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Fas Signal Promotes the Immunosuppressive Function of Regulatory Dendritic Cells via the ERK/β-Catenin Pathway*[S]

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Cheng Qian[‡1], Li Qian[‡1], Yizhi Yu[‡1], Huazhang An[‡], Zhenhong Guo[‡], Yanmei Han[‡], Yongjian Chen[‡], Yi Bai[‡], Qingqing Wang[§], and Xuetao Cao[‡§¶2]

From the [‡]National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, Shanghai 200433, China, the [§]Institute of Immunology, Zhejiang University School of Medicine, Hangzhou 310058, China, and the [¶]National Key Laboratory of Medical Molecular Biology, Chinese Academy of Medical Sciences, Beijing 100730, China

Background: Regulatory DCs are important in tolerance maintenance, whereas the mechanisms for maintaining their immunosuppressive function in immune microenvironment remain unclear.
Results: Fas signal enhances the immunosuppressive function of splenic stroma-educated regulatory DCs via the ERK/β-catenin pathway.
Conclusion: Fas signal, at least from activated T cells, enhances regulatory function of regulatory DCs.
Significance: This study provides new mechanistic insight for immune homeostasis.

Dendritic cells (DCs) play important roles in the initiation of immune response and also in the maintenance of immune tolerance. Now, many kinds of regulatory DCs with different phenotypes have been identified to suppress immune response and contribute to the control of autoimmune diseases. However, the mechanisms by which regulatory DCs can be regulated to exert the immunosuppressive function in the immune microenvironment remain to be fully investigated. In addition, how T cells, once activated, can feedback affect the function of regulatory DCs during immune response needs to be further identified. We previously identified a unique subset of CD11b^{hi}Ia^{low} regulatory DCs, differentiated from mature DCs or hematopoietic stem cells under a stromal microenvironment in spleen and liver, which can negatively regulate immune response in a feedback way. Here, we show that CD11b^{hi}Ia^{low} regulatory DCs expressed high level of Fas, and endothelial stromal cell-derived TGF-β could induce high expression of Fas on regulatory DCs via ERK activation. Fas ligation could promote regulatory DCs to inhibit CD4$^+$ T cell proliferation more significantly. Furthermore, Fas ligation preferentially induced regulatory DCs to produce IL-10 and IP-10 via ERK-mediated inactivation of GSK-3 and subsequent up-regulation of β-catenin. Interestingly, activated T cells could promote regulatory DCs to secrete more IL-10 and IP-10 partially through FasL. Therefore, our results demonstrate that Fas signal, at least from the activated T cells, can promote the immunosuppressive function of Fas-expressing regulatory DCs, providing a new manner for the regulatory DCs to regulate adaptive immunity.

Dendritic cells (DCs),[3] professional antigen-presenting cells (APCs) with a unique capacity to prime naive T cells, play an essential role in linking innate and adaptive immune responses (1). Recently, subsets of regulatory DCs have been identified to be important in maintaining immune homeostasis and play important roles in the pathogenesis or treatment of autoimmune diseases (2, 3). Some kinds of regulatory DCs negatively regulate immune responses by inducing generation of regulatory T (Treg) cells or preferentially programming Th2 response (4). However, the regulatory DCs, commonly used in most current work, are prepared *in vitro* by culturing DC progenitors in the presence of immunosuppressive agents, including IL-10 or TGF-β, or other substances, such as vitamin D receptor ligands and galectin-1 (5, 6). How the immunosuppressive function of regulatory DCs is maintained in the immune microenvironment, especially being feedback-regulated during their interaction with other kinds of immune cells, such as activated T cells, remains to be fully investigated.

The roles of DCs in regulating T cell activation and T cell tolerance have been abundantly documented (7). DCs provide at least two signals required for T cell activation: a signal via the TCR-CD3 complex that is transmitted upon recognition of antigen and an additional signal(s) delivered through one or more costimulatory molecule interactions, like B7-CD28 or LFA-ICAM (8, 9). Once activated, T cells also provide signals to activate APCs. For example, CD40 ligand up-regulated on CD4 T cells after exposure to antigen is an important stimulus for DC activation (10). However, the feedback effect of the T cells, once activated, on the function of regulatory DCs during their interaction and the underlying mechanism have remained unclear up to now.

The microenvironment in lymphoid organs has been found to be important in regulating the development and function of immune cells (11). Although many studies have shown that

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[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed. Tel.: 86-21-5562-0605; Fax: 86-21-6538-2502; E-mail: caoxt@immunol.org.

[3] The abbreviations used are: DC, dendritic cell; APC, antigen-presenting cell; maDC, mature DC; imDC, immature DC; diffDC, differentiated DC; TCR, T cell receptor; Ab, antibody; ESSC, endothelial-like splenic stromal cell; 7-AAD, 7-aminoactinomycin D.

several subsets of DCs display unique functions in large part due to the local microenvironment in different organs or tissues (12), little is known about the role that microenvironment plays in the DC subset and T cell interactions. Our previous studies show that stromal cells, which mimic the lymph organ microenvironment of spleen and liver can drive mature DCs (maDCs) or hematopoietic stem cells to proliferate and further differentiate into a unique subset of $CD11b^{hi}Ia^{low}$ regulatory DCs (diffDCs, DCs differentiated from mature DCs), which express a higher level of IL-10 but minimal IL-12p70 and inhibit maDC-initiated T cell proliferation (13–15). Overactivation of ERK and suppression of p38 MAPK pathways contribute to the unique cytokine profile of regulatory DCs (16). Moreover, the regulatory DCs can chemoattract more Th1 cells through IP-10 in favor of their suppression of Th1 response, enhance NK cell cytotoxicity via IL-10, and also program generation of Th2 memory CD4 T cells as well as regulatory B cells, thus providing a new manner for negative feedback control of immune response and maintenance of immune homeostasis (16–19). However, whether signals emanating from T cells of the adaptive immune system may modulate the function of regulatory DCs at the late stage of the immune response remains unknown.

In this study, we show that endothelial stromal cell-derived TGF-β contributes to the preferential Fas expression of regulatory DCs via an ERK-dependent pathway. Furthermore, Fas ligation induced regulatory DCs to preferentially secret IL-10 and IP-10 through ERK-mediated inactivation of GSK-3 and subsequent up-regulation of β-catenin. Therefore, our data demonstrate that Fas signal can enhance the immunosuppressive function of regulatory DCs in the immune microenvironment, providing a new feedback route for the negative regulation of immune response and maintenance of immune homeostasis by regulatory DCs.

