes $CD4^+CD25^+$Treg cells were isolated from mouse splenocytes and were enriched using a $CD4^+CD25^+$Treg Cells Isolation Kit with MidiMACS™ Separator according to the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany).

Briefly, splenocytes were incubated with a Biotin-antibody cocktail against CD8α (Ly2), CD11b (Mac-1), CD45R (B220), CD49B (DX5), and Ter-119, for 20 min at 4°C, and then with microbead-conjugated antibiotin mAb (Bio318E7.2) and PE-labeled anti-CD25 mAb. The cell suspension was loaded on a LD column, which is placed in magnetic field of a MACS Separator. The remaining fraction in the column is the enriched $CD4^+$cells. For the isolation of $CD4^+CD25^+$cells, the PE-labeled $CD25^+$cells in the enriched $CD4^+$cells fraction were magnetically labeled with anti-PE MicroBeads. The magnetically labeled $CD4^+CD25^+$cells were enriched from the $CD4^+$cells fraction by MACS sorting. The purity for $CD4^+CD25^+$Treg cells was more than 95% and the purity for $CD4^+CD25^-$T cells was more than 98% as determined by flow cytometry (FCM) in each experiment. Purified cells were suspended in complete RPMI 1640 medium.

Immunofluorescence staining and flow cytometry PBMCs, lymphocytes from LNs, splenocytes, or thymocytes were incubated with 2.4G2 to block FcRs and then incubated with an optimal concentration of fluorochrome-labeled mAbs for 30 min at 4°C in the dark. Cells were washed three times and resuspended by FCM buffer (PBS with 0.1% BSA and 0.1% NaN_3). At least 10,000 cells were assayed using a FASCalibur flow cytometry (Becton Dickinson, CA), and data were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA). In some experiments, nonviable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained nonspecifically with the control mAb from staining in the same dot-plot region with the anti-mouse mAbs.

For the intracellular Foxp3 staining, cells were incubated with Cy-chrome-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs first. After washing, these cells were then fixed and stained with anti-mouse Foxp3 mAb, according to the instruction offered by the manufactory (eBioscience, San Diego, CA).

The proliferation of T cells to Con A $CD4^+CD25^-$T cells (4×10^4 cells/well) from BALB/c mice were cultured in U-bottom, 96-well plate with BALB/c spleen cells as accessory cells (4×10^4 cells/well, pretreated with 30 μg/ml mitomycin c at 37°C for 30 min), 3 μg/ml Con A and the indicated numbers of BALB/c $CD4^+CD25^+$Treg cells isolated from either control or indirubin-treated mice for 72 h at 37°C, 5%CO_2. For the last 12 h, 1 μCi [^{3}H] thymidine was added to each well [25]. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland) and were dried in an oven. Samples were determined in a Liquid Scintillation Analyzer (Beckmon Instruments, America). Values are presented as counts per minute (cpm) of triplicate wells.

Mixed lymphocyte reaction (MLR) BALB/c splenic $CD4^+CD25^+$Treg cells were isolated from either control or indirubin-treated mice as described above. BALB/c $CD4^+CD25^-$T cells were used as responder T cells. BALB/c or C57BL/6 splenocytes, which were pretreated with mitomycin C at the concentration of 30 μg/ml at 37°C for 30 min, were used as stimulator cells. In general, 8×10^4 responder cells (BALB/c $CD4^+CD25^-$T cells) and 8×10^4 stimulator cells (C57BL/6 splenocytes) per well in RPMI1640 medium supplemented with 10% FCS were added in 96-well round-bottomed plates [24]. $CD4^+CD25^+$Treg cells were subsequently added to each well in different ratios to $CD4^+CD25^-$T cells. Cells were cultured at 37°C and 5% CO_2 for 5 days. For the last 16 h, 1 μCi [^{3}H] thymidine (185GBq/mmol; Atomic Energy Research Establishment, China) was added. Cells were harvested with an automatic cell harvester. The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer. Values are expressed as counts per minute (cpm) of triplicate wells.

Statistical analysis All data are presented as the mean+SD. Student's unpaired *t* test for comparison of means was used to compare groups. $P<0.05$ was considered to be statistically significant.

