HHS Public Access

Author manuscript

Immunol Res. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Immunol Res. 2016 February ; 64(1): 73–81. doi:10.1007/s12026-015-8676-7.

Apoptotic Cell-treated Dendritic Cells Induce Immune Tolerance by Specifically Inhibiting Development of CD4+ Effector Memory T Cells

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Abstract

CD4+ memory T cells play an important role in induction of autoimmunity and chronic inflammatory responses; however, regulatory mechanisms of CD4+ memory T cell-mediated inflammatory responses are poorly understood. Here we show that apoptotic cell-treated dendritic cells inhibit development and differentiation of CD4+ effector memory T cells *in vitro* and *in vivo*. Simultaneously, i.v. transfer of apoptotic T cell-induced tolerogenic dendritic cells can block development of experimental autoimmune encephalomyelitis (EAE), an inflammatory disease of the central nervous system in C57 BL/6J mouse. Our results imply that it is effector memory CD4+ T cells, not central memory CD4+ T cells, which play a major role in chronic inflammatory responses in mice with EAE. I.V. transfer of tolerogenic dendritic cells induced by apoptotic T cells leads to immune tolerance by specifically blocking development of CD4+ effector memory T cells compared with results of EAE control mice. These results reveal a new mechanism of apoptotic cell-treated dendritic cell-mediated immune tolerance *in vivo*

Keywords

Dendritic cell; Immune tolerance; Immunotherapy; Memory T cell; Autoimmunity; Inflammation; Apoptosis

Introduction

Memory T cells play a central role in immune surveillance and chronic inflammation [1]. Memory T cells can be divided by two groups including CD4+ and CD8+ memory T cells according to subpopulation of T cells [2]. However, memory T cells are also composed of central memory T cells (T_{CM}s), effector memory T cells (T_{EM}s) and tissue-resident memory T cells (T_{RM}s) according to distribution *in vivo* [3, 4]. It is known that the phenotypes of the three subsets of memory T cells are different. For example, T_{CM}s are $CD44^{hi}CD62L^{+}$ T cells and are mainly located in spleen, lymph nodes and blood. T_{EM}s are $CD44^{hi}CD62L^{-}$ T cells and are mainly distributed in spleen. There are rare T_{EM}s in lymph nodes and blood.

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T_{RM}s are also $CD44^{hi}CD62L^{-}$ T cells [5], but CD103 molecules are expressed on T_{RM}s [6]. In contrast, there are no CD103 molecules expressed on T_{CM}s and T_{EM}s [5]. T_{RM}s are mainly located in peripheral tissue, for example, $CD8^{+}$ T_{RM}s are in the epithelium of skin, brain and ganglia; there are $CD4^{+}$ T_{RM}s in lung parenchyma. Immune function of $CD4^{+}$ T_{RM}s in the central nervous system are still obscure [5]. To date, cellular regulatory mechanisms of memory T cell development and differentiation *in vivo* have not been fully elucidated. Our research is focused on dendritic cell-mediated memory T cell development and differentiation. Our results imply that apoptotic cell-treated dendritic cells inhibit chronic inflammatory responses by specifically blocking development of $CD4^{+}$ effector memory T cells *in vivo*.

Dendritic cells (DCs) are important regulatory cells in the immune system and play a critical role in induction of immune tolerance and autoimmunity [7-9]. There are at least four subpopulations of DCs, including conventional DCs (cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs and myeloid DCs (mDCs) [10-12]. Moreover, DCs are also divided into inflammatory and tolerogenic DCs depending on their different functions [13, 14]. Production of inflammatory and tolerogenic DCs can be induced in several ways. For example, tolerogenic DCs can be induced by anti-inflammatory cytokines such as IL-10 and TGF-β and by some biological molecules such as vitamin D_3 and apoptotic cells [15-17]. Our project is focused on tolerogenic DCs induced by apoptotic T cells [18]. Our results suggest that apoptotic T cell-induced tolerogenic DCs inhibit development of $CD4^{+}$ memory T cells, leading to immune tolerance *in vivo*.

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS) [19]. EAE is also an inflammatory disease induced by $CD4^{+}$ T cells [20]. Regulatory mechanisms of chronic inflammatory responses mediated by $CD4^{+}$ memory T cells in EAE have not yet been elucidated. Our project is focused on how tolerogenic DCs induced by apoptotic T cells inhibit chronic inflammatory responses mediated by $CD4^{+}$ memory T cells in EAE. Our results suggest that apoptotic T cell-induced tolerogenic DCs inhibit development of EAE by specifically suppressing chronic inflammatory responses mediated by $CD4^{+}$ effector memory T cells.

Materials and Methods

Peptide

Mouse $MOG_{35\text{-}55}$ peptide (MEVGWYRSPFSRVVHLYRNGK), a component of myelin oligodendrocyte glycoprotein (MOG), was purchased from Invitrogen (Invitrogen, Carlsbad, California, USA).

Isolation of Spleen DCs

Splenocytes were isolated from naïve C57 BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA). Cells were collected and erythrocytes 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 $CD11c^{+}$ cells (DCs) were sorted using

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fluorescence-activated cell sorting (FACS; BD Biosciences, San Jose, CA, USA) for co-culture assay [21].