MATERIALS AND METHODS

Mice and Reagents—C57BL/6J mice were obtained from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China). OVA(323–339)-specific TCR-transgenic DO11.10 mice, Mx-Cre mice, *Ctnnb1*$^{fl/fl}$ mice, Fas-deficient B6.MRL-Tnfrsf6lpr mice, and FasL-deficient B6.Smn.C3-Tnfsf6gld mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred under specific pathogen-free conditions. Transgenic mice expressing Cre recombinase under control of the interferon-inducible Mx1 promoter (Mx-Cre) were crossed with mice in which essential portions of the gene encoding β-catenin were flanked by *lox*P sequences (*Ctnnb1*$^{fl/fl}$ mice; Jackson Laboratory). Mx-Cre × *Ctnnb1*$^{fl/fl}$ mice were treated with five intraperitoneal injections of 250 μg of poly(I:C) (Amersham Biosciences) at 2-day intervals to induce *Ctnnb1*-deficient mice (20). 2 days after the last injection, mice were killed, and genomic DNA was prepared from bone marrow cells using standard protocols and assessed by PCR to confirm that it was deleted in Mx-Cre × *Ctnnb1*$^{fl/fl}$ mice. Poly(I:C)-treated Mx-Cre × *Ctnnb1*$^{fl/fl}$ mice are referred to as "β-catenin-deficient mice" here; poly(I:C)-treated Mx-Cre mice and poly(I:C)-treated *Ctnnb1*$^{fl/fl}$ mice served as controls. Animal experiments were performed 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 the Second Military Medical University (Shanghai, China). Recombinant mouse GM-CSF and IL-4 were from PerproTech (London, UK). Anti-mouse TGF-β Ab was from R&D Systems (Minneapolis, MN). Anti-mFas Ab Jo-2 (15400D) and isotype Ab (11150D) were from BD PharMingen (San Diego, CA). PD98059, an inhibitor of MEK1, was from Calbiochem. Anti-phospho-ERK and anti-β-catenin Ab were from Santa Cruz Biotechnology, Inc. Anti-phospho-STAT3 mAb and anti-phospho-GSK3α/β(Ser21/9) mAb were from Cell Signaling (Beverly, MA).

Preparation of Mouse Immature DCs, Mature DCs, and Regulatory DCs—Bone marrow-derived imDCs and maDCs from C57BL/6J mice were generated as described previously (18). Briefly, bone marrow progenitors were cultured in 10 ng/ml GM-CSF and 1 ng/ml IL-4. Nonadherent cells were gently washed out on day 4 of culture; the remaining loosely adherent clusters were cultured for an additional 4–5 days in the presence of 10 ng/ml LPS. At day 8, maDCs were positively selected using CD11c magnetic microbeads (Miltenyi Biotec, Auburn, CA). The cells cultured only with the same concentrations of GM-CSF and IL-4 for 6 days were harvested as imDCs. Regulatory DCs (designated as diffDCs in our previous studies) derived from mature DCs were generated by coculturing with splenic stroma as described previously (15). Once monolayers of endothelial-like splenic stromal cells (ESSCs) had reached 50–60% confluence, maDCs were seeded at a density of 2×10^6 cells/5 ml/well in 6-well plates in RPMI 1640 medium supplemented with 5% FCS for at least 7 days. Regulatory DCs were washed off the layer using 0.1% trypsin and 5 mM EDTA and purified using CD11c magnetic microbeads.

Immunofluorescence Microscopy and Analysis—Cells were cultured on microscope coverglasses placed on a 6-well plate for 3 h, and glass coverslips were precoated with 0.1% polylysine. For immunofluorescence staining, cells were stained with the indicated Abs. Cells on slides were fixed in 4% paraformaldehyde for 30 min at 4 °C and counterstained with Hoechst. Then slides were visualized through a ×40 lens with an inverted Leica TCS-NT confocal laser-scanning microscope as described (21).

Flow Cytometry—Cell surface marker and intracellular cytokine detection, proliferating T cell counts, and cell death analysis were carried out as described before (13, 22, 23). To analyze Fas expression, DCs were stained with anti-Fas, followed by incubation with SAv-phycoerythrin for 30 min at 4 °C. Staining was carried out in the presence of purified antibody to CD16/CD32 (rat IgG2b; clone 2.4G2) to block Fc receptor binding. To test T cell proliferation in 96-well plates after 7 days of culture, cells were double-stained with anti-CD4-FITC and 7-aminoactinomycin D (7-AAD) and resuspended in exactly 300 μl of PBS, and cellular data were acquired for 56 s using flow cytometry. The number of $CD4^+$ $7\text{-}AAD^-$ live cells was calculated to represent the altitude of CD4 T cell proliferation. For intracellular staining, brefeldin A (Sigma-Aldrich) was added to a coculture system at a final concentration of 10 μg/ml simultaneously. After 24 h of coculture, cells were harvested and stained intracellularly for IL-10 expression detected by flow cytometry (cytokine, CD4, and CD11c triplicate staining). Cells were analyzed by flow cytometry using a BD LSR II flow cytom-

eter, and data were analyzed with CellQuest version 3.3 software (BD Biosciences) or FlowJo version 5.7.2 software (Tree Star).

Assay for IL-10, IP-10, and NO—The culture supernatants were detected for IL-10 and IP-10 by ELISA kits (R&D, Minneapolis, MN) and for NO by measurement of the nitrite concentration with the Griess assay as described (15, 24).

Western Blot Analysis of ERK, GSK3, and STAT3 Activation—Cells were lysed with M-PER™ protein extraction reagent (Pierce) supplemented with protease inhibitor mixture, and protein concentrations of the extracts were measured by a BCA assay (Pierce). Proteins were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (25).

Assessment of β-Catenin Expression and Nuclear Translocation—Cytoplasmic, nuclear, and total cell extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents and M-TER mammalian protein extraction reagent (Pierce) containing protease inhibitor mixture (Calbiochem). Blots were probed for 1 h with β-catenin (1:1000) and then incubated with 1:5000 diluted HRP-conjugated secondary antibody for 1 h at room temperature. Proteins were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted.

Isolation, Purification, and Activation of $CD4^+$ T Cells—Resting splenic $CD4^+$ T cells from wild-type (WT) or $FasL^{-/-}$ C57BL/6J mice were enriched using CD4-conjugated microbeads (Miltenyi Biotec). For T cell activation, freshly isolated $CD4^+$ T cells (5.0×10^5/well) were cultured in 96-well round bottom plates in the presence of plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml).

Regulatory DC-activated T Cell Coculture—Coculture experiments were performed with activated T cells and DCs from WT or $Fas^{-/-}$ C57BL/6J mice. Purified imDCs, maDCs, regulatory DCs, or $Fas^{-/-}$ regulatory DCs were resuspended in RPMI 1640 supplemented with 10% FCS and plated in 96 U-bottom well plates, and then activated T cells were seeded to DCs in a total volume of 200 μl/well.

Transwell Separation Assay—24-well transwell chambers with 1.0-μm pore size polycarbonate filters (BD Biosciences) were used. For IL-10 and IP-10 determination, activated T cells were seeded to the upper chamber at 0.1 ml/well, and various DCs were plated at the bottom at 0.5 ml/well. After coculture for the indicated time, supernatants at the bottom were collected to measure IL-10 and IP-10 production.

Sorting of a Natural Counterpart of Regulatory DCs in Vivo—$CD11c^+$ cells enriched by CD11c-conjugated microbeads from spleen were stained with fluorescently labeled anti-Ia-FITC, and then $CD11c^+Ia^{low}$ cells were sorted as described previously (15).

Statistical Analysis—Data are shown as mean ± S.D. for separate experiments. Statistical significance was determined by Student's *t* test.

RESULTS

TGF-β-induced ERK Activation Is Responsible for the Higher Expression of Fas by Regulatory DCs—TNF superfamily receptors are expressed on a variety of cell types and play important roles in cell-cell interactions in the immune system (26). Confocal microscopy showed that regulatory DCs could express a significantly higher level of Fas than imDCs and maDCs (Fig. 1*A*). Our previous studies showed that ESSCs could secrete TGF-β, which was important for the ESSC-driven generation of regulatory DCs in the coculture system (15). Because TGF-β has been shown to be important in Fas expression and ERK activation, we tested whether TGF-β was responsible for the higher Fas expression on regulatory DCs compared with that on imDCs or maDCs. The neutralizing antibody against TGF-β could decrease the Fas expression and ERK activation in regulatory DCs (Fig. 1, *B* and *C*). In addition, regulatory DCs expressed a high level of activated ERK at the steady state. To confirm the role of ERK in the higher expression of Fas in regulatory DCs compared with that on imDCs or maDCs, we assayed the level of Fas by regulatory DCs in the presence of 25 μM ERK inhibitor PD98059 and found that ERK inhibitor decreased Fas expression in regulatory DCs (Fig. 1*D*). These results demonstrate that the increased ERK activation by TGF-β derived from ESSCs contributes to a high level of Fas expression in regulatory DCs.

