

## Apoptotic Cell-induced Tolerogenic Dendritic Cells Facilitate Development of CD4<sup>+</sup> and CD8<sup>+</sup> Regulatory T cell Subsets

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### Abstract

**Objective:** Dendritic cells (DCs) play an important role in regulating T cell-mediated immune responses; however, the mechanisms of DC-mediated immune responses have not been fully elucidated. Regulatory T cells (T<sub>regs</sub>) including CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>regs</sub> play an essential role in induction of immune tolerance *in vivo*. It is unclear how DCs regulate development of T<sub>regs</sub>. Our objective is to observe whether or not apoptotic cell-induced tolerogenic DCs can affect development and differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> regulatory T cells.

**Methods:** CD11c<sup>+</sup> DCs were sorted and isolated from spleen of C57BL/6J mice. DCs were incubated with apoptotic or fresh T cells for 24 hrs at 37°C. MOG-specific CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from 2D2 transgenic mice. Apoptotic or fresh T cell-treated DCs were loaded with MOG peptide and co-cultured with MOG-primed CD4<sup>+</sup>CD25<sup>-</sup> T cells for 72 hrs at 37°C. Expression of signal molecules and cytokines in DCs and Tregs was detected using flow cytometry.

**Results:** Apoptotic T cell-treated DCs express CD40, CD80, CD86 and MHC class II at low levels compared with those on DCs co-cultured with fresh T cells. Treatment of DCs with apoptotic T cells inhibits production of inflammatory cytokines such as IL-12 and IL-23, but facilitates production of anti-inflammatory cytokines including IL-10 and TGF-β, indicating a tolerogenic phenotype. In co-culture, apoptotic T cell-induced tolerogenic DCs facilitate development of multiple subsets of CD4<sup>+</sup> and CD8<sup>+</sup> regulatory T cells (T<sub>regs</sub>) such as CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD127<sup>+</sup> and CD8<sup>+</sup>CD122<sup>+</sup> regulatory T cells, and modulate expression of multiple signal molecules including GARP, CD152, CD44, CD62L, CCR6 and CCR7 on CD4<sup>+</sup> T<sub>regs</sub>.

**Conclusions:** It can be concluded that apoptotic cell-induced tolerogenic DCs exert an immune-regulatory effect by facilitating development of both CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>reg</sub> subpopulations. These results may reveal a new cellular mechanism of tolerogenic DCs induced by apoptotic cells to modulate inflammatory responses.

**Keywords:** Dendritic cell; Immune tolerance; Immunotherapy; Regulatory T cell; Autoimmunity; Inflammation; Apoptosis

### Abbreviations

CCR6/7: C-C chemokine receptor type 6/7; CD: Cluster of Differentiation; DC: Dendritic Cell; EAE: Experimental Autoimmune Encephalomyelitis; FACS: Fluorescence-Activated Cell Sorting; FCS: Fetal Calf Serum; FoxP3: Forkhead box P3; GARP: **Glycoprotein A Repeats Predominant**; GITR: Glucocorticoid-Induced Tumor Necrosis Factor Receptor-related Protein; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; GVHD: Graft Versus Host Disease; IL: Interleukin; MHC: Major Histocompatibility Complex; MOG: Myelin Oligodendrocyte Glycoprotein; MS: Multiple Sclerosis; PBS: Phosphate Buffered Saline; SD: Standard Deviation; TCR: T cell Receptor; TGF-β: Transforming Growth Factor *Beta*; Tregs: Regulatory T cells

### Introduction

Auto-reactive regulatory T cells (T<sub>regs</sub>) play an important role in induction of peripheral tolerance [1]. A great deal of research is currently focused on T<sub>reg</sub> development, and multiple subsets of regulatory T cells have been found [2]. In addition to conventional CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub>, CD4<sup>+</sup>CD127<sup>low</sup> T<sub>regs</sub> have recently been shown to inhibit T cell-mediated autoimmunity [3,4]. CD8<sup>+</sup> T<sub>regs</sub>, as a subpopulation like CD4<sup>+</sup> T<sub>regs</sub>, are also necessary for induction of immune tolerance [5]. For example, CD8<sup>+</sup>CD122<sup>+</sup> T<sub>regs</sub> can inhibit development of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) [6], T cell-mediated colitis [7] and skin graft rejection *in vivo* [8]. Regulatory mechanisms of T<sub>reg</sub> development have not yet been elucidated.

CD4<sup>+</sup> regulatory T cells play an important role in induction of peripheral tolerance. Multiple sub-populations of CD4<sup>+</sup> regulatory T cells such as CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub>, CD4<sup>+</sup>CD127<sup>+</sup>Tregs and CD4<sup>+</sup>GITR<sup>+</sup>T<sub>regs</sub> have been found [9-11]. CD4<sup>+</sup> T<sub>regs</sub> produce anti-

inflammatory cytokines such as IL-10 to inhibit effector T cell-mediated inflammatory responses [12]. We proposed that apoptotic T cell-induced tolerogenic dendritic cells may regulate development and differentiation of CD4<sup>+</sup> T<sub>regs</sub> through modulating protein expression of T<sub>reg</sub>-associated markers and cytokines such as FoxP3, CD25, GITR and IL-10. To test this hypothesis, apoptotic cell-treated and fresh cell-treated dendritic cells were pulsed with MOG peptide and co-cultured with MOG-specific CD4<sup>+</sup>CD25<sup>-</sup> T cells. Expression of FoxP3, CD25, GITR and IL-10 in CD4<sup>+</sup> T cells was detected using flow cytometry. Our results indicated that apoptotic cell-treated dendritic cells facilitate expression of FoxP3, CD25, GITR and IL-10 in CD4<sup>+</sup> T cells. These results suggest that apoptotic cell-induced tolerogenic dendritic cells may elicit development and differentiation of CD4<sup>+</sup> T<sub>regs</sub> through up-regulating expression of T<sub>reg</sub>-associated markers and cytokines *in vivo*.

In addition, there are many signal molecules such as GARP, CD44, CD62L, CD152, CCR6 and CCR7 expressed on CD4<sup>+</sup> T<sub>regs</sub>. CD4<sup>+</sup> T<sub>regs</sub> perform immune function via signal transduction pathways-mediated by these molecules on CD4<sup>+</sup> T<sub>regs</sub> [13-18]. We proposed that apoptotic cell-induced tolerogenic dendritic cells may affect T<sub>reg</sub>-mediated immune function through modulating expression of these signal molecules on CD4<sup>+</sup> T<sub>regs</sub>. To test this hypothesis, apoptotic cell-treated dendritic cells were loaded with MOG peptide and incubated with MOG-specific CD4<sup>+</sup> T cells. Our results demonstrated that co-culture with apoptotic cell-induced tolerogenic dendritic cells leads to modulation of expression of GARP, CD44, CD62L, CD152, CCR6 and CCR7 on CD4<sup>+</sup>CD25<sup>+</sup> T cells. These results imply that apoptotic cell-induced tolerogenic dendritic cells may regulate CD4<sup>+</sup> T<sub>reg</sub>-mediated immune function via modulating expression of signal molecules on CD4<sup>+</sup> T<sub>regs</sub>.

