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*Original Article*

# CD24 is expressed on FoxP3$^+$ regulatory T cells and regulates their function

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**Abstract:** CD24 is a glycosyl-phosphatidylinositol (GPI) anchored cell surface glycoprotein with a variety of immunomodulatory functions such as inhibition of thymic generation of autoreactive T cells, regulation of antigen presenting cell functions, and mediation of autoimmunity. Given the autoimmune nature of FoxP3$^+$ regulatory T cells and their importance in autoimmune diseases, we hypothesize that CD24 regulates the generation and functions of Treg cells. Through the analysis of the Treg repertoire in two strains of CD24-deficient mice, we found that CD24 does not globally affect the thymic generation of Treg cells. However, CD24 is abundantly expressed on Treg cells, and CD24 antibody treatment of Treg cells enhances their suppressive functions. Concurrently, we observed CD24-deficient Treg cells exhibit increased suppressive functions and produce more IL-10 compared to their wild type counterparts. In addition, CD24-deficient Treg cells exhibited more potent suppressive capacity in inhibiting the development of experimental autoimmune encephalomyelitis (EAE) in mice. Thus, CD24 on Treg cells regulates their suppressive functions. Our findings can partially explain the resistance of EAE development in CD24-deficient mice and CD24 polymorphism-associated susceptibility of human autoimmune diseases. Further investigations regarding mechanisms of CD24 regulation of Treg function may lead to a new approach for the immunotherapy of human autoimmune diseases.

**Keywords:** CD24, regulatory T cells, experimental autoimmune encephalomyelitis

## Introduction

CD24 is a glycosyl-phosphatidylinositol (GPI) anchored cell surface glycoprotein [1, 2]. It is expressed on immature thymocytes, mature B lymphocytes and a variety of other types of cells, such as dendritic cells (DC) [3-6]. CD24 expression reduces from T cells after maturation and upregulates rapidly after T cell activation [7, 8]. CD24 expression on T cells is required for their optimal homeostatic proliferation [9]. In the thymus, CD24 is expressed on immature thymocytes, mTEC and DC [3-6]. CD24 is broadly used as a maturation marker of thymocytes and cross-linking the murine CD24 induced apoptosis of thymocytes [10]. One study suggested transgenic expression of CD24 in thymocytes results in thymic atrophy [11]; however, we did not observe this in our transgenic model [7].

A variety of studies have revealed that CD24 plays a role in regulating the functions of antigen presenting cells (APC). For instance, CD24 on APC has been shown to mediate CD28 independent co-stimulation of CD4 and CD8 T cells [4, 12-16]. CD24 expression on DC has been shown to control the rapid homeostatic proliferation of syngenic T cells in a lymphopenic model [17]. A previous study [18] revealed that CD24 interacts with Siglec G on DCs and binds to danger-associated molecular patterns (DAMP) to form a tri-molecular complex on DC to regulate DC function. Thus, one of the major roles of CD24 is regulating APC functions.

We have reported [19-21] that targeted mutation of CD24 reduces the development of experimental autoimmune encephalomyelitis (EAE), an experimental model of human multiple sclerosis. Our initial works showed that CD24 on APCs contribute to myelin specific T cell expansion and persistence in the peripheral lymphoid organs and in the CNS [19-21]. Our subsequent investigations further revealed that CD24 is also required for the thymic generation

of myelin antigen-specific T cells [5, 6]. These studies suggest that CD24 acts both intra-thymically and extra-thymically. In the thymus, CD24 protects thymocytes from autoantigen-mediated clonal deletion; in the peripheral lymphoid organs and in the CNS, CD24 is required for the optimal activation and survival of myelin antigen-specific T cells.

FoxP3$^+$ regulatory T cells (Tregs) are generated in the thymus (nTreg) and converted from conventional T cells during immune response (iTreg). These cells are considered the key regulators of autoimmunity [22, 23]. Increasing evidence suggests that Tregs manifest their function through a myriad of mechanisms including the secretion of immunosuppressive soluble factors such as IL-10 and TGF beta [24, 25]. Given the autoreactive nature of Tregs [26, 27] and their essential roles in autoimmunity, we hypothesized that Treg cell dysregulation may be related to CD24-mediated autoimmunity. In this study, we have tested this hypothesis by evaluating Treg generation, repertoire, and their suppressive functions in two strains of CD24-deficient mice. We found that CD24 does not globally affect generation of Treg cells in the thymus and peripheral lymphoid organs. However, CD24-deficient Tregs exhibit increased suppressive activity and IL-10 production, making them more potent in inhibiting EAE development.

## Materials and methods

### *Mice*

C57BL6, BALB/c and Rag1$^{-/-}$C57BL/6 mice were purchased from the Jackson Laboratory. CD24$^{-/-}$ mice in the C57BL/6 background have been described [19, 20]. CD24$^{-/-}$Rag1$^{-/-}$ mice were generated by breeding CD24$^{-/-}$ C57BL6 mice with Rag1$^{-/-}$C57BL/6 mice for two generations. CD24$^{-/-}$ BALB/c mice were generated by breeding CD24$^{-/-}$ C57BL/6 mice with BALB/c mice for 20 generations before they were used for this study. 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ mice has been described before [5]. All mice were bred and maintained in the animal facilities of The Ohio State University that are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

### *Antibodies and flow cytometry*

The following antibodies were used in the experiments according to the manufacturer's recommendations: FITC-, PE-, PerCp-, allophycocyanin-labeled anti-Vα3.2 (RR3-16), -Vβ2 (B20.6), -Vβ3 (KJ25), -Vβ4 (KT4), -Vβ5.1/5.2 (MR9-4), -Vβ6 (RR4-7), -Vβ7 (TR310), -Vβ8 (F23.1), -Vβ9 (MR10-2), -Vβ10 (B21.5), -Vβ11 (RR3-15), -Vβ12 (MR11-1), -Vβ13 (MR12-3), -Vβ14 (14-2), -CD4 (GK1.4), -CD8α (53-6.7), -CD24 (M1/69), -CD25 (7D4) and -FoxP3 (NRRF-30). These antibodies were purchased from eBioscience (West San Diego, CA) or BD Biosciences (San Diego, CA). For flow cytometry analysis, thymocytes, splenocytes or lymph node cells were incubated with antibodies on ice for 30 min or by following established protocol (FoxP3 staining, eBiosciences). Cells were collected on a FACScalibur cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed using Flowjo software (Tree star).

