Published in final edited form as:

Immunobiology. 2013 August ; 218(8): 1069–1076. doi:10.1016/j.imbio.2013.02.003.

Intravenous Transfer of Apoptotic Cell-treated Dendritic Cells Leads to Immune Tolerance by Blocking Th17 Cell Activity

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Abstract

Apoptotic cell-induced tolerogenic dendritic cells (DCs) play an important role in induction of peripheral tolerance *in vivo*; however, the mechanisms of immune tolerance induced by these DCs are poorly understood. Here we show that treatment of apoptotic cells modulates expression of inflammation- and tolerance-associated molecules including Gr-1, B220, CD205 and galectin-1 on bone marrow-derived DCs. In addition, apoptotic cell-treated DCs suppress secretion of cytokines produced by Th17 cells. Our data also demonstrate that i.v. transfer of apoptotic cell-treated DCs blocks EAE development and down-regulates production of inflammatory cytokines such as IL-17A and IL-17F in CD4⁺ T cells. These results suggest that apoptotic cell-treated DCs may inhibit activity of Th17 cells via down-regulation of inflammatory cytokine production, thereby affecting EAE development *in vivo*. Our results reveal a potential mechanism of immune tolerance mediated by apoptotic cell-treated DCs and the possible use of apoptotic cell-treated DCs to treat autoimmune diseases such as MS/EAE.

Keywords

Dendritic cell; EAE; immune tolerance; immunotherapy; Th17 cell

Introduction

Dendritic cells (DCs) play an important role in both autoimmunity and immune tolerance (Langlois and Legge, 2007; Lutz and Kurts, 2009). Although DC-based immunotherapy has been used to treat autoimmune disease, the regulatory mechanisms controlling DC-mediated immune responses have not been fully elucidated. Understanding these regulatory mechanisms may facilitate the development of immunotherapy to treat autoimmune diseases.

Apoptotic cell-induced tolerogenic DCs play an important role in induction of immune tolerance (da Costa et al., 2011; Gleisner et al., 2011). However, the molecular mechanisms

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The authors have no financial conflict of interest.

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of immune tolerance mediated by these tolerogenic DCs have not been elucidated. It has been reported that uptake of apoptotic cells by immature DCs causes generation of tolerogenic DCs producing transforming growth factor-beta (TGF- β) (Danese et al., 2008). TGF- β -producing tolerogenic DCs facilitate development of regulatory T cells (Tregs) in the microenvironment (Gleisner et al., 2011; Huang et al., 2010). Moreover, digestion of apoptotic cells by immature DCs up-regulates expression of programmed cell death protein ligand 1 (PDL-1) on DCs. PDL-1 binds to programmed cell death protein 1 (PD-1) expressed on T cells and leads to inhibition of T cell proliferation and up-regulation of T cell apoptosis. Peripheral inflammatory responses are therefore inhibited and immune tolerance is induced (Sumpter and Thomson, 2011).

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS) (Furlan et al., 2009). It has been reported that T helper 17 (Th17) cells play an important role in EAE/MS development (Brereton et al., 2009; Hofstetter et al., 2009; Sutton et al., 2009); however, it is unclear whether uptake of apoptotic cells by viable DCs can affect Th17 activity and block EAE/MS development. We hypothesized that apoptotic cell-induced tolerogenic DCs might modulate expression of inflammation- and tolerance-associated molecules on viable DCs and generate tolerogenic DCs to block Th17 cell activity that is necessary for induction of autoimmune diseases such as EAE/MS. To test this hypothesis, bone marrow-derived DCs were co-cultured with apoptotic cells to induce tolerogenic DCs and were intravenously (i.v.) transferred into EAE mice. Our results show that apoptotic cell treatment modulates expression of Gr-1, B-220, CD205 and galectin-1 on DCs and i.v. injection of apoptotic cell-treated DCs inhibits EAE development. Moreover, apoptotic cell-treated DCs can suppress Th17 activity *in vitro* and *in vivo*, suggesting the potential possibility of using tolerogenic DCs in the treatment of autoimmune diseases such as EAE/MS.