FoxP3, CD25 and IL-10 are also expressed on CD8<sup>+</sup> T<sub>regs</sub> like those presented on CD4<sup>+</sup> T<sub>regs</sub> [19-21]. Moreover, CD122 is a specific marker of CD8<sup>+</sup> T<sub>regs</sub> [5]. We proposed that apoptotic cell-induced tolerogenic dendritic cells may also regulate differentiation and development of CD8<sup>+</sup> T<sub>regs</sub> through affecting expression of CD8<sup>+</sup> T<sub>reg</sub>-associated markers and cytokines on them. To test this hypothesis, apoptotic cell- and fresh cell-treated dendritic cells were pulsed with MOG peptide and co-cultured with MOG-specific CD4<sup>+</sup>CD25<sup>-</sup> T cells. Expression of FoxP3, CD25, IL-10 and CD122 on CD4<sup>+</sup>CD8<sup>+</sup> T cells was detected using flow cytometry. Our results indicated that apoptotic cell-induced tolerogenic dendritic cells up-regulate expression of FoxP3, CD25, CD122 and IL-10 on CD4<sup>+</sup>CD8<sup>+</sup> T cells. It suggests that apoptotic cell-induced tolerogenic dendritic cells not only facilitate development of CD4<sup>+</sup> T<sub>regs</sub>, but also elicit differentiation of CD8<sup>+</sup> T<sub>regs</sub> through improving expression of T<sub>reg</sub>-associated markers and cytokines.

Dendritic cells (DCs) are important immune cells in the innate immune system. DCs not only regulate autoimmunity, but also play an important role in induction of immune tolerance [22]. For instance, DCs modulate CD4<sup>+</sup> T cell-mediated inflammatory responses through three major pathways: (1). Epitope /MHC class II complexes presented on DCs bind to T cell receptor of CD4<sup>+</sup> T cells to initiate activation of CD4<sup>+</sup> T cells (Signal 1). (2). There are some co-stimulatory and signal molecules such as CD40, CD80 and CD86 expressed on DCs. DCs interact with CD4<sup>+</sup> T cells via co-stimulatory molecules which associate with their ligands expressed on CD4<sup>+</sup> T cells to facilitate or inhibit activity of effector cells (Signal 2). (3). DCs secrete some pro- and anti-inflammatory cytokines such as IL-10, IL-12, IL-23 and TGF-β to modulate activation of CD4<sup>+</sup> T cells (Signal 3) [23-26]. To test

whether or not treatment with apoptotic cells can affect protein expression of signal 1, 2 and 3-associated molecules on DCs, DCs were co-cultured with apoptotic or fresh T cells as a control. Our results showed that co-culture with apoptotic T cells leads to generation of tolerogenic DCs with low expression of CD40, CD80, CD86 and MHC class II. Incubation with apoptotic cells inhibits production of inflammatory cytokines including IL-12 and IL-23, but elicits production of anti-inflammatory cytokines such as IL-10 and TGF-β in DCs. Our results imply that tolerogenic DCs-induced by apoptotic cells may regulate CD4<sup>+</sup> T cell-mediated immune responses and lead to immune tolerance through modulating protein expression of signal 1, 2 and 3-associated molecules on DCs.

The multiple subsets of dendritic cells perform different functions *in vivo*. For example, immunogenic DC subpopulations up-regulate T cell-mediated immune responses [27], while tolerogenic ones inhibit function of effector T cells [28]. Some immunosuppressive signals are transferred into DCs via receptors and co-stimulatory molecules expressed on DCs and lead to generation of tolerogenic DCs [22,29-32]. Moreover, DCs engulf apoptotic cells via the CD205-mediated signal transduction pathway and cause generation of immunosuppressive DCs [33]. DCs capture self-antigens and present epitopes on their surface, leading to T cell homeostasis, anergy [22,29-32], and differentiation of T<sub>regs</sub> [34,35]; however, details of regulatory mechanisms of tolerogenic DCs in T cell-mediated immune responses are still poorly understood. In the present study we investigated the effect of tolerogenic DCs induced by apoptotic T cells on development of T<sub>regs</sub>. Our results show that apoptotic T cell-induced tolerogenic DCs facilitate differentiation of multiple subsets of T<sub>regs</sub>. These results may reveal a new mechanism whereby apoptotic T cell-treated DCs can lead to immune tolerance.

## Materials and Methods

### Mice

Female C57 BL/6J and 2D2 T cell receptor (TCR) specific to MOG peptide/MHC class II complex transgenic mice (8-12 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred in the Thomas Jefferson Animal Care facilities. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University.

### Immunogen and peptide

Mouse MOG<sub>35-55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK), a component of myelin oligodendrocyte glycoprotein (MOG), was ordered from Invitrogen (Invitrogen, Carlsbad, California, USA).

### Isolation of spleen DCs

Splenocytes were isolated from naïve C57 BL/6J mice. Cells were harvested and red cells were depleted using red cell lysis buffer (Biolegend, San Diego, CA, USA). Lymphocytes were then stained using anti-mouse cluster of differentiation (CD)11c antibody (Biolegend), and CD11c<sup>+</sup> cells (DCs) were sorted using fluorescence-activated cell sorting (FACS; BD Biosciences, San Jose, CA, USA) for co-culture assay [36,37].

## Induction of apoptotic thymocyte-induced tolerogenic DCs

As described previously [37], thymocytes were isolated from C57 BL/6J mice, then irradiated at 1500 Rad to induce apoptosis. Fresh thymocytes without irradiation were collected as a control. Irradiated and fresh thymocytes were co-cultured with splenic CD11c<sup>+</sup> DCs (thymocytes: DCs=10:1) for 24 hrs at 37°C. These DCs were also primed in the presence of MOG<sub>35-55</sub> peptide (0.1 μM) in the medium. Cells were then harvested for conducting flow cytometry.