### *Purification of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from spleen and lymph node cells*

CD4$^+$ T cells were purified from CD24-deficient BALB/c or C57BL6 mice or their relative WT controls by negative selection. Briefly, spleen and lymph node cells from donor mice were incubated with a cocktail of mAbs (anti-CD8 mAb TIB210, anti-FcR mAb 2.4G2 and anti-CD11c mAb N418). After removing the unbound antibodies, the cells were incubated with anti-rat IgG coated magnetic beads (Dynal Biotech). A magnet was used to remove the Ab-bound cells. The remaining cells were CD4$^+$ T cells. To isolate CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells, the purified CD4$^+$ T cells were stained with anti-CD4-FITC and anti-CD25-PE, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were subsequently separated by high-speed sorting using a flow cytometer. In some experiments, the purified CD4$^+$ T cells were further stained with anti-CD25-PE antibody (7D4, BD Biosciences) followed by separation of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells using anti-PE MACS bead technology.

### *Lymphocyte proliferation and Treg-mediated suppression assay*

For Treg-mediated suppression assay, 0.5×10$^6$/ml purified CD4$^+$CD25$^-$ T cells from WT or CD24$^{-/-}$ mice were co-cultured with graded numbers of CD4$^+$CD25$^+$ Treg cells from WT or EBI3$^{-/-}$ mice in the presence of irradiated splenocytes (2×10$^6$/ml) from Rag1$^{-/-}$ or CD24$^{-/-}$Rag1$^{-/-}$ (C57BL6) or Rag2$^{-/-}$ (BALB/c) mice and 0.1 μg/ml of anti-CD3 mAb (2C11). After

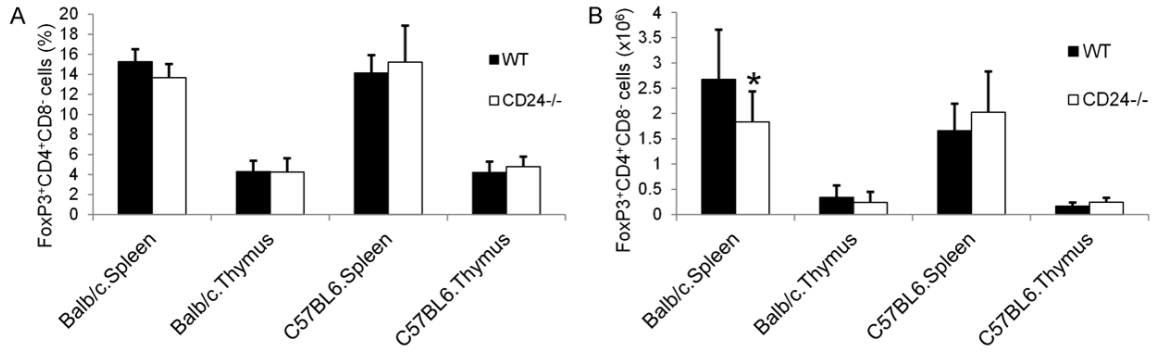


**Figure 1.** Impact of CD24-deficiency on numbers of Treg cells. Thymocytes and splenocytes from WT and CD24$^{-/-}$ BALB/c or C57BL/6 mice were stained for CD4, CD8α, and Foxp3. Percentages (%) of Treg cells (A) and their absolute numbers (B) were quantified by flow cytometry. Six pairs of age and sex-matched BALB/c-CD24$^{-/-}$BALB/c mice and 6 pairs of C57BL/6-CD24$^{-/-}$C57BL6 mice were used. *P<0.05 by student's t test.

48 h, 1 μCi/well $^{3}$H-Tritium was pulsed into the cultures and incorporation of $^{3}$H-Tritium was measured in a liquid scintillation counter 12 h later.

*Isolation of total RNA from Treg cells and qRT-PCR*

Total RNA was isolated from purified Treg cells by using the Trizol method (Invitrogen). The first strand cDNA of each sample was synthesized using a reverse transcription kit (Invitrogen). Quantitative real time PCR was performed to detect the expression of IL-2, IL-10, IFN-γ, and TGF-β1 as we described before [28].

*Cytokine ELISA*

ELISA kits for the detection of IL-2, IL-10 and IFN-γ were purchased from eBiosciences. Standard procedures were followed to detect release of cytokines in culture supernatants in a variety of settings (detailed in figure legends to each experiment).

*Induction and assessment of EAE*

Myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide was purchased from Genemed Synthesis, Inc (San Antonio, TX), was used as the immunogen. Rag1$^{-/-}$ mice (8-12 weeks old) received 5×10$^{6}$ CD4$^{+}$ T cells from sex and age-matched C57BL6 mice or 1×10$^{6}$ CD4$^{+}$ T cells from 2D2$^{+}$CD24$^{-/-}$MOG$^{-/-}$ mice [5]. The recipient mice were then immunized for the induction of EAE as we described [29]. Briefly, the T cell-recipient Rag1$^{-/-}$ mice were immunized subcutaneously with 200 μg MOG peptide in CFA (containing 400 μg of Mycobacterium tuberculosis) in a total volume of 100 μL. Mice were also injected with 150 ng of pertussis toxin (List Biological, Campbell, CA) in 200 μL PBS via the tail vein immediately after the immunization and again 48 hours later. EAE scores were evaluated on a scale of 0 to 5: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, moribund; and 5, death.

*Statistics*

Wilcoxon signed-rank test was used to compare EAE scores in the time course experiments. Student's t-test was used for all other comparisons. In all cases, the alpha level was set at P<0.05.

## Results

*CD24 does not globally affect the generation of Treg cells*

In our previous study, we found that CD24 plays a critical role in thymic generation of MOG-specific 2D2 T cells but not OVA-specific OT2 T cells [5, 6]. Frequencies of viral super antigen reactive T cells (autoreactive) were also reduced in CD24-deficient mice [6]. Given the autoreactive nature of CD4$^{+}$FoxP3$^{+}$ Treg cells [26, 27] and their essential roles in autoimmunity [22, 23], we sought to determine if CD24 affected the generation of Treg cells.