Materials and Methods

Mice

C57 BL/6J female mice (8–12 weeks) were ordered 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 of Thomas Jefferson University.

Immunogen and Peptide

Mouse MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) is part of myelin oligodendrocyte glycoprotein (MOG) and was purchased from Invitrogen (Invitrogen, Carlsbad, California, USA).

Bone Marrow-derived DC Culture

As described previously (Lutz et al., 1999; Zhang et al., 2002), femurs and tibiae of mice were isolated from muscle tissue by rubbing with Kleenex tissues. The intact bones were then put into 70% ethanol for 5 min for disinfection and washed with phosphate-buffered saline (PBS). Both ends of the bones were cut with scissors and the marrow was flushed with PBS by using a syringe with 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting and then washed with PBS. These cells were then fed in bacteriological 100 mm Petri dishes (Falcon, Becton Dickinson, Heidelberg, Germany) at 2×10^6 cells per dish. Cells were cultured in RPMI1640 complete medium (Gibco-BRL, Eggenstein, Germany) including penicillin (100 U/ml, Sigma, St. Louis, MO, USA), streptomycin (100 U/ml, Sigma), L-glutamine (2 mM, Sigma), 2-mercaptoethanol (2-ME, 50 μ M, Sigma), 10% heated inactivated and filtered (0.22 μ m,

Millipore, Inc., Bedford, MA, USA) Fetal Calf Serum (FCS, Sigma) and granulocyte-macrophage colony-stimulating factor (GM-CSF, Pepro Tech, Rocky Hill, NJ, USA) at 20 ng/ml at day 0 (10 ml medium per dish). At day 3, 10 ml fresh medium with GM-CSF at 20 ng/ml was added to each dish and at day 6, half of the medium (about 10ml supernatant) was collected and centrifuged at 300 g for 5 min. Subsequently, cells were resuspended in 10 ml fresh medium with GM-CSF (20 ng/ml) and were then re-fed in the original dish. DCs were collected at day 8 of culture by gentle pipetting, washed with PBS at 300 g for 5 min., and then counted for flow cytometry.

Generation of apoptotic cell-induced tolerogenic DCs

Apoptotic cell-induced tolerogenic DCs were generated as previously described (da Costa et al., 2011; Gleisner et al., 2011; Kushwah et al., 2010). Briefly, thymocytes were isolated from C57 BL/6J mice and then irradiated at 1500 Rad. Fresh thymocytes without irradiation were harvested as a control. Irradiated and fresh T cells were co-cultured with bone marrow-derived DCs as described above for 24 hrs. Cells were then collected for conducting flow cytometry or i.v. transferred into EAE mice.

Flow Cytometry

Cultured DCs were incubated with anti- mouse CD11c, B220, Gr-1, CD205 and galectin-1 antibodies. MOG-primed T lymphocytes were isolated from EAE mice and incubated with anti-mouse anti-CD4 and, for intracellular staining, anti-mouse- interleukin (IL)-17A, IL-21, IL-22, interferon gamma (IFN- γ), Retinoic acid-related orphan receptor (ROR) gamma t (ROR- γt) and -IL-17F antibodies (Biolegend, San Diego, CA, USA) at 4°C for 1 hr. Cells were stimulated with leukocyte activator (BD) before conducting intracellular staining. Cells were then washed twice with 5% FCS in PBS at 300 g for 5 min., and then fixed with 5% formalin in PBS. Fixed cells were run on a FACSaria (BD Biosciences, San Jose, CA, USA) and data were analysed with FlowJo software (Treestar, Ashland, OR, USA).

ELISA

Anti-mouse IL-17A Enzyme-linked immuno-sorbent assay (ELISA) kit 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 analysed with DSJV ELISA software (Fisher Scientific).

