European Journal of Immunology

CD24 on thymic APCs regulates negative selection of myelin antigen-specific T lymphocytes

Xuejun Zhang[*1,2], *Jin-Qing Liu*[*1], *Yun Shi*[3], *Hugh H. Reid*[4], *Richard L. Boyd*[5], *Mazin Khattabi*[1], *Hani Y. El-Omrani*[1], *Pan Zheng*[6], *Yang Liu*[6] *and Xue-Feng Bai*[1]

[1] Department of Pathology and Comprehensive Cancer Center, The Ohio State University Medical Center, Columbus, OH, USA
[2] Department of Immunology, Basic Medical College, Tianjin Medical University, Tianjin, P. R. China
[3] Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou, P. R. China
[4] The Protein Crystallography Unit, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia
[5] Monash Immunology and Stem Cell Laboratories, Monash University, Victoria, Australia
[6] Department of Surgery and Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI, USA

Negative selection plays a key role in the clonal deletion of autoreactive T cells in the thymus. However, negative selection is incomplete; as high numbers of autoreactive T cells can be detected in normal individuals, mechanisms that regulate negative selection must exist. In this regard, we previously reported that CD24, a GPI-anchored glycoprotein, is required for thymic generation of autoreactive T lymphocytes. The CD24-deficient 2D2 TCR transgenic mice (2D2$^+$CD24$^{-/-}$), whose TCR recognizes myelin oligodendrocyte glycoprotein (MOG), fail to generate functional 2D2 T cells. However, it was unclear if CD24 regulated negative selection, and if so, what cellular mechanisms were involved. Here, we show that elimination of MOG or Aire gene expression in 2D2$^+$CD24$^{-/-}$ mice — through the creation of 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ or 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$mice — completely restores thymic cellularity and function of 2D2 T cells. Restoration of CD24 expression on DCs, but not on thymocytes also partially restores 2D2 T-cell generation in 2D2$^+$CD24$^{-/-}$ mice. Taken together, we propose that CD24 expression on thymic antigen-presenting cells (mTECs, DCs) down-regulates autoantigen-mediated clonal deletion of autoreactive thymocytes.

Key words: Autoimmunity · CD4$^+$ T Cell · TCR · Thymic selection

Introduction

Autoreactive thymocytes are eliminated through apoptosis in a process termed negative selection. It is generally considered that negative selection occurs at the double positive (DP) and semi-mature single positive ((SP), CD24$^+$) stages [1, 2]. It is increasingly clear that medulla epithelial cells (mTECs) known for the expression of tissue-specific antigens and Autoimmune Regulator (Aire)-mediated negative selection [3–5]. Aire has been shown to regulate autoantigen expression in mTECs, thereby shaping autoimmune

Correspondence: Dr. Xue-Feng Bai
e-mail: Xue-Feng.Bai@osumc.edu

*These authors contributed equally to this work.

T-cell generation in the thymus [5–7]. In addition to mTECs, DCs have also been implicated to play important roles in negative selection [8, 9]. The antigenic signals, presumably mediated by mTECs and DCs, play pivotal roles in the negative selection of autoreactive thymocytes. For instance, TCR-mediated activation of c-Jun NH2-terminal kinase (JNK) pathway is required for the deletion of DP thymocytes [10–12].

Negative selection has always been considered important in preventing autoimmunity. Human patients suffering from autoimmune polyendocrinopathy (APECED) were identified as having a defective expression of Aire [13, 14]. Similar to human APECED patients, Aire-deficient mice exhibit autoimmunity in multiple organs due to diminished negative selection [5, 6]. However, despite negative selection, significant numbers of autoreactive T cells can easily be detected [15, 16] and expanded [17] even in normal individuals. While lack of self antigen expression has been largely attributed as a key factor [18–20], we have reported that even T cells specific for P1A, a self antigen expressed in mTEC [4], can escape clonal deletion [21]. Thus, other than TCR signaling, there must be other mechanisms that actively regulate negative selection. Investigation of such mechanisms may hold a key to understanding pathogenesis of autoimmune diseases.

CD24 is a glycosyl-phosphatidylinositol (GPI) anchored cell-surface glycoprotein [22, 23] and is broadly used as a maturation marker of thymocytes. Cross-linking of the murine CD24 using antibodies induced apoptosis of thymocytes [24]. One study reported thymus atrophy due to CD24 transgenic expression in thymocytes [25]; however, we demonstrated that transgenic expression of CD24 in the thymocyte had no effect on the overall thymic cellularity [26]. More recently, we have reported [27] that CD24 is required for the thymic generation of myelin antigen-specific T lymphocytes. The CD24-deficient 2D2 TCR transgenic mice ($2D2^+CD24^{-/-}$) have been found to have atrophic thymi with a dramatic reduction of $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes. In the peripheral lymphoid organs of these mice, mature 2D2 T cells are essentially absent. Since this phenotype was not observed in mice with transgenic T cells specific for foreign antigen, we suggested that CD24-regulated negative regulation of autoreactive T cells. However, since the requirement for antigenic signaling in this model was not demonstrated, it remains possible that CD24 may regulate T-cell maturation by mechanisms unrelated to negative selection. Here, we demonstrate that thymic deletion of 2D2 T cells in $2D2^+CD24^{-/-}$ mice is myelin oligodendrocyte glycoprotein (MOG) antigen- and Aire-dependent. Restoration of CD24 on DCs, but not on thymocytes was sufficient to protect autoreactive T cells against clonal deletion.

