Splenic Stroma-Educated Regulatory Dendritic Cells Induce Apoptosis of Activated CD4 T Cells via FasL-Enhanced IFN-γ and Nitric Oxide

Xiongfei Xu, Hai Yi, Zhenhong Guo, Cheng Qian, Sheng Xia, Yushi Yao and Xuetao Cao

*J Immunol* published online 28 December 2011
http://www.jimmunol.org/content/early/2011/12/28/jimmunol.1101696

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/12/29/jimmunol.1101696.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Splenic Stroma-Educated Regulatory Dendritic Cells Induce Apoptosis of Activated CD4 T Cells via FasL-Enhanced IFN-γ and Nitric Oxide

Xiongfei Xu,*†,1 Hai Yi,*† Zhenhong Guo,*† Cheng Qian,* Sheng Xia, † Yushi Yao,* and Xuetao Cao*†

Stromal microenvironments of bone marrow, lymph nodes, and spleen have been shown to be able to regulate immune cell differentiation and function. Our previous studies demonstrate that splenic stroma could drive mature dendritic cells (DC) to further proliferate and differentiate into regulatory DC subset that could inhibit T cell response via NO. However, how splenic stroma-educated regulatory DC release NO and whether other molecules are involved in the suppression of T cell response remain unclear. In this study, we show that splenic stroma educates regulatory DC to express high level of FasL by TGF-β via ERK activation. The findings, that inhibition of CD4 T cell proliferation by regulatory DC required cell-to-cell contact and FasL deficiency impaired inhibitory effect of regulatory DC, indicate that regulatory DC inhibit CD4 T cell proliferation via FasL. Then, regulatory DC have been found to be able to induce apoptosis of activated CD4 T cells via FasL in caspase 8- and caspase 3-dependent manner. Interestingly, FasL on regulatory DC enhanced IFN-γ production from activated CD4 T cells, and in turn T cell-derived IFN-γ and NO could reduce the apoptosis induction. Therefore, our results demonstrated that splenic stroma-educated regulatory DC induced T cell apoptosis via FasL-enhanced T cell IFN-γ and DC NO production, thus outlining a new way for negative regulation of T cell responses and maintenance of immune homeostasis by regulatory DC and splenic stromal microenvironment. The Journal of Immunology, 2012, 188: 000–000.

Received for publication June 15, 2011. Accepted for publication November 27, 2011.

This work was supported by National Natural Science Foundation of China Grants 30771963, 30872303, and 31100630; Shanghai Rising-Star Program Grant 10QA1408300; and National Key Basic Research Program of China Grant 2009CB521902.

Address correspondence and reprint requests to Prof. Xuetao Cao, National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, 800 Xiangyang Road, Shanghai 200433, China. E-mail address: caoxt@immunol.org

The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; DC, dendritic cell; dTIDC, differentiated DC; ESSC, endothelial-like splenic stromal cell; iNOS, inducible NO synthase; L-NAME, nitro-L-arginine methyl ester; mDC, mature DC; MLN, mesenteric lymph node; siRNA, small interfering RNA; Treg, regulatory T.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/S16.00
disorders attract much attention. Now, regulatory DC can be generated in vitro by coculture with stromal cells or immunosuppressive agents, including IL-10 or TGF-β, or other substances such as vitamin D receptor ligands, vasoactive intestinal peptide, and thymic stromal lymphopoietin (5, 17–21). Recently, it was demonstrated that the surface protein TIGHT could bind poliovirus receptor on human DC and induce IL-10 production to suppress T cell activation, exerting immunosuppressive effects (22). Galectin-1 could endow DCs with regulatory potential via producing IL-27 and then inducing IL-10–producing T cells (23). The data indicate that many factors, known or unknown, are involved in the induction of regulatory DC. Regulatory DC can suppress T cell activation and proliferation via inducing Treg cell expansion or generation, or T cell anergy (5, 6). Because tolerogenic or regulatory DC can induce Treg cell expansion or generation, they are loosely designated as regulatory DC. In addition to the induction of Treg cell expansion or generation, regulatory DC may also regulate directly the fate of effector CD4 T cells via other manners such as induction of T cell anergy, which is being widely investigated. It has been shown that splenic stromal cells can regulate DC differentiation and function (21, 24, 25). Our previous data showed that splenic stroma could drive mDC or hematopoietic stem cells to differentiate into regulatory DC subset, designated as differentiated DC (diffDC), which strongly inhibited T cell response via NO production (17, 18), indicating the important role of splenic microenvironment in the negative control of T cell response. However, how NO is induced from regulatory DC, whether splenic stroma-educated regulatory DC can regulate T cell response through other molecules, and whether regulatory DC can induce apoptosis of activated CD4 T cells remain unclear.

In this study, we reported that splenic stroma-educated regulatory DC could express high level of FasL and induce apoptosis of activated CD4 T cells via FasL. Stroma-derived TGF-β was responsible for the induction of high-level FasL expression on regulatory DC via ERK activation. Importantly, FasL–enhanced IFN-γ from activated CD4 T cells could in turn induce high-level NO production from regulatory DC, being involved in apoptosis induction of activated CD4 T cells. Thus, our results indicated that splenic stroma could educate regulatory DC to highly express FasL to induce T cell apoptosis, and FasL-enhanced IFN-γ and NO contributed to induction of T cell apoptosis. The data indicated a new way for negative regulation of T cell response and maintenance of immune homeostasis by regulatory DC and splenic stromal microenvironment.