Generation of Effector T cells for *in vitro* assay

C57 BL/6J mice were immunized with MOG (35–55) peptide (Invitrogen) 200 μ g, QuilA (Sigma) 20 μ g, Keyhole limpet hemocyanin (KLH, Sigma) 20 μ g per mouse at day 0. Spleen cells were then isolated at day 10 after immunization. T lymphocytes were purified with mouse CD4 subset column kit (R&D Systems). CD4⁺ T cells (1×10^6 cells/per well) were co-cultured with DCs at 5:1 (T cells: DCs) and pulsed with MOG (35–55) peptide at 0.1 μ M in complete medium with mouse IL-2 (Pepro Tech) at 1 ng/ml for 5 days. Cultured cells were harvested for flow cytometry.

EAE induction and DC treatment

C57BL/6J mice (female, 8–12 weeks of age) were immunized with MOG peptide/Complete Freund's adjuvant (CFA, Sigma) at 200 μ g/200 μ l/per mouse (subcutaneous injection, s.c.). Pertussis toxin (PT, Sigma) was intraperitoneally (i. p.) injected at 200 ng/per mouse at days 0 and 2 post immunization (p. i.). EAE disease was evaluated as following standard (clinical score): 0.5: half of tail paralysis, 1: whole tail paralysis, 2: tail and one leg paralysis, 3: tail and two legs paralysis, 4: moribund, 5: death.

DCs (5×10^5 cells/per mouse/per time) treated with apoptotic T cells or fresh T cells were pulsed with MOG peptide (0.1 μ M), washed, and then intravenously injected into EAE mice on days 11, 14, and 17 p. i. EAE mice treated with i.v. PBS are control.

Generation of Effector T cells for *ex vivo* assay

At day 21 p. i., spleen cells were isolated from mice described above, and stimulated with MOG peptide at 0.1 μ M and mouse IL-2 at 1 ng/ml for 3 days. Cells were collected for flow cytometry and supernatants were harvested for ELISA.

Statistical Analysis

Experimental data were analysed using Prism software (GraphPad, La Jolla, CA, USA). A *t* test was conducted. Results were regarded as showing a significant difference if the P value was less than 0.05.

Results

1. Apoptotic cell treatment modulates protein expression of inflammation- and tolerance-associated molecules on CD11c⁺ DCs

To determine whether treatment of apoptotic cells can affect expression of inflammation- and tolerance-associated molecules on DCs, DCs were co-cultured with apoptotic cells or fresh cells for 24 hrs. The numbers of CD11c⁺Gr-1⁺, CD11c⁺B220⁺, CD11c⁺CD205⁺ and CD11c⁺galectin-1⁺ DCs were counted. The results showed that treatment with apoptotic cells up-regulate the number of CD11c⁺CD205⁺ and CD11c⁺galectin-1⁺ DCs compared with that of DCs co-cultured with fresh cells (Fig. 1). By contrast, treatment of apoptotic cells down-regulate the number of CD11c⁺Gr-1⁺ and CD11c⁺B220⁺ DCs compared with that of DCs co-cultured with fresh cells (Fig. 1). These results suggest that treatment of apoptotic cells may affect the balance of immune responses mediated by CD11c⁺CD205⁺ and CD11c⁺galectin-1⁺ DCs vs. CD11c⁺B220⁺ and CD11c⁺Gr-1⁺ DCs.

2. DCs treated with apoptotic cells down-regulate expression of multiple inflammatory cytokines in CD4⁺ T cells

To determine whether apoptotic cell-treated DCs can affect the activity of Th17 cells, DCs treated with apoptotic or fresh cells were co-cultured with MOG-primed CD4⁺ T cells. Analysis by flow cytometry showed that apoptotic cell-treated DCs down-regulate expression of ROR- γ t, and intracellular secretion of IL-17A, IL-17F, IL-21, IL-22 and IFN- γ in CD4⁺ T cells (Fig. 2). These results suggest that apoptotic cell-treated DCs down-regulate the expression of multiple cytokines produced by Th17 cells *in vitro*.