Results

MOG antigen-dependent deletion of thymocytes in $2D2^+CD24^{-/-}$ mice

We have previously generated CD24-deficient 2D2 TCR transgenic mice ($2D2^+CD24^{-/-}$ mice). Compared with $2D2^+CD24^{+/+}$ mice, the $2D2^+CD24^{-/-}$ mice have dramatically withered thymi and reduced cellularity. In the peripheral lymphoid organs, CD24-deficient 2D2 T cells were CD4-negative and were not functional [27]. Despite the dramatic impacts of CD24 on the thymic generation of MOG-specific T cells, the generation of OT2 T cells, which are CD4 T cells specific for the foreign antigen OVA, were not affected by CD24-deficiency. Based on these observations, we hypothesized that CD24 inhibits autoantigen-mediated deletion of immature thymocytes. To test this issue, we crossed MOG-deficient mice [28] with $2D2^+CD24^{-/-}$ mice and generated 2D2 transgenic, double knock out mice ($2D2^+CD24^{-/-}MOG^{-/-}$). As shown in Fig. 1A and B, $2D2^+CD24^{-/-}MOG^{+/+}$ mice had almost completely withered thymi, and their thymi contained much fewer $V\alpha3.2^+V\beta11^+$ (MOG-specific) $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes. In contrast, $2D2^+CD24^{+/+}MOG^{-/-}$ mice had normal thymi with large populations of specific $V\alpha3.2^+V\beta11^+$, $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes (Fig. 1A and B). $2D2^+CD24^{-/-}MOG^{-/-}$ mice had similar thymic cellularity and numbers of $V\alpha3.2^+V\beta11^+CD4^+CD8^-$ thymocytes as that seen in $2D2^+CD24^{+/+}MOG^{-/-}$ mice (Fig. 1A and B). We observed a slightly larger population of $V\alpha3.2^+V\beta11^+CD4^+CD8^+$ thymocytes in $2D2^+CD24^{-/-}MOG^{-/-}$ mice compared with $2D2^+CD24^{+/+}MOG^{-/-}$ mice (Fig. 1B). In the peripheral lymphoid organs, similar numbers of 2D2 ($V\alpha3.2^+V\beta11^+CD4^+$) T cells were detected in $2D2^+CD24^{-/-}MOG^{-/-}$ and $2D2^+CD24^{+/+}MOG^{-/-}$ mice, which was in dramatic contrast to that of $2D2^+CD24^{-/-}MOG^{+/+}$ mice (Fig. 2A). Splenocytes from $2D2^+CD24^{-/-}MOG^{-/-}$ and $2D2^+CD24^{+/+}MOG^{-/-}$ mice were fully responsive to MOG antigen stimulation (Fig. 2B and C), whereas splenocytes from $2D2^+CD24^{-/-}MOG^{+/+}$ mice failed to respond to MOG antigen. Thus, disruption of the MOG gene completely restored thymic cellularity and generated functional $CD4^+$ 2D2 T lymphocytes in $2D2^+CD24^{-/-}$ mice.

To determine whether MOG antigen mediates thymocyte deletion in $2D2^+CD24^{-/-}$ mice, we compared the expression of activation and maturation markers in different subsets of thymocytes between $2D2^+CD24^{-/-}MOG^{+/+}$ mice and $2D2^+CD24^{-/-}MOG^{-/-}$ mice (Fig. 3A). The double negative (DN) 2D2 thymocytes in $2D2^+CD24^{-/-}MOG^{+/+}$ and $2D2^+CD24^{-/-}MOG^{-/-}$ mice expressed high levels of CD5 and TCR, and thus are matured thymocytes [29, 30]. At the DN stage, thymocytes from $2D2^+CD24^{-/-}$ mice had higher expression of CD44 and CD5. Expression of other activation markers was similar between the two types of DN thymocytes. At the DP and particularly the SP stage, thymocytes from $2D2^+CD24^{-/-}MOG^{+/+}$ mice had much higher expression of CD25, CD44 and down-regulated TCR compared with thymocytes from $2D2^+CD24^{-/-}MOG^{-/-}$ mice. Higher Annexin V-positive cells were also observed in DP and SP thymocytes in $2D2^+CD24^{-/-}MOG^{+/+}$ mice (Fig. 3B). These results suggest that MOG antigen-mediated activation and deletion, rather than blockade of thymocyte development was responsible for the observed phenotype in $2D2^+CD24^{-/-}$ mice.