Materials and Methods

Mice and reagents

C57BL/6 mice (B6) were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China) or from C57BL/6J-transgenic DO11.10 mice or OT-2 mice. B6.SJL-Ptprca Pep3b Boy1 mice (CD45.1 mice), IFN-γ R1-deficient mice (C57BL/6 background), IFN-γ-deficient mice (C57BL/6 background), and B6Smn.C3H-FasL(B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in specific pathogen-free conditions. All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University. Recombinant mouse GM-CSF and IL-4 were purchased from PeproTech (London, U.K.). Mouse rIFN-γ, anti-FasL mAb, anti-mouse IFN-γ mAb, anti-mouse TGF-β mAb, and isotype control mAbs were from R&D Systems (Minneapolis, MN). Fluorescence-conjugated mAbs to CD4, F4/80, CD11b, CD11c, 1A-8, TRAIL, B7-1H, B7–DC, annexin V, biotin-conjugated mAb to FasL, and PE-conjugated streptavidin (Sigma–Aldrich, St. Louis, MO). Anti–phospho-ERK mAb, anti-ERK polyclonal Ab, caspase 1 inhibitor (Z-LETD-FMK), and caspase 8 inhibitor (Z-DEV-FMK) were from Calbiochem (San Diego, CA). RPMI 1640 medium (PAA Laboratories, Linz, Austria) was supplemented with 10% FBS (PAA Laboratories), 2 mM l-glutamine, 1% sodium pyruvate, and 2 × 10−5 M 2-ME, all from Sigma-Aldrich. IFN-γ ELISA kit was from R&D Systems. OV A323–339-specific TCR-transgenic C57BL/6J mice (B6) were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). OV A323–339-specific TCR-transgenic R1-deficient mice (C57BL/6J background), IFN-γ R1-deficient mice (C57BL/6 background), and their respective H-2Kb homozygous secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-caspase inhibitor (Z-VAD-FMK), caspase 3 inhibitor (Z-IEET-FMK), and caspase 8 inhibitor (Z-DEV-FMK) were from Calbiochem (San Diego, CA). RPMI 1640 medium (PAA Laboratories, Linz, Austria) was supplemented with 10% FBS (PAA Laboratories), 2 mM l-glutamine, 1% sodium pyruvate, and 2 × 10−5 M 2-ME, all from Sigma-Aldrich. OV A323–339-specific TCR-transgenic R1-deficient mice (C57BL/6J background), IFN-γ R1-deficient mice (C57BL/6 background), and their respective H-2Kb homozygous secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-caspase inhibitor (Z-VAD-FMK), caspase 3 inhibitor (Z-IEET-FMK), and caspase 8 inhibitor (Z-DEV-FMK) were from Calbiochem (San Diego, CA). RPMI 1640 medium (PAA Laboratories, Linz, Austria) was supplemented with 10% FBS (PAA Laboratories), 2 mM l-glutamine, 1% sodium pyruvate, and 2 × 10−5 M 2-ME, all from Sigma-Aldrich. OV A323–339-specific TCR-transgenic R1-deficient mice (C57BL/6J background), IFN-γ R1-deficient mice (C57BL/6 background), and their respective H-2Kb homozygous secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-caspase inhibitor (Z-VAD-FMK), caspase 3 inhibitor (Z-IEET-FMK), and caspase 8 inhibitor (Z-DEV-FMK) were from Calbiochem (San Diego, CA). RPMI 1640 medium (PAA Laboratories, Linz, Austria) was supplemented with 10% FBS (PAA Laboratories), 2 mM l-glutamine, 1% sodium pyruvate, and 2 × 10−5 M 2-ME, all from Sigma-Aldrich. OV A323–339-specific TCR-transgenic R1-deficient mice (C57BL/6J background), IFN-γ R1-deficient mice (C57BL/6 background), and their respective H-2Kb homozygous secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-caspase inhibitor (Z-VAD-FMK), caspase 3 inhibito
Assay for IFN-γ and NO

The supernatants from coculture system on day 4 were detected for IFN-γ by ELISA kit and NO by measurement of the nitrite concentration with the Griess assay (18).

Western blotting

Cells were lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor mixture, and protein concentrations of the extracts were measured by bichinchoninic acid assay (Pierce). Proteins were subjected to NaDodSO₄-PAGE (SDS-PAGE), transferred onto nitrocellulose membranes, and then blotted, as described previously (26).

Assay for the inhibitory function of regulatory DC in vivo

OVA 323–339-specific TCR-transgenic splenic CD4 T cells (2 × 10⁶) from OT-2 × CD45.1 F1 hybrid mice were i.v. injected into C57BL/6J mice (2 × 10⁶ cells per mouse). After 24 h, 2 × 10⁶ OVA 323–339-pulsed mDC together with or without the same number of OVA 323–339-pulsed diffDC, IFN-γ R1-deficient diffDC (IFN-γ R1⁻/⁻ diffDC), or Fas-deficient diffDC (gld diffDC) were transferred i.p. into the mice. OVA 323–339-pulsed DC were prepared by pulsing mDC or diffDC with 2 μM OVA 323–339 peptide at 37°C for 6 h, and then DC were washed with cold PBS three times. In some experiments, L-NAME (20 mg/kg body weight) was injected i.p. into mice together with transfer of mDC. After 5 d, single-cell suspensions of spleen and mesenteric lymph nodes (MLN) were double stained with CD4-PerCP Cy5.5 and CD45.1 allophycocyanin for analysis of flow cytometry, and the ratio of CD45.1⁺ cells to CD4 cells was calculated, as described previously (27).

Assay for FasL expression on splenic DC subsets in vivo

OVA 323–339-specific TCR-transgenic splenic CD4 T cells (2 × 10⁶) from OT-2 × CD45.1 F1 hybrid mice were i.v. injected into C57BL/6J mice (2 × 10⁶ cells per mouse). After 24 h, 100 μg OVA protein was transferred i.p. into the mice. Then splenic mononuclear cells were prepared from the mice 5 d later and stained with F4/80 FITC, I-A⁺/⁻ PE, CD11b PerCP Cy5.5, CD11c PE Cy7, FasL, and streptavidin allophycocyanin. After gating F4/80 FITC-negative cells, the FasL expression on mDC (CD11c⁺/⁻ I-A⁺/⁻ cells) and diffDC (CD11c⁺/⁻ I-A⁺/⁻/CD11b⁺/⁻ cells) was assessed by flow cytometry and analyzed by Software FlowJo (Tree Star).