## Isolation and cell culture of MOG peptide specific CD4<sup>+</sup> T cells

Splenocytes were isolated from TCR specific to MOG<sub>35-55</sub> peptide/MHC class II complex transgenic mice (2D2; Jackson Laboratory), and stained by anti-mouse CD4 and CD25 antibodies (Biolegend). Effector T cells (CD4<sup>+</sup>CD25<sup>-</sup>) were collected using FACS (BD), then co-cultured for 3 days with CD11c<sup>+</sup> DCs that had been pre-treated with apoptotic or fresh thymocytes, in the presence of mouse IL-2 (1 ng/ml) and MOG peptide (0.1 μM). Cells were washed at 300 g x 5 min twice using phosphate buffered saline (PBS) for flow cytometry [37,38].

## Flow cytometry

CD11c<sup>+</sup> DCs treated with apoptotic or fresh thymocytes were incubated with anti-mouse CD11c, CD40, CD80, CD86 and *major histocompatibility complex* (MHC) II antibodies (Biolegend) for 24 hrs at 4°C. MOG-reactive T cells were isolated from 2D2 mice and co-cultured with 0.1 μM MOG peptide-pulsed DCs treated with apoptotic or fresh thymocytes for 24 hrs at 37°C. Cells were washed at 300 g x 5 min twice with 5% fetal calf serum (FCS) in PBS, then incubated with anti-mouse CD4, CD8, CD25, CD44, CD62L, CD152, CD122, CD127, **glycoprotein A repetitions predominant** (GARP), C-C chemokine receptor type 6 (CCR6), CCR7 and glucocorticoid-induced tumor-necrosis-factor-receptor-related protein (GITR) antibodies (Biolegend) for 24 hrs at 4°C. These cells were washed using FACS sorting buffer (5% FCS in PBS) at 300 g x 5 min twice and collected for intracellular staining [38].

## Intracellular staining

As described previously [37,38], DCs or MOG-reactive T cells were stimulated by leukocyte activator (BD) at 37°C for 6 hrs before conducting intracellular staining. Cells were then washed twice with 5% FCS in PBS at 300 g for 5 min. Cells were fixed with 5% formalin (Biolegend) in PBS for 30 min at room temperature after completion of surface staining as described above, then washed by permeabilization buffer (Biolegend) twice at 300 g x 10 min.

DCs were stained by anti-mouse IL-10, IL-12, IL-23 and transforming growth factor *beta* (TGF-β) antibodies (Biolegend); MOG-reactive T cells were stained by anti-mouse forkhead box P3 (FoxP3) and IL-10 antibodies (Biolegend) for 24 hrs at 4°C. Cells were washed with 5% FCS in PBS twice at 300 g x 5 min and harvested for flow cytometry.

Fixed cells were run on a FACS Aria (BD) and data were analyzed with FlowJo software (Treestar, Ashland, OR, USA).

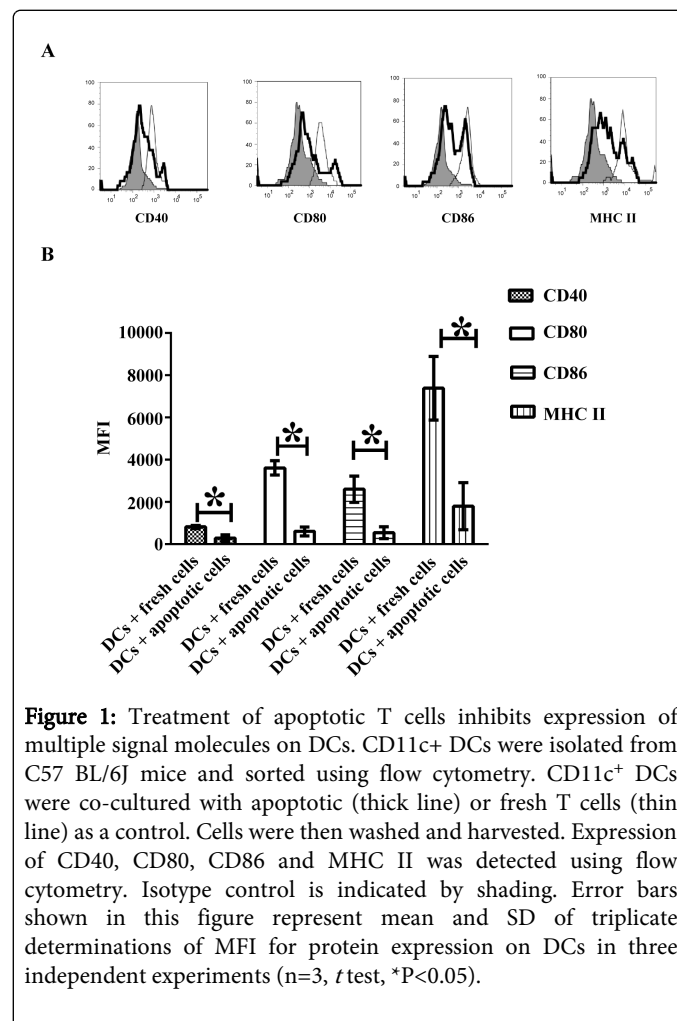
## Statistical analysis

Experimental data were analysed using Prism software (GraphPad, La Jolla, CA, USA). A *t* test was conducted. Error bars shown in this paper represent the mean and standard deviation (SD). Results are regarded as showing a significant difference if the P value is less than 0.05.