Induction of apoptotic thymocyte-induced tolerogenic DCs

As described previously, thymocytes were isolated from C57 BL/6J mice, and 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 $CD11c^+$ DCs (thymocytes: DCs = 10:1) for 24 hrs at 37°C. These DCs were also primed in the presence of $MOG_{35\text{-}55}$ peptide (0.1 μM) in the medium. Cells were then harvested for conducting co-culture experiments [21].

Isolation and cell culture of MOG peptide-specific $CD4^+$T cells

Splenocytes were isolated from TCR specific to MOG/MHC class II complex transgenic mice (2D2, Jackson Laboratory) or mice immunized with MOG peptide/ complete Freund's adjuvant (CFA, Sigma, St. Louis, MO, USA). T cells were collected and co-cultured at 37 °C for 3 days with $CD11c^+$ DCs that had been pre-treated with apoptotic or fresh thymocytes, in the presence of mouse IL-2 (1 ng/ml) and MOG peptide (0.1μM). Cells were washed at 300 g × 5 min twice using phosphate buffered saline (PBS) for flow cytometry [21].

Flow Cytometry

MOG-specific T cells were isolated from 2D2 mice or mice immunized with MOG peptide/CFA and co-cultured with 0.1 μM MOG peptide-pulsed DCs treated with apoptotic or fresh thymocytes for 72 hrs at 37 °C. Cells were washed at 300 g × 5 min twice with 5% fetal calf serum (FCS) in PBS, then incubated with anti-mouse CD4, CD25, CD44 and L-Selectin (CD62L) antibodies (Biolegend) for 24 hrs at 4 °C. These cells were washed using FACS sorting buffer (5% FCS in PBS) at 300 g × 5 min twice and collected for intracellular staining [21].

Intracellular Staining

As described previously, MOG-specific 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 × 10 min [21-23].

MOG-primed T cells were stained by anti-mouse Forkhead box P3 (FoxP3) and IFN-gamma (IFN-γ) antibodies (Biolegend) for 24 hrs at 4 °C. Cells were washed with 5% FCS in PBS twice at 300 g × 5 min and harvested for flow cytometry [21-23].

Fixed cells were run on a FACSAria (BD) and data were analyzed with FlowJo software (Treestar, Ashland, OR, USA) [21-23].

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EAE induction and treatment

C57BL/6J mice (female, 8-12 week) were immunized with MOG_{35-55} peptide/complete Freund's adjuvant (CFA, Sigma) at 200μg/200μl/per mouse (subcutaneous injection, s.c.). Pertussis toxin (PT, Sigma) was simultaneously injected at 200ng/per mouse (intraperitoneal injection, i.p.) and the second PT injection was conducted after 48 hrs. EAE was assessed by standard clinical scores: 0.5: paralysis of half the tail, 1: paralysis of whole tail, 2: paralysis of tail and one leg, 3: paralysis of tail and two legs, 4: moribund, 5: death [21, 22, 24].

Mice were divided into three groups. DCs were washed with PBS twice and were immediately injected via tail vein (3×10^5 cells/per mouse/per time) on days 11, 14 and 17 post-immunization (p.i): 1) injected with un-pulsed DCs incubated with apoptotic cells; 2) injected with fresh cell-treated DCs pulsed with MOG peptide; 3) injected with apoptotic cell-treated DCs pulsed with MOG peptide.

At day 50 p.i., splenocytes were isolated from immunized-mice and stimulated with MOG peptide (0.1μM) and mouse IL-2 (1ng/ml) for 3 days at 37 °C. Cells were then harvested for flow cytometry and the supernatant was assayed by enzyme-linked immunosorbent assay (ELISA)

ELISA

Anti-mouse ELISA kit to target IFN-γ was purchased from R&D Systems (Minneapolis, MN, USA). Assays were conducted according to the manufacturer's instructions. Plates were read out in Labsystems Multiskan MCC/340 (Fisher Scientific, Suwanee, GA, USA) and data were analyzed using DSJV ELISA software (Fisher Scientific) [21-24].

Statistical Analysis

Experimental data were analyzed using Prism software (GraphPad, La Jolla, CA, USA). Clinical scores of EAE were analyzed using *two-way ANOVA* test. A *t* test was conducted for analysis of flow cytometry and ELISA data. Error bars shown in this paper represent the mean and standard deviation (SD). Results were regarded as showing a significant difference if the P value was less than 0.05 [21-24].

Results

1. Apoptotic cell-treated DCs block development of central and memory $CD4^+$ T cells *in vitro*

To investigate whether or not apoptotic cell-treated DCs can modulate differentiation of memory $CD4^+$ T cells *in vitro*, DCs were incubated with apoptotic or fresh T cells as a control. MOG-primed $CD4^+$ T cells were isolated from 2D2 transgenic mice and co-cultured with apoptotic or fresh T cell-treated DCs loaded with MOG peptide. Experimental data showed that apoptotic T cell-treated DCs block development of both effector and central memory $CD4^+$ T cells (Figs.1-2). Our results imply that co-culture with apoptotic T cells leads to generation of tolerogenic DCs, which can inhibit development of central and effector memory $CD4^+$ T cells.