3. Apoptotic cell-treated DCs block EAE development induced by MOG/CFA immunization

To investigate whether apoptotic cell-treated DCs also affect EAE development, C57 BL/6J mice were immunized with MOG/CFA to induce EAE, and DCs treated with apoptotic or fresh cells were transferred into EAE mice. The results showed that apoptotic cell-treated DCs can significantly block EAE development; in contrast, mice that received fresh cell-treated DCs develop EAE with a similar clinical course and severity as PBS-injected control EAE mice (Fig. 3A).

ELISA results also showed that the production of IL-17A by CD4⁺ T cells isolated from mice that have received apoptotic cell-treated DCs is less than that of CD4⁺ T cells derived from mice i.v. transferred with DCs treated with fresh cells (Fig. 3B). However, the amount of IL-17A produced by CD4⁺ T cells isolated from mice i.v. transferred with fresh cell-

treated DCs is similar to that of CD4⁺ T cells derived from PBS-injected control mice with EAE (Fig. 3B).

4. *In vivo* treatment by apoptotic cell-treated DCs down-regulates expression of multiple inflammatory cytokines in CD4⁺ T cells

To investigate whether apoptotic cell-treated DCs can affect protein expression of pro-inflammatory molecules, the expression of ROR- γ t, IL-17A, IL-22, IFN- γ , IL-21 and IL-17F was detected using flow cytometry. Results demonstrated that the expression of these molecules in CD4⁺ T cells isolated from mice i.v. transferred with apoptotic cell-treated DCs is lower than that of CD4⁺ T cells derived from mice i.v. transferred with fresh cell-treated DCs and that of CD4⁺ T cells isolated from PBS-treated mice (Fig. 4). There is no significant difference in production of these molecules between CD4⁺ T cells isolated from mice i.v. transferred with fresh cell-treated DCs and those isolated from PBS-treated mice (Fig. 4). Our results suggest that apoptotic cell-treated DCs down-regulate the amount of inflammatory cytokine produced by Th17 cells in EAE mice.

Discussion

DCs are professional antigen presenting cells that play an important role in both autoimmunity and immune tolerance. Different signal molecules expressed on DCs perform diverse immune functions. For example, Gr-1, B220, CD205 and galectin-1 expressed on DCs have different immune functions (Fukaya et al., 2012; Ilarregui et al., 2009; Nakano et al., 2001; Nikolic et al., 2002; Perone et al., 2006). It is not clear whether treatment of apoptotic cells can modulate the expression of these molecules on DCs. Our study shows that treatment of apoptotic cells up-regulates the numbers of CD11c⁺galectin-1⁺ and CD11c⁺CD205⁺ DCs. It is known that galectin-1 and CD205 expressed on DCs facilitate development of immune tolerance (Fukaya et al., 2012; Ilarregui et al., 2009; Perone et al., 2006). These changes may underlie the observed inhibition of EAE development following transfer of apoptotic cell-treated DCs.

CD11c⁺Gr-1⁺ DCs facilitate T-cell-mediated immune response. For example, differentiation of CD11c⁺Gr-1⁺ DC facilitates the activity of cytotoxic T lymphocytes (CTLs) and inhibits tumor growth (Li et al., 2004). Our results indicated that treatment of apoptotic cell down-regulates the number of CD11c⁺Gr-1⁺ DCs. This may inhibit CD11c⁺Gr-1⁺ DCs-mediated immune response induced by CTLs in mice with EAE and lead to immune tolerance after transfer of DCs treated with apoptotic cells.

B220 is expressed on plasmacytoid dendritic cells and GM-CSF is an inhibitory factor of B220 expression on DCs. For example, Gilliet et al. reported that there is rare expression of B220 on bone marrow-derived DCs incubated with GM-CSF at 100 ng/ml (Gilliet et al., 2002). In contrast, our data indicated that B220 is still expressed on 10–20% of bone marrow-derived DCs cultured in GM-CSF (20 ng/ml) medium, using a different protocol (Lutz et al., 1999; Zhang et al., 2002). CD11c⁺B220⁺ DCs play a critical role in rejection of xenoengraftment (Ito et al., 2012), indicating that CD11c⁺B220⁺ DCs may facilitate T-cell-mediated inflammatory response. Our study showed that treatment of apoptotic cell causes down-regulation of CD11c⁺B220⁺ DCs numbers. This may block CD11c⁺B220⁺ DCs-mediated inflammatory responses induced in EAE mice, and thus inhibit EAE development.