RNA interference

diffDC were transfected with INTERFERin small interfering RNA (siRNA) transfection reagent (Polyplus-Transfection SA), according to the manufacturer’s instructions. iNOS-specific siRNA (sense, 5′-GGCCAAAACAUAACAGAAGATT-3′; antisense, 5′-UCAUCCUUGAUUUGGCGTCT-3′) were used to suppress iNOS expression; nonsense sequence (sense, 5′-UUUCUGCAAGCAGUUGUAG-3′; antisense, 5′-AACGUGACACG-UUCGGAGAATT-3′) was used as a negative control siRNA. siRNA sequences were designed and synthesized by GenePharma RNAi.

Statistical analysis

Data were compared using the Student t test. A p value < 0.05 was considered statistically significant.

Results

Splenic stroma-educated regulatory DC induce apoptosis of activated CD4 T cells

Our previous data showed that splenic stroma could drive mDC to differentiate into a new regulatory DC subset (diffDC), which strongly inhibited T cell response via NO production (18). To demonstrate whether splenic stroma-educated regulatory DC can induce apoptosis of activated CD4 T cells to exert their inhibitory effects, we cocultured diffDC with the activated CD4 T cells whose activation was initiated by mDC/Ag, and then observed the apoptosis of the activated CD4 T cells. As shown in Fig. 1A and 1B, diffDC could significantly increase the percentage of early and late apoptotic CD4 T cells, indicating diffDC could induce apoptosis of activated CD4 T cells. Besides, we used other viability dyes, including JC-1 and R123 (detecting the decreased mitochondrial membrane potential), to detect T cell apoptosis in our system. We found that the percentage of JC-1low 7-AAD⁺ apoptotic cells or R123low 7-AAD⁺ apoptotic cells in diffDC/mDC/T group was higher than that of mDC/T group (Fig. 1C, 1D). Furthermore, diffDC could significantly induce apoptosis of CD4 T cells that were activated by anti-CD3/mDC (Fig. 1E, 1F). These data indicate that splenic stroma-educated regulatory DC could induce apoptosis of Ag-specific and nonspecific activated CD4 T cells. To exclude the possibility that diffDC interrupted T cell activation and caused T cell death, we first induced activation of naïve CD4 T cells by mDC/Ag for 24 h, and then added diffDC or mDC into the coculture system. As shown in Fig. 1G, diffDC could increase the percentage of early and late apoptosis of the activated CD4 T cells, whereas mDC failed, further confirming that splenic stroma-educated regulatory DC could induce apoptosis of activated CD4 T cells. In addition, we found that diffDC could inhibit the proliferation of CD4 T cells initiated by anti-CD3/anti-CD28 via inducing CD4 T cell apoptosis (Fig. 1H, 1I), indicating regulatory DC-induced apoptosis of the activated CD4 T cells is independent of the presence of mDC.

FasL on regulatory DC mediates apoptosis induction of activated CD4 T cells

To investigate the mechanisms underlying diffDC-induced apoptosis of activated CD4 T cells, we determined whether soluble factors from diffDC or cell-cell contact between diffDC and activated CD4 T cells were required for the apoptosis induction of activated CD4 T cells. Using Transwell assay, we found that both diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation were reversed once diffDC were separated from mDC/CD4 T cell coculture system (Fig. 2). The data suggest that regulatory DC-induced apoptosis of activated CD4 T cells requires cell-cell contact.

Next, we wondered what membrane molecule(s) on diffDC mediated apoptosis induction of activated CD4 T cells. It is well known that various death receptor ligands, such as Fas ligand, TRAIL, B7-H1, and B7-DC, can trigger apoptosis of activated CD4 T cells (28). Therefore, we detected whether diffDC expressed these molecules. As shown in Fig. 3A, diffDC expressed higher levels of FasL and B7-H1 on their surface than that on mDC, but diffDC did not express TRAIL and B7-DC. We prepared diffDC derived from FasL-deficient gld mice, and found that diffDC from gld mice (gld diffDC) failed to induce apoptosis of activated CD4 T cells and lost their ability to inhibit CD4 T cell proliferation almost completely (Fig. 3B, 3C). Furthermore, blockade of FasL significantly reversed diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation (Fig. 3D, 3E), whereas blockade of FasL did not affect CD4 T cell proliferation in mDC/T control group (data not shown). In addition, we found that B7-H1 on diffDC could not mediate apoptosis of activated CD4 T cells induced by diffDC (data not shown). These results demonstrated that FasL on diffDC played a vital role in diffDC-induced apoptosis of activated CD4 T cells and could mediate apoptosis of activated CD4 T cells effectively. Importantly, we demonstrated that FasL-deficient diffDC could not inhibit mDC-induced activated CD4 T cell proliferation in vivo (Fig. 4), further indicating the importance of FasL on diffDC for the inhibitory function of diffDC.

Then, we investigated whether caspases played essential role in diffDC-induced apoptosis of activated CD4 T cells. First, Z-VAD-FMK (pan-caspase inhibitor) could significantly reverse diffDC-induced apoptosis of activated CD4 T cells and the inhibition of CD4 T cell proliferation by ∼50% (Fig. 3D, 3E), indicating that diffDC-induced apoptosis of activated CD4 T cells was caspase dependent. Moreover, we found that both Z-IETD-FMK (caspase 3 inhibitor) and Z-DEVD-FMK (caspase 8 inhibitor) could signifi-
cantly reverse diffDC-induced apoptosis of activated CD4 T cells and subsequent inhibition of CD4 T cell proliferation (Fig. 3D, 3E).

In contrast, these caspase inhibitors did not interrupt CD4 T cell proliferation in mDC/T control group (data not shown).

These results demonstrated that diffDC-induced apoptosis of activated CD4 T cells was dependent on caspase-3 and caspase-8.