Two strains (BALB/c and C57BL6) of CD24$^{+/+}$ and CD24$^{-/-}$ mice were sacrificed and their thymocytes and splenocytes were prepared, three color staining (CD4, CD8 and Foxp3) was performed and frequencies of CD4$^{+}$CD8$^{-}$Foxp3$^{+}$ T cells were quantified using flow cytometry. As

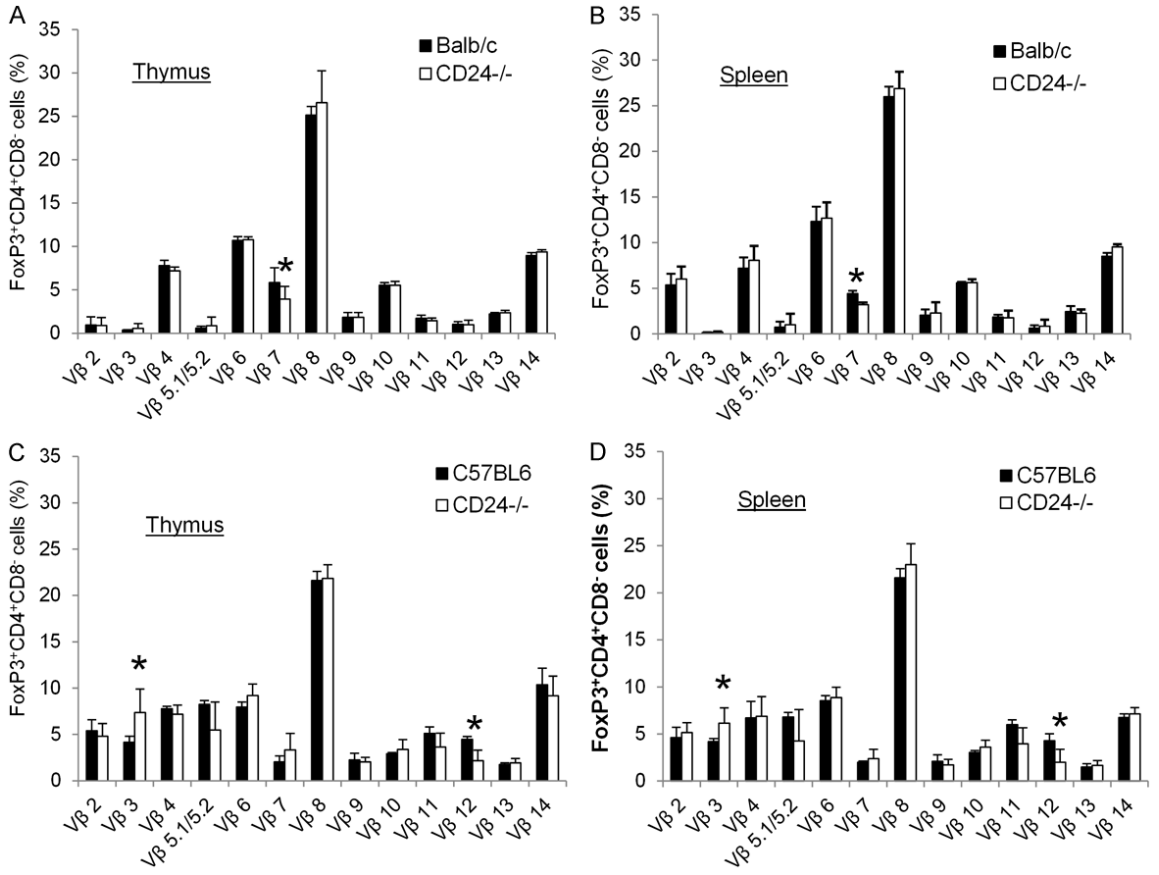


**Figure 2.** TCR Vβ subsets of Treg cells in WT and CD24$^{-/-}$ mice. Thymocytes and splenocytes from WT and CD24$^{-/-}$ BALB/c or C57BL/6 mice were stained for CD4, CD8α and FoxP3 or one of the Vβ chains. Percentages of each subset of Treg cells in thymus (A, C) and spleen (B, D) were quantified by flow cytometry. Age and sex -matched 6 pairs of BALB/c/CD24$^{-/-}$BALB/c mice and 6 pairs of C57BL/6/CD24$^{-/-}$C57BL6 mice were used. $^{*}$P<0.05 and $^{**}$P<0.01 by student's t test.

shown in **Figure 1**, in the thymus, the percentages of Treg cells among CD4 single positive cells were about 4% and were similar between WT and CD24$^{-/-}$ mice in both strains (**Figure 1A**). However, slightly reduced absolute numbers of Treg cells were observed in CD24$^{-/-}$ BALB/c mice but not in CD24$^{-/-}$C57BL6 mice (**Figure 1B**).

To determine whether CD24-deficiency affects T cell receptor repertoire of Tregs, antibodies specific to different Vβ chains, CD4, CD8α and Foxp3 were used to stain thymocytes and splenocytes, and flow cytometry was used for analysis. As shown in **Figure 2**, Tregs bearing Vβ3, Vβ5.1/5.2, Vβ11 and Vβ12 were largely absent in WT and CD24$^{-/-}$BALB/c mice but not in CD24-deficient or sufficient C57BL/6 mice, presumably due to viral super antigen-mediated deletion of these subsets [30]. Tregs from CD24$^{-/-}$ BALB/c mice had similar T cell receptor repertoire with WT BALB/c mice with slightly decreased Vβ7$^{+}$ cells in both thymus and spleens (**Figure 2A** and **2B**). Increased Vβ3 and decreased Vβ12 positive Treg cells were observed in CD24$^{-/-}$C57BL/6 mice compared to WT controls (**Figure 2C** and **2D**). CD24-deficiency did not lead to differential generation of other subsets of Tregs (**Figure 2**). Thus, CD24-deficiency differentially affects subsets of Treg cells in different strains of mice without globally affecting Treg cell generation.