Results

Significantly enhanced percentages of $CD4^+CD25^+$Treg cells in the periphery of indirubin-treated BALB/c mice

To assess the effect of indirubin on $CD4^+CD25^+$Treg cells in the periphery, we firstly compared the cell number and

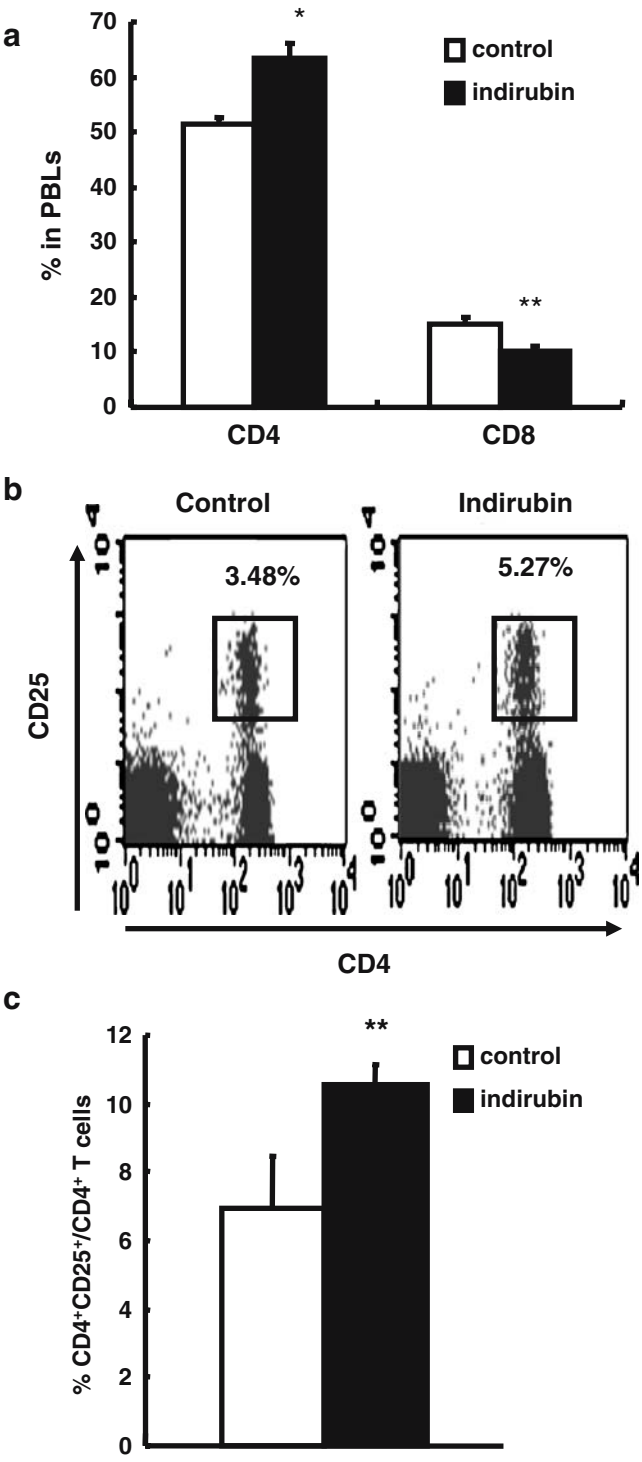

Fig. 1 The enhanced percentages of $CD4^+CD25^+$T cells in the peripheral blood of indirubin-treated BALB/c mice. BALB/c mice were treated with indirubin as described in the "Materials and methods." After treatment with indirubin for 4 weeks, PBMCs were stained with FITC-labeled anti-CD4 mAb and PE-labeled anti-CD25 mAb and assayed by FCM. **a** The percentages of $CD4^+$ or $CD8^+$T cells in the control and indirubin-treated mice. **b** One representative for the staining with FITC-labeled anti-CD4 mAb and PE-labeled anti-CD25 mAb. *Numbers* in the *dot plot* indicate the percentage of $CD4^+CD25^+$Treg cells in PBMCs. **c** The percentages of $CD4^+CD25^+$T cells in $CD4^+$T cells in control and indirubin-treated mice. Data are shown as mean±SD (N=5). *Asterisk*, $P<0.05$, *double asterisk*, $P<0.01$ compared with the control mice. The data are one representative of three separated experiments with similar data

percentage of $CD4^+CD25^+$Treg cells in the periphery blood of indirubin-treated BALB/c mice to those in control mice. By 4 weeks after the treatment with indirubin, the total number of PBMCs did not show detectable changes (data not shown). However, significantly increased percentages of $CD4^+$T cells and decreased percentages of $CD8^+$T cells in the peripheral blood of indirubin-treated mice were observed (Fig. 1a; $P<0.05$ compared with control mice). The percentages of B cells in the peripheral blood were not significantly altered after the treatment with indirubin (data not shown). Interestingly, the percentages of $CD4^+CD25^+$Treg cells were significantly enhanced in indirubin-treated mice (Fig. 1b and c).