**Stroma-derived TGF-β is responsible for high-level FasL expression on regulatory DC via ERK activation**

We went further to investigate the underlying mechanism of high-level FasL expression by diffDC. Because stroma-derived TGF-β plays an important role in the differentiation of mDC into diffDC driven by splenic stroma (18) and it is reported that TGF-β could upregulate FasL expression of lung epithelial cells or stromal cells (29, 30), we wondered whether stroma-derived TGF-β could induce upregulation of FasL expression on diffDC. As shown in Fig. 5A, blockade of TGF-β could significantly downregulate FasL expression of diffDC during differentiation from mDC driven by splenic stromal cells. This indicates that high-level FasL expression on diffDC is induced by stroma-derived TGF-β. Our previous study showed that diffDC had increased ERK activation, which was responsible for the preferential expression of IL-10 in diffDC (28). We found that blockade of TGF-β significantly inhibited ERK activation in diffDC (Fig. 5B), and interestingly, ERK inhibitor PD98059 could significantly downregulate FasL expression of diffDC (Fig. 5C). These results demonstrate that stroma-derived TGF-β induces high-level FasL expression of diffDC via promoting ERK activation in diffDC.

**FIGURE 1.** Induction of apoptosis of activated CD4 T cells by splenic stroma-educated regulatory DC. A, Naive CD4 T cells (1 × 10^6/well) from DO11.10 × C57 F1 mice were cocultured with mDC and/or diffDC (1 × 10^6/well) in the presence of OVA323–339 peptide for 4 d, and then the apoptosis of CD4 T cells was assayed by flow cytometry. Numbers in plots indicate the percentage of annexin V+ 7-AAD+ CD4+ T cells (late apoptotic cells) gated in CD4+ T cells. B, The data in A were analyzed statistically and summarized. C and D, Naive CD4 T cells (1 × 10^5/well) from DO11.10 × C57 F1 mice were cocultured with mDC and/or diffDC (1 × 10^6/well) in the presence of OVA323–339 peptide. After 4 d, cells were stained with JC-1 (or R123), CD4 allophycocyanin, and 7-AAD, and then the apoptosis of CD4 T cells was assayed by flow cytometry. Numbers in plots indicate the percentage of JC-1 (or R123)high 7-AAD+ CD4+ T cells (live cells), annexin V+ 7-AAD+ CD4+ T cells (early apoptotic cells), or annexin V+ 7-AAD+ CD4+ T cells (live apoptotic cells) gated in CD4+ T cells. **Data represent one of at least three experiments with similar results.**

**FIGURE 2.** Cell-cell contact is required for regulatory DC-induced apoptosis of the activated CD4 T cells. A and B, In the experiments using Transwell system, diffDC (1 × 10^5/well) were cultured in the upper chamber of culture inserts with 0.4 μm pore size; mDC (1 × 10^5/well) and CD4 T cells (1 × 10^5/well) were added to the lower chamber and incubated on 24-well flat-bottom plate. On day 4, the proliferation and apoptosis of CD4 T cells were assayed by flow cytometry. The number of viable OVA323–339-specific CD4 T cells (A) and the percentage of early and late apoptotic CD4 T cells (B) were shown. Data represent one of at least three experiments with similar results. **p < 0.01. “Transwell-diffDC” represents the group in which diffDC were separated from mDC/CD4 T cell coculture system.
The number of viable OVA323–339-specific CD4 T cells (B) and the percentage of early and late apoptotic CD4 T cells (C) were shown. *p < 0.01. D and E, Neutralizing anti-FasL mAb (10 μg/ml) or various caspase inhibitors, including pan-caspase inhibitor (Z-VAD-FMK, 20 μM), caspase 3 inhibitor (Z-IETD-FMK, 20 μM), or caspase 8 inhibitor (Z-DEVD-FMK, 20 μM), were added into diffDC/mDC/CD4 T cell coculture system, respectively. On day 4, the number of viable OVA323–339-specific CD4 T cells (D) and the percentage of early and late apoptotic CD4 T cells (E) were detected by flow cytometry. Data represent one of at least three independent experiments with similar results. *p < 0.05, **p < 0.01. Data represent one of at least three independent experiments with similar results.

High expression of FasL by in vivo counterpart of regulatory DC

As high-level FasL expression is one of the important characteristics of diffDC, we wonder whether the in vivo counterpart of diffDC also expresses high-level FasL. As shown in Fig. 5D, expressed FasL on regulatory DC mediates apoptosis induction of activated CD4 T cells. A, Expression of FasL, B7-H1, TRAIL, and B7-DC on diffDC and mDC was assayed by flow cytometry. Dotted lines, background staining. Numbers in histograms indicate the geometric mean fluorescence. B and C, Naïve CD4 T cells (1 × 10⁵ well) from DO11.10 × C57 F1 mice were cocultured with wild-type mDC or FasL-deficient (gld) mDC, and/or wild type diffDC or gld diffDC in the presence of OVA323–339 peptide for 4 d, and then the proliferation and apoptosis of CD4 T cells were assayed by flow cytometry. The number of viable OVA323–339-specific CD4 T cells (B) and the percentage of early and late apoptotic CD4 T cells (C) were shown. **p < 0.01. Data represent one of at least three independent experiments.

IFN-γ and NO contribute to regulatory DC-induced apoptosis of activated CD4 T cells

As observed above, cell-cell contact between diffDC and mDC/CD4 T cells was required for diffDC-induced apoptosis of activated CD4 T cells. We found higher levels of IFN-γ and NO in the supernatants of diffDC/mDC/CD4 T cell coculture system compared with that in the supernatants of mDC/CD4 T cell coculture system (Fig. 6A). Recent studies indicate that IFN-γ plays important roles in inducing apoptosis or loss of CD4 T cells during infectious diseases, antitumor responses, and experimental autoimmune encephalomyelitis (31–33). Then we wanted to know whether the cell contact-dependent IFN-γ and NO production was involved in diffDC-induced apoptosis of activated CD4 T cells. As shown in Fig. 6B and 6C, blockade of IFN-γ significantly reversed diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation. Also, addition of mouse rIFN-γ (10 ng/ml) further enhanced diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation (Fig. 6B, 6C). These results indicated that IFN-γ was involved in diffDC-induced apoptosis of activated CD4 T cells.