Fas Ligation Promotes Regulatory DCs to Produce More IL-10 and IP-10 and Suppression of T Cell Proliferation—It is well known that resistance to Fas-induced apoptosis is of great biological significance for DCs in their roles as professional APCs. Thus, we tested whether Fas ligation could induce apoptosis of regulatory DCs. Ligation of Fas with 1 μg/ml Jo-2 for 8 h could effectively induce apoptosis of mouse thymocytes but failed to induce apoptosis of regulatory DCs (Fig. 2*A*). As reported previously, regulatory DCs secreted significantly higher levels of IL-10 and IP-10 than imDCs and maDCs (16, 18). In light of these results, we wondered whether Fas ligation could influence the unique cytokine secretion of regulatory DCs. As shown in Fig. 2, *B* and *C*, Fas ligation could stimulate regulatory DCs to produce more IL-10 and IP-10 than imDCs or maDCs in a dose- and time-dependent manner.

Our previous studies showed that ligation of Fas could induce maturation of DCs and enhance the capability of DCs to prime T cell proliferation and, as a result, positively regulate the immune response. Therefore, we observed whether Fas ligation can induce regulatory DCs to be active in T cell proliferation or promote the inhibitory function of regulatory DCs for T cell proliferation. OVA(323–339)-specific TCR transgenic $CD4^+$ T cells purified from DO11.10×C57BL/6 F1 mice were used as responders to imDCs or maDCs and/or regulatory DCs derived from wild-type or Fas-deficient mice in the presence of OVA(323–339) peptides. After 7 days, viable $CD4^+$ T cells present in T cell/DC cultures were counted by flow cytometry. As shown in Fig. 2*D*, regulatory DCs could not promote T cell proliferation but significantly inhibited maDC-induced antigen-specific $CD4^+$ T cell proliferation. Fas ligation could further enhance the inhibitory function of regulatory DCs for T cell proliferation. Furthermore, regulatory DCs from Fas-deficient mice lost their ability to inhibit T cell proliferation. These data indicate that Fas ligation can enhance the immunosuppressive function of regulatory DCs on T cell proliferation.

In our previous study (15), we found that the inhibitory function of regulatory DCs was partially mediated by NO. Therefore, we asked whether Fas endowed regulatory DCs with NO-dependent

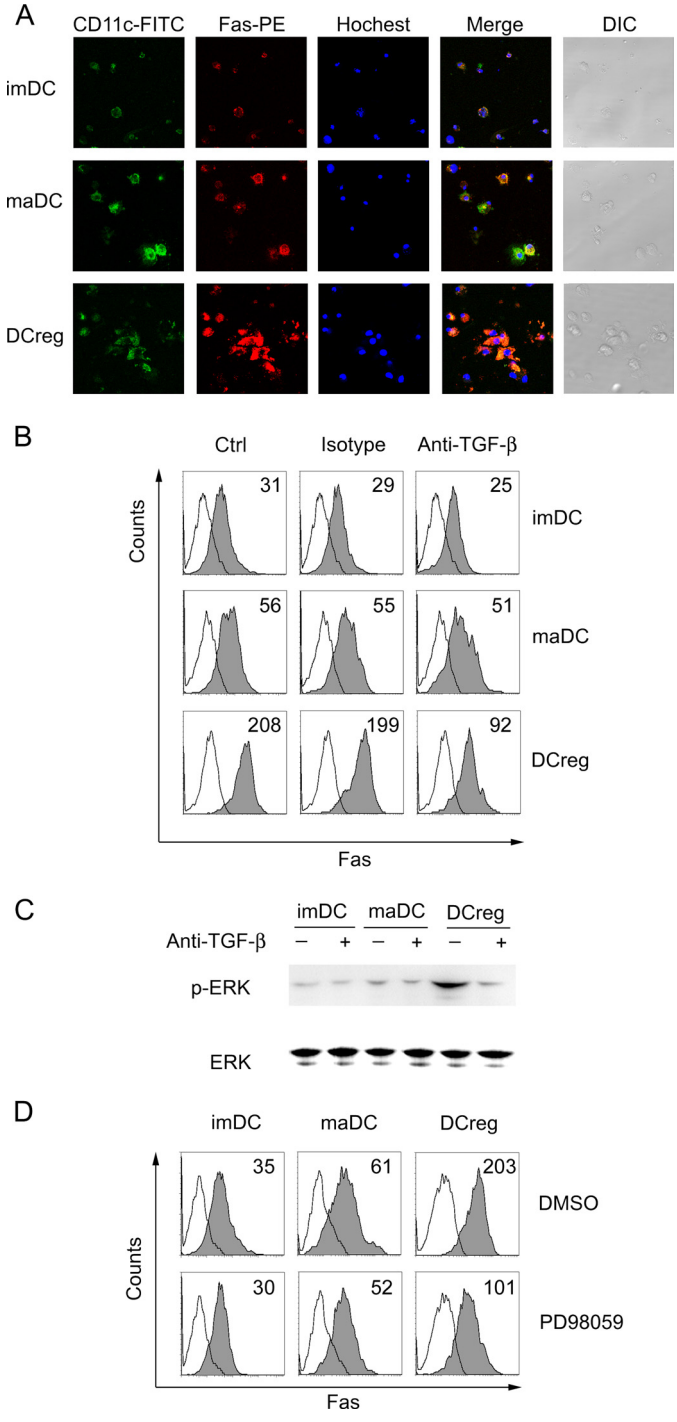


FIGURE 1. **TGF-β-induced ERK activation is responsible for the increased Fas expression of regulatory DCs.** *A*, confocal analysis of Fas expression by regulatory DCs. Representative images of immunofluorescence were stained with CD11c-FITC, Fas-phycoerythrin, and Hoechst staining (nuclei) of imDCs, maDCs, and regulatory DCs. *B*, endothelial stromal cell-derived TGF-β was involved in the Fas expression of regulatory DCs. At the beginning of the culture, neutralizing antibody against mouse TGF-β (5 μg/ml) or isotype antibody was added into the culture system of imDCs, maDCs, or regulatory DCs. After 7 days, imDCs, maDCs, and regulatory DCs were collected to detect the Fas expression by flow cytometry. *C*, phosphorylated ERK in imDCs, maDCs, and regulatory DCs in the presence or absence of neutralizing antibody against TGF-β was detected by Western blotting. imDCs, maDCs, and regulatory DCs in medium alone were used as controls. *D*, imDCs, maDCs, and regulatory DCs were treated with 25 μM PD98059 (ERK inhibitor) for 12 h, and then the Fas expression was analyzed by flow cytometry. Equal amounts of DMSO contained in medium were used as negative controls. Data represent one of at least three experiments with similar results. *DIC*, differential interference contrast.

immunosuppressive function. Compared with that in the supernatant of the DCreg/maDC/T cell coculture system, the concentration of NO in supernatant of the $Fas^{-/-}$DCreg/maDC/T cell coculture system decreased significantly along with the impaired ability of Fas-deficient regulatory DCs to inhibit T cell proliferation (Fig. 2*E*). This indicated that a high level of NO in DCreg/activated T cell interaction was induced by Fas on regulatory DCs, and NO was involved in DCreg-induced inhibition of T cell proliferation. As shown in Fig. 2*F*, the selective NO synthase inhibitor dihydrobromide (*PBIT*) could effectively reverse DCreg-induced inhibition of maDC-primed T cell proliferation, whereas blockade of IL-10 did not affect T cell proliferation in the DCreg/maDC/T coculture system. These results further confirmed the inhibitory action of NO from regulatory DCs on T cell proliferation.