*CD24 is expressed on Treg cells and inhibits Treg functions*

CD24 on T cells has been shown to affect their function [9]. We hypothesized that CD24 on Treg cells also affected the function of Tregs. We first asked whether Treg cells express CD24.

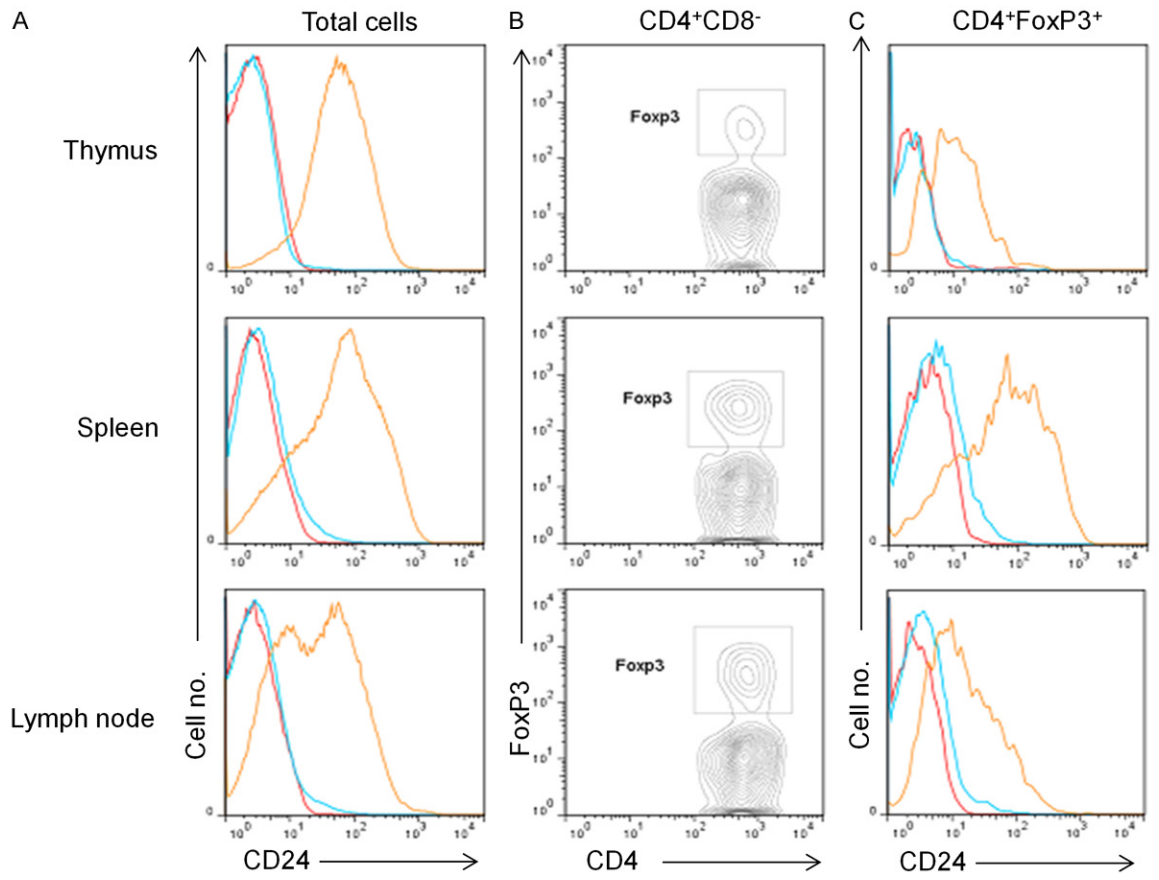


**Figure 3.** Expression of CD24 on Treg cells. Thymus, spleen, and lymph nodes from WT and CD24$^{-/-}$C57BL6 mice were disassociated into single cell suspensions and were stained for CD24, CD4, CD8α, and Foxp3. Cells were then analyzed by flow cytometry. (A) Cellular expression of CD24 on total cell populations. (B) FoxP3 expression among CD4$^{+}$ single positive T cells and (C) CD24 expression on FoxP3$^{+}$CD4$^{+}$ Treg cells. Red lines represent control mAb stained WT cells; blue lines represent anti-CD24 mAb stained cells from CD24$^{-/-}$ mice; and yellow lines represent anti-CD24 mAb stained cells from WT mice. Data shown represent three to five experiments with similar results.

Thymocytes, splenocytes, and lymph node cells from WT and CD24$^{-/-}$ mice were stained for CD24, CD4, CD8, and FoxP3 followed by flow cytometry analysis. As shown in **Figure 3A**, thymocytes, splenocytes, and lymph node cells globally expressed CD24. Among the FoxP3$^{+}$CD4$^{+}$ Tregs (**Figure 3B**), CD24 was readily detectable on Tregs from WT mice (**Figure 3C**), with spleen Treg cells having higher CD24 expression compared to thymic Tregs and lymph node Tregs (**Figure 3C**).

To determine whether CD24 expression on Treg cells affects their effector functions, CD4$^{+}$CD25$^{+}$ Treg cells were purified from WT and CD24$^{-/-}$ mice and their suppressive effects onCD4$^{+}$CD25$^{-}$ T cell proliferation and cytokine production was assessed. As shown in **Figure 4A**, CD24-deficient Treg cells exhibited stronger suppressive activity on T cell proliferation compared to their WT counterparts. CD24-deficient Treg cells also exhibited more potent suppression of IL-2 and IFN-γ but not IL-10 production by T cells (**Figure 4B**). To determine whether CD24 on Treg cells directly affects Treg function, a CD24-specific mAb (M1/69) was used in the coculture and we found that CD24 mAb dose-dependently increased the suppressive activity of WT Treg cells (**Figure 4C**).