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2. Apoptotic cell-treated DCs inhibit development of EAE *in vivo*

To investigate whether or not apoptotic cell-treated DCs also inhibit autoimmunity in mice with EAE, apoptotic or fresh cell-treated DCs pulsed with MOG peptide were i.v. transferred into mice immunized with MOG peptide/CFA. Apoptotic cell-treated DCs without loading MOG peptide were also i.v. transferred into mice with EAE as a control. Experimental results indicated that i.v. transfer of apoptotic cell-treated DCs pulsed with MOG peptide can significantly inhibit EAE development *in vivo* compared to mice treated with DCs incubated with apoptotic cells, but without loading MOG peptide, or to fresh cell-treated DCs pulsed with MOG peptide (Fig.3A). Our results suggest that immune tolerance induced by apoptotic cell-treated DCs is specific to MOG peptide. Apoptotic cell-induced tolerogenic DCs can block autoimmune responses *in vivo*.

To test whether or not apoptotic cell-treated DCs can affect production of IFN-γ in lymphocytes, spleen cells were isolated from mice treated with apoptotic cell or fresh cell-treated DCs and re-stimulated respectively with MOG peptide (0.1μM) and mice IL-2 (1ng/ml). Supernatant was collected and an ELISA assay was conducted. Our results demonstrated that i.v. transfer of apoptotic cell-treated DCs pulsed with MOG peptide can significantly down-regulate production of IFN-γ in T lymphocytes compared with cells isolated from mice i.v. transferred with apoptotic cell-treated DCs without loading MOG peptide or with fresh cell-treated DCs pulsed with MOG peptide (Fig. 3B). Experimental data indicate that treatment with apoptotic cells leads to generation of suppressive DCs, which can block production of IFN-γ by T lymphocytes.

3. Apoptotic cell-treated DCs inhibit development of effector memory $CD4^+$ T cells *ex vivo*

To investigate whether or not apoptotic cell-treated DCs also inhibit development of effector and central memory $CD4^+$ T cells *in vivo*, splenocytes were isolated from mice treated with fresh or apoptotic cell-incubated DCs pulsed with MOG peptide or apoptotic cell-treated DCs without loading MOG peptide as a control. Frequency of effector and central memory $CD4^+$ T cells was detected using flow cytometry (Fig.4). Our results indicated that i.v. transfer of apoptotic cell-treated DCs pulsed with MOG peptide can significantly inhibit development of effector memory $CD4^+$ T cells compared with those in mice i.v. transferred with apoptotic cell-treated DCs without loading MOG peptide or fresh cell-treated DCs pulsed with MOG peptide (Fig. 5A). In contrast, there is no significant difference in frequency of central memory $CD4^+$ T cells between mice i.v. transferred with apoptotic cell-treated DCs pulsed with MOG peptide and mice i.v. transferred with apoptotic cell-incubated DCs without loading MOG peptide (EAE control group) (Fig. 5A) , although there is significant difference in frequency of central memory $CD4^+$ T cells between mice i.v. transferred with apoptotic cell-treated DCs pulsed with MOG peptide and mice i.v. transferred with fresh cell-treated DCs loaded with MOG peptide, (Fig. 5B). Our results suggest that tolerogenic DCs induced by apoptotic T cells cause immune tolerance by blocking effector memory $CD4^+$ T cell-mediated chronic inflammatory responses *in vivo*.

In addition, to test whether or not apoptotic cell-induced tolerogenic DCs can also inhibit production of IFN-γ by $CD4^+$ T cells *ex vivo*, splenocytes were isolated from mice shown in Fig. 3A and re-stimulated with leukocyte activator, MOG peptide (0.1 μM) and mouse IL-2

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(1ng/ml). Production of IFN-γ by $CD4^+$ T cells was detected using flow cytometry. Experimental data demonstrated that i.v. transfer of apoptotic cell-treated DCs pulsed with MOG peptide can significantly down-regulate production of IFN-γ by $CD4^+$ T cells compared with $CD4^+$ T cells derived from mice i.v. transferred with fresh cell-treated DCs loaded with MOG peptide or mice i.v. transferred with apoptotic cell-treated DCs without loading MOG peptide (Fig. 5C). Our results suggest that apoptotic cell-induced tolerogenic DCs also inhibit activation of MOG-primed $CD4^+$ T cells by affecting production of IFN-γ *in vivo*.

Discussion

DCs play a central role in regulating immune responses mediated by T cells and in maintaining balance between autoimmunity and tolerance. For example, tolerogenic DCs induced by apoptotic cells induce immune tolerance by suppressing activity of T helper 17 (Th17) cells, but by facilitating development of regulatory T cells [21, 22]. However, it is still unclear whether or not apoptotic cell-induced tolerogenic DCs also inhibit development of $CD4^+$ memory T cells. Given that $CD4^+$ memory T cells play an important role in chronic inflammatory responses and autoimmunity, in order to develop further studies, it is necessary to investigate whether or not apoptotic cell-treated DCs also block differentiation of $CD4^+$ memory T cells.