In our previous study (18), we found that the inhibitory function of diffDC was partially mediated by NO. NO is reported to be involved in apoptosis of CD4 T cells (31, 34, 35). So, we asked compared with CD11chigh I-Ahigh cells in the spleen (the in vivo counterpart of mDC), CD11clow I-Alow glddiffDC-deficient cells (the in vivo counterpart of diffDC) express higher level of FasL. Furthermore, in the immunized mice, which were adoptively transferred with Ag-specific T cells and then immunized with Ag to induce T cell responses, FasL expression of CD11clow I-Alow glddiffDC-deficient cells was upregulated significantly in addition to the increase of the proportion of CD11chigh I-Alow glddiffDC-deficient cells in the spleen. The data further indicate that FasL might contribute to the negative immune regulation in vivo by diffDC.

FIGURE 3. FasL on regulatory DC mediates apoptosis induction of activated CD4 T cells. A, Expression of FasL, B7-H1, TRAIL, and B7-DC on diffDC and mDC was assayed by flow cytometry. Dotted lines, background staining. Numbers in histograms indicate the geometric mean fluorescence. B and C, Naïve CD4 T cells (1 × 10⁵ well) from DO11.10 × C57 F1 mice were cocultured with wild-type mDC or FasL-deficient (gld) mDC, and/or wild type diffDC or gld diffDC in the presence of OVA323–339 peptide for 4 d, and then the proliferation and apoptosis of CD4 T cells were assayed by flow cytometry. The number of viable OVA323–339-specific CD4 T cells (B) and the percentage of early and late apoptotic CD4 T cells (C) were shown. **p < 0.01. Data represent one of at least three independent experiments.

FIGURE 4. Inhibition of CD4 T cell proliferation by regulatory DC via FasL and NO. A and B, OVA323–339-specific TCR-transgenic splenic CD4 T cells (2 × 10⁵) from OT-2 × CD45.1 F1 hybrid mice were i.v. injected into C57BL/6J mice (2 × 10⁵ cells per mouse). After 24 h, 2 × 10⁵ OVA323–339-pulsed mDC together with or without the same number of OVA323–339-pulsed diffDC or FasL-deficient diffDC (gld diffDC) were transferred i.p. into the mice. In some groups, L-NAME (20 mg/kg body weight) was injected i.p. into mice together with mDC. After 5 d, single-cell suspensions of spleen and MLN were double stained with CD4-PerCP Cy5.5 and CD45.1 aliphycocyanin for flow cytometry. Numbers in CD4-gated plots indicated the percentage of early and late apoptotic CD4 T cells (A) and the data were analyzed statistically and summarized (B). Each group contained three mice. **p < 0.01. Similar results were obtained in at least three independent experiments. compared with CD11chigh I-Ahigh cells in the spleen (the in vivo counterpart of mDC), CD11clow I-Alow glddiffDC-deficient cells (the in vivo counterpart of diffDC) express higher level of FasL. Furthermore, in the immunized mice, which were adoptively transferred with Ag-specific T cells and then immunized with Ag to induce T cell responses, FasL expression of CD11clow I-Alow diffDC-deficient cells was upregulated significantly in addition to the increase of the proportion of CD11chigh I-Alow diffDC-deficient cells in the spleen. The data further indicate that FasL might contribute to the negative immune regulation in vivo by diffDC.

IFN-γ and NO contribute to regulatory DC-induced apoptosis of activated CD4 T cells

As observed above, cell-cell contact between diffDC and mDC/CD4 T cells was required for diffDC-induced apoptosis of activated CD4 T cells. We found higher levels of IFN-γ and NO in the supernatants of diffDC/mDC/CD4 T cell coculture system compared with that in the supernatants of mDC/CD4 T cell coculture system (Fig. 6A). Recent studies indicate that IFN-γ plays important roles in inducing apoptosis or loss of CD4 T cells during infectious diseases, antitumor responses, and experimental autoimmune encephalomyelitis (31–33). Then we wanted to know whether the cell contact-dependent IFN-γ and NO production was involved in diffDC-induced apoptosis of activated CD4 T cells. As shown in Fig. 6B and 6C, blockade of IFN-γ significantly reversed diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation. Also, addition of mouse rIFN-γ (10 ng/ml) further enhanced diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation (Fig. 6B, 6C). These results indicated that IFN-γ was involved in diffDC-induced apoptosis of activated CD4 T cells.
Whether NO was also involved in diffDC-induced apoptosis of activated CD4 T cells. As shown in Fig. 6D and 6E, both the selective NO synthase inhibitor dihydrobromide (1,4 PBIT) and an analog of arginine (L-NAME) could effectively reverse diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation. Also, L-NAME injection could partially reverse the inhibitory function of diffDC in vivo (Fig. 4). The results demonstrated that NO was also involved in diffDC-induced apoptosis of activated CD4 T cells. As iNOS inhibitor may act on each kind of cell in coculture system, we used siRNA interference to knock down iNOS expression in diffDC and then detected the function of diffDC. We found that iNOS siRNA could inhibit NO production of diffDC induced by LPS, although the efficiency of inhibition was not very high (Fig. 6F). Furthermore, the ability of iNOS siRNA-treated diffDC to inhibit T cell proliferation decreased, along with the lower NO production in the supernatant of iNOS-siRNA diffDC/mDC/T coculture (Fig. 6G), demonstrating the inhibitory action of NO from diffDC on T cell proliferation.

**FasL on regulatory DC is responsible for the increased production of IFN-γ and NO during interaction of regulatory DC and activated T cells**

As FasL on diffDC mediated apoptosis of CD4 T cells and both IFN-γ and NO contributed to diffDC-induced apoptosis of CD4 T cells, we wondered whether high level of IFN-γ and NO production in diffDC/mDC/CD4 T cell coculture system was medi-
**FIGURE 7.** FasL on regulatory DC enhances the increased production of IFN-γ and NO. Naive CD4 T cells (1 × 10⁵/well) from DO11.10 × C57 F₁ mice were cocultured with wild-type mDC or gld mDC and/or wild-type diffDC or gld diffDC in the presence of OVA₃₂₃₋₃₃⁹ peptide for 4 d, and the concentrations of IFN-γ and NO in each group were tested by ELISA and Griess assay, respectively. Data represent one of at least three experiments with similar results. *p < 0.05.