The thymus atrophy/T-cell deletion phenotype in $2D2^+CD24^{-/-}$ mice suggests that CD24 expression in the thymus provides protection for MOG antigen-mediated deletion of thymocytes. To

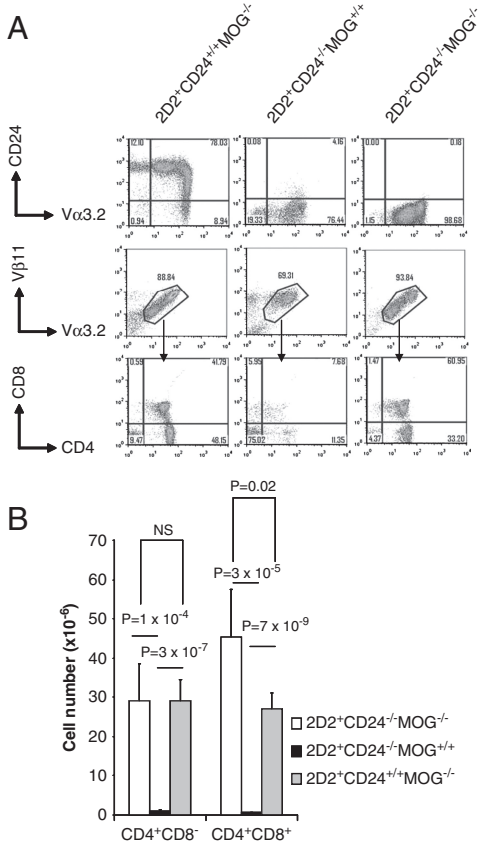

Figure 1. Phenotypic analysis of the thymi in $2D2^+CD24^{-/-}MOG^{-/-}$ mice and controls. (A) Flow cytometry analysis of thymocytes from mice of three different genotypes. Data from one representative mouse of each genotype (representing five to six mice per genotype) is shown. (B) Numbers of thymocyte $CD4^+CD8^-$ and $CD4^+CD8^+$ subsets in mice of different genotypes. Numbers were calculated based on total thymocytes and percentages of each subset of thymocytes and data shown are mean+SD of $n = 5$–6 mice per genotype. Data shown are representative of three experiments involving over 20 mice per group with similar results. Student's *t*-test was used for the statistical analysis.

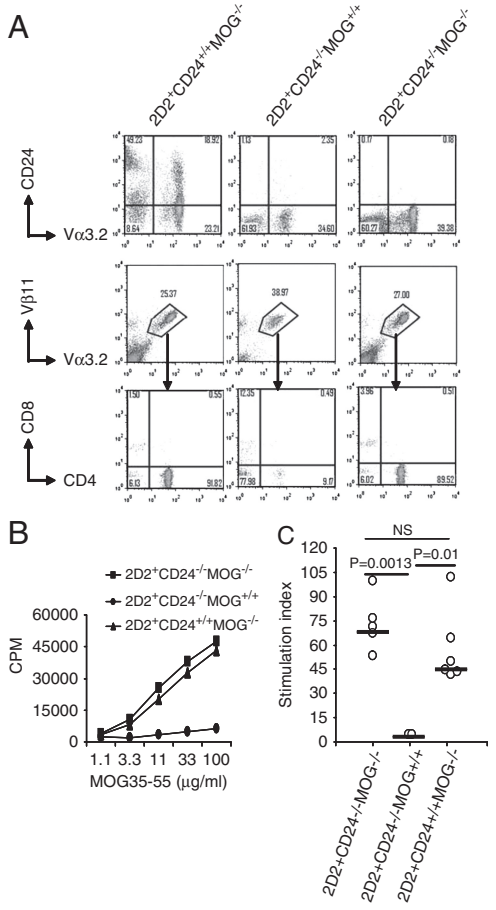

Figure 2. Phenotype analysis of the splenocytes in $2D2^+CD24^{-/-}MOG^{-/-}$ mice and controls. (A) Flow cytometry analysis of splenocytes from mice of three different genotypes. Data from one representative mouse of each genotype (representing five to six mice per genotype) is shown. (B) Proliferation of splenocytes from mice of three genotypes. 1×10^6/mL splenocytes were used in the proliferation assay. Data from one representative mouse of each genotype (representing five to six mice per genotype) are shown. Mean of counts from triplicate wells are shown. Three experiments were performed with similar results. (C) Summary of proliferation assay. Each circle represents stimulation index from a single mouse. Stimulation index = Counts of MOG (100 μg/mL) stimulated cells/counts of cells in the absence of MOG. Bars represent median numbers. Data shown are representative of three experiments involving over 20 mice per group with similar results. Student's *t*-test was used for the statistical analysis.

test this hypothesis, we injected 1 mg of MOG 35–55 into each $2D2^+CD24^{-/-}MOG^{-/-}$ or $2D2^+CD24^{+/+}MOG^{-/-}$ mouse intravenously, and after 24 h, we sacrificed the mice and counted thymocyte numbers. As shown in Fig. 3C, equal numbers of thymocytes were harvested from untreated $2D2^+CD24^{-/-}MOG^{-/-}$ or $2D2^+CD24^{+/+}MOG^{-/-}$ mice. We harvested reduced numbers of thymocytes from MOG-peptide-treated mice. The thymocytes harvested from $2D2^+CD24^{-/-}MOG^{-/-}$ mice were only 50% of that from $2D2^+CD24^{+/+}MOG^{-/-}$ mice. Thus, CD24-deficiency renders thymocytes more susceptible to antigen-mediated deletion.