After 4 weeks of indirubin injection, the percentage of $CD4^+$T cells in lymph nodes (LNs; axillary, inguinal, and cervical regions) was significantly decreased (Fig. 2a; $P<0.01$). However, the percentages of B cells and NK cells in LNs of indirubin-treated mice did not show significant difference compared with the control mice (data not shown). Consistent with the peripheral blood, the percentage of

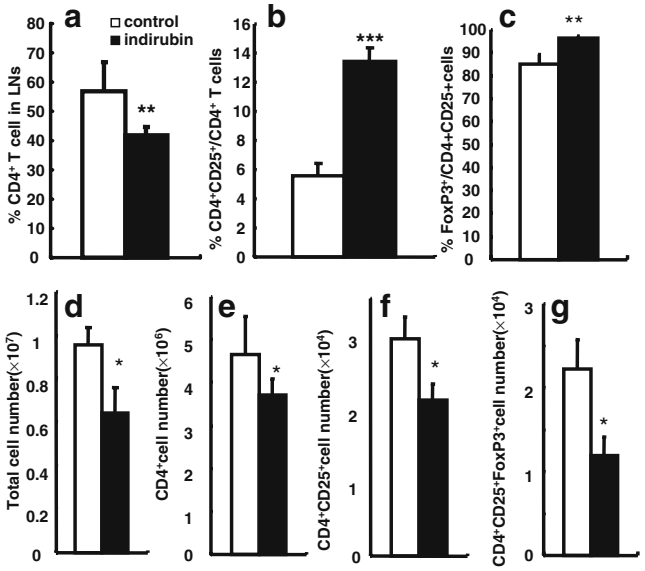

Fig. 2 The percentage and total cell numbers of $CD4^+CD25^+$T cells in LNs of mice treated with or without indirubin. The cells in LNs of control or indirubin-treated mice were analyzed for the expression of CD4 and CD25 by FCM. The proportion of $CD4^+$T cells (**a**), the ratio of $CD4^+CD25^+$T cells to $CD4^+$T cells (**b**), and the percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+CD25^+$T cells (**c**) in LNs of control and indirubin-treated mice were shown. The total cell numbers (**d**), $CD4^+$T cells (**e**), $CD4^+CD25^+$T cells (**f**), and $CD4^+CD25^+Foxp3^+$T cells (**g**) in LNs of control and indirubin-treated mice. Data are shown as mean±SD (N=5). *Asterisk*, $P<0.05$; *double asterisk*, $P<0.01$; *triple asterisk*, $P<0.001$ compared with the control mice. The data are one representative of three separated experiments with similar data

$CD4^+CD25^+$Treg cells in $CD4^+$T cells was remarkably increased after indirubin treatment (Fig. 2b; $P<0.01$). Foxp3, as a unique marker for naturally occurring $CD4^+CD25^+$Treg cells, is predominantly expressed in the $CD4^+CD25^+$Treg cells [7, 26]. Therefore, the expression of Foxp3 in $CD4^+CD25^+$T cells in these mice was detected using three-color staining FCM as reported previously [27], the percentage $CD4^+CD25^+Foxp3^+$Treg cells in $CD4^+CD25^+$Treg cells was increased after indirubin treatment (Fig. 2c; $P<0.01$), although the total cell number, the $CD4^+$T cell number, $CD4^+CD25^+$Treg cells, $CD4^+CD25^+Foxp3^+$Treg cells were significantly decreased compare to those of nontreated mice (Fig. 2d–g; $P<0.05$).