**FIGURE 8.** IFN-γ from activated CD4 T cells induces NO production by regulatory DC to feedback inhibit T cell response

It is reported that DC can secrete IFN-γ (36). We asked whether DC or activated CD4 T cells were responsible for high-level secretion of IFN-γ in the coculture system. We found that both diffDC and mDC did not secrete IFN-γ regardless of stimulation by 500 ng/ml LPS (data not shown). Next, we used diffDC or mDC derived from wild-type or IFN-γ-deficient mice in the coculture system, and found that high IFN-γ secretion in diffDC/mDC/CD4 T cell coculture system remained unchanged regardless of IFN-γ deficiency of diffDC or mDC (Fig. 8A). Meanwhile, diffDC-induced apoptosis of activated CD4 T cells and the inhibition of CD4 T cell proliferation were not affected by IFN-γ deficiency in diffDC or mDC (data not shown). These results demonstrated that production of high-level IFN-γ in coculture system was from the activated CD4 T cells, but not from DC. To demonstrate this directly, we cocultured diffDC with IFN-γ⁻/⁻ CD4 T cells activated by mDC/anti-CD3. As shown in Fig. 8B and 8C, when CD4 T cells were deficient in IFN-γ, IFN-γ secretion in coculture system was completely lost and diffDC-induced apoptosis of activated CD4 T cells and inhibition of CD4 T cell proliferation were reversed significantly. The data demonstrated that high-level IFN-γ in the coculture system was from the activated CD4 T cells.

It is well known that myeloid cells, including macrophages and DC, produce NO via iNOS in response to LPS, TNF-α, or IFN-γ. So, we investigated whether diffDC or mDC were responsible for production of high-level NO. As shown in Fig. 8D, diffDC produced a lot of NO after IFN-γ stimulation, indicating that high-level NO in the coculture system was from diffDC.

As IFN-γ can induce NO production in a variety of cells, we determined whether T cell-derived IFN-γ contributed to NO production from diffDC in diffDC/mDC/CD4 T cell coculture system. As shown in Fig. 8E, addition of anti–IFN-γ neutralizing Ab could inhibit NO production in diffDC/mDC/CD4 T cell coculture system, whereas addition of mouse rIFN-γ further increased NO production. In addition, when CD4 T cells were deficient in IFN-γ, NO production decreased significantly in diffDC/mDC/CD4 T cell coculture system (Fig. 8C). To demonstrate that IFN-γ from activated CD4 T cells acts on diffDC rather than mDC or T cells to produce NO and inhibit T cell proliferation, we used IFN-γ R1-deficient diffDC in the coculture system. As expected, IFN-γ R1-deficient diffDC could not produce NO after IFN-γ stimulation (data not shown). As shown in Fig. 9A and 9B, we demonstrated that the ability of IFN-γ R1-deficient diffDC to inhibit T cell proliferation and induce T cell apoptosis decreased significantly along with NO level in IFN-γ R1-deficient diffDC/mDC/T coculture system, decreasing to the level in mDC/T (Fig. 9C), indicating that IFN-γ produced by activated T cells mainly acted on diffDC to induce NO production in diffDC/mDC/T coculture system. In addition, we found that the ability of IFN-γ R1-deficient diffDC to inhibit T cell proliferation decreased significantly along with NO level in IFN-γ R1-deficient diffDC/anti-CD3 plus anti-CD28/T coculture system, decreasing to the level in...
anti-CD3 plus anti-CD28/T (Fig. 9D). Furthermore, we found that the ability of IFN-γ R1−/− diffDC to inhibit mDC-induced activated CD4 T cell proliferation in vivo decreased significantly (Fig. 9E). These results demonstrated that IFN-γ from activated CD4 T cells was responsible for induction of NO from diffDC, thus forming a network to jointly inhibit T cell response (Fig. 10).
Discussion
After recognizing Ag presented by APCs such as DC, peripheral naive CD4 T cells are activated, proliferate, and differentiate into effector T cells to initiate immune responses. When the pathogenic Ag is deleted, effector T cells are induced to undergo apoptosis to avoid disturbing T cell homeostasis and immune disorders such as autoimmune diseases. Only a few T cells remain and develop into memory T cells. Apoptosis of T cells is well regulated to maintain immune homeostasis, which is regulated by several mechanisms, as follows: extrinsic cell death receptor-mediated and caspase-dependent apoptosis, intrinsic mitochondria- and caspase-dependent apoptosis, or caspase-independent cell death (1). Extrinsic cell death receptor-mediated apoptosis, that is, activation-induced cell death, is mediated by cell death receptors (such as Fas) triggering via caspase 8- and caspase 3-dependent manners. During the process, the expression of the ligands of cell death receptors (such as FasL) is induced on activated T cells. However, emerging evidence demonstrated that the expression of the ligands of cell death receptors on nonactivated T cells, including various DC subsets, also actively triggered death of activated T cells via extrinsic cell death receptor-mediated apoptosis (37–39). Even some cells such as T regulatory (Treg), liver sinusaloidal endothelial cells, and hepatic satellite cells can induce apoptosis or death of activated T cells via other effector molecules such as perforin and granzymes or the negative molecules such as B7-H1 or TGF-β (7, 40, 41). Regulatory CD4 T cells also induce cytokine deprivation-mediated apoptosis of effector CD4 T cells by inducing proapoptotic protein Bim and Bad (42). In this study, we demonstrated that splenic stroma could educate regulatory DC to induce apoptosis of activated CD4 T cells via induction of FasL and subsequently FasL-enhanced T cell IFN-γ and DC NO production.