*Increased IL-10 production by CD24-deficient Treg cells*

To determine the mechanisms of increased suppressive functions in CD24-deficient Tregs, expression of cytokine genes in the purified WT and CD24$^{-/-}$ Treg cells and CD4$^{+}$CD25$^{-}$ conventional T cells were compared. As shown in **Figure 5A**, expression of IL-2 and IFN-γ genes

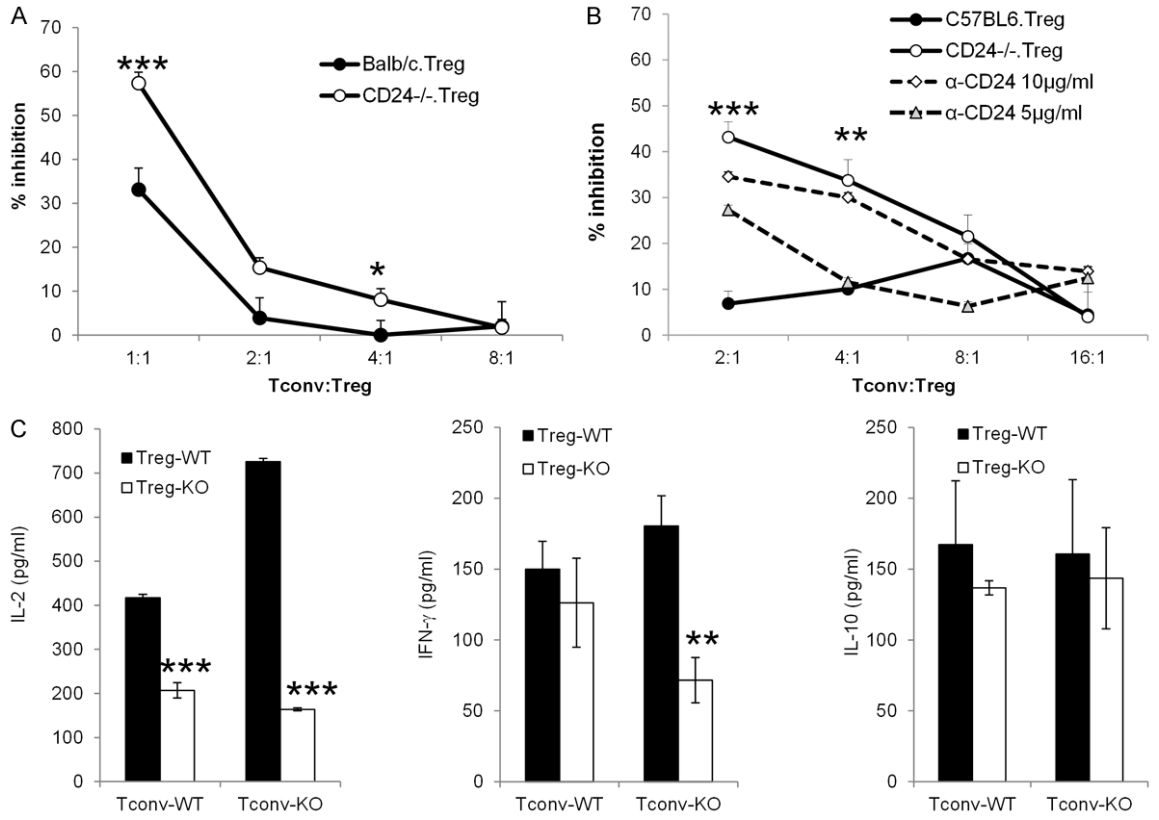


**Figure 4.** Increased suppressive functions of CD24-deficient Tregs. $CD4^+CD25^+$ (Treg) and $CD4^+CD25^-$ T cells (Tconv) were purified from spleen and lymph node cells by fluorescence-activated cell sorting. T cell suppression assay and ELISA were set up as described in materials and methods. A. A representative experiment using Tconv cells from $CD24^{-/-}$BALB/c mice as responders are shown. B. Concentrations of cytokines in the supernatants of co-cultures. C. A representative proliferation assay using Tconv cells from $CD24^{-/-}$C57BL6 mice as responder, irradiated splenocytes from $CD24^{-/-}Rag1^{-/-}$ mice as APC in the presence and absence of different concentrations of anti-CD24 mAb (M1/69). Data shown represent at least three experiments with similar results.

was similar between WT and $CD24^{-/-}$ Treg cells and did not show a difference between Treg cells and conventional T cells. However, expression of IL-10 gene was significantly higher in Treg cells compared to conventional T cell. Notably, CD24-deficient Treg cells had significantly increased expression of the IL-10 gene compared to WT Tregs (**Figure 5A**). Increased expression of TGF-β1 gene was also detected in CD24-deficient Treg cells. However, the overall expression of TGF-β1 gene in Tregs was much lower than that in conventional T cells (**Figure 5A**).

Consistent with the gene expression data, we found that plate-bound anti-CD3-activated $CD24^{-/-}$ Treg cells produce significantly higher amounts of IL-10 than WT Treg cells (**Figure 5B**). However, the productions of IL-2 and IFN-γ were low and did not differ between WT and $CD24^{-/-}$ Treg cells. Conventional T cells (Tconv) produced high amounts of IL-2 and IFN-γ and low amounts of IL-10. However, no significant differences were observed between WT and $CD24^{-/-}$ Tconv cells in production of cytokines (**Figure 5B**).

*CD24-deficient Treg cells are more inhibitory in vivo*

To determine whether CD24-deficiency affects Treg function during the development of EAE, an experimental model of human multiple sclerosis, we performed a series of adoptive transfer experiments. First, we isolated Tregs from $CD24^{-/-}$ and WT C57BL/6 mice and adoptively transferred them into $Rag1^{-/-}$C57BL/6 mice. $CD4^+CD25^-CD24^{-/-}$ T cells from C57BL/6 mice were used as effectors. The recipient mice were then immunized for EAE development. We