To demonstrate whether regulatory DC would induce apoptosis of activated T cells to exert their inhibitory effects, we cocultured DO11.10 $CD4^+$ T cells with maDCs and/or regulatory DCs derived from wild-type or Fas-deficient mice in the presence of OVA(323–339) peptide, and then the apoptosis of T cells was assayed by flow cytometry. As shown in Fig. 2*G*, regulatory DCs could increase the percentage of the apoptosis of the activated T cells whose activation was initiated by maDCs. Furthermore, regulatory DCs from Fas-deficient mice failed to induce apoptosis of activated T cells. Carboxyfluorescein diacetate succinimidyl ester-labeled OVA(323–339) peptide-specific $CD4^+$ T cells were used as responders, and the results demonstrated that regulatory DCs suppressed $CD4^+$ T cell divisions, and the ability of Fas-deficient regulatory DCs to inhibit the activated T cell proliferation decreased significantly (supplemental Fig. S1). These results demonstrated that Fas on regulatory DCs mediated apoptosis induction and halted proliferation of activated T cells.

Fas Ligation Induces Regulatory DCs to Preferentially Produce IL-10 and IP-10 through the β-Catenin Pathway—Next, we wanted to know the mechanisms by which regulatory DCs secrete high levels of IL-10 and IP-10 in response to Fas ligation. As we previously reported (15), ESSCs could induce maDCs to undergo proliferation and differentiation by way of cell-cell contact, fibronectin, and TGF-β. Also, it is well known that the Wnt signaling pathway plays key roles in many cellular processes, such as proliferation, differentiation, motility, and survival/apoptosis. β-Catenin transmits the Wnt signal into the nucleus, where it acts as a transcriptional coactivator by binding to members of the lymphoid enhancer factor/T cell factor (LEF/TCF) family of transcription factors and influences the secretion of many cytokines (27, 28). Thus, we tested the activation of the Wnt/β-catenin pathway in regulatory DCs. As shown in Fig. 3*A*, a higher level of β-catenin could be detected in the nuclear and cytoplasmic extracts of regulatory DCs than that in imDCs. As expected, regulatory DCs expressed sharply higher nuclear β-catenin at the resting state, and the nuclear translocation of β-catenin was also more significant following Fas ligation as compared with that in imDCs. To further confirm the role of β-catenin in the elevated production of IL-10 and IP-10 by regulatory DCs, we assayed IL-10 and IP-10 production by regulatory DCs and imDCs prepared from β-catenin-deficient mice. We found that β-catenin deficiency