## Results

### Apoptotic cells down-regulate expression of signal molecules and MHC class II on DCs

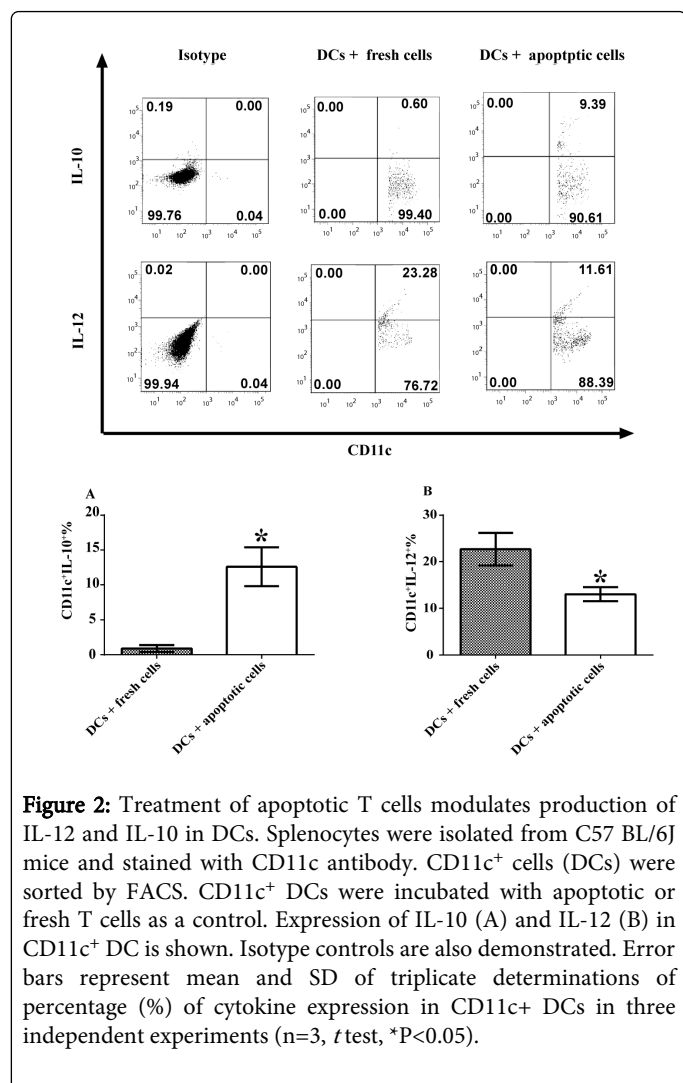
To investigate whether or not treatment with apoptotic cells can affect expression of signal molecules on DCs, DCs were cultured with apoptotic thymocytes, those cultured with fresh thymocytes served as a control. Expression of CD40, CD80, CD86 and MHC class II on DCs was determined using flow cytometry. The results showed that treatment with apoptotic cells leads to down-regulation of expression of CD40, CD80, CD86 and MHC class II on DCs, compared with that on DCs co-cultured with fresh cells (Figure 1). These results imply that treatment with apoptotic cells induces generation of tolerogenic DCs by inhibiting expression of signal and co-stimulatory molecules on DCs.



**Figure 1:** Treatment of apoptotic T cells inhibits expression of multiple signal molecules on DCs. CD11c<sup>+</sup> DCs were isolated from C57 BL/6J mice and sorted using flow cytometry. CD11c<sup>+</sup> DCs were co-cultured with apoptotic (thick line) or fresh T cells (thin line) as a control. Cells were then washed and harvested. Expression of CD40, CD80, CD86 and MHC II was detected using flow cytometry. Isotype control is indicated by shading. Error bars shown in this figure represent mean and SD of triplicate determinations of MFI for protein expression on DCs in three independent experiments (n=3, *t* test, \*P<0.05).

### Apoptotic cells modulate cytokine production of DCs

To determine whether or not treatment with apoptotic cells can modulate DC cytokine production, DCs were cultured with apoptotic thymocytes, and those cultured with fresh thymocytes served as a control. Production of IL-10, IL-12, IL-23 and TGF- $\beta$  was determined by flow cytometry. Our results indicated that treatment with apoptotic T cells facilitates production of anti-inflammatory cytokines, including IL-10 and TGF- $\beta$ , but suppresses secretion of inflammatory cytokines such as IL-12 and IL-23 in DCs (Figures 2 and 3), compared with DCs co-cultured with fresh thymocytes. This cytokine profile of DCs suggests that treatment with apoptotic cells drives DCs into a tolerogenic phenotype.

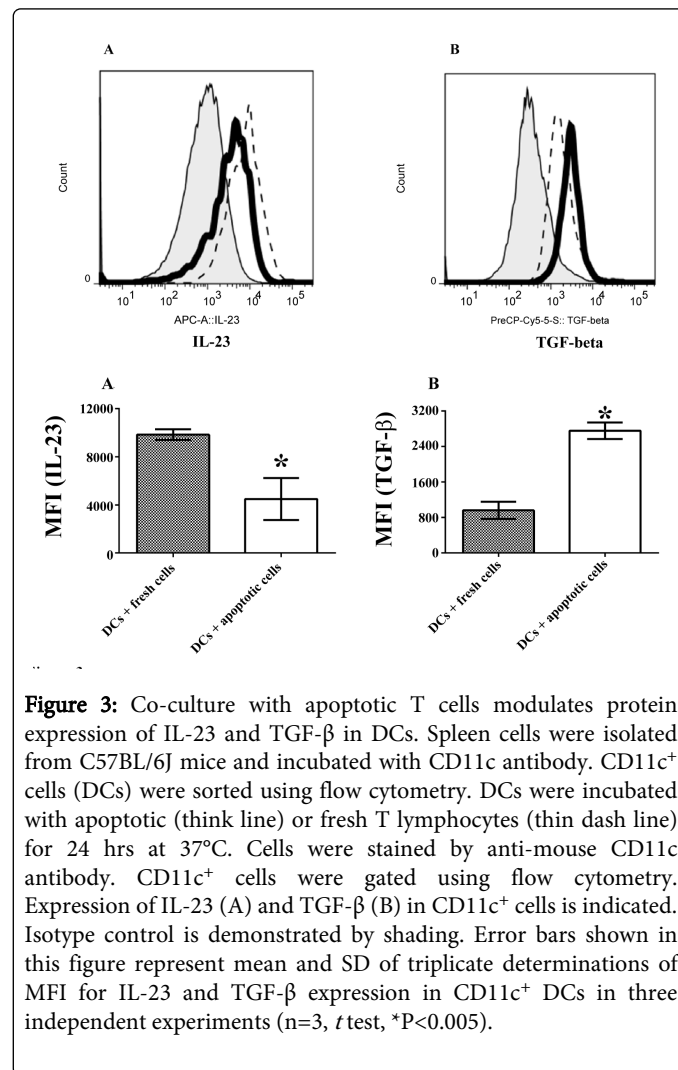


**Figure 2:** Treatment of apoptotic T cells modulates production of IL-12 and IL-10 in DCs. Splenocytes were isolated from C57 BL/6J mice and stained with CD11c antibody. CD11c<sup>+</sup> cells (DCs) were sorted by FACS. CD11c<sup>+</sup> DCs were incubated with apoptotic or fresh T cells as a control. Expression of IL-10 (A) and IL-12 (B) in CD11c<sup>+</sup> DC is shown. Isotype controls are also demonstrated. Error bars represent mean and SD of triplicate determinations of percentage (%) of cytokine expression in CD11c<sup>+</sup> DCs in three independent experiments (n=3, *t* test, \*P<0.05).

