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

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J Immunol published online 28 December 2011
http://www.jimmunol.org/content/early/2011/12/28/jimmunol.1101696
Splenic Stroma-Educated Regulatory Dendritic Cells Induce Apoptosis of Activated CD4 T Cells via FasL-Enhanced IFN-γ and Nitric Oxide

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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 and NO could reduce the apoptosis induction. Therefore, our results demonstrated that splenic stroma-educated regulatory DC with regulatory function have been defined to control T cell response in recent years.

A fter elimination of invading pathogens, the innate and adaptive immune response is controlled at the appropriate level so as to prevent host tissue damage from excessive immune cell activation. Even in the physiological conditions, activation of immune cells, including T cells, is tightly regulated to avoid pathogenesis of autoimmune diseases. In addition to the well-studied negative regulation of T cell response via activation-induced cell death at late phase of immune responses (1), actively negative regulation of T cell response by many kinds of suppressive cells and inhibitory molecules also attracts much attention in recent years. Many kinds of suppressive cell populations with regulatory function have been defined to control T cell response in the latest decade, such as regulatory T (Treg) cells (2), regulatory B cells (3), alternatively activated macrophage (M2) (4), and regulatory dendritic cells (DC) (5, 6). The detailed mechanisms for the negative regulation of T cell response by these regulatory cells need to be fully investigated.

Increasing evidence demonstrates that stromal microenvironment may regulate the development and function of immune cells in the lymphoid and nonlymphoid organs. The microenvironment in parenchymatous organs contributes to the regulation of local immune response. For example, human liver sinusoidal endothelial cells have been found to tolerize T cells and induce apoptosis of the activated T cells, providing the mechanistic explanation for the liver as a tolerogenic organ (7). Our previous studies show that liver fibroblast stromal cells can program hematopoietic stem cells to differentiate into regulatory DC (8), and pulmonary stromal cells can also induce the generation of regulatory DC (9). Moreover, the microenvironment in lymphoid organs has been found to be important in regulating immune response (10, 11). For example, bone marrow stromal cells suppress T cell proliferation (12). Lymph node stroma promotes T cell tolerance to intestinal self (13). The spleen is an important secondary lymphoid organ in which immune cell development and function are generated and regulated, and also mature DC (mDC) present Ags to T cells and initiate T cell response. However, the mechanisms for how splenic stroma can regulate T cell response need to be further identified.

DC are the most potent professional APCs that play pivotal roles in the initiation of primary immune responses and induction and maintenance of immunological tolerances (14–16). Different functions of DC may be explained by existence of different subsets of DC or DC at different developmental/stages (15). In recent years, identification and characterization of phenotype and cytokine profile of DC with regulatory properties (so-called regulatory or tolerogenic DC), and investigation of their roles in the immune regulation and the pathogenesis of immune...
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 remains 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/6J mice (B6) were obtained from Joint Ventures Sipper BK Enterprises, Animal Laboratory, Graz, Austria. OVAp1-specific Tg transgenic, DO11.10 mice or OT-2 mice. B6.SJL-Ptprca Pep3b/B6 mice (CD45.1 mice), IFN-γ R1-deficient mice (C57BL/6J background), IFN-γ-deficient mice (C57BL/6J background), and B6Smn.C3H-FasL(B6/gld) 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-Fas mouse 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-2, TRAIL, B7-1, B7-2, annexin V, biotin-conjugated mAb to FasL, and PE-conjugated streptavidin, were from BD Pharmingen (San Diego, CA), BioLegend (San Diego, CA), or eBioscience (San Diego, CA). Microbead-conjugated mAbs to CD4 and CD11c were from Miltenyi Biotec (Bergisch Gladbach, Germany). Functional grade-purified anti-CD3 mAb and anti-CD28 mAb were from eBioscience. The 2.4G2 for blocking mouse CD16/CD32 was from BD Pharmingen. Rat sera were from Life Technologies (Grand Island, NY). The 7-aminocinocymycin D (7-AAD), LPS, 1,4 PBIT, PD98059, and nitro-L-arginine methyl ester (L-NAME) were from Sigma-Aldrich (St. Louis, MO). JC-1, R123, and CFSE were from Invitrogen (Eugene, OR). Anti-phospho-ERK mAb, anti-ERK polyclonal Ab, and their respective HRP-coupled secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Pos-caspase inhibitor (Z-VAD-FMK), caspase 3 inhibitor (Z-DEVD-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-glutamate, 1% sodium pyruvate, and 2 × 10⁻⁵ M 2-ME, all from Sigma-Aldrich. IFN-γ ELISA kit was from R&D Systems.