The effect of tolerogenic DCs on activation of $CD4^+$ memory T cells in human diseases has been widely investigated in the past few years. For example, Torres-Aguilar et al. reported that tolerogenic DCs induced by IL-10 and TGF-β block anti-phospholipid syndrome-derived effector/memory $CD4^+$ T cell-mediated immune responses *in vivo*. Moreover, IL-10/TGF-β-treated DCs inhibit activation of insulin-specific $CD4^+$ effector/memory T cells in diabetic patients. Tolerogenic DCs cause immune tolerance by inducing anergy of $CD4^+$ effector memory T cells and facilitating development of memory regulatory T cells [15, 25, 26]. Nasreen's data also indicated that steady-state antigen-expressing DCs suppress $CD4^+$ memory T cell-mediated immune responses [27]. These results suggest the possibility of immunotherapy using tolerogenic DCs that can inhibit $CD4^+$ memory T cell-mediated autoimmune and inflammatory diseases such as EAE/MS. It is not known, however, whether or not apoptotic cell-induced tolerogenic DCs can block development of autoimmune diseases such as EAE by inhibiting development of $CD4^+$ memory T cells.

Interferon gamma (IFN-γ) is an important cytokine which can modulate multiple immune functions *in vivo*. IFN-γ can be produced by Th1 cells [28, 29]. IFN-γ is also secreted by T_{EM}s; it is, however, unclear whether or not apoptotic cell-induced tolerogenic DCs block T_{EM}s activity by modulating its production of IFN-γ.

The relation between development of $CD4^+$ memory T cells and induction of chronic inflammatory responses in human diseases has been thoroughly studied. For example, Olson found that the number of $CD4^+$ memory T cells is increased in atherosclerosis [30]. Moreover, Egwuagu et al. reported that auto-reactive memory $CD4^+$ T cells play an important role in chronic uveitis and their immigration to bone marrow is dependent on signal transducer and activator of transcription 3 (STAT3)-mediated pathways [31].

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Moreover, Haines' results demonstrated that autoimmune memory Th17 cell-mediated inflammatory responses in EAE mice are regulated by IL-23-mediated pathways [32]. Elyaman et al. found that development of EAE can be facilitated by pro-inflammatory memory $CD4^+$ T cells [33]. However, it is still unclear whether or not apoptotic cell-induced tolerogenic DCs can inhibit autoimmune memory Th17 cell-mediated chronic inflammatory responses in EAE mice.

In summary, we observed the effect of apoptotic cell-treated DCs on development and differentiation of $CD4^+$ memory T cells *in vitro* and *in vivo*. Apoptotic cell-induced tolerogenic DCs can block development of differentiation of T_{CMs} and T_{EM}s *in vitro* and *in vivo*. However, immune tolerance induced by apoptotic cell-treated DCs is mainly dependent on $CD4^+$ effector memory T cells, not on $CD4^+$ central memory T cells. Our results suggest a new mechanism of immune tolerance induced by apoptotic cell-treated DCs *in vivo*.

Abbreviations

CD	Cluster of differentiation
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FoxP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL	Interleukin
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
PBS	Phosphate buffered saline
SD	Standard deviation
SEM	Standard error of arithmetic mean
$\mathbf{T_{CM}}$	Central memory T cell
TCR	T cell receptor
$\mathbf{T_{EM}}$	Effector memory T cell
$\mathbf{T_{RM}}$	Tissue resident memory T cell

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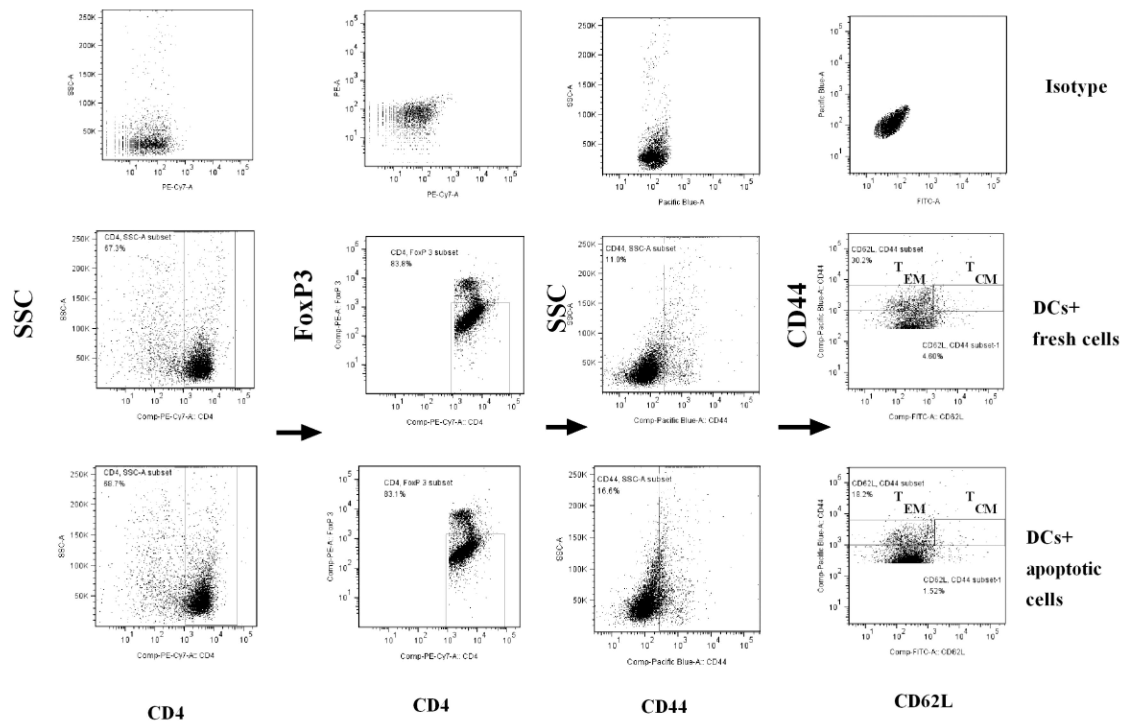