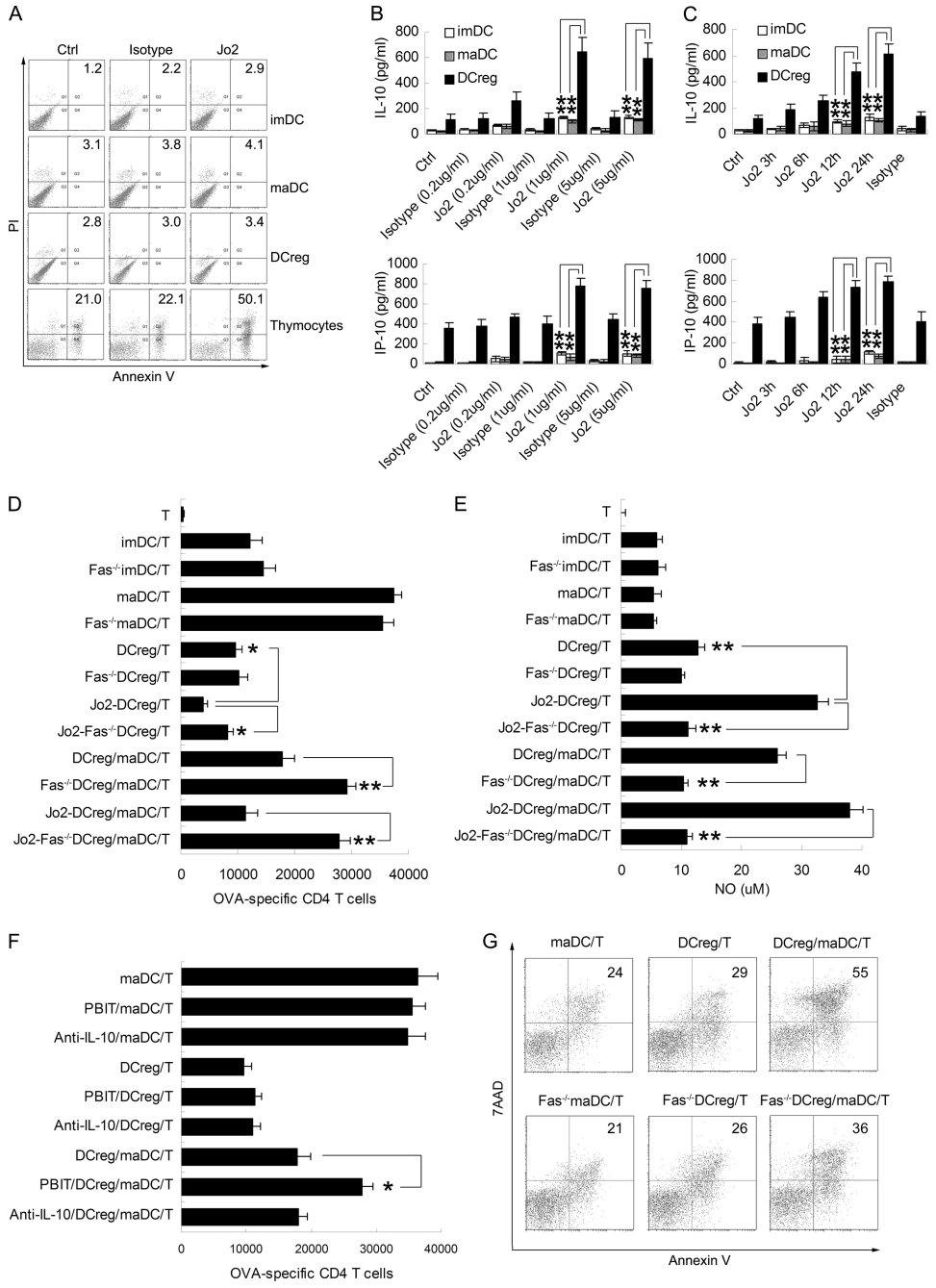


FIGURE 2. **Fas ligation stimulates regulatory DCs to produce higher levels of IL-10 and IP-10 than imDCs.** *A*, the resistance of regulatory DCs to Fas-mediated apoptosis. imDCs, maDCs, regulatory DCs, and mouse thymocytes were incubated in the presence or absence of Jo2 or isotype control for 8 h. Then the percentage of apoptotic cells was assessed by FACS by staining with Annexin V-FITC and propidium iodide. Data are shown as the sums of Annexin V-single positive cells, Annexin V and PI-double positive cells, and PI-single positive cells. Data represent one of at least three independent experiments with similar results. *B*, IL-10 (*top*) and IP-10 (*bottom*) production by imDCs, maDCs, or regulatory DCs stimulated with various concentrations of Jo2 (0.1 μg-5 μg/ml) or isotype antibody for 24 h. Unstimulated DCs were used as controls. *C*, IL-10 (*top*) and IP-10 (*bottom*) production by imDCs, maDCs, or regulatory DCs following stimulation with 1 μg/ml Jo2 for various lengths of time. *D–E*, regulatory DCs inhibited maDC-induced antigen-specific T cell proliferation via Fas-enhanced NO production. Purified DO11.10 $CD4^+$ T cells (1×10^5) were cocultured with wild-type imDCs or Fas-deficient ($Fas^{-/-}$) imDCs, wild-type maDCs or $Fas^{-/-}$ maDCs, and/or wild-type regulatory DCs or $Fas^{-/-}$ regulatory DCs (with or without 1 μg/ml Jo2 stimulation for 24 h) in the presence of OVA(323–339) at a ratio of 1:10 (DC/T). After 7 days, cells were collected and double-stained with anti-CD4-FITC and 7-AAD and counted by FACS. The number of $CD4^+$ $7\text{-}AAD^-$ live cells (*D*) was calculated to represent the altitude of T cell proliferation. The concentrations of NO (*E*) in supernatants in each group were tested by a Griess assay. *F*, iNOS inhibitor dihydrobromide (*PBIT*) (5 μg/ml) or neutralizing antibody against mouse IL-10 (10 μg/ml) was added into a maDC/T cell or DCreg/maDC/T cell coculture system. On day 7, the number of viable OVA(323–339)-specific T cells was detected by flow cytometry. *G*, purified DO11.10 $CD4^+$ T cells were cocultured with maDCs and/or regulatory DCs derived from wild-type or Fas-deficient mice in the presence of OVA(323–339) peptide for 7 days, and then the apoptosis of T cells was assayed by flow cytometry. Data are shown as the sums of Annexin V-single positive cells, Annexin V and 7AAD-double positive cells, and 7AAD-single positive cells. Data represent one of at least three independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

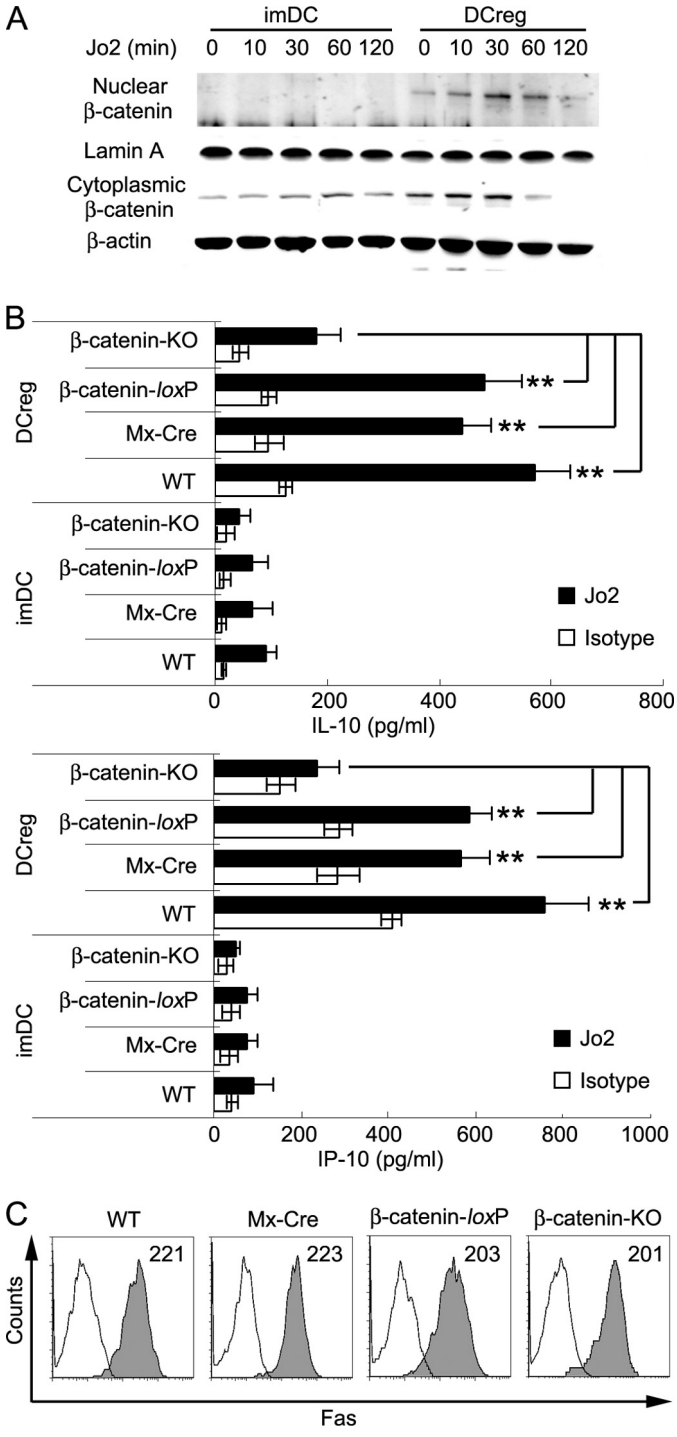


FIGURE 3. **Up-regulated β-catenin is responsible for the preferential IL-10 and IP-10 by regulatory DCs.** *A*, nuclear or cytoplasmic extracts from imDCs and regulatory DCs cultured stimulated with or without Jo2 for 10, 30, 60, and 120 min were prepared, blotted, and probed with β-catenin-specific antibodies, respectively. Lamin A or β-actin serves as a loading control. *B*, imDCs and regulatory DCs from β-catenin-deficient (β-catenin-KO) and control (wild-type, Mx-Cre, β-catenin-*lox*P) mice were stimulated with 1 μg/ml Jo2 for 24 h. Supernatant was measured using an ELISA kit for IL-10 (*top*) and IP-10 (*bottom*). Untreated DCs were used as a control. *C*, expression of Fas on imDCs and regulatory DCs derived from β-catenin-deficient (β-catenin-KO) and control (wild-type, Mx-Cre, β-catenin-*lox*P) mice. Data represent one of at least three independent experiments with similar results. **, $p < 0.01$.

impaired the secretion of IL-10 and IP-10 in regulatory DCs after Jo2 stimulation (Fig. 3*B*). Furthermore, we wondered whether a lack of β-catenin would affect the Fas expression in