CD205 is an important tolerance-associated molecule that is expressed on DCs. DCs engulf apoptotic cells via CD205-mediated pathway and lead to self-tolerance *in vivo* (Shrimpton et al., 2009; Zhou et al., 2012). For instance, CD205⁺ DCs up-regulate development of regulatory T cells, which are necessary for immune tolerance *in vivo* (Yamazaki et al., 2008). Our results demonstrated that the expression of CD205 is enhanced after treatment of

apoptotic cells, suggesting that apoptotic cell treatment may facilitate endocytosis of apoptotic cells by DCs and then lead to self-tolerance mediated by CD11c⁺CD205⁺ DCs in mice with EAE.

Galectin-1 expressed on DCs also plays an important role in induction of tolerance *in vivo* (Xu et al., 2010); it induces generation of tolerogenic DC and inhibits EAE development (Ilarregui et al., 2009). We have in the current study shown that treatment of apoptotic cells facilitates expression of galectin-1 on DCs. This may lead to tolerance mediated by galectin-1 in mice with EAE after i.v. transfer of apoptotic cell-treated DCs.

Th helper 17 cells (Th17) are IL-17 producing CD4⁺ T cells (Glader et al., 2010). ROR- γ t is a transcriptional factor that is necessary for the differentiation of Th17 cells (Manel et al., 2008). Th17 cells play an important role in development of autoimmune diseases such as EAE (Jin et al., 2009; Segal, 2010). However, the regulatory mechanisms of Th17 differentiation are poorly understood. For instance, it is not known whether or not apoptotic cell-treated DCs can affect the activity of Th17 cells. DCs treated with apoptotic cells down-regulate expression of ROR- γ t in CD4⁺ T cells, suggesting that apoptotic cell-treated DCs may block differentiation of Th17 cells. Moreover, apoptotic cell-treated DCs modulate cytokine expression by Th17 cells, and i.v. transfer of apoptotic cell-treated DCs inhibits EAE development. These findings suggest that apoptotic cell-induced tolerant DCs may affect activity of Th17 cells by modulating their cytokine secretion.

Th17 cells perform their immune function via secretion of multiple inflammatory cytokines such as IL-17A and IL-17F (Korn et al., 2009; Miossec, 2009). Apoptotic cell-treated DC transfer resulted in suppression of IL-17A and IL-17F in CD4⁺ T cells, thereby affecting EAE development.

IL-21 is a pro-inflammatory cytokine produced by Th17 cells (Jang et al., 2009; Wei et al., 2007), which is known to play a role in EAE development (Liu et al., 2008; Vollmer et al., 2005). Our results showed that apoptotic cell-treated DCs down-regulated IL-21 in CD4⁺ T cells and blocked EAE development. Thus, these data suggest that IL-21 produced by Th17 may regulate Th-17-mediated inflammatory responses and apoptotic cell-induced tolerant DCs may down-regulate IL-21 secretion in Th17 cells and then inhibit IL-21-mediated Th17 immune responses in EAE development.

IL-22 is produced by Th17 cells and is necessary for Th17-mediated inflammatory responses (Kreymborg et al., 2007; Liang et al., 2006). Apoptotic cell-treated DCs down-regulate expression of IL-22 in CD4⁺ T cells, suggesting that apoptotic cell-induced tolerogenic DCs block IL-17 cell-mediated inflammatory responses through inhibition of IL-22 secretion by Th17 cells. However, IL-22 is also produced by Th22 cells, which are IL-22 producing CD4⁺ T cells (Zhuang et al., 2012). Apoptotic cell-induced tolerogenic DCs may also affect the activity of Th22 cells via down-regulation of IL-22 expression. The role of Th22 in EAE development is still unclear. It is necessary to investigate whether Th22 cells also play an important role in EAE development so that we can evaluate whether apoptotic cell-treated DCs block EAE development by suppressing Th22 cell activity.