### DCs treated with apoptotic cells facilitate development of T<sub>regs</sub>

We then investigated the functional property of DCs treated with apoptotic cells by testing whether or not these DCs can elicit development of T<sub>regs</sub>. CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from MOG-TCR transgenic mice (2D2 mice) and co-cultured with DCs treated with apoptotic or fresh thymocytes. Expression of FoxP3, GITR, CD25, CD127 and IL-10 in CD4<sup>+</sup> T cells was determined by flow

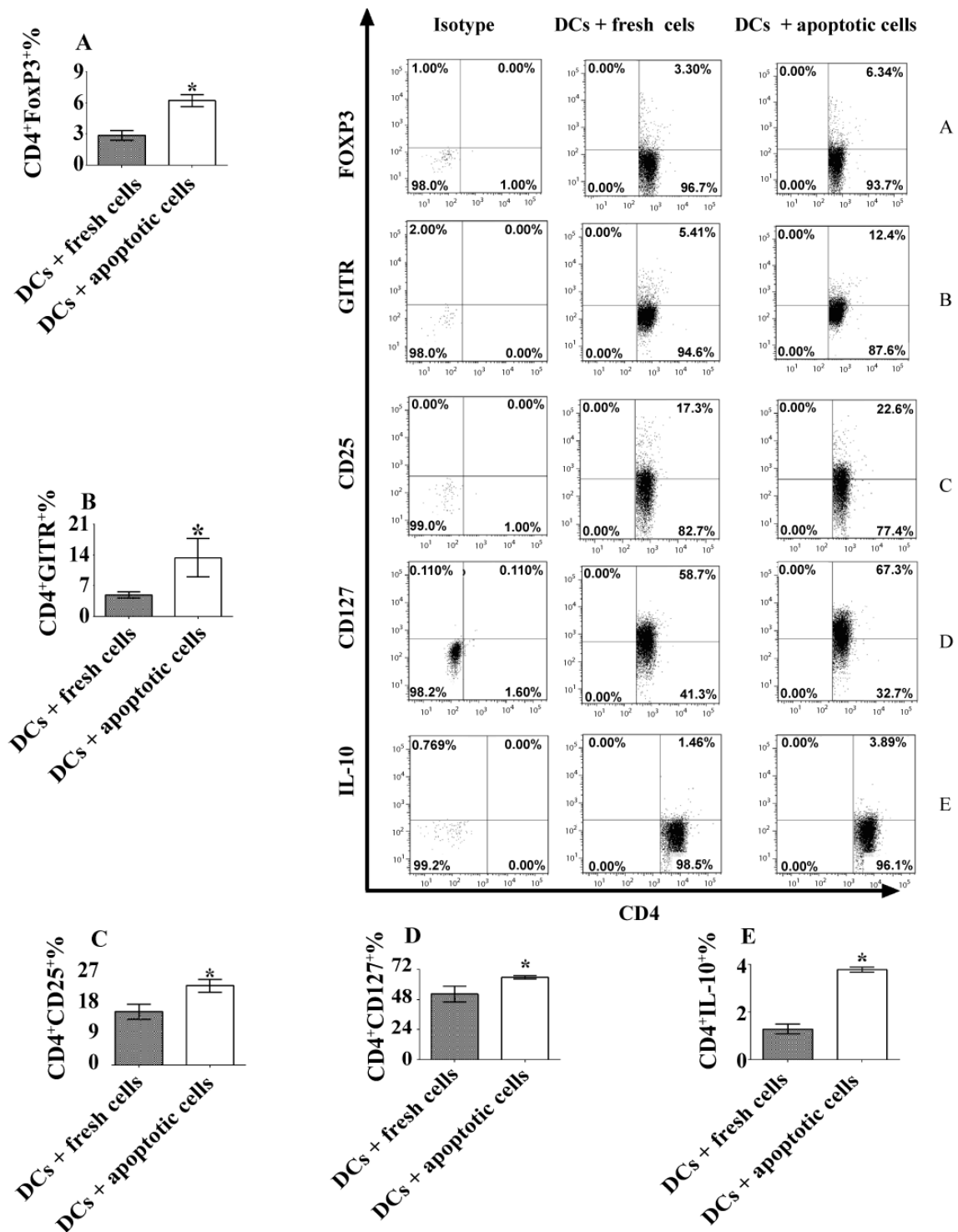
cytometry. Our results demonstrated that treatment with apoptotic cell-induced tolerogenic DCs leads to improvement of expression of FoxP3, GITR, CD25, CD127 and IL-10 on CD4<sup>+</sup> T cells, compared with expression of these molecules on CD4<sup>+</sup> T cells co-cultured with fresh thymocyte-treated DCs (Figure 4). These results suggest that apoptotic cell-treated DCs are tolerogenic ones, which can facilitate development of T<sub>regs</sub>.