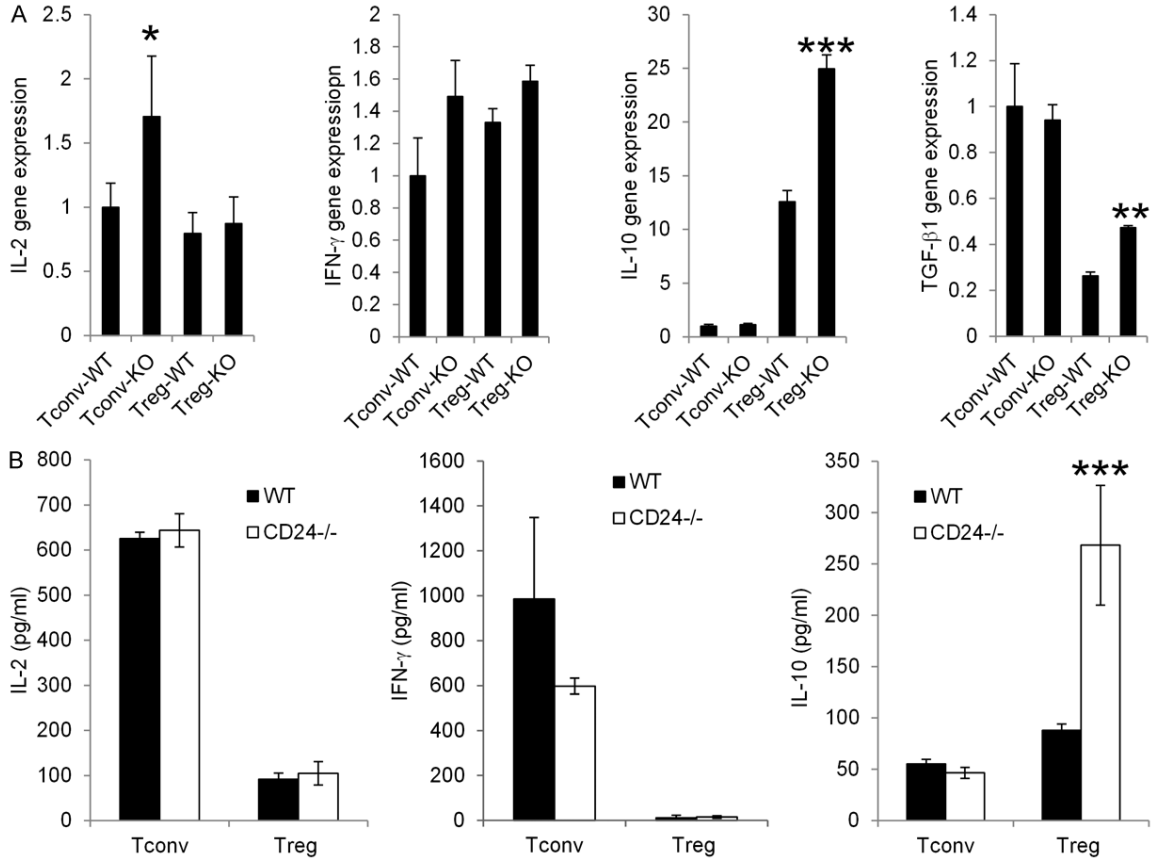


**Figure 5.** Increased IL-10 production by CD24-deficient Treg cells. FACS-sorted Treg and Tconv cells from WT and CD24$^{-/-}$ mice were analyzed for cytokine production by qRT-PCR (A) and ELISA (B). Data are expressed as mean + SD of five samples and represent three experiments with similar results.

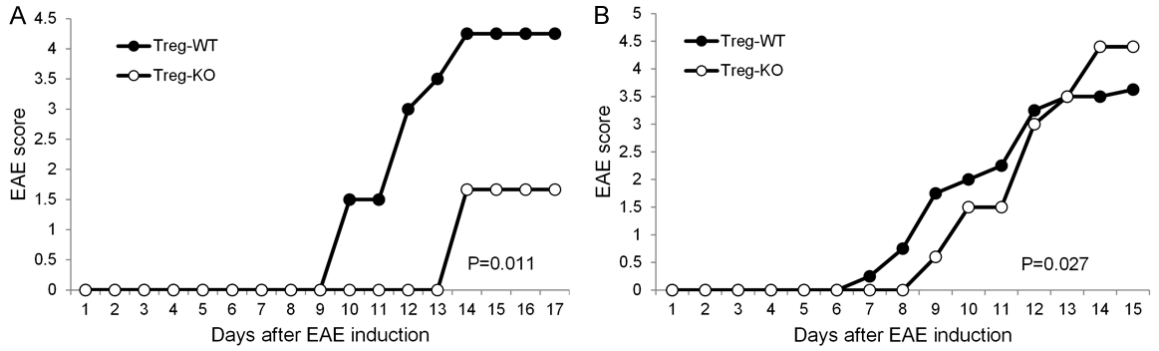


**Figure 6.** Increased suppressive functions of CD24-deficient Treg cells *in vivo*. A. 5×10$^6$ CD4$^+$CD25$^-$ T cells from CD24$^{-/-}$ mice were injected into each Rag1$^{-/-}$ mouse i.v. Each recipient mouse also received 0.7×10$^6$ Treg-WT cells or Treg-CD24$^{-/-}$. The recipient mice were then immunized with MOG35-55/CFA/p.t. Data shown represent two experiments with similar results. B. 1×10$^6$ CD4$^+$CD25$^-$ T cells from 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ mice were injected into each Rag1$^{-/-}$ mouse i.v. Each recipient mouse also received 0.5×10$^6$ Treg-WT cells or Treg-CD24$^{-/-}$. The recipient mice were then immunized with MOG35-55/CFA/p.t. Wilcoxon signed-rank test was used for comparison.

found that mice receiving CD24$^{-/-}$ Treg cells had diminished EAE development compared with mice receiving WT Treg cells (**Figure 6A**). In a second series of experiments, we injected CD4$^+$CD25$^-$ 2D2 T cells from 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ mice into Rag1$^{-/-}$ mice. The recipient mice also received Treg cells from CD24$^{-/-}$ or WT mice followed by EAE induction. Mice receiving CD24-

deficient Tregs developed less severe EAE, further supporting our hypothesis (**Figure 6B**). Thus, CD24-deficient Tregs have a more potent capacity in inhibiting EAE development *in vivo*.

## Discussion

CD24 expression on Tregs has been implicated in some recent studies [31, 32]. However, its functional role remains unclear. In this study we have evaluated the roles of CD24 in Treg cell development and function. We made the following two notable observations. First, CD24 does not globally affect the generation of Treg cells. Second, CD24 is expressed on Treg cells and regulates their function *in vitro* and *in vivo*.