Figure 1.

Gate scheme of T_{EM}s and T_{CM}s. Spleen cells were isolated from 2D2 transgenic mice. $CD4^{+}CD25^{-}$ T cells and $CD11c^{+}$ DCs were respectively sorted using flow cytometry. T lymphocytes were re-stimulated by MOG peptide (0.1μM) and mouse IL-2 (1ng/ml) and co-cultured with MOG peptide (0.1μM)-pulsed DCs treated with fresh or apoptotic cells for 72 hrs at 37 °C. Cells were then stained with anti-mouse CD4, CD44, CD62L and FoxP3 antibodies. $CD4^{+}FoxP3^{-}CD44^{+}$ cells were gated. T_{EMs} ($CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{-}$ cells) and T_{CMs} ($CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{+}$ cells) are shown. Further studies and statistical analysis are indicated in Figure 2.

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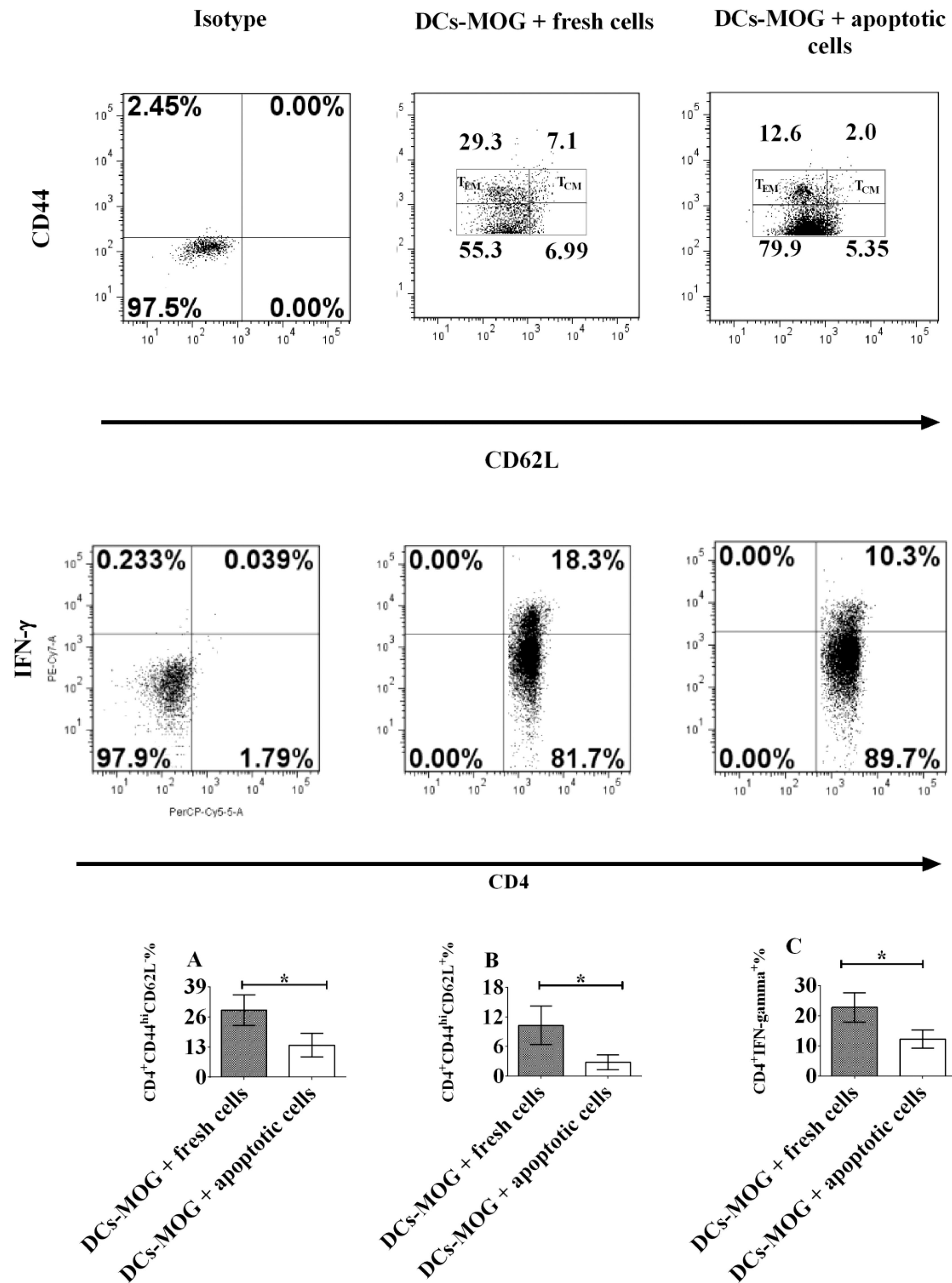

Figure 2.