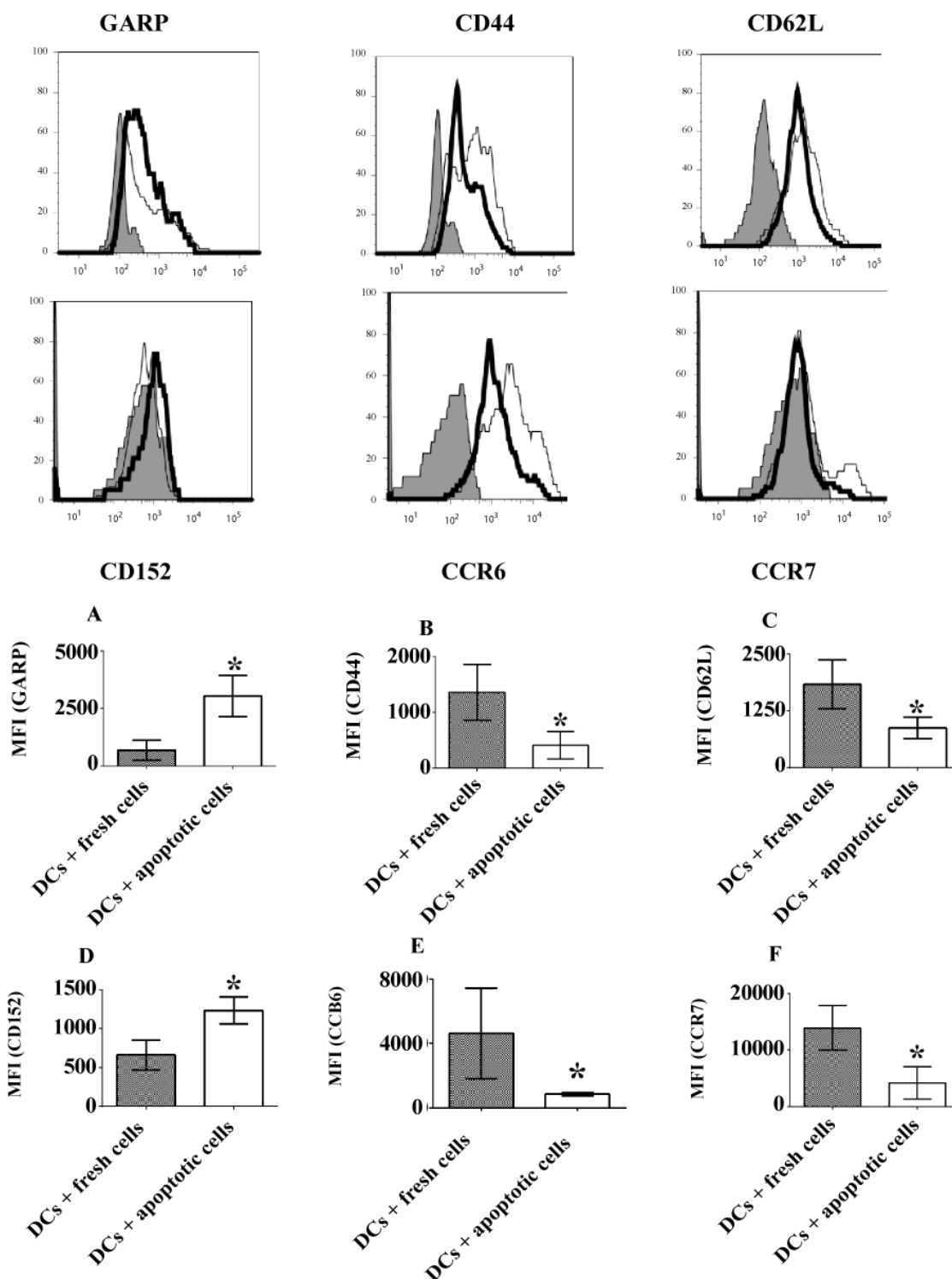
**Figure 3:** Co-culture with apoptotic T cells modulates protein expression of IL-23 and TGF- $\beta$  in DCs. Splenocytes were isolated from C57BL/6J mice and incubated with CD11c antibody. CD11c<sup>+</sup> cells (DCs) were sorted using flow cytometry. DCs were incubated with apoptotic (thin line) or fresh T lymphocytes (thin dash line) for 24 hrs at 37°C. Cells were stained by anti-mouse CD11c antibody. CD11c<sup>+</sup> cells were gated using flow cytometry. Expression of IL-23 (A) and TGF- $\beta$  (B) in CD11c<sup>+</sup> cells is indicated. Isotype control is demonstrated by shading. Error bars shown in this figure represent mean and SD of triplicate determinations of MFI for IL-23 and TGF- $\beta$  expression in CD11c<sup>+</sup> DCs in three independent experiments (n=3, *t* test, \*P<0.005).

### Apoptotic cell-induced tolerogenic DCs modulate expression of multiple T<sub>reg</sub>-related signal molecules

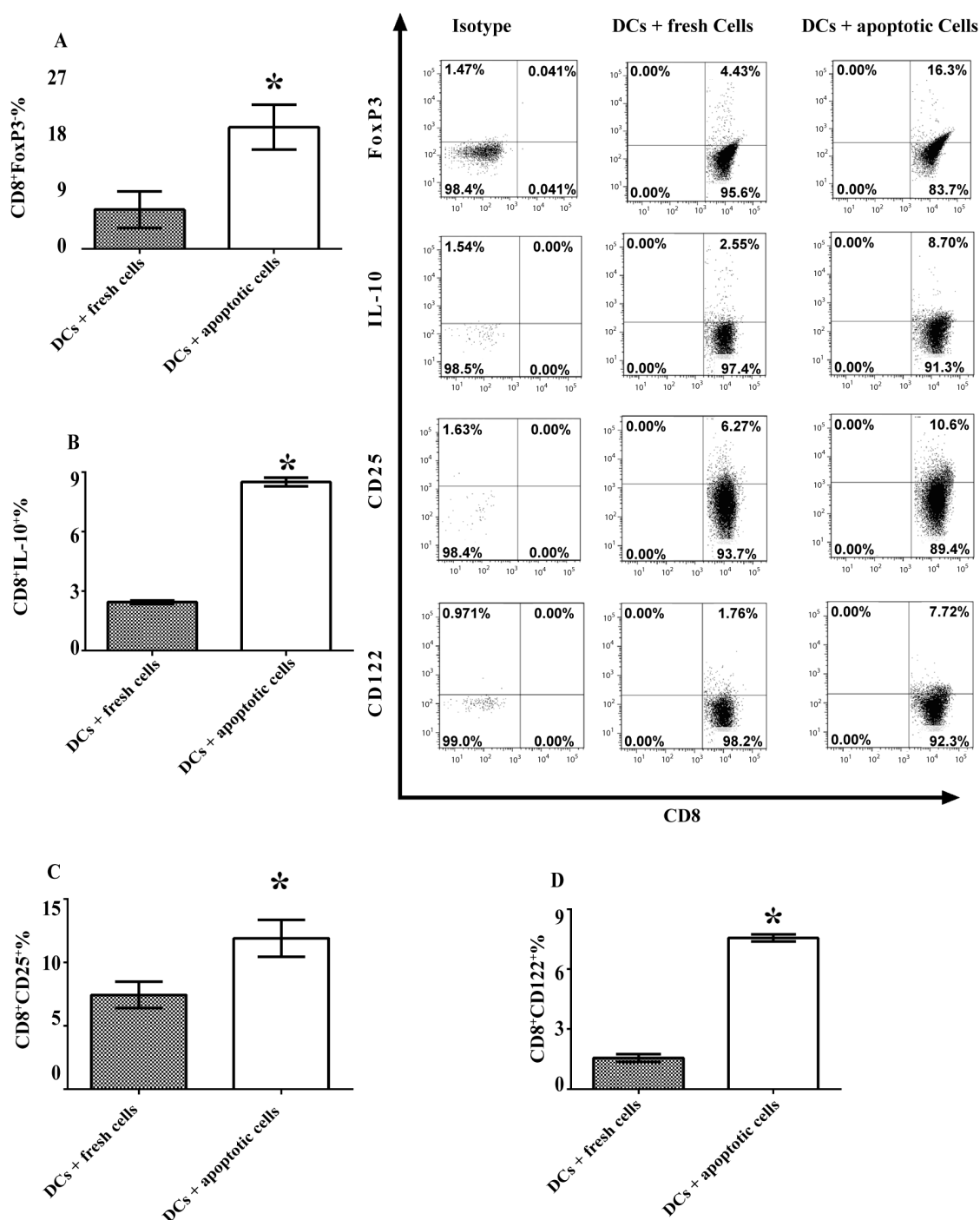
To confirm our finding that DCs treated with apoptotic cells drive T<sub>reg</sub> development, we further examined the effect of these DCs on expression of T<sub>reg</sub>-related signal molecules. CD4<sup>+</sup> T cells were co-cultured with DCs treated with apoptotic or fresh thymocytes, and expression of GARP, CD44, CD62L, CD152, CCR6 and CCR7 was detected by flow cytometry. Our data showed that co-culture with apoptotic T cell-treated DCs leads to up-regulation of GARP and CD152 expression on CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub>, with down-regulated expression of CD44, CD62L, CCR6 and CCR7 (Figure 5). These results show that apoptotic cell-induced tolerogenic DCs indeed affect expression of T<sub>reg</sub>-related signal molecules, thus modulating the development and function of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub>.