IFN- γ is an important regulator of immune responses (Weyand et al.). Recent research has shown that IFN- γ not only plays an important role in autoimmunity, but is also necessary for immune tolerance (Malmberg et al., 2002; Weyand et al.; Zhou, 2009). Th17 cells also produce IFN- γ (Zielinski et al., 2012); however, the role of IFN- γ in the immune function of Th17 cells is unclear. IFN- γ may facilitate Th17-mediated inflammatory responses in EAE. Apoptotic cell-treated DCs down-regulate the amount of IFN- γ in CD4⁺ T cells. This effect may block IFN- γ -mediated Th17 immune responses in EAE mice and inhibit EAE development. Moreover, IFN- γ is primarily produced by Th1 cells, which also play an

important role in EAE (Yura et al., 2001). Apoptotic cell-treated DCs may affect the activity of Th1 cells via down-regulation of IFN- γ production. Further studies to investigate whether or not apoptotic cell-treated DCs inhibit EAE development by blocking the activity of Th1 cells should be carried out in the future.

Molecular mechanisms of DC-mediated immune tolerance have not been fully elucidated. It has been reported that multiple molecules expressed on DCs and CD4⁺ T cells are related to induction of tolerance. Our recent data demonstrate that i.v. transfer of tolerogenic DCs modulates expression of multiple tolerance-associated molecules on CD4⁺ T cells (Zhou, et al. 2013). Results from other laboratories have also shown that DCs induce immune tolerance via multiple pathways. For example, plasmacytoid DCs induce tolerance through the (C-C motif) chemokine receptor type 9 (CCR9)/chemokine (C-C motif) ligand 25 (CCL25)-mediated signal pathway. Tolerogenic DCs facilitate the development of regulatory T cells via CCR9 expressed on DCs (Hadeiba et al., 2008). Tolerogenic DCs also induce T cell anergy through the inducible T cell costimulator (ICOS)/ICOS ligand-mediated signal transduction pathway. Deficiency in expression of ICOS on T cells leads to inhibition of T cell anergy (Tuettenberg et al., 2009). Moreover, galectin-1 expressed on DCs is necessary for development of regulatory T cells and induction of tolerance (Blois et al., 2007). DCs also regulate IL-27-mediated activity of IL-10 producing CD4⁺ T cells via the galectin-1-mediated signal transduction pathway (Ilarregui et al., 2009). These results, together with our findings in the current study, imply that apoptotic cell-induced tolerogenic DCs may inhibit the activity of CD4⁺ T cells such as Th17 through multiple pathways. Extensive further studies using antibodies or gene knock-out mice to target individual tolerance-associated molecules expressed on DCs will help to determine the molecular mechanisms of immune tolerance mediated by apoptotic cell-induced tolerogenic DCs.

In conclusion, we designed an immunotherapy using apoptotic cell-treated DCs to block EAE development *in vivo*. Treatment of apoptotic cells down-regulates expression of inflammatory cytokines in CD4⁺ T cells. *Ex vivo* data showed that apoptotic cell-treated DCs affect expression of multiple cytokines in CD4⁺ T cells. These effects may lead to inhibition of EAE development *in vivo*. Our results suggest that apoptotic cell-induced tolerant DCs may provide a potential immunotherapy to treat MS.

Acknowledgments

This study was supported by the National Institutes of Health and the National Multiple Sclerosis Society. We thank Katherine Regan for editorial assistance.