The size and weight of the spleen were significantly reduced in mice treated with indirubin for 4 weeks (data not shown). Consistently, the number of $CD4^+$ T cells was obviously decreased (Fig. 3a; $P<0.01$), while that of $CD4^+CD25^+$Treg cells showed no significant difference in indirubin-treated mice (Fig. 3b). The percentage of $CD4^+CD25^+$Treg cells in $CD4^+$T cells was significantly increased after treatment with indirubin (Fig. 3c; $P<0.01$). Consistent with the observation in LNs, enhanced percentage of $CD4^+CD25^+Foxp3^+$T cells in $CD4^+CD25^+$Treg cells was detected in mice treated with indirubin (Fig. 3d and e).

Normal immunosuppressive ability of $CD4^+CD25^+$Treg cells in indirubin-treated mice

To further assess whether indirubin has the ability to alter the function of $CD4^+CD25^+$Treg cells, we compared the immunosuppressive effects of $CD4^+CD25^+$Treg cells sorted by bead separation from mice treated with or without indirubin in an in vitro culture system [24]. As shown in Fig. 4, both $CD4^+CD25^+$Treg cells from mice treated with indirubin or not showed markedly suppressive function on the proliferation of $CD4^+CD25^-$T cells induced by allogeneic antigens or mitogen Con A without detectable significant difference (Fig. 4a and b). These data suggested