Aire-dependent deletion of thymocytes in $2D2^+CD24^{-/-}$ mice

mTECs express tissue-specific antigens/Aire and mediate negative selection [3–6]. Aire regulates autoantigen expression in mTECs, thereby shaping autoimmune T-cell generation in the thymus [5–7]. Although there are conflicting views regarding MOG antigen expression in the thymus [31–33], one published report clearly demonstrated that MOG mRNA is present in mTECs [4]. Since we have clearly demonstrated that MOG antigen mediates deletion of 2D2 T cells in the thymi of CD24-deficient mice, it is conceivable to hypothesize that Aire-regulated MOG expression in mTEC mediates deletion of 2D2 T cells in the absence of CD24. To test this hypothesis, we have bred $Aire^{-/-}$ mice with $2D2^+CD24^{-/-}$ mice and generated $2D2^+CD24^{-/-}Aire^{-/-}$ mice. As shown in Fig. 4A and B, $2D2^+CD24^{-/-}Aire^{+/+}$ mice had almost completely withered thymi, and their thymi contained much fewer $V\alpha3.2^+V\beta11^+$, $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes. In contrast, $2D2^+CD24^{+/+}Aire^{+/+}$ mice had normal thymi with large

populations of Vα3.2$^+$Vβ11$^+$, CD4$^+$CD8$^+$ and CD4$^+$CD8$^-$ thymocytes (Fig. 4A and B). 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ mice had similar thymic cellularity and numbers of Vα3.2$^+$Vβ11$^+$CD4$^+$CD8$^-$ thymocytes as that seen in 2D2$^+$CD24$^{+/+}$Aire$^{+/+}$ mice (Fig. 4A and B). In the peripheral lymphoid organs, similar numbers of 2D2 (Vα3.2$^+$Vβ11$^+$CD4$^+$) T cells were detected in 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ and 2D2$^+$CD24$^{+/+}$Aire$^{+/+}$ mice, which was in dramatic contrast to that of 2D2$^+$CD24$^{-/-}$Aire$^{+/+}$ mice (Fig. 5A). Splenocytes from 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ and 2D2$^+$CD24$^{+/+}$Aire$^{+/+}$ mice were fully responsive to MOG antigen stimulation (Fig. 5B and C), whereas splenocytes from 2D2$^+$CD24$^{-/-}$Aire$^{+/+}$ mice failed to respond to MOG antigen. Thus, Aire gene deficiency completely restored thymic cellularity and generation of functional CD4$^+$ 2D2 T lymphocytes in 2D2$^+$CD24$^{-/-}$ mice.

The fact that elimination of Aire, a transcription factor that regulates tissue antigen expression in mTECs, also rescued thymus atrophy/T-cell deletion phenotype in the 2D2$^+$CD24$^{-/-}$ mice suggests that Aire-mediated antigen expression in mTECs plays an essential role in the thymic deletion of 2D2 T cells in the absence of CD24. To test this possibility, we performed real-time PCR analysis on MOG gene expression in whole thymic stromal cells in WT, CD24$^{-/-}$ and CD24$^{-/-}$Aire$^{-/-}$ mice. As shown in Fig. 6A, while MOG gene expression in whole thymic stroma of WT mice were readily detected, MOG gene expression in the whole thymic stroma of CD24$^{-/-}$ mice were slightly decreased (~30%) and MOG gene expression in CD24$^{-/-}$Aire$^{-/-}$ mice was almost undetectable (>10-fold reduction). Since a recent study [34] has reported that 2D2 T cells also recognize another neuronal cytoskeletal self antigen, namely neurofilament-M (NF-M), we tested whether NF-M is expressed in the thymus and whether its expression is regulated by CD24 and Aire. As shown in Fig. 6B, we observed a slight reduction of NF-M gene in the whole thymic stroma of CD24$^{-/-}$ mice (~30%), however, NF-M gene expression in CD24$^{-/-}$Aire$^{-/-}$ mice was dramatically reduced (>10-fold reduction). Thus, Aire and CD24 deficiency regulate the expression of both MOG and NF-M genes in the thymic stroma.