Regulatory DC can inhibit T cell proliferation by inducing Treg cells or T cell anergy (5, 6). We also found that regulatory DC could increase the number of conventional Treg in vitro rather than in vivo during primary T cell response (Supplemental Fig. 2), although increasing Treg were not involved in the inhibition of T cell proliferation by regulatory DC (18). However, rare studies reported that regulatory DC could induce T cell apoptosis or death, although some DC subsets were reported to be able to induce T cell apoptosis or death. Splenic CD8α+ DC highly expressing FasL could kill CD4 T cells via Fas/FasL-induced apoptosis (38). Interestingly, FasL-expressing regulatory DC did not express CD80 (data not shown). It was reported that Langerhans cells could express FasL and induce apoptosis of Jurkat cells after activation through CD40 ligation (43). Bone marrow DC grown in GM-CSF and IL-4 could express FasL and also induce Jurkat T cell apoptosis in a Fas-dependent pathway in vitro (39). Plasmacytoid DC were recently found to express TRAIL and induce CD4 T cell apoptosis in HIV-1 viremic patients (37). Together with our findings, we propose that induction of T cell apoptosis is one of the manners for regulatory DC to exert their regulatory function.

IFN-γ can directly act on activated T cells to induce apoptosis (44). It is reported that only naive CD4 T cells and Th2 cells express both types of IFN-γ receptor chains (IFN-γ R1 and IFN-γ R2), whereas expression of the IFN-γ R2 is lost in Th1 cells (45, 46). In our system, we used LPS-activated mDC to prime T cells, so the activated CD4 T are mainly Th1 cells. Furthermore, by using IFN-γ R1-deficient dDC, we demonstrated that IFN-γ from activated CD4 T cells acts on dDC rather than mDC or T cells to produce NO and then inhibits T cell proliferation. Hence, IFN-γ from activated CD4 T cells contributed to apoptosis induction of activated CD4 T cells in a paracrine manner, via inducing large amounts of NO production by regulatory dDC. Then NO, as an effector molecule, triggered apoptosis of activated T cells. The indirect mechanism is just like recent reports (47, 48). NO, a pleiotropic molecule, especially NO produced by iNOS, has been found to mediate T cell apoptosis or death directly, thus playing important roles in negative regulation of immune response (34, 35). Our previous study demonstrated that dDC-derived soluble factors are partially involved in inhibitory functions of dDC, and one of the soluble factors is NO (18). Besides NO, in this study we found other molecules were involved in regulating T cell response by splenic stroma-educated regulatory DC, as follows. Splenic stroma educates regulatory DC to express high level of FasL. Inhibition of CD4 T cell proliferation by regulatory DC requires cell-to-cell contact, and FasL is responsible for regulatory DC to induce apoptosis of activated CD4 T cells and subsequently inhibit CD4 T cell proliferation. Furthermore, FasL on regulatory DC enhances IFN-γ production from activated CD4 T cells, and in turn IFN-γ induces NO production from regulatory DC, then working jointly to induce apoptosis of activated CD4 T cells (Fig. 10A). The results will further enrich the immunoregulatory function of splenic stromal microenvironment and clarify the mechanisms for negative control of T cell response by regulatory DC at the late stage of immune response. However, how FasL enhances IFN-γ production from activated CD4 T cells and then IFN-γ induces NO production from regulatory DC remain to be further studied in the future.

Interestingly, we found that splenic stroma could even suppress mDC-initiated T cell response via inducing CD4 T cell apoptosis (Supplemental Fig. 3), indicating the new way for direct regulation of T cell response by splenic stromal environment. Furthermore, the suppression of T cell response by splenic stroma was, at least partially, mediated by NO production from mDC induced by T cell-derived IFN-γ, and the process was triggered by splenic stroma (Supplemental Fig. 4). The data indicate that splenic stroma can negatively control T cell response by triggering NO production from mDC at the early stage of immune response (Fig. 10B). During the revision of this work, Lukacs-Kornek et al. (49) reported that lymph node stromal cells inhibit T cell proliferation through a tightly regulated mechanism dependent on NO production, indicating the important roles of lymph node and spleen stroma in the direct regulation of T cell response.

In sum, our data demonstrate that splenic stromal microenvironment educates regulatory DC to highly express FasL and induce apoptosis of activated CD4 T cells via FasL-enhanced T cell IFN-γ and regulatory DC NO production at the late stage of immune response. Our results indicate a new way for negative regulation of T cell response by regulatory DC and splenic stromal microenvironment.

Acknowledgments
We thank Qian Li and Sibiao Yue for helpful discussion and Rui Zhang for excellent technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References
FasL-EXPressING REGULATORY DC INDUCE T CELL APOPTOSIS


26. Ogdon, C., and U. Schleicher. 2006. Production of interferon-gamma by my-


33. Chu, C. A., S. Wittmer, and D. K. Dalton. 2000. Failure to suppress the ex-


Downloaded from http://www.jimmunol.org/ by guest on April 30, 2017
Supplemental materials:

Supplemental Figure 1. Characteristics of ESSC-derived diffDC from adult mice

(A) diffDC prepared by coculture of ESSC derived from adult mice and mDC for 7 d (Adult ESSC-derived diffDC) or diffDC (prepared by coculture of ESSC derived from newborn mice and mDC for 7 d) were stained with antibodies to CD11b, CD11c, I-A^b, CD40, CD80 and CD86 and analysed by flow cytometry. Shaded histograms, background staining. Bold solid line histograms, specific antibodies. Numbers in histograms indicate the geometric mean fluorescence of each DC population. (B)

Xu X, et al. Fig. S1

IL-10 (pg/ml)

IL-12 p70 (pg/ml)

NO (µM)

C

Adult ESSC-derived diffDC/mDC/T

diffDC/mDC/T

Adult ESSC-derived diffDC/T

diffDC/T

mDC/T

0 10 20 30 40 50 60 70

OVA-specific CD4 T cells

(1×10^4)