Our recent studies have revealed that CD24 is required for the thymic generation of myelin antigen specific T lymphocytes [5, 6]. Given the autoreactive nature of Treg cells [26, 27], we hypothesized that CD24 in the thymus might affect Treg cell development. To test this hypothesis, in this study we examined numbers and TCR repertoire of Tregs in two strains (BALB/c and C57BL/6) of CD24-deficient mice. We found that CD24-deficiency does not globally affect Treg generation in the thymus. This conclusion is supported by the observation that the percentages of $FoxP3^+CD4^+$ cells are similar between $CD24^{-/-}$ and WT mice in thymus and spleen of both strains of mice. In addition, the percentage of the majority of Treg TCR subsets are also similar between $CD24^{-/-}$ and WT mice in thymus and spleen. However, we observed a few minor differences in Treg cells between $CD24^{-/-}$ and WT mice. First, we found that the total numbers of Treg cells in the spleens of $CD24^{-/-}$BALB/c mice were lower than that in WT BALB/c mice (**Figure 1B**). Studies show that CD24 expression is required for homeostatic proliferation of T cells [9]. Thus it is likely that reduced homeostatic proliferation of CD24-deficient Treg cells may be responsible for the Treg cell number reduction. Intriguingly, this phenomenon was not observed in C57BL/6 mice. It remains unclear whether the strain difference is because BALB/c mice are more lymphopenic, for some subsets of T cells, such as Vβ3, Vβ5, Vβ11 and Vβ12 or if they are missing in BALB/c mice due to viral super antigen mediated T cell depletion [30]. Either way, homeostatic proliferation is more dependent on the lymphopenic environment. Second, we found some minor differences in some subsets of Tregs from WT and $CD24^{-/-}$ mice. In the BALB/c strain, CD24-deficiency caused a minor reduction of Vβ7-specific Treg cell populations; while in the C57BL/6 mice, CD24-deficiency resulted in increased numbers of $Vβ3^+$ Treg cells and decreased $Vβ12^+$ Treg cells. While it remains unclear how $Vβ3^+$ Treg cells are increased, reduced $Vβ7^+$ and $Vβ12^+$ Treg cells are consistent with our previous observation that in the absence of CD24, autoreactive thymocytes are more sensitive to negative selection [5, 6].

This is the first work that shows CD24 on Treg regulates Treg cell functions. This conclusion is supported by the following evidence: first, CD24-deficient Tregs are more suppressive for T cell proliferation and production of IL-2 and IFN-γ. Second, anti-CD24 mAb dose-dependently increases the suppressive functions of Treg cells. Third, CD24-deficient Treg cells produce more IL-10. Fourth, CD24-deficient Tregs are more potent in inhibiting EAE development. Since plate-bound anti-CD3-activated, CD24-deficient Treg cells produce more IL-10, it is unlikely that CD24 on Treg cells are engaging its receptors on other cells. In this regard, recent studies have revealed that CD24 forms a tri-molecular complex with Siglec G and HMGB1 on APCs, allowing it to regulate functions of APC [18]. It is reasonable to hypothesize that CD24 on Treg cells also forms a tri-molecular complexes with Siglec G and HMGB1, regulating Treg IL-10 production. It is also tempting to hypothesize that anti-CD24 antibody treatment increases the suppressive activity of Treg cells by blocking CD24/Siglec G/HMGB1 complex formation on Treg cells.

Treg cell production of TGF-β and IL-10 has been shown to contribute to their suppressive functions [24, 25]. In this study, we found that TGF-β1 mRNA expression was increased in CD24-deficient Treg cells compared to WT Treg cells. However, the overall expression of TGF-β1 mRNA in Treg cells was much lower than Tconv cells. Thus, it is unlikely that TGF-β1 production is a major contributor of increased suppressive functions of CD24-deficient Tregs. In contrast, we found that IL-10 production was significantly increased in CD24-deficient Treg cells compared to WT Treg cells, and the amounts of IL-10 production in Tregs was much higher

than Tconv cells. Thus, it is most likely that increased IL-10 production by CD24-deficient Treg cells contributes to increased suppressive functions of CD24-deficient Treg cells.

Taken together, we have demonstrated that CD24 is abundantly expressed on Treg cells and regulates functions of Tregs. This finding can partially explain the resistance of EAE development in CD24-deficient mice [19-21] and CD24 polymorphism-associated susceptibility of human autoimmune diseases [33, 34]. Further investigation of the mechanisms of CD24 regulation of Treg function may present a new venue for the immunotherapy of human autoimmune diseases.

## Disclosure of conflict of interest

None.

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## References

[1] Williams LA, McLellan AD, Summers KL, Sorg RV, Fearnley DB and Hart DN. Identification of a novel dendritic cell surface antigen defined by carbohydrate specific CD24 antibody cross-reactivity. Immunology 1996; 89: 120-125.

[2] Kay R, Rosten PM and Humphries RK. CD24, a signal transducer modulating B cell activation responses, is a very short peptide with a glycosyl phosphatidylinositol membrane anchor. J Immunol 1991; 147: 1412-1416.

[3] Nielsen PJ, Lorenz B, Muller AM, Wenger RH, Brombacher F, Simon M, von der Weid T, Langhorne WJ, Mossmann H and Köhler G. Altered erythrocytes and a leaky block in B-cell development in CD24/HSA-deficient mice. Blood 1997; 89: 1058-1067.

[4] Liu Y, Jones B, Brady W, Janeway CA Jr, Linsley PS and Linley PS. Co-stimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. Eur J Immunol 1992; 22: 2855-2859.

[5] Zhang X, Liu JQ, Shi Y, Reid HH, Boyd RL, Khattabi M, El-Omrani HY, Zheng P, Liu Y and Bai XF. CD24 on thymic APCs regulates negative selection of myelin antigen-specific T lymphocytes. Eur J Immunol 2012; 42: 924-935.

[6] Carl JW Jr, Liu JQ, Joshi PS, El-Omrani HY, Yin L, Zheng X, Whitacre CC, Liu Y and Bai XF. Autoreactive T cells escape clonal deletion in the thymus by a CD24-dependent pathway. J Immunol 2008; 181: 320-328.

[7] Zhou Q, Wu Y, Nielsen PJ and Liu Y. Homotypic interaction of the heat-stable antigen is not responsible for its co-stimulatory activity for T cell clonal expansion. Eur J Immunol 1997; 27: 2524-2528.