Apoptotic cell-treated dendritic cells (DCs) inhibit development of effector and central memory CD4^{+} T cells *in vitro*. Splenocytes were isolated from C57BL/6J mice and stained by anti-mouse CD11c antibody. CD11c^{+} cells (DCs) were sorted using flow cytometry. DCs were then co-cultured with apoptotic or fresh T lymphocytes for 24 hrs at 37 °C as a control. MOG-primed T lymphocytes were isolated from 2D2 transgenic mice and stained by anti-mouse CD4 and CD25 antibodies. Only CD4^{+}CD25^{-} cells (effector T cells) were sorted and collected using flow cytometry. MOG-specific T lymphocytes were incubated with

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apoptotic or fresh T cell-treated DCs pulsed with MOG peptide (0.1μM) for 3 days at 37 °C. Cells were washed and harvested with PBS. Lymphocytes were stained with anti-mouse CD4, CD44, CD62L, IFN-γ and FoxP3 antibodies. Only $CD4^{+}CD44^{+}FoxP3^{-}$ cells were gated like those in Figure 1. Expression of CD44, CD62L on $CD4^{+}CD44^{+}FoxP3^{-}$ cells and that of IFN-γ in $CD4^{+}$ T cells are shown. $CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{-}$ (T_{EMs}) and $CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{+}$ (T_{CMs}) cells are gated. Error bars indicated in this figure represent mean and SD of triplicate determinations of percentage (%) of T_{EMs} (A), T_{CMs} (B) and IFN-γ (C) in three independent experiments (*$P<0.05$, n=3, *t* test).

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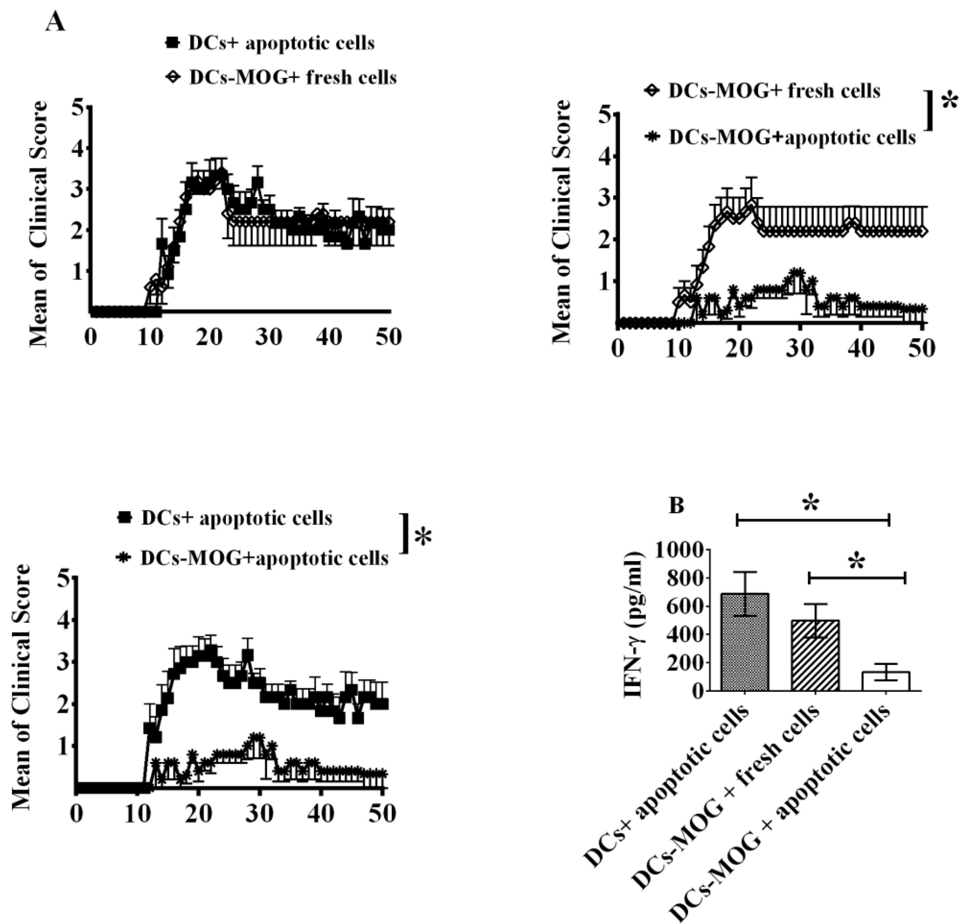