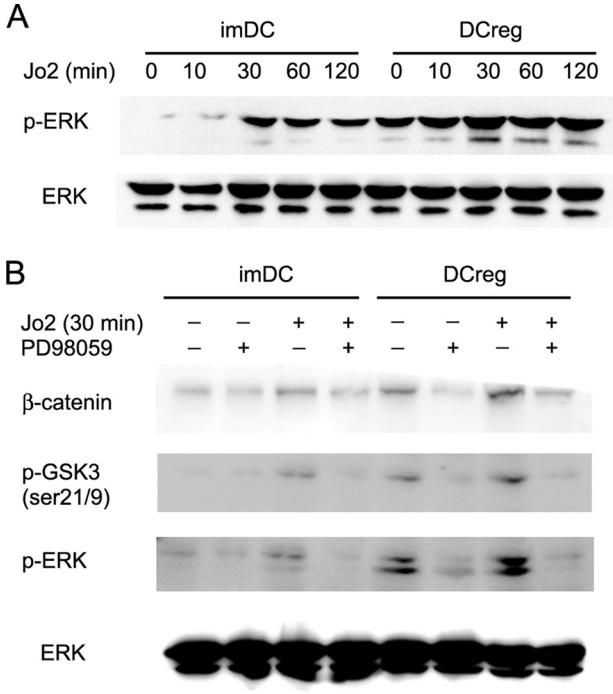


FIGURE 4. **ERK overactivation-induced GSK-3 inactivation increases β-catenin expression in regulatory DCs.** *A*, 1×10^6 imDCs and regulatory DCs were treated with 1 μg/ml Jo2 for 0–120 min and then lysed. The phosphorylation of ERK (*p-ERK*) was detected by Western blotting. Total ERK in each sample was used as the equal loading control. *B*, regulatory DCs and imDCs were pretreated with 25 μM ERK inhibitor for 30 min and then stimulated with Jo2 for 30 min. ERK and GSK-3 (*p-GSK3*) phosphorylation and β-catenin expression were examined by immunoblotting of cell lysates. Similar results were obtained from three independent experiments.

regulatory DCs during coculture and found that Fas was comparably expressed in β-catenin-deficient regulatory DCs and in control cells (Fig. 3*C*). Such data demonstrate that the increased β-catenin in regulatory DCs contributes to high production of IL-10 and IP-10 in response to Jo2 stimulation.

Fas-mediated ERK Activation Is Responsible for Inactivation of GSK-3 and Subsequent Up-regulation of β-Catenin—We had previously found that ERK is overactivated in regulatory DCs. ERK has been suggested to serve as a scaffold to hold GSK-3β, which affects the stability of β-catenin (29). Thus, we also examined the phosphorylation of ERK in regulatory DCs after Jo2 treatment and found that ERK was more significantly phosphorylated following Jo2 stimulation in comparison with that in imDCs (Fig. 4*A*). To further investigate the relationship between ERK and β-catenin, regulatory DCs and imDCs were stimulated with Jo2 in the presence of 25 μM PD98059. As shown in Fig. 4*B*, PD98059 pretreatment could inhibit the phosphorylation of GSK-3 at Ser-21 and Ser-9 and the up-regulation of β-catenin. These results suggest that Fas-induced ERK activation is responsible for inactivation of GSK-3 and the resulting up-regulation of β-catenin.

Fas/FasL Interaction Contributes to the Promotion of Regulatory DC-derived IL-10 and IP-10 Production by Activated T Cells—In addition to signals being delivered by APCs to T cells, T cells also provide signals to "condition" APCs (10, 30). It has been reported that activated T cells may express levels of FasL sufficient to provide signals to condition effector cells (31). Therefore, whether activated T cells can cooperate with regu-

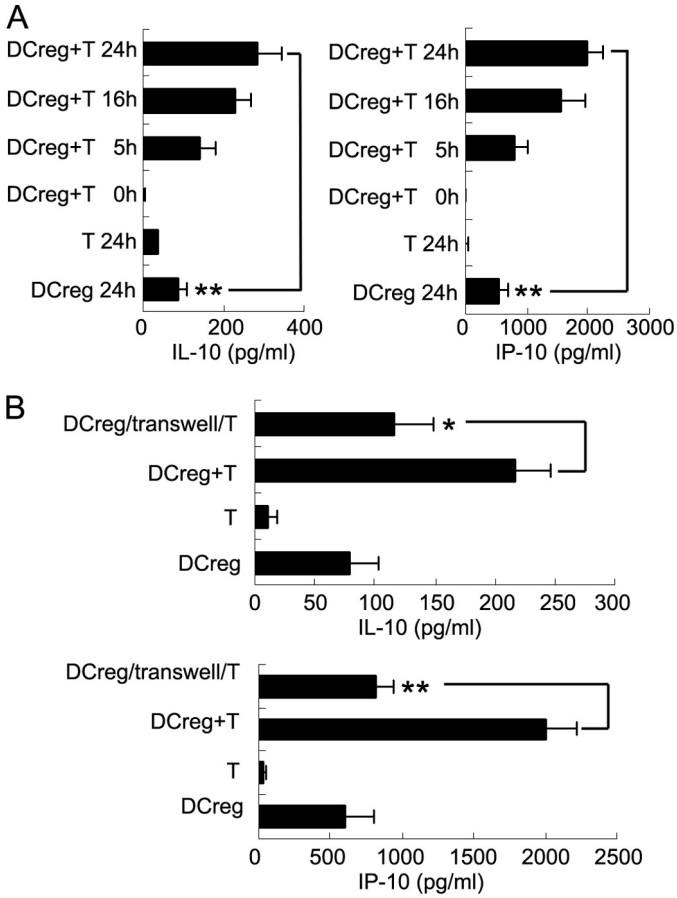


FIGURE 5. **Activated T cells promote regulatory DCs to secrete IL-10 and IP-10.** *A*, IL-10 (*left*) and IP-10 (*right*) production in the supernatant of regulatory DCs/T coculture was determined by ELISA at the indicated time point (5, 16, or 24 h). *B*, 1×10^5 regulatory DCs were cocultured with activated T cells separately by a transwell membrane. After 24 h of coculture, the supernatants were collected to measure IL-10 and IP-10 production by ELISA. Data are shown as means ± S.D. (*error bars*) of triplicate wells. *, $p < 0.05$; **, $p < 0.01$.

latory DCs is crucial for the fulfillment of regulatory functions. Expression of the activation markers CD69 and CD25 was used to identify activated T cells, and as expected, they were up-regulated by 5 h and continued to be expressed at 24 h. We also found that T cells highly expressed FasL and still survived when T cells were stimulated with CD3/CD28 for 24 h (supplemental Fig. S2). We then examined IL-10 and IP-10 production in supernatant of regulatory DCs/T cocultures. As shown in Fig. 5*A*, significant IL-10 and IP-10 production in regulatory DC/T cell cocultures appeared as soon as 5 h after the coculture, indicating that IL-10 and IP-10 production could be induced rapidly. These results suggest that activated T cells could condition regulatory DCs to increase IL-10 and IP-10. We then used a transwell separation assay to confirm that cell-to-cell direct contact from activated T cells was required for the increased production of IL-10 and IP-10 by regulatory DCs, suggesting that cell-to-cell direct contact was involved in the enhancement of regulatory function of DCs by the activated T cells (Fig. 5*B*).