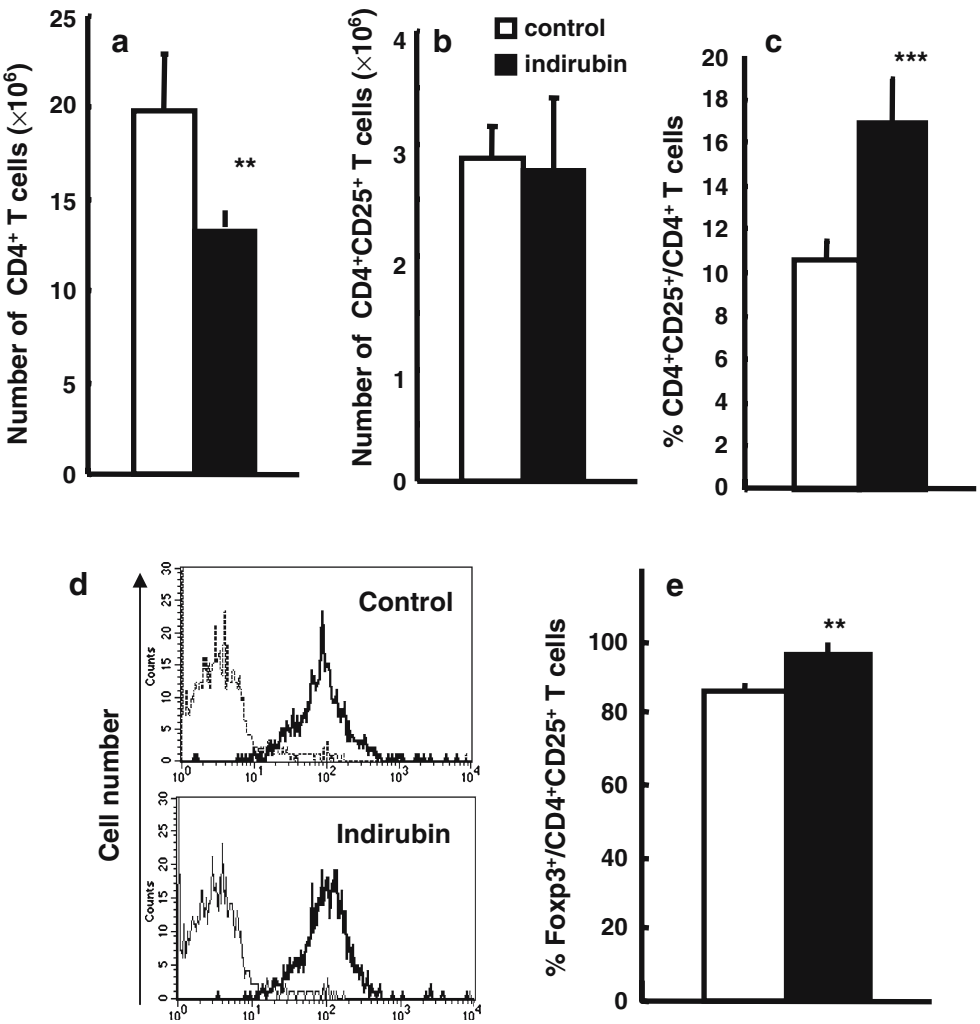

Fig. 3 The enhanced proportion of $CD4^+CD25^+$T cells in $CD4^+$T cells in spleens of mice treated with indirubin. Splenocytes were isolated from BALB/c mice after treatment with indirubin for 4 weeks. The cells were analyzed for the expressions of CD4, CD25, and Foxp3 by FCM. The numbers of $CD4^+$ T cells (**a**), $CD4^+CD25^+$T cells (**b**) in spleens of control and indirubin-treated mice were summarized. The significantly enhanced ratio of $CD4^+CD25^+$ T cells to $CD4^+$T cells in indirubin-treated mice (**c**). One representative for Foxp3 staining in gated $CD4^+CD25^+$T cells in control and indirubin-treated mice (**d**). The percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+CD25^+$T cells in control and indirubin-treated mice were shown (**e**). Results were shown as mean±SD, which is one representative of three independent experiments ($N=5$ in each group). *Asterisk*, $P<0.05$; *double asterisk*, $P<0.01$; *triple asterisk*, $P<0.001$ compared with the control mice

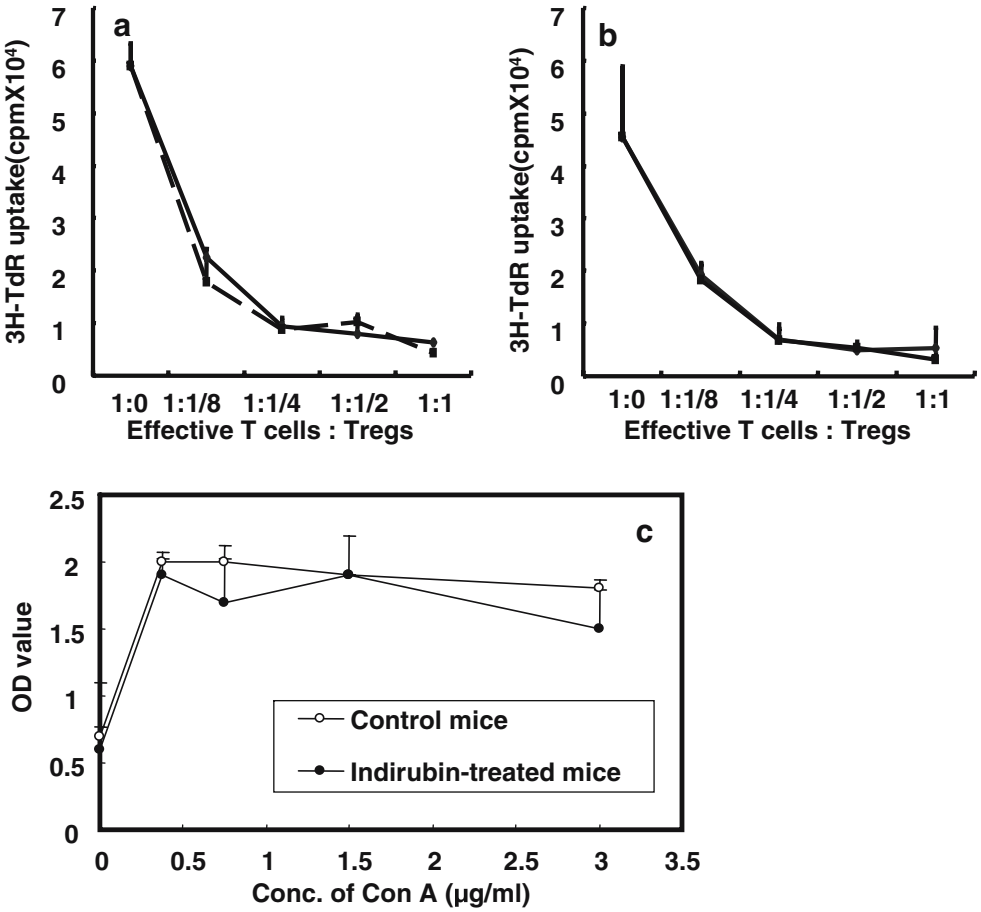

Fig. 4 The immune function of $CD4^+CD25^+$Treg cells and $CD4^+CD25^-$T effector cells in indirubin-treated mice. The immunosuppressive ability of $CD4^+CD25^+$Treg cells was determined using the traditional in vitro assay. Both $CD4^+CD25^+$Treg cells in control and indirubin-treated mice significantly inhibited the proliferation of effector $CD4^+CD25^-$T cells to mitogen Con A (**a**) or to alloantigen (**b**). No significance was observed between $CD4^+CD25^+$Treg cells from control and indirubin-treated mice at the same dose ($P>0.05$). **c** The unchanged response to Con A of $CD4^+CD25^-$T cells separated from indirubin-treated mice in vitro. Data are shown as mean±SD, which is one representative of two independent experiments