Figure 3.

i.v. transfer of apoptotic cell-treated DCs leads to inhibition of EAE development. C57 BL/6J mice were immunized with MOG/CFA on day 0 and PT was injected (i.p) at days 0 and 2. DCs were pulsed with MOG peptide (0.1μM) (DC-MOG) or without loading MOG peptide (DC). DCs were incubated with apoptotic or fresh T cells as a control for 24 hrs at 37 °C. DCs treated with apoptotic or fresh cells were i.v. transferred into mice with EAE at days 11, 14 and 17 (3×10^5 cells/per mouse/per time). Clinical scores of EAE are shown (A). Splenocytes were isolated from mice shown in A and re-stimulated with MOG peptide (0.1μM) and mouse IL-2 (1ng/ml) for 3 days at 37° C. Supernatant was harvested and an ELISA assay to target IFN-γ was carried out. Concentration of IFN-γ is indicated (B). Error bars shown in A represent mean and SEM of clinical score of EAE in one experiment (*P<0.05, n=6, *two-way ANOVA* test). Error bars shown in B represent mean and SD of triplicate determinations of concentration of IFN-γ in three independent experiments (*P<0.05, n=3, *t* test).

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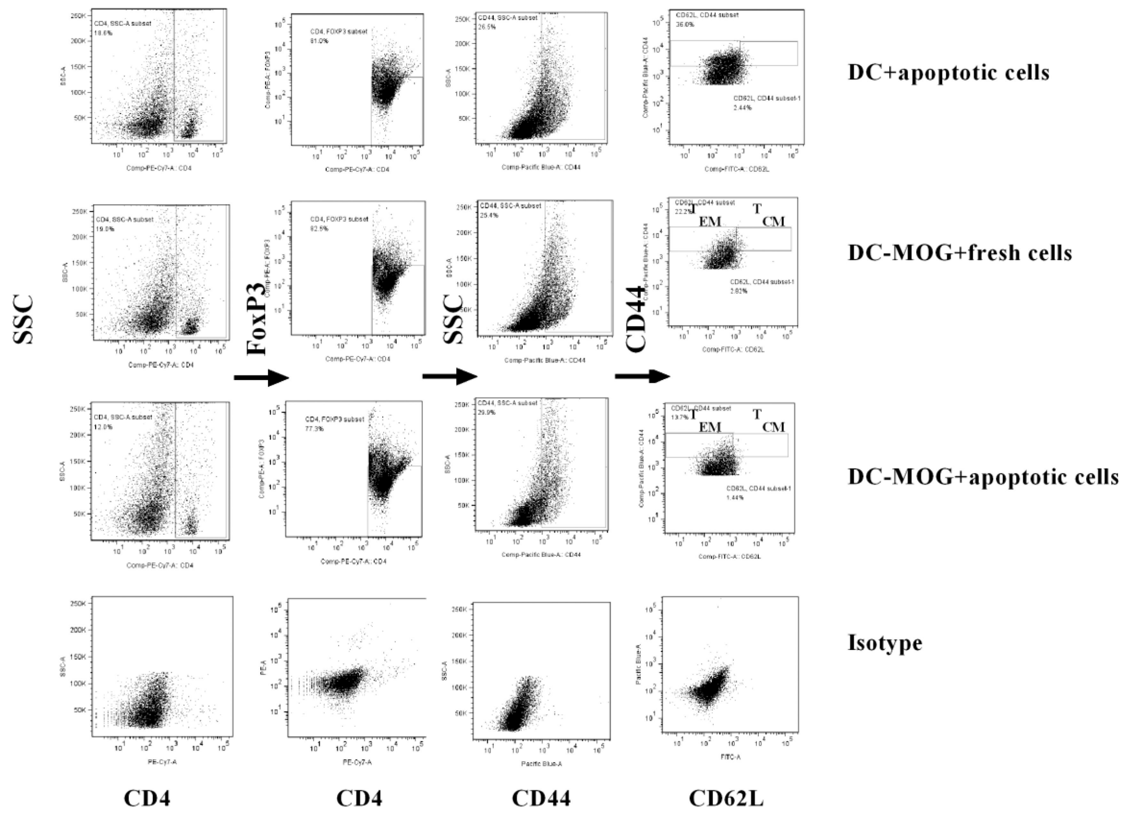

Figure 4.

Gate strategy of T_{EMs} and T_{CMs} *ex vivo*. Splenocytes were isolated from mice immunized with MOG/CFA shown in figure 3A. T lymphocytes were re-stimulated with MOG peptide (0.1μM) and mouse IL-2 (1ng/ml). Cells were collected and washed with PBS twice at 300g X 5min. Splenocytes were stained by anti-mouse CD4, CD44, CD62L and FoxP3 antibodies. $CD4^{+}FoxP3^{-}CD44^{+}$ cells were gated. T_{EMs} ($CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{-}$ cells) and T_{CMs} ($CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{+}$ cells) are indicated. Further studies and statistical analysis are demonstrated in figure 5.