**Figure 4:** Tolerogenic DCs induced by apoptotic T cells facilitate development of CD4<sup>+</sup> regulatory T cells. CD11c<sup>+</sup> DCs were isolated from C57BL/6J mice and sorted using flow cytometry. CD11c<sup>+</sup> DCs were co-cultured at 37°C overnight with apoptotic or fresh T cells as a control. MOG specific CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from 2D2 transgenic mice and incubated with 0.1 μM MOG peptide-pulsed DCs treated with apoptotic or fresh T cells as a control for 3 days at 37°C. Cells were then harvested and a flow cytometry assay was carried out. Expression of FoxP3 (A), GITR (B), CD25 (C), CD127 (D) and IL-10 (E) is shown. Isotype controls are also indicated. Error bars represent mean and SD of triplicate determinations of percentage (%) of expression of T<sub>reg</sub>-associated markers on CD4<sup>+</sup> T cells in three independent experiments (n=3, *t* test, \*P<0.05).



**Figure 5:** Tolerogenic DCs induced by apoptotic T cells modulate expression of signal molecules on CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. CD11c<sup>+</sup> DCs were isolated from C57 BL/6J mice and sorted using flow cytometry. CD11c<sup>+</sup> DCs were incubated with apoptotic or fresh T cells overnight at 37°C. MOG-specific CD4<sup>+</sup> CD25<sup>-</sup> T cells were isolated from 2D2 transgenic mice. CD4<sup>+</sup>CD25<sup>-</sup> T cells were co-cultured with 0.1 μM MOG peptide-pulsed CD11c<sup>+</sup> DCs treated with apoptotic (thick line) or fresh T cells (thin line) as a control, and an assay of flow cytometry was carried out. CD4<sup>+</sup>CD25<sup>+</sup> cells were gated. Expression of GARP (A), CD44 (B), CD62L (C), CD152 (D), CCR6 (E) and CCR7 (F) is indicated. Isotype controls are also shown by shading. Error bars represent mean and SD of triplicate determinations of MFI of expression of signal molecules on CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells in three independent experiments (n=3, *t* test, \*P<0.05).



**Figure 6:** Apoptotic T cell-induced tolerogenic DCs block development of CD8<sup>+</sup> regulatory T cells. CD11c<sup>+</sup> DCs were isolated from spleen of C57 BL/6J mice and sorted using flow cytometry. CD11c<sup>+</sup> DCs were incubated overnight with apoptotic or fresh T cells at 37°C. MOG-specific CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from 2D2 mice and co-cultured with DCs pulsed with 0.1 μM MOG peptide and treated with apoptotic or fresh T cells as a control for 3 days at 37°C. A flow cytometry assay was done. CD4<sup>+</sup>CD8<sup>+</sup> cells were gated. Expression of FoxP3 (A), IL-10 (B), CD25 (C) and CD122 (D) is shown. Isotype controls are also demonstrated. Error bars represent mean and SD of triplicate determinations of percentage (%) of expression of T<sub>reg</sub>-associated markers on CD4<sup>+</sup>CD8<sup>+</sup> T cells in three independent experiments (n=3, t test, \*P<0.05).

## DCs incubated with apoptotic cells elicit development of CD8<sup>+</sup> Tregs

To determine whether or not DCs treated with apoptotic cells can also induce CD8<sup>+</sup> T<sub>regs</sub>, we examined expression of CD8<sup>+</sup> T<sub>reg</sub>-associated markers, including FoxP3, IL-10, CD25 and CD122, on CD4<sup>+</sup>CD8<sup>+</sup> T cells using flow cytometry. We found that expression of all these markers increased on CD8<sup>+</sup> T cells after co-culture with apoptotic cell-treated DCs, compared with CD8<sup>+</sup> T cells incubated with fresh cell-treated DCs (Figure 6). Our data thus suggest that apoptotic cell-induced tolerogenic DCs have the capacity to induce development of not only CD4<sup>+</sup> T<sub>regs</sub>, but also CD8<sup>+</sup> T<sub>regs</sub>.

## Discussion

DCs, as important regulatory immune cells in the innate immune system, modulate T cell activity through multiple pathways [22]. For example, peptide/MHC complexes on DCs, the major population of antigen presenting cells, bind to TCR on effector T cells and present the peptide to T cells (Signal 1). Co-stimulation molecules such as CD80 and CD86 expressed on DCs associate with ligands expressed on effector T cells to activate or inhibit T cell activity (Signal 2). Moreover, DCs produce inflammatory and anti-inflammatory cytokines such as IL-12 and IL-10, which are associated with receptors expressed on T cells to promote or suppress T cell-mediated immune responses (Signal 3). The foregoing shows the diversity of DC function in induction of autoimmunity or immune tolerance [39-44].

Uptake of apoptotic cells by dendritic cells leads to generation of tolerogenic DCs [45], which facilitate development and differentiation of regulatory T cells and induction of peripheral tolerance [35]. Moreover, tolerogenic DCs inhibit activity of effector T cells such as Th17 and block autoimmunity. Our data indicate that apoptotic cell-induced tolerogenic DCs facilitate development and differentiation of multiple subsets of regulatory T cells including CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>regs</sub>. These results imply diversity in mechanisms of dendritic cell-mediated immune tolerance [37].

Signal molecules expressed on DCs such as CD40, CD80 and CD86 play an important role in DC-mediated immune response. MHC class II is necessary for MHC class II antigen processing and presentation. The epitope of CD4<sup>+</sup> T cells is associated with MHC class II molecules and is presented on the surface of DCs. Epitope/MHC class II complexes interact with TCR on CD4<sup>+</sup> T cells and then initiate activation of CD4<sup>+</sup> T cells [46-48]. Treatment of apoptotic cell leads to down-regulation of CD40, CD80, CD86 and MHC class II expression on DCs. This may attenuate signal 1- and 2-initiated CD4<sup>+</sup> T cell activity and cause immune tolerance.