that indirubin did not alter the immunosuppressive function of $CD4^+CD25^+$Treg cells as determined by the standard assays. On the other hand, $CD4^+CD25^-$T cells separated from mice treated with indirubin for 4 weeks did show normal proliferation response to Con A stimulation in vitro compared with those from nontreated mice (Fig. 4c).

Significantly enhanced percentages of $CD4^+CD8^-CD25^+$Treg cells in the thymus after indirubin treatment

It was well known that the majority of natural $CD4^+CD25^+$Treg cells were produced in the thymus [1, 8, 25, 28]. We thus observed the percentages of $CD4^+CD8^-CD25^+$Treg cells in the thymi of indirubin-treated mice. After the treatment with indirubin for 4 weeks, the size and weight of the mouse thymus were obviously withered (data not shown). The total cell number of thymocytes was remarkably decreased (Fig. 5a; $P<0.01$). Although the percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, and other thymocyte subsets did not show significant changes in indirubin-treated mice (Fig. 5b and d), the percentages of $CD4^+CD8^-CD25^+$Treg cells in $CD4^+CD8^-$T cells or $CD4^+Foxp3^+$Treg cells in $CD4^+CD8^-$T cells in indirubin-treated mice were significantly higher than those of the control mice (Fig. 5e and f; $P<0.05$).

Discussion

Studies on the effects of indirubin on immune cells were limited. In the present study, we observed that indirubin could remarkably decrease the cell numbers of total immune cells and $CD4^+$T cells in thymus, spleen, and LNs in mice, indicating the immunosuppressive ability of indirubin. The percentages of B cells and NK cells in spleens and LNs were not significantly altered after the treatment with indirubin for 4 weeks. Importantly, the treatment with indirubin significantly enhanced the percentages of immunocompetent $CD4^+CD25^+$Treg cells in the periphery (blood, spleen, and LNs) and central (thymus) immune system in mice. The balance between effector T cells and $CD4^+CD25^+$Treg cells predominately control the direction and quality of the host immune response. Thus, the ideal immunosuppressive drugs for the treatment of autoimmune

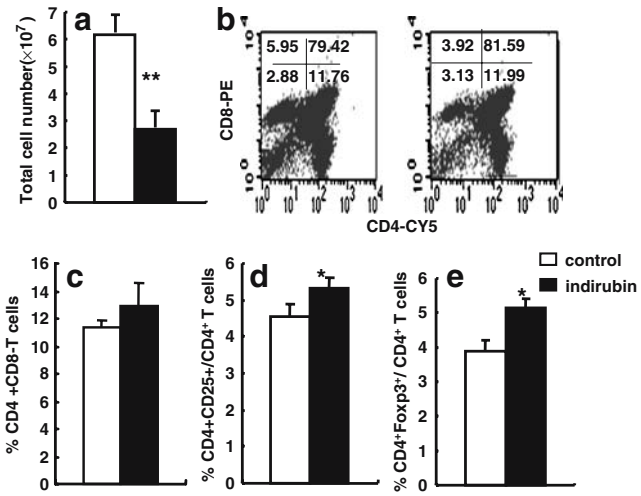

Fig. 5 The significantly enhanced percentages of $CD4^+CD25^+$Treg cells in the thymus of indirubin-treated mice. Thymocytes were stained with PE-Cy5-labeled anti-CD4 mAb, PE-labeled anti-CD25 mAb, and FITC-labeled anti-CD8 mAb, and analyzed by FCM after injection of indirubin for 4 weeks. **a** The total cell numbers of thymus in control and indirubin-treated mice are shown. **b** One representative for the staining with CY5-labeled anti-CD4 mAb and PE-labeled anti-CD8 mAb is shown. *Numbers* in the *dot plot* indicate the percentages of $CD4^+CD8^-$, $CD8^+CD4^-$, $CD4^+CD8^+$, or $CD4^-CD8^-$ thymocytes in control and indirubin-treated mice. The percentages of $CD4^+CD8^-$T cells (**c**), $CD4^+CD25^+$T cells in $CD4^+CD8^-$T cells (**d**), and $CD4^+Foxp3^+$T cells in $CD4^+CD8^-$T cells (**e**) are summarized. *Asterisk*, $P<0.05$; *double asterisk*, $P<0.01$ compared with the control mice. Results were shown as mean±SD ($N=5$), which is one representative of three independent experiments