2D2 T cells from MOG- and Aire-deficient 2D2$^+$CD24$^{-/-}$ mice are pathogenic

The peripheral 2D2 T cells from 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ and 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ mice have very similar levels of TCR

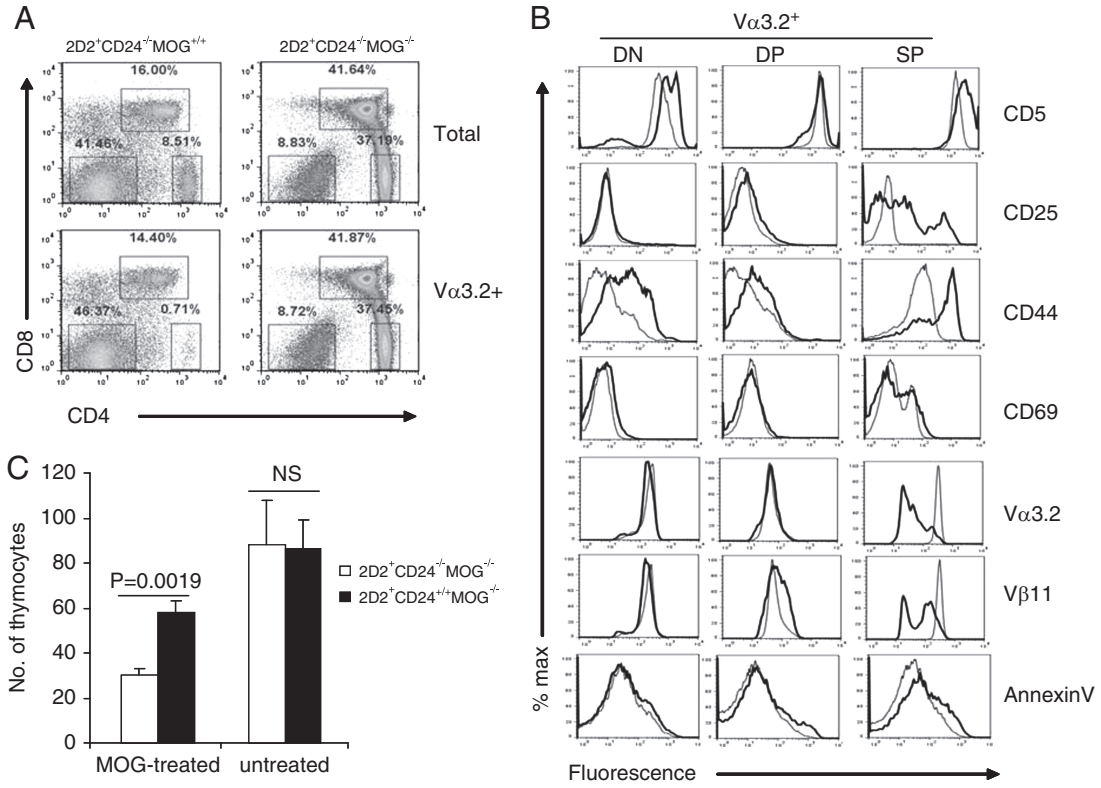

Figure 3. Expression of activation markers in thymocyte subsets and thymocyte susceptibility to MOG-mediated deletion. (A) Flow cytometry analysis of CD4 and CD8 expression on thymocytes from 2D2$^+$CD24$^{-/-}$MOG$^{+/+}$ and 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ mice. (B) Expression of activation and maturation markers on DN, DP and SP thymocytes from sex- and age-matched 2D2$^+$CD24$^{-/-}$MOG$^{+/+}$ and 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ mice. Data shown are representative of two experiments with similar results. Dotted line histograms represent 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ mice and solid line histograms represent 2D2$^+$CD24$^{-/-}$MOG$^{+/+}$ mice. (C) CD24-deficiency renders thymocytes more susceptible to deletion signal. About 1 mg of MOG 35–55 was injected into each mouse intravenously. Twenty four hours after MOG peptide injection, thymocytes were harvested from each mouse and numbers of live thymocyte were quantified. Data are shown as mean+SD of $n = 5$ mice and are representative of two experiments with similar results. Student's *t*-test was used for the comparison.