**Preparation of splenic stromal cells, mDC, and regulatory DC (diffDC)**

Mouse endothelial-like splenic stromal cells (ESSC) derived from 1-wk-old newborn wild-type C57BL/6J mice or 6-wk-old adult wild-type C57BL/6J mice were prepared and maintained in long-term culture in RPMI 1640/20% FCS by passaging to new plates each week, as described previously (18). Bone marrow-derived mDC from C57BL/6J or B6.gld/gld mice were generated, as described previously (18). Regulatory DC (diffDC) were generated by coculturing mDC with splenic stroma with minor modification, as described previously (18). Once ESSC monolayers derived from 1-wk-old newborn mice had reached 80–90% confluence, mDC were seeded at a density of 2 × 10⁶ per 5 ml/6-well plates in RPMI 1640 medium supplemented with 5% FCS for at least 7 d. Loosely adhering cells from stromal cell–DC coculture were washed off gently without using 0.1% trypsin and 5 mM EDTA to avoid the contamination of ESSC, and then purified using anti-CD11c magnetic microbeads. The isolated cells were diffDC used for the following experiments, including T cell assays. In our experiments, we found ESSC prepared from 1-wk-old adult mice could also drive the development of regulatory DC (named as adult ESSC-derived diffDC), which had similar characteristics with diffDC (Supplemental Fig. 1), but we selected ESSC derived from 1-wk-old newborn mice in our following experiments because this kind of ESSC had more potent proliferation in vitro and was easy to be used to prepare diffDC.

**Flow cytometry**

For analysis of FasL, TRAIL, B7-1, and B7-2 expression of ESSC, mDC, or diffDC, the cells were stained with biotin-conjugated mAb to FasL and PE-conjugated streptavidin, PE-conjugated mAbs to TRAIL, B7-1, H7-1, and B7-CD, and then assayed by flow cytometry, as described previously (18). Flow cytometry was carried out using a FACSCalibur or LSR II (BD Biosciences), and data were analyzed with CellQuest Version 3.3 software (BD Biosciences) or FlowJo Version 5.7.2 software (Tree Star).

**Assay for T cell proliferation and apoptosis**

Splenic CD4 T cells from DO11.10 × C57BL/6J F1 hybrid mice were positively selected with anti-CD4 microbeads (Miltenyi Biotec) and used as Ag-specific responder. The purity of CD4 T cells was confirmed by flow cytometry to be >95%. CD4 T cells were cocultured for 4 d with mDC and/or ESSC or diffDC in the presence of 200 nM OVA23–33 and peptide in 96-well round-bottom plates. On day 4, supernatants were collected for assay of cytokines by ELISA, and cells were stained with anti-CD4 PE, 7-AAD, and annexin V F-ITC resuspended in 300 μl binding buffer containing 0.1% calcium ion, and cellular data were acquired for 56 s with flow cytometry. For analysis of CD4 T cell proliferation, the number of CD4⁷⁻ 7-AAD⁻ cells was counted, and the total cells in each well were calculated, as described previously (18). For analysis of CD4 T cell apoptosis, annexin V⁺ 7-AAD⁻ CD4⁺ T cells were early apoptotic cells and annexin V⁺ 7-AAD⁺ CD4⁺ T cells were late apoptotic cells gated in CD4⁺ T cells. In some experiments, cells were stained with 2 μg JC-1 or R123, CD4, and PE-conjugated streptavidin, and then the apoptosis cells were assayed by flow cytometry. JC-1 or R123 (7-AAD⁻) CD4⁺ T cells were early apoptotic cells, and JC-1 or R123 (7-AAD⁺) CD4⁺ T cells were late apoptotic cells gated in CD4⁺ T cells. In Transwell experiments, ESSC or diffDC (1 × 10⁵/well) were cultured in the upper chamber of culture inserts with 0.4 μm pore size, and mDC (1 × 10⁵/well) and CD4 T cells (1 × 10⁵/well) were added to the lower chamber and incubated on 2-well flat-bottom plate. In some experiments, inducible NO synthase (iNOS) inhibitor 1,4 PBIT, arginine analog L-NAME, mouse rIFN-γ, or neutralizing Abs (anti-FasL, anti–IFN-γ, anti–B7-H1, or anti–TGF-β) were added to the experimental system, respectively.
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 bicinchoninic acid assay (Pierce). Proteins were subjected to Nanodot PAGE (SDS-PAGE), transferred onto nitrocellulose membranes, and then blotted, as described previously (26).