DCs produce inflammatory cytokines such as IL-12 and IL-23 to up-regulate CD4<sup>+</sup> T cell activities, e.g., to drive Th1 [49] and Th17 [50,51] polarization, respectively. In addition, DCs produce anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  to inhibit effector T cell development and function [52,53]. IL-10 and TGF- $\beta$  produced by DCs are necessary for T<sub>reg</sub> development [54], while this development is inhibited after neutralization of IL-10 or TGF- $\beta$  by IL-10 or TGF- $\beta$  specific antibody [55]. Our results show that treatment of apoptotic cells improves production of IL-10 and TGF- $\beta$ , but down-regulates production of IL-12 and IL-23 in DCs, thus facilitating development and differentiation of T<sub>regs</sub> while inhibiting activity of effector T cells such as Th1/Th17 cells.

T<sub>regs</sub> play an important role in induction of immune tolerance. The two types of T<sub>regs</sub>, natural and antigen-induced, are both CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells [56,57]. CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> can inhibit T and B cell-mediated immune responses [58], and i.v. transfer of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> before the onset of thrombocytopenia can prevent later induction of murine autoantibody-mediated thrombocytopenia [58-60]. It has been found that GITR is expressed on CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> and is an important regulator for their function. CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup> T<sub>regs</sub> inhibit both autologous and allogeneic CD4<sup>+</sup> and CD8<sup>+</sup> T cell-mediated immune responses [61]. Similarly, CD127 is also an important biomarker expressed on CD4<sup>+</sup> T<sub>regs</sub> when the function and development of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T<sub>regs</sub> are impaired in multiple sclerosis (MS) patients [62]. T<sub>regs</sub> inhibit effector T cell-mediated inflammatory responses by producing IL-10, a potent anti-inflammatory cytokine [63,64]. Importantly, we found that expression of T<sub>reg</sub>-related molecules, such as FoxP3, CD25, GITR, CD127 and IL-10, could be up-regulated in CD4<sup>+</sup> T cells when these cells were co-cultured with apoptotic cell-induced tolerogenic DCs. We also found that apoptotic cell-treated DCs blocked development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS, by inhibiting development of Th17 cells [37]. Together, these observations suggest that apoptotic cell-treated DCs can elicit development of T<sub>regs</sub> and enhance T<sub>reg</sub>-mediated immune tolerance.

Multiple signal molecules have been found to be expressed on T<sub>regs</sub>, which may play an important role in T<sub>reg</sub> development or function. It has been reported that GARP, a membrane protein expressed on activated T<sub>regs</sub>, is a receptor of TGF- $\beta$  and GARP-TGF- $\beta$  complexes that modulate development and differentiation of T<sub>regs</sub> and Th17 [65,66]. CD44 [14], as well as CD62L [67], are expressed on T<sub>regs</sub> and play an important role in modulation of T<sub>reg</sub> function. CD62L<sup>+</sup> T<sub>regs</sub> also increase the odds of transplantation survival [68,69]. CD152 is also expressed on T<sub>regs</sub> and plays an important role in their development and function. For example, human plasmacytoid DC-induced CD152<sup>+</sup> T<sub>regs</sub> can suppress memory T cell activity [70]. CD152 is necessary for FoxP3<sup>+</sup> T<sub>reg</sub> function [71], and over-stimulation to target CD152 using specific anti-CD152 antibody facilitates T<sub>reg</sub> development [72]. CCR6 plays an important role in T<sub>reg</sub>-mediated immune function. While development of CD4<sup>+</sup> T<sub>regs</sub> is inhibited in the absence of CCR6 expression [73], an early decrease in T<sub>regs</sub> leads to enhancement of autoimmunity in CCR6-deficient mice [74]. Development of CCR6<sup>+</sup> T<sub>regs</sub> is dependent on TGF- $\beta$  produced by DCs [17]. Another important signal molecule expressed on T<sub>regs</sub> is CCR7, which also plays a critical role in T<sub>reg</sub>-mediated immune function [18]. Our data show that treatment of DCs with apoptotic cells leads to modulation of expression of multiple signal molecules on T<sub>regs</sub>, suggesting that apoptotic cell-induced tolerogenic DCs may facilitate development of T<sub>regs</sub> by regulating their expression of these signal molecules.

CD8<sup>+</sup> T<sub>regs</sub> are a new subset of T<sub>regs</sub> that also plays an important role in induction of peripheral tolerance. CD25, FoxP3 and IL-10 are expressed in CD8<sup>+</sup> T<sub>regs</sub> as they are in CD4<sup>+</sup> T<sub>regs</sub>. CD8<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> suppress activation of allo-reactive T cell responses in graft versus host disease (GVHD) [19]. Moreover, CD8<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> may play an important role in tolerance of patients with remitting MS [75]. Our recent research demonstrates that i.v. transfer of apoptotic cell-treated DCs inhibits development of EAE, an animal model of MS, by blocking Th17 cell activity [37]. CD8<sup>+</sup> T<sub>regs</sub> also produce anti-inflammatory cytokine IL-10 to inhibit T cell-mediated inflammatory responses, e.g., allograft rejection [5], indicating that IL-10 is necessary for CD8<sup>+</sup> T<sub>reg</sub>-mediated peripheral tolerance. As is the case with CD4<sup>+</sup>



T<sub>regs</sub>, CD25 is also a biological marker expressed on CD8<sup>+</sup> T<sub>regs</sub>. CD8<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> have a highly suppressive function in effector T cell-mediated immune response [76]. Further, CD8<sup>+</sup>CD122<sup>+</sup> T<sub>regs</sub>, a subpopulation of CD8<sup>+</sup> T<sub>regs</sub>, plays a critical role in maintenance of T cell homeostasis [77], inhibits proliferation of CD8<sup>+</sup> T cells by producing anti-inflammatory cytokine IL-10 [21], and blocks development of EAE [78]. Our present study shows that apoptotic cell treatment up-regulates expression of CD25, FoxP3, CD122 and IL-10 in CD8<sup>+</sup> T cells, suggesting that apoptotic cell-induced tolerogenic DCs may elicit development and differentiation of CD8<sup>+</sup> T<sub>regs</sub> by increasing expression of multiple T<sub>reg</sub>-associated molecules.

In summary, the present study demonstrates that apoptotic cell-treated DCs possess biological features of tolerogenic DCs and facilitate development of multiple subsets of CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>regs</sub>. Our results may help to reveal cellular mechanisms of immune tolerance induced by DC-treated with apoptotic cells *in vivo*.

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