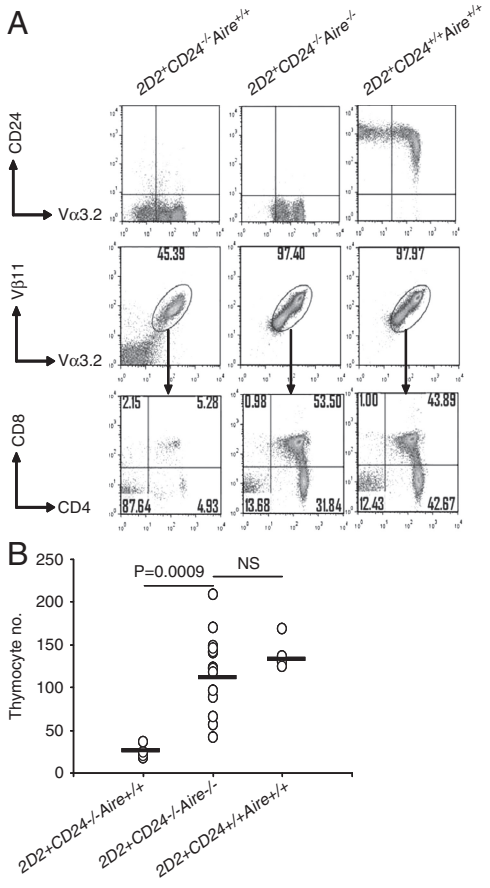

Figure 4. Phenotype analysis of the thymi in 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ mice and controls. (A) Phenotypic analysis of thymocytes from mice with three different genotypes. Data from one representative mouse of each genotype is shown. (B) Numbers of thymocytes in mice with different genotypes. Each circle in (B) represents data from one single mouse. Bars represent medians. Student's *t*-test was used for the comparison.

expression, which is higher than 2D2 T cells from 2D2$^+$CD24$^{-/-}$ mice (Fig. 6C). To test whether 2D2 T cells from 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ and 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ mice are pathogenic, we adoptively transferred purified 2D2 T cells from these mice to RAG-1$^{-/-}$ mice. The recipient mice were then immunized for the induction of EAE. As shown in Fig. 6D, RAG-1$^{-/-}$ mice receiving 2D2 T cells from 2D2$^+$CD24$^{-/-}$MOG$^{-/-}$ and 2D2$^+$CD24$^{-/-}$Aire$^{-/-}$ mice developed progressive EAE, which was lethal by week 3 after immunization. No mice receiving 2D2 T cells from 2D2$^+$CD24$^{-/-}$ mice developed EAE.

Restoration of CD24 expression on DCs partially rescues 2D2 T-cell generation in 2D2$^+$CD24$^{-/-}$ mice

We have previously found that CD24 expression on thymocytes alone does not rescue the thymic atrophy/T-cell deletion phenotype in 2D2$^+$CD24$^{-/-}$ mice [27]. To determine whether CD24 on DCs is required for the generation of functional 2D2 T cells, we have generated CD24 transgenic mice in whom CD24 expression is under the control of CD11c promotor (designated as DC^{CD24TG} mice) (Fig. 7). CD24 transgene expression was detected in DCs from transgenic mice as determined by flow cytometry (Fig. 7A). To determine the exclusive effects of CD24 on DCs in T-cell generation, we bred CD24 transgenic mice with CD24$^{-/-}$ mice and generated mice with CD24 expression only on DCs (DC^{CD24}CD24$^{-/-}$ mice, Fig. 7B). Immunostaining of frozen sections of thymi suggested that in the thymi of DC^{CD24}CD24$^{-/-}$ mice, CD24 expression was strictly overlapping with CD11c (Fig. 7C).

To test whether CD24 expression on DCs is required for the generation of 2D2 T cells, we further crossed DC^{CD24}CD24$^{-/-}$ mice with 2D2$^+$T^{CD24}CD24$^{-/-}$ mice [27] and generated mice of three genotypes, i.e. 2D2$^+$T^{CD24}CD24$^{-/-}$, 2D2$^+$DC^{CD24}CD24$^{-/-}$ and 2D2$^+$CD24$^{-/-}$ (Fig. 8A). As we demonstrated before [27], restoration of CD24 on thymocytes failed to rescue the thymic cellularity and failed to generate Vα3.2$^+$Vβ11$^+$CD4$^+$CD8$^+$ and Vα3.2$^+$Vβ11$^+$CD4$^+$CD8$^-$ thymocytes in the majority of mice. In contrast, expression of CD24 on DCs restored thymic cellularity and generation of Vα3.2$^+$Vβ11$^+$CD4$^+$CD8$^+$ and Vα3.2$^+$Vβ11$^+$CD4$^+$CD8$^-$ thymocytes in about 50% of mice (mostly male mice) (Fig. 8A and B). In spleens of about 50% 2D2$^+$DC^{CD24}CD24$^{-/-}$ mice, CD4$^+$ 2D2 T cells were predominant (Fig. 9A) and were fully responsive to MOG antigen stimulation (Fig. 9B), in dramatic contrast to that of 2D2$^+$CD24$^{-/-}$ mice (Fig. 9B). In about 18% of 2D2$^+$T^{CD24}CD24$^{-/-}$ mice, we observed improved thymic cellularity and restoration of T-cell function in peripheral lymphoid organs (Fig. 9B). Thus, restoration of CD24 expression on DCs, but not on thymocytes partially cured thymus atrophy/T-cell deletion phenotype in 2D2$^+$CD24$^{-/-}$ mice.