diseases or graft reaction should have strong inhibiting ability on effector T cells but not on $CD4^+CD25^+$Treg cells so that their treatment makes hosts be more susceptible to immune tolerance induction. The different effects of indirubin on $CD4^+CD25^-$T effector and $CD4^+CD25^+$Treg cells opened the possibility for indirubin to be one of the favorable reagents to treat autoimmune disease or transplant tolerance induction.

Indirubin significantly enhanced the percentages of $CD4^+CD25^+$Treg cells in peripheral lymphoid tissues in mice, while the total numbers of $CD4^+CD25^+$ Treg cells were decreased. The different effects of indirubin on $CD4^+$T cells in different tissues (PBL, spleens, and LNs) and on different immune cell subsets (T, B, and NK cells) were observed. However, the reasons for the distinct effects of indirubin are not addressed at the present study. Consistent with the peripheral immune system, the treatment with indirubin significantly decreased the cell number of thymocytes in mice, but the percentages of $CD4^+CD25^+$Treg cells or $CD4^+Foxp3^+$Treg cells in the thymus were significantly enhanced. Thus, the newly developed $CD4^+CD25^+$Treg cells in the thymus were more resistant to indirubin than other thymocyte subsets.

It has been well known that Foxp3 is predominantly expressed in the $CD4^+CD25^+$Treg cells and Foxp3 expression in naïve T cells convert these cells to a regulatory T cell phenotype functionally similar to naturally occurring $CD4^+CD25^+$Treg cells [7, 26]. Foxp3 is widely recognized as a specific marker for $CD4^+CD25^+$Treg cells [29]. Significantly higher percentages of $CD4^+CD25^+Foxp3^+$ Treg cells in peripheral blood, spleen, LNs were detected in indirubin-treated mice, supporting indirubin selectively and relatively enhanced a $CD4^+CD25^+$T cell subset with regulatory function. Furthermore, the efficient immunosuppressive function of $CD4^+CD25^+$Treg cells separated from indirubin-treated mice indicates that indirubin does not bother the immune function of $CD4^+CD25^+$Treg cells. In our studies, identical phenotype of $CD4^+CD25^+$Treg cells in indirubin-treated and untreated mice were observed, except of lower percentages of $CD62L^+$ cells in $CD4^+CD25^+$Treg cells in indirubin-treated mice than those in control mice (data not shown). On the other hand, the $CD4^+CD25^-$T effector cells separated from indirubin-treated mice showed normal response to Con A stimulation in vitro. However, our preliminary data indicated that indirubin could directly inhibit the immune response to Con A of mouse T cells in vitro (Zhang AJ and Zhao Y, unpublished data). The direct role of indirubin on different T cell subsets and the related mechanisms request determination.

In summary, the treatment with indirubin significantly decreased the total cell numbers of immune cells in thymus, spleen, and LNs in mice. Indirubin could significantly enhance the percentages of functional $CD4^+CD25^+Foxp3^+$ Treg cells. These data may have potential impacts on clinical application of indirubin in the treatment of patients with autoimmune diseases, grafts, or cancers.

Acknowledgements The authors wish to thank Ms. Jing Wang and Ms. Jianxia Peng for their expertise technical assistance, Ms. Qinghuan Li for her excellent laboratory management. This work was supported by grants from 973 Program 2006 CB 503803, National Natural Science Foundation of China (no. 30300312, no. 30470742, no. 30579779, and no. 30600259; M.H.), and Key Clinical Research Project of Public Health Ministry of China 2004-2006 (M.H.), National Natural Science Foundation for Distinguished Young Scholars (C03020504, Y.Z.), 100 Quality Vocational Colleges of Chinese Academy of Sciences (2003-85, Y.Z.), and the Scientific Research Foundation for the Returned Overseas Chinese Scholars of State Education Ministry (2005-546, Y.Z.).

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