Assay for the inhibitory function of regulatory DC in vivo

OVA323–339-specific TCR-transgenic splenic CD4 T cells (2 × 10⁶) from OT-2 × CD45.1 F1 hybrid mice were i.v. injected into C57BL/6j mouse (2 × 10⁶ cells per mouse). After 24 h, 2 × 10⁶ OVA323–339-pulsed mDC, together with or without the same number of OVA323–339-pulsed diffDC, IFN-γ R1-deficient diffDC (IFN-γ R1−/− diffDC), or FasL-deficient diffDC (gld diffDC) were transferred i.p. into the mice. OVA323–339-pulsed DC were prepared by pulsing mDC or diffDC with 2 μM OVA323–339 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

OVA323–339-specific TCR-transgenic splenic CD4 T cells (2 × 10⁶) from OT-2 × CD45.1 F1 hybrid mice were i.v. injected into C57BL/6J mouse (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^V PE, CD11b PerCP Cy5.5, CD11c PE Cy7, FasL, biotin, and streptavidin allophycocyanin. After gating F4/80 FITC-negative cells, the FasL expression on mDC (CD11c^high I-A^high cells) and diffDC (CD11c^low I-A^low/CD11b^low cells) was assayed by flow cytometry and the ratio of CD45.1+ cells to CD4 cells was calculated. As shown in Fig. 1, FasL expression is induced on activated CD4 T cells induced by mDC or diffDC (data not shown). These results suggest that regulatory DC-induced apoptosis of activated CD4 T cells requires cell-cell contact.

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′-GGCCAACAACAUCAAGAGATT-3′; antisense, 5′-UCAUCUUGUAUUUUGGCTT-3′) were used to suppress iNOS expression; nonsense sequence (sense, 5′-UUUCGCAACUGACUGAUCCUGTT-3′; antisense, 5′-ACUGUAACAGG-UUCCUCAGGTT-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-

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Induction of apoptosis of activated CD4 T cells by splenic stroma-educated regulatory DC. 

**FIGURE 1.** Naive CD4 T cells (1 × 10^5/well) from DO11.10 × C57 F1 mice were cocultured with mDC and/or diffDC (1 × 10^5/well) in the presence of OVA323–339 peptide for 4 d, and then the apoptosis of CD4 T cells was assayed by flow cytometry. The percentage of early and late apoptotic CD4 T cells was shown. Data represent one of at least three experiments with similar results. **p < 0.01.

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 diffDC 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.
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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, Naive 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. 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 wondered whether the in vivo counterpart of diffDC also expresses high-level FasL. As shown in Fig. 5D, compared with CD11chigh I-Ahigh cells in the spleen (the in vivo counterpart of mDC), CD11clow I-Alow/− CD11bhigh 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/− CD11bhigh cells was upregulated significantly in addition to the increase of the proportion of CD11clow I-Alow/− CD11bhigh 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.