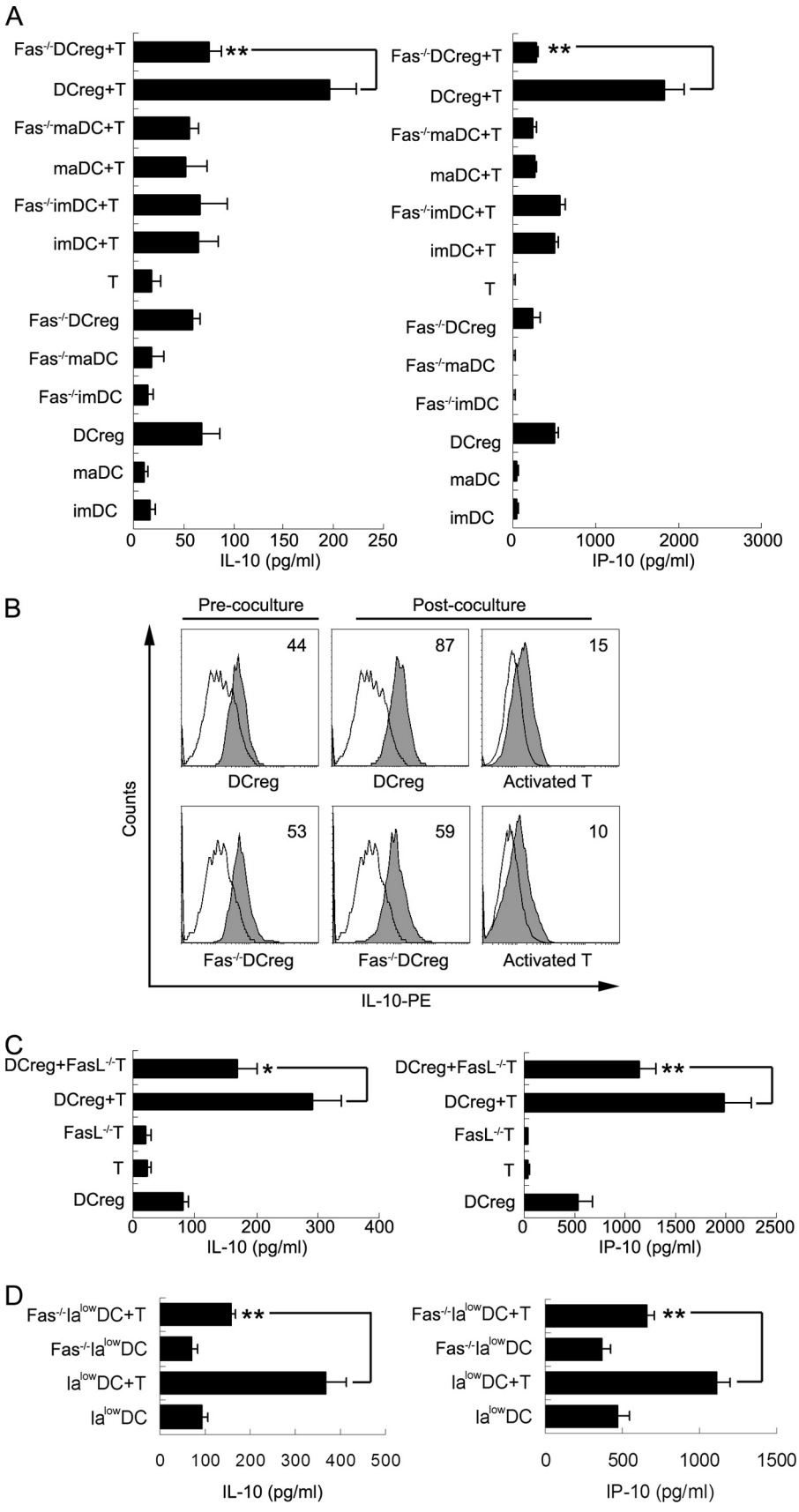
Because regulatory DCs expressed a high level of Fas, we examined the role of the Fas signal in the phenomenon observed above. Using Fas-deficient mice, we surprisingly found that lack of Fas impaired regulatory DC production of IL-10 and IP-10 during coculture with the activated T cells (Fig. 6*A*). To verify that IL-10 and IP-10 were mainly produced by regulatory DCs or activated T cells, the presence of IL-10-positive cells was confirmed by intracellular staining after 24 h of coculture in the presence of 10 μg/ml brefeldin A. Because there is no commercial antibody to detect IP-10 for intracellular staining, we cannot perform the experiments for IP-10. We found that regulatory DCs produced more IL-10, whereas Fas-deficient regulatory DCs produced less IL-10 after coculture with activated T cells. Meanwhile, IL-10-positive T cells were also detected in the coculture, but IL-10 expression was relatively very low (Fig. 6*B*). We then cocultured regulatory DCs with FasL-deficient T cells and found that FasL deficiency in activated T cells could also decrease IL-10 and IP-10 production in the coculture system (Fig. 6*C*). We went further to investigate whether activated FasL-expressing T cells might induce apoptosis of different DC subsets. As shown in supplemental Fig. S3, activated T cells could partially induce apoptosis of maDCs, whereas blockade of FasL failed to reverse the activated T cell-induced apoptosis of maDCs. By contrast, apoptosis of imDCs and regulatory DCs was not remarkably affected by activated T cells. These observations implied that interaction of Fas and FasL was not involved in activated T cell-induced apoptosis of DCs.

Because high levels of IL-10 and IP-10 were markers of the regulatory function of regulatory DCs, we wondered whether the *in vivo* counterpart of regulatory DCs could also produce high level IL-10 and IP-10 by Fas and FasL interaction during coculture with the activated T cells. As shown in Fig. 6*D*, lack of Fas impaired production by $CD11c^+Ia^{low}$ cells (the *in vivo* counterpart of regulatory DCs) of IL-10 and IP-10 during coculture with the activated T cells. The data further indicated that Fas might contribute to the negative immune regulation *in vivo* by regulatory DCs.

We went further to investigate whether regulatory DCs could induce inhibition of the activated T cell proliferation via Fas/FasL interaction. As shown in supplemental Fig. S4, regulatory DCs could inhibit the proliferation of activated T cells initiated by anti-CD3/anti-CD28. Meanwhile, the inhibition of $CD4^+$ T cell proliferation was reversed significantly by Fas deficiency in regulatory DCs or FasL deficiency in T cells. These results demonstrated that Fas and FasL interaction contributed to the inhibitory action of regulatory DCs on the activated T cell proliferation independent of the presence of maDCs. Collectively, these data demonstrated that Fas and FasL interaction contributed, at least partially, to the preferential production of IL-10 and IP-10 as well as inhibition of T cell proliferation by regulatory DCs during coculture with the activated T cells.

DISCUSSION

Regulatory DCs have been considered to be important in maintaining immune homeostasis. Inhibition of T cell proliferation is the functional hallmark of regulatory DCs (5, 32). However, most evidence supporting this viewpoint has been obtained from experiments using DC only *in vitro* cultures, ignoring the involvement of microenvironments in lymph organs that might be important in determining the immune cell functions (32, 33). In our previous report, splenic stromal cells secrete a wide variety of cytokines, whereas only TGF-β is ver-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IL-10 (pg/ml)
0 1000 2000 3000
IP-10 (pg/ml)
B
Pre-coculture
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44
87
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DCreg
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10
Fas-/-DCreg
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IL-10 (pg/ml)
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ified to be involved in the induction of regulatory DCs (15). Given this observation, we went further to investigate the influence of splenic stromal cells on maDCs with a view to elucidating more clearly the fates of DCs after they home to the spleen, where they interact with T cells after antigen uptake or exposure to inflammatory stimuli in the periphery.

We found that regulatory DCs expressed a higher level of Fas than imDCs and maDCs, and stroma-derived TGF-β was responsible for the high expression of Fas on regulatory DCs. It has been proposed that in some cell types, TGF-β inhibits Fas-mediated apoptosis by down-regulating Fas expression or by up-regulating cFLIP, a molecule known to regulate caspase-8 activation, whereas in other cells, such as mast cells, stem cell factor, TGF-β, and FcϵRI aggregation enhances Fas expression (34, 35). In our coculture system, ESSCs secreted stem cell factor, TGF-β, and other soluble or cell surface factors and might provide complex signals to maDCs to modulate Fas expression. Meanwhile, regulatory DCs could also produce TGF-β, albeit at a low level, in this coculture. Thus, the reduced Fas expression might be caused partially by a reduction in DCreg-derived TGF-β (15). Moreover, it has been recently shown that rapid activation of the Ras-ERK pathway is observed when cells are treated with TGF-β, although detailed mechanisms are not yet fully understood (36). This finding is confirmed by our finding that block of TGF-β could markedly decrease the ERK overactivation in regulatory DCs. In addition, we found that ERK activation was required for Fas expression of regulatory DCs. This finding is contrary to the general view that ERK inhibits the expression of Fas on both lymphomas and in solid tumor cells and is only consistent with the study in which the ERK MAPK cascade plays a partial role in regulating TCR-CD3-triggered Fas expression (37). A possible explanation was that ESSCs might confer complicated signals to regulatory DCs and reprogram ERK for a diverse function in Fas expression. Therefore, these results suggest that splenic stromal cells might secrete TGF-β to induce ERK activation, leading to up-regulation of Fas expression in regulatory DCs.