Discussion

Negative selection has been shown to play key roles in clonal deletion of autoreactive T cells in the thymus [3–6]. However, negative selection is incomplete, as high numbers of autoreactive T cells can be easily detected [15, 16] in normal individuals. Mechanisms that can regulate negative selection must exist. In this regard, we have shown that CD24 signaling may inhibit negative selection [27]. However, the cellular mechanisms and the requirement for antigenic signaling in this model were not fully demonstrated. In this study, we have investigated this issue by using a series of genetic models, and we have found that the deletion of MOG antigen-specific T cells observed in CD24-deficient 2D2 mice is MOG antigen- and Aire-dependent. In addition, we showed that CD24 expression on DCs could also contribute to the generation of functional autoreactive T cells. Based on these observations, we make the following two points regarding the roles of CD24 in negative selection.

First, CD24 regulates negative selection signaling. We have found that removal of the cognate antigen, MOG, completely rescued the thymus atrophy/T-cell deletion phenotype in the

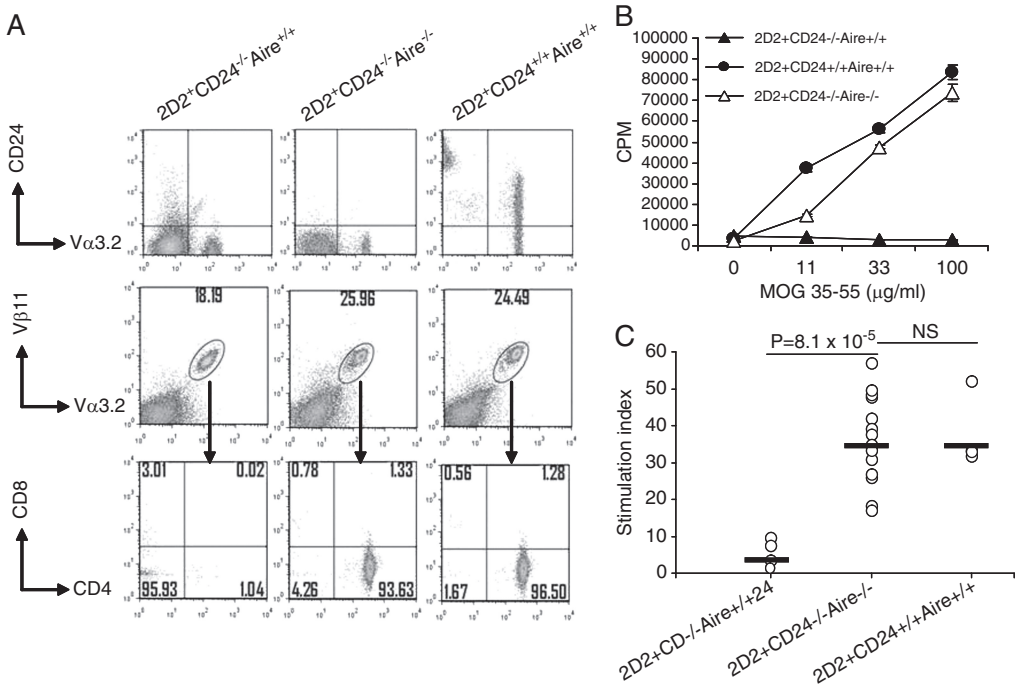

Figure 5. Phenotypic analysis of the splenocytes in $2D2^{+}CD24^{-/-}Aire^{-/-}$ mice and controls. (A) Cell surface markers on splenocytes from mice of three different genotypes. Data from one representative mouse of each genotype is shown. (B) Proliferation of splenocytes from mice of three genotypes. 1×10^{6}/mL splenocytes were used in the proliferation assay. Data are shown as mean ± SD of triplicate cultures from cells of one mouse and are representative of over 20 mice per group from at least three experiments. (C) Summary of proliferation assay. Each circle represents stimulation index from a single mouse. Stimulation index = Counts of MOG (100 μg/mL) stimulated cells/counts of cells in the absence of MOG. Bars represent medians. Student's *t*-test was used for the comparison.

$2D2^{+}CD24^{-/-}$ mice and restored T-cell pathogenicity. This observation suggests that the deletion of autoreactive thymocytes in the thymus of CD24-deficient mice is MOG antigen dependent. Studies [1, 2] have revealed that both DP as well as semi-mature SP thymocytes are targets of negative selection. The remaining thymocytes in MOG-positive $2D2^{+}CD24^{-/-}$ mice, particularly those in DP and SP stage, had enhanced expression of activation markers, down-regulated TCRs and more prone to apoptosis (Annexin V^{+}), suggesting that those thymocytes had seen MOG antigen and were activated. The fact that elimination of Aire, a transcription factor that promotes tissue antigen expression in mTEC also rescues T-cell deletion phenotype in the $2D2^{+}CD24^{-/-}$ mice and restores T-cell pathogenicity, suggests that MOG antigen expression in mTEC plays an essential role in the thymic deletion of 2D2 T cells in the absence of CD24. This conclusion is supported by the finding that in the whole thymic stroma of $CD24^{-/-}Aire^{-/-}$ mice, MOG gene expression is essentially undetectable (Fig. 6A). Thus, our data support the viewpoint that MOG is not only expressed in mTEC, it deletes specific T cells in the absence of CD24. 2D2 T cells also recognize NF-M, another neural antigen [34]. Neurofilaments were found to be expressed in thymic epithelial tumor cells but little in normal thymic epithelial cells [35]. In this study, we have found that in the whole thymic stroma of $CD24^{-/-}Aire^{-/-}$ mice, NF-M gene expression was also dramatically reduced. However, since NF-M gene expression was not affected in MOG-deficient mice (Fig. 6B) and MOG-deletion completely restored thymic cellularity and generation of 2D2 T cells, NF-M does not seem to play a major role in the selection of 2D2 T cells in the thymus.