NS

**

Supplemental Figure 1. Characteristics of ESSC-derived diffDC from adult mice

(A) diffDC prepared by coculture of ESSC derived from adult mice and mDC for 7 d (Adult ESSC-derived diffDC) or diffDC (prepared by coculture of ESSC derived from newborn mice and mDC for 7 d) were stained with antibodies to CD11b, CD11c, I-A^b, CD40, CD80 and CD86 and analysed by flow cytometry. Shaded histograms, background staining. Bold solid line histograms, specific antibodies. Numbers in histograms indicate the geometric mean fluorescence of each DC population. (B)
diffDC or adult ESSC-derived diffDC (5×10^5/ml) were stimulated with or without 500 ng/ml LPS for 24 hours and then the concentrations of cytokines and nitric oxide were tested by ELISA and Griess assay respectively. (C) Naive CD4 T cells (1×10^5/well) from DO11.10×C57 F1 mice were cocultured with mDC and/or diffDC or adult ESSC-derived diffDC (1×10^4/well) in the presence of OVA_{323-339} peptide for 4 days and then the proliferation of CD4 T cells was assayed by flow cytometry. The number of viable OVA_{323-339}-specific CD4 T cells was shown. Data represent one of at least three experiments with similar results. NS indicates no statistical significance; **, P<0.01.
Supplemental Figure 2. Influence of splenic stroma-educated regulatory DC on the number of conventional regulatory T cells in vitro and in vivo

(A) Naive CD4 T cells (1×10^5/well) from DO11.10×C57 F1 mice were cocultured with mDC and/or diffDC(1×10^4/well) in the presence of OVA_{323-339} peptide. After 4 days, cells were stained with Foxp3-PE, CD25-APC and CD4-FITC. Intracellular Foxp3 expression of CD4^+ T cells was assayed by flow cytometry. Numbers in plots indicate the percentage of CD25^+ Foxp3^+ cell in CD4^+ T cells. Data represent one of at least three independent experiments with similar results. (B) OVA_{323-339}-specific TCR-transgenic splenic CD4 T cells (2×10^6) from OT-2×CD45.1 F1 hybrid mice were intravenously injected into C57BL/6J mice (2×10^6 cells per mouse). After 24 h, 2×10^6 OVA_{323-339}-pulsed mDC together with or without the same number of
OVA$_{323-339}$-pulsed dDC were transferred \textit{i.p} into the mice. After 5 days, single-cell suspensions of spleen and mesenteric lymph nodes (MLN) were stained with Foxp3-PE, CD4-Percp Cy5.5 and CD45.1-APC for flow cytometry. Intracellular staining of Foxp3 expression was analyzed in CD45.1$^+$ CD4$^+$ OT-2 T cells and endogenous CD45.1$^-$ CD4$^+$ T cells. Numbers in plots indicate the percentage of Foxp3$^+$ cells among gated CD45.1$^+$ CD4$^+$ OT-2 cells or endogenous CD45.1$^-$ CD4$^+$ T cells. Three mice in each group were used. Similar results were obtained in at least three independent experiments.
Supplemental Figure 3. Splenic stroma inhibits mDC-initiated antigen-specific CD4 T cell proliferation by inducing T cell apoptosis

(A) OVA323-339-specific naïve CD4 T cells (1×10^5/well) were co-cultured with mDC (1×10^4/well) and OVA323-339 peptide in the presence or absence of graded numbers (10^4, 10^3, 10^2) of ESSCs in 96-well flat bottom plate. The relative number of 7AAD− viable OVA323-339-specific CD4 T cells in each well was measured on day 4 by flow cytometry. Values are the means ± S.D. of triplicate wells. * P<0.05. Data are representative of at least three independent experiments. (B) OVA323-339-specific naïve CD4 T cells were labeled with 5μM CFSE and co-cultured with mDC and ESSC in the presence of OVA323-339 peptide for 4 days, and then the divisions of CFSE-labeling CD4^+ T cells were assessed by flow cytometry. (C) OVA323-339-specific naïve CD4 T
cells \((1 \times 10^5/\text{well})\) were cocultured with mDC \((1 \times 10^4/\text{well})\) and/or splenic stroma ESSC \((1 \times 10^4/\text{well})\) in the presence of OVA\textsubscript{323-339} peptide for 4 days and then the apoptosis of CD4\(^+\) T cells was assayed by flow cytometry. Numbers in plots indicate the percentage of Annexin \textsuperscript{V} \textsuperscript{-} 7AAD\textsuperscript{-} CD4\(^+\) T cells (live cells), Annexin \textsuperscript{V} \textsuperscript{+} 7AAD\textsuperscript{-} CD4\(^+\) T cells (early apoptotic cells) or Annexin \textsuperscript{V} \textsuperscript{+} 7AAD\textsuperscript{+} CD4\(^+\) T cells (late apoptotic cells) gated in CD4\(^+\) T cells.
Supplemental Figure 4. IFN-γ from activated CD4 T cells and NO from mDC contribute to the suppression of CD4 T cell response by splenic stroma

(A) OVA323-339-specific naïve CD4 T cells were co-cultured with mDC and OVA323-339 peptide in the presence or absence of ESSC in 24-well flat bottom plate. In Transwell experiments, ESSC were cultured in the upper chamber of Transwell with 0.4 μm pore size and mDC/CD4T in the lower chamber. The relative number of 7AAD− viable OVA323-339-specific CD4 T cells was measured on day 4 by flow cytometry. (B) Naïve CD4 T cells (1×10^5/well) from wild-type or IFN-γ deficient mice were co-cultured with anti-CD3 mAb (1μg/ml) and mDC (1×10^4/well) in the presence of ESSC (1×10^4/well). 4 days later, the number of 7AAD− viable CD4^+ T cells was measured by flow cytometry and NO concentration was measured by Griess assay. (C) OVA323-339-specific naïve CD4 T cells (1×10^5/well) were co-cultured with
mDC (1×10⁴/well) and/or splenic stroma ESSC (1×10⁴/well) in the presence of OVA₃₂₃-₃₃₉ peptide. Arginine analog L-NAME (250μg/ml) was added into ESSC/mDC/CD4T co-culture system. 4 days later, the relative number of 7AAD⁻ viable OVA₃₂₃-₃₃₉-specific CD4⁺ T cells in each well was measured by flow cytometry and NO concentration was measured by Griess assay. (D) mDC were co-cultured with or without ESSC for 1, 2, or 3 days. Then ESSC-treated mDC were stimulated with IFN-γ (10ng/ml) for 24 hours, and NO concentration of collected supernatants was measured by Griess assay. Data represent one of at least three experiments with similar results. *, P<0.05.