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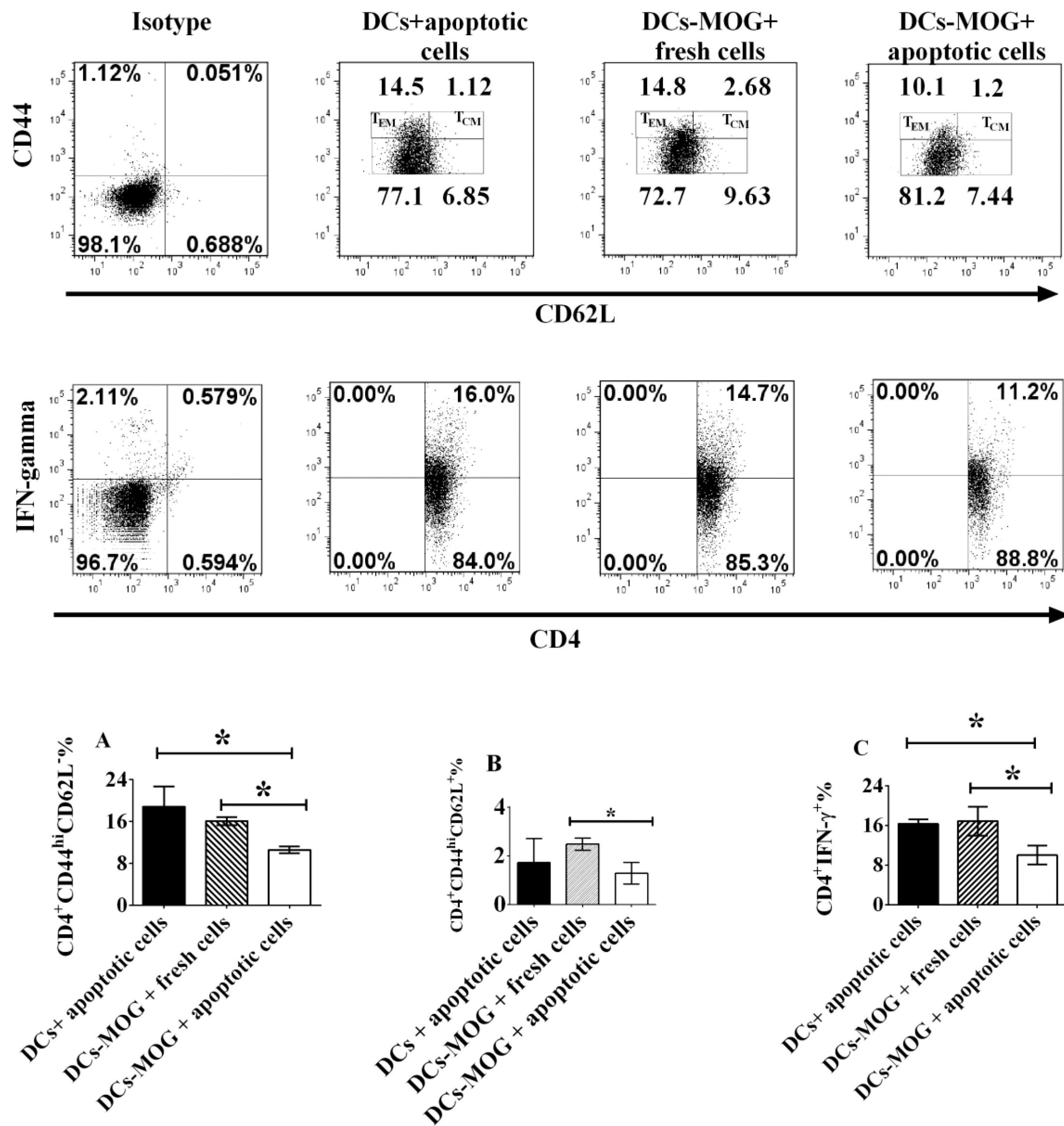

Figure 5.

Apoptotic cell-treated DCs inhibit differentiation of effector memory CD4^{+} T cells *ex vivo*. Splenocytes were isolated from mice immunized with MOG/CFA shown in Fig. 3A. Cells were re-stimulated with mouse IL-2 (1ng/ml) and MOG peptide (0.1μM) for 3 days at 37 °C. Splenocytes incubated with leukocyte activator were then harvested and washed with PBS. Cells were stained by anti-mouse CD4, CD44, CD62L, FoxP3 and IFN-γ antibodies. CD4^{+}CD44^{+}FoxP3^{-} cells were gated for further studies like those in figure 4. Frequencies of CD4^{+}FoxP3^{-}CD44^{hi}CD62L^{-} (T_{EMs}) and CD4^{+}FoxP3^{-} CD44^{hi}CD62L^{+} (T_{CMs}) cells are demonstrated. The frequencies of T_{EMs} (A), T_{CMs} (B) and IFN-γ (C) in CD4^{+} T cells were determined using flow cytometry. Error bars indicated in this figure represent mean and SD of triplicate determinations of frequency of T_{EMs} (A), T_{CMs} (B) and IFN-γ (C) in CD4^{+} T cells in three independent experiments (*P<0.05, n=3, *t* test).