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...
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 dihydroubromide (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.

**FIGURE 6.** Involvement of IFN-γ and NO in the apoptosis induction of the activated CD4 T cells by regulatory DC. **A,** 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 into the lower chamber and incubated on 24-well flat-bottom plate. On day 4, the concentrations of IFN-γ and NO in the supernatants of each group were detected by ELISA and Griess assay, respectively. **B** and **C,** Neutralizing anti-IFN-γ mAb (10 μg/ml) or mouse iNOS (10 ng/ml) was added into mDC/C4D T cell or diffDC/mDC/C4D T cell coculture system. On day 4, the number of viable OVA323–339-specific CD4 T cells (B) and the percentage of early and late apoptotic CD4 T cells (C) were detected by flow cytometry. **D** and **E,** iNOS inhibitor PBIT (5 μg/ml) or arginine analog L-NAME (250 μg/ml) was added into mDC/C4D T cell or diffDC/mDC/C4D T cell coculture system. 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. **F,** diffDC (5 × 10^5/ml) were transfected with negative control siRNA (NC-siRNA) or iNOS-specific siRNA (iNOS-siRNA) in 24-well flat-bottom plate. After 6 h, cell culture medium was depleted and cells were cocultured with mDC and naive CD4 T cells from DO11.10 × C57 F1 mice in the presence of OVA323–339. On day 4, the proliferation of CD4 T cells was assayed by flow cytometry. The number of viable OVA323–339-specific CD4 T cells was shown. Concentrations of NO in the supernatants were detected by Griess assay. Data represent one of at least three independent experiments with similar results. *p < 0.05, **p < 0.01.

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/C4D T cell coculture system was medi-
diffDC or 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 7. FasL on regulatory DC enhances the increased production of IFN-γ and NO. Naive CD4 T cells (1 × 10^5/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 from regulatory DC. A, Naive CD4 T cells (1 × 10^5/well) from DO11.10 × C57 F1 mice were cocultured with wild-type mDC or IFN-γ−/− mDC and/or wild-type diffDC or IFN-γ−/− diffDC in the presence of OVA323–339 peptide for 4 d. On day 4, the concentrations of IFN-γ in the supernatants in each group were measured by ELISA. B and C, Wild-type or IFN-γ−/− naive CD4 T cells (1 × 10^5/well) were cocultured with mDC and/or diffDC (1 × 10^5/well) in the presence of anti-CD3 (1 μg/ml). On day 4, the percentage of early and late apoptotic CD4 T cells (B) and the number of viable CD4 T cells (C) were detected by flow cytometry. The concentrations of IFN-γ and NO and C (C) were measured by Griess assay, respectively. D, diffDC or mDC (5 × 10^5/ml) were stimulated with or without 10 ng/ml rIFN-γ for 24 h, and then the concentrations of NO were tested by Griess assay. E, Neutralizing anti–IFN-γ mAb (10 μg/ml) or mouse rIFN-γ (10 ng/ml) were added into diffDC/mDC/CD4 T cell coculture system. On day 4, the concentrations of NO in the supernatants were measured by Griess assay. Data represent one of at least three experiments with similar results. NS indicates no statistical significance. *p < 0.05, **p < 0.01.
anti-CD3 plus anti-CD28/T (Fig. 9). 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).
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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) trigerring 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 sinusoidal 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, recent 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, high expressing FasL, could kill CD4 T cells via Fas/FasL-induced apoptosis (38). Interestingly, FasL-expressing regulatory DC did not express CD8α (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 DC, we demonstrated that IFN-γ from activated DC T cells acts on mDC rather than mDC or T cells to produce NO and then inhibits T cell proliferation. Hence, IFN-γ from activated DC T cells contributed to apoptosis induction of activated CD4 T cells in a paracrine manner, via inducing large amounts of NO production by regulatory DC.

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 diffDC-derived soluble factors are partially involved in inhibitory functions of diffDC, 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. Spleen 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 DC 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

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