NO is a pleiotropic molecule that has been found to mediate T cell apoptosis or death directly, thus playing important roles in the negative regulation of immune response. Our previous study demonstrated that splenic stroma could drive mature DCs to differentiate into regulatory DC subsets, which could strongly inhibit T cell response via NO production (15). We have found that a high level of NO in DCreg/activated $CD4^+$ T cell interaction was induced by Fas on regulatory DCs, and NO was involved in DCreg-induced inhibition of $CD4^+$ T cell proliferation. These results further confirmed the inhibitory action of NO from regulatory DCs on T cell proliferation. Splenic stroma educates regulatory DCs to express a high level of Fas. Furthermore, the Fas signal is involved in activated T cell-promoted NO production from regulatory DCs, and Fas-induced NO subsequently inhibits CD4 T cell proliferation. 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 DCs at the late stage of immune response. However, how Fas enhances NO production from regulatory DCs remains to be further studied in the future.

IL-10 is generally considered to limit immune and inflammatory responses and plays an important role in maintaining the balance between Th1 and Th2 responses. The Th2-biased cytokine profile characterized by high IL-10 production was found as one of characteristics of regulatory DCs we identified (15). As we have previously reported, regulatory DCs could program generation of memory CD4 T cells with suppressive activity via IL-10, indicating that regulatory DCs display the inhibitory function by directly secreting IL-10 (17). Moreover, regulatory DCs could chemoattract more Th1 cells through IP-10 and then inhibit their proliferation, thus providing a new manner of negative feedback control of immune responses (18). These results showed that regulatory DCs could display the inhibitory function by directly secreting IL-10 and IP-10. In this study, we went further to understand the manner in which regulatory DCs negatively regulate immune responses by finding that the Fas signal could promote the immunosuppressive function of regulatory DCs as markers of IL-10 and IP-10 in the immune microenvironment.

As to the mechanisms for preferential IL-10 and IP-10 production by regulatory DCs, with more production in response to Fas ligand, we showed that the ERK-mediated inactivation of GSK-3, resulting in up-regulation of β-catenin, was responsible for the unique cytokine secretion. It has been reported that ERK docks to and phosphorylates GSK-3β, resulting in inactivation of GSK-3β and in turn inhibition of β-catenin ubiquitination (29). GSK-3 is negatively regulated by phosphorylation of an N-terminal serine (Ser-21 for GSK-3α and Ser-9 for GSK-3β) (38–40). Recent work has shown that GSK-3 inhibitors can enhance both β-catenin expression and GSK-3-Ser-21/9 phosphorylation, resulting in elevated IL-10 secretion and decreased IL-12p40, IL-6, and TNF-α secretion of human monocytes (41–43). Our data showed that ERK was more activated in resting regulatory DCs than that in imDCs, and ERK activation was more significant in regulatory DCs following Fas ligation. Treatment with ERK inhibitor resulted in inhibition of GSK-3 phosphorylation and β-catenin expression. Thus, it is likely that ERK-induced inactivation of GSK-3 was responsible for up-regulation of β-catenin during Fas ligation. Once β-catenin is accumulated in the cytoplasm, it can translocate to the nucleus, where it binds to Tcf/Lef and acts together with its coactivator to stimulate the transcription of target genes (44,

FIGURE 6. **Fas signal is involved in activated T cell-promoted IL-10 and IP-10 production by regulatory DCs.** *A*, similar to DCs/T coculture, 1×10^5 $Fas^{-/-}$ DCs were cocultured with activated T cells. After 24 h of coculture, the supernatants were collected to measure IL-10 and IP-10 production by ELISA. Data are shown as means ± S.D. (*error bars*) of triplicate wells. **, $p < 0.01$. *B*, intracellular staining for IL-10 expression by regulatory DCs, $Fas^{-/-}$ regulatory DCs, or activated T cells (cytokine, CD11c, and CD4 triplicate staining). *Numbers* in the histograms indicate the geometric mean fluorescence of the test samples. Similar results were obtained in at least three independent experiments. *C*, 1×10^5 regulatory DCs were cocultured with wild-type or $FasL^{-/-}$ activated T cells. After 24 h of coculture, the supernatants were collected to measure IL-10 and IP-10 production by ELISA. *D*, $CD11c^+Ia^{low}$ cells from the spleen of wild-type or Fas-deficient mice were sorted by flow cytometry. Ia^{low} DCs or Fas-deficient Ia^{low} DCs were cocultured with activated $CD4^+$ T cells. After 24 h of coculture, the supernatants were collected to measure IL-10 and IP-10 production by ELISA. Data are shown as means ± S.D. of triplicate wells. *, $p < 0.05$; **, $p < 0.01$.

45). We found that regulatory DCs expressed a high level of β-catenin, and Fas ligation could increase translocation of β-catenin to the nucleus in regulatory DCs as compared with that in imDCs. Moreover, β-catenin-deficient regulatory DCs are defective in the production of IL-10 and IP-10 with or without Fas ligation. These results suggest that ERK/GSK-3/β-catenin pathways might be responsible for the increased IL-10 and IP-10 production in regulatory DCs. However, determination of why coculture of maDCs with splenic stromal cells could program generation of regulatory DCs with high β-catenin expression and how Fas ligation is linked to ERK activation will require further investigation in the future.

Fas and FasL are typical members of the TNF receptor and TNF ligand family, respectively. They play pivotal roles in the regulation of apoptotic processes, including activation-induced cell death, T cell-induced cytotoxicity, immune privilege, and escape (46, 47). Fas has been shown to mediate proliferation and activation signals in a variety of cell types and to contribute to inflammatory responses (48). Our results showed that Jo2 could provoke regulatory DCs to secret more IL-10 and IP-10, which was in agreement with our observation that Fas ligation could trigger DCs to secrete proinflammatory cytokines and cytokines (49), indicating that Fas ligation might amplify the regulatory function of regulatory DCs as markers of IL-10 and IP-10. The Fas-FasL system is important in the regulation of the immune system in several ways under different physiologic or pathologic conditions, such as autoimmune diseases and inflammation (50–53). We thus hypothesized that regulatory DCs might sense the danger signals provided by FasL through a higher expression level of Fas on their membrane and then by feedback exert a more potent regulatory function on naive T cells, NK cells, or Th1 cells.

In conclusion, we demonstrate that stroma-derived TGF-β induces ERK activation, which is responsible for the high expression of Fas on regulatory DCs. The Fas signal preferentially induces secretion of IL-10 and IP-10 by regulatory DCs via ERK/GSK-3/β-catenin pathways. The Fas signal from the FasL on activated T cells can promote regulatory DCs to secret more IL-10 and IP-10. In this way, the Fas signal in regulatory DCs may be involved in maintaining the immune homeostasis in a feedback manner.

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