Abbreviations

CCL25	Chemokine (C-C motif) ligand 25
CCR9	(C-C motif) chemokine receptor type 9
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immuno-sorbent assay
FCS	Fetal Calf Serum

Fig	Figure
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICOS	Inducible T cell costimulator
IL	Interleukin
IFN-γ	Interferon gamma
i.p	Intraperitoneally
i.v	Intravenous
KLH	Keyhole limpet hemocyanin
MOG	Myelin oligodendrocyte glycoprotein
2-ME	2-mercaptoethanol
MS	Multiple sclerosis
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PDL-1	Programmed cell death protein ligand1
PT	Pertussis toxin
ROR-γ	Retinoic acid-related orphan receptor (ROR) gamma t
s.c	Subcutaneous
TGF-β	Transforming growth factor-beta
Th	T helper cells
Tregs	Regulatory T cells

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Figure 1. Treatment of apoptotic cells modulates the expression of inflammation- and tolerance-associated molecules on CD11c⁺ DCs *in vitro*

Bone marrow-derived DCs were isolated and cultured in GM-CSF (20ng/ml) medium for 8 days. DCs were co-cultured with fresh T cells or apoptotic T cells for 24 hrs. DCs were harvested and flow cytometry was conducted. Cells were stained with anti-mouse CD11c antibody and CD11c⁺ cells were gated. Expression of Gr-1 (A), B220 (B), CD205 (C) and galectin-1 (D) was detected on CD11c⁺ DCs. Data represent the mean and SD of triplicate determinations of percentage (%) in three independent experiments (n=3, *t* test, * P<0.05).

Figure 2. Apoptotic cell-treated DCs down-regulate expression of ROR- γ t, IL-17A, IL-17F, IL-21, IL-22 and IFN- γ in CD4⁺ T cells *in vitro*

Bone marrow DCs were treated with fresh T cells or apoptotic T cells for 24 hrs. DCs were pulsed with MOG peptide at 0.1 μ M and co-cultured with MOG-primed CD4⁺ T cells for 5 days. CD4⁺ T cells were harvested and gated. The expression of ROR- γ t (A), IL-17A (B), IL-17F (C), IL-21 (D), IL-22 (E) and IFN- γ (F) on CD4⁺ T cells co-cultured with DCs treated with fresh T cells or apoptotic T cells was detected. Data represent the mean and SD of triplicate determinations of percentage (%) in three independent experiments (n=3, *t* test, * P<0.05).

Figure 3. Intravenous (i.v.) transfer of apoptotic cell-treated DCs inhibits EAE development *in vivo*

C57BL/6J mice were (s.c.) immunized with MOG/CFA at day 0. Pertussis toxin was i.p. injected at day 0 and day 2 p. i. DCs co-cultured with fresh T cells or apoptotic T cells had been pulsed with MOG peptide (0.1 μ M) for 24 hrs. Apoptotic cell- and fresh cell-treated DCs were then i.v. injected into EAE mice at days 11, 14, and 17 p. i. (3×10^5 cells/per mouse/per time). EAE mice treated with i.v. PBS are control. EAE development with clinical score is shown (A). This experiment was repeated three times with similar results. Data represent the mean and SEM of EAE clinical score (n=3–6, *t* test, * P<0.05). (B): Apoptotic cell-treated DCs modulated expression of IL-17A produced by MOG-primed T

lymphocytes. Spleen cells were isolated from mice shown in (A) and re-stimulated with MOG peptide (0.1 μ M) for 3 days in complete medium with IL-2 at 1 ng/ml. The supernatant was collected and an ELISA assay was conducted. Expression of IL-17A is shown. The experiments shown in this figure were repeated three times with similar results. Data represent the mean and SD of triplicate determinations of IL-17A concentration from one experiment (n=3, *t* test, * *P*<0.05).

Figure 4. Apoptotic cell-treated DCs inhibit the activity of Th17 cells *in vivo*

Spleen cells were isolated from mice with EAE or tolerance shown in Fig. 3. Cells were re-stimulated with MOG peptide (0.1 μ M) and mouse IL-2 (1 ng/ml) for 3 days. Spleen cells were collected and stained by anti-mouse CD4 antibody. CD4⁺ cells were gated, and the expression of ROR- γ t (A), IL-17A (B), IL-17F (C), IL-21 (D), IL-22 (E) and IFN- γ (F) on CD4⁺ T cells derived from mice i.v. transferred with fresh thymocyte- or apoptotic cell-treated DCs, or PBS only was determined. Data represent the mean and SD of triplicate determinations of percentage (%) in three independent experiments (n=3, *t* test, * *P*<0.05).