[8] Hubbe M and Altevogt P. Heat-stable antigen/CD24 on mouse T lymphocytes: evidence for a costimulatory function. Eur J Immunol 1994; 24: 731-737.

[9] Li O, Zheng P and Liu Y. CD24 expression on T cells is required for optimal T cell proliferation in lymphopenic host. J Exp Med 2004; 200: 1083-1089.

[10] Jung KC, Park WS, Kim HJ, Choi EY, Kook MC, Lee HW and Bae Y. TCR-independent and caspase-independent apoptosis of murine thymocytes by CD24 cross-linking. J Immunol 2004; 172: 795-802.

[11] Hough MR, Takei F, Humphries RK and Kay R. Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. J Exp Med 1994; 179: 177-184.

[12] Liu Y, Jones B, Aruffo A, Sullivan KM, Linsley PS and Janeway CA Jr. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. J Exp Med 1992; 175: 437-445.

[13] Enk AH and Katz SI. Heat-stable antigen is an important costimulatory molecule on epidermal Langerhans' cells. J Immunol 1994; 152: 3264-3270.

[14] De Bruijn ML, Peterson PA and Jackson MR. Induction of heat-stable antigen expression by phagocytosis is involved in in vitro activation of unprimed CTL by macrophages. J Immunol 1996; 156: 2686-2692.

[15] Wang YC, Zhu L, McHugh R, Sell KW and Selvaraj P. Expression of heat-stable antigen on tumor cells provides co-stimulation for tumor-specific T cell proliferation and cytotoxicity in mice. Eur J Immunol 1995; 25: 1163-1167.

[16] Wu Y, Zhou Q, Zheng P and Liu Y. CD28-independent induction of T helper cells and immunoglobulin class switches requires costimulation by the heat-stable antigen. J Exp Med 1998; 187: 1151-1156.

[17] Li O, Chang X, Zhang H, Kocak E, Ding C, Zheng P and Liu Y. Massive and destructive T cell response to homeostatic cue in CD24-deficient lymphopenic hosts. J Exp Med 2006; 203: 1713-1720.

[18] Chen GY, Tang J, Zheng P and Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science 2009; 323: 1722-1725.

[19] Bai XF, Liu JQ, Liu X, Guo Y, Cox K, Wen J, Zheng P and Liu Y. The heat-stable antigen deter-

mines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. J Clin Invest 2000; 105: 1227-1232.
[20] Bai XF, Li O, Zhou Q, Zhang H, Joshi PS, Zheng X, Liu Y, Wang Y, Zheng P and Liu Y. CD24 controls expansion and persistence of autoreactive T cells in the central nervous system during experimental autoimmune encephalomyelitis. J Exp Med 2004; 200: 447-458.
[21] Liu JQ, Carl JW Jr, Joshi PS, Raychaudhury A, Pu XA, Shi FD and Bai XF. CD24 on the resident cells of the central nervous system enhances experimental autoimmune encephalomyelitis. J Immunol 2007; 178: 6227-6235.
[22] Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J and Sakaguchi S. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 1998; 10: 1969-1980.
[23] Shevach EM. Regulatory T cells in autoimmmunity*. Annu Rev Immunol 2000; 18: 423-449.
[24] Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 2009; 30: 636-645.
[25] Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR Jr, Muller W and Rudensky AY. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 2008; 28: 546-558.
[26] Ribot J, Enault G, Pilipenko S, Huchenq A, Calise M, Hudrisier D, Romagnoli P and van Meerwijk JP. Shaping of the autoreactive regulatory T cell repertoire by thymic cortical positive selection. J Immunol 2007; 179: 6741-6748.
[27] Simons DM, Picca CC, Oh S, Perng OA, Aitken M, Erikson J and Caton AJ. How specificity for self-peptides shapes the development and function of regulatory T cells. J Leukoc Biol 2010; 88: 1099-1107.
[28] Liu JQ, Liu Z, Zhang X, Shi Y, Talebian F, Carl JW Jr, Yu C, Shi FD, Whitacre CC, Trgovcich J and Bai XF. Increased Th17 and regulatory T cell responses in EBV-induced gene 3-deficient mice lead to marginally enhanced development of autoimmune encephalomyelitis. J Immunol 2012; 188: 3099-3106.
[29] Zhu J, Liu JQ, Liu Z, Wu L, Shi M, Zhang J, Davis JP and Bai XF. Interleukin-27 gene therapy prevents the development of autoimmune encephalomyelitis but fails to attenuate established inflammation due to the expansion of CD11b(+)Gr-1(+) myeloid cells. Front Immunol 2018; 9: 873.
[30] Abe R, Foo-Phillips M and Hodes RJ. Genetic analysis of the Mls system. Formal Mls typing of the commonly used inbred strains. Immunogenetics 1991; 33: 62-73.
[31] Herppich S, Toker A, Pietzsch B, Kitagawa Y, Ohkura N, Miyao T, Floess S, Hori S, Sakaguchi S and Huehn J. Dynamic imprinting of the Treg Cell-specific epigenetic signature in developing thymic regulatory T Cells. Front Immunol 2019; 10: 2382.
[32] Zhang Z, Zhang W, Guo J, Gu Q, Zhu X and Zhou X. Activation and functional specialization of regulatory T cells lead to the generation of Foxp3 instability. J Immunol 2017; 198: 2612-2625.
[33] Wang L, Lin S, Rammohan KW, Liu Z, Liu JQ, Liu RH, Guinther N, Lima J, Zhou Q, Wang T, Zheng X, Birmingham DJ, Rovin BH, Hebert LA, Wu Y, Lynn DJ, Cooke G, Yu CY, Zheng P and Liu Y. A dinucleotide deletion in CD24 confers protection against autoimmune diseases. PLoS Genet 2007; 3: e49.
[34] Zhou Q, Rammohan K, Lin S, Robinson N, Li O, Liu X, Bai XF, Yin L, Scarberry B, Du P, You M, Guan K, Zheng P and Liu Y. CD24 is a genetic modifier for risk and progression of multiple sclerosis. Proc Natl Acad Sci U S A 2003; 100: 15041-15046.