Because significant numbers of DP cells exist in some $2D2^{+}CD24^{-/-}$ mice, CD24 does not seem to be blocking the transition of DN to DP thymocytes. Those DN thymocytes display matured phenotype by expressing CD5 and TCRs. The reason that DN cells are not deleted may be due to their lack of co-receptors (CD4/CD8) and therefore being non-responsive to MOG antigen. Thus, the requirement for MOG/Aire expression for deletion of 2D2 thymocytes, and the activated phenotype of the remaining thymocytes in $2D2^{+}CD24^{-/-}MOG^{+/+}$ mice clearly establish that deletion of 2D2 T cells, rather than blockade of maturation, is responsible for the thymus atrophy/T-cell deletion phenotype in the $2D2^{+}CD24^{-/-}$ mice.

Second, CD24 on thymic APCs plays a key role in regulating negative selection. mTECs can synthesize peripheral antigens and thereby are predicted to play a central role in negative selection [4, 6]. Our previous studies have demonstrated the importance of radio-resistant TEC in rescuing DP thymocytes, however, CD4 SP thymocytes were not rescued in BM chimeric mice with CD24 expression only on TEC [27]. In this study, we found that elimination of Aire gene in $2D2^{+}CD24^{-/-}$ mice completely rescued 2D2 T-cell generation. This observation suggests that Aire-mediated expression of MOG in mTEC mediates clonal deletion of 2D2 thymocytes and CD24 inhibits this process. Thus, CD24 on mTEC is likely to play the most important role in protecting autoantigen-mediated deletion of autoreactive thymocytes. When

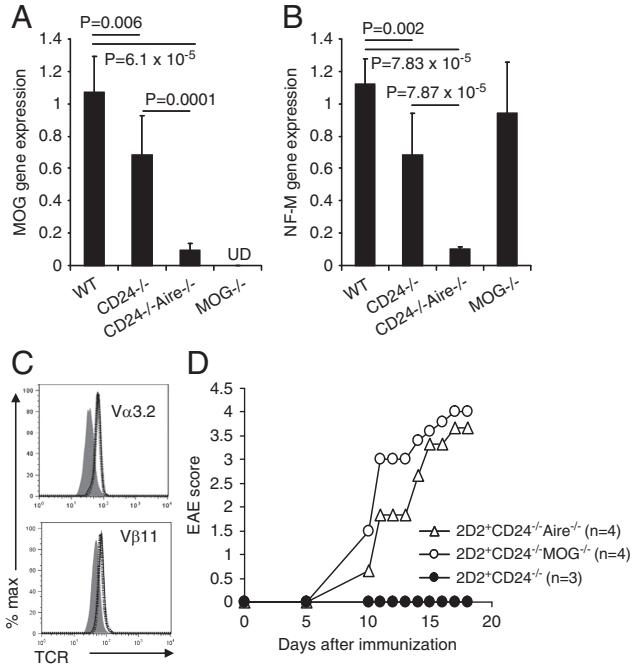

Figure 6. The impacts of MOG or Aire deficiency on the pathogenicity of 2D2 T cells. (A) and (B) Real-time PCR was used to analyze expression of MOG or NF-M genes in whole thymic stromal cells from mice with different genotypes. The relative amounts of mRNA were calculated by plotting the Ct (cycle number), and average relative expression was determined by the comparative method ($2^{-\Delta\Delta Ct}$). Expression of MOG or NF-M genes in one WT sample was set as 1. Data shown are mean+SD of three samples in each group, and the experiments were repeated three times with similar results. MOG gene expression was undetectable (UD) in whole thymic stromal cells from $MOG^{-/-}$ mice. Student's *t*-test was used for the comparison in each group. (C) TCR expression in peripheral 2D2 T cells (spleens) from $2D2^{+}CD24^{-/-}MOG^{+/+}$ (gray shaded histogram), $2D2^{+}CD24^{-/-}MOG^{-/-}$ (solid line histogram) and $2D2^{+}CD24^{-/-}Aire^{-/-}$ (dotted line histogram) mice. Data shown are representative of at least three experiments involving 10 mice per group. (D) EAE development in RAG-$1^{-/-}$ mice receiving 2D2 T cells from mice of different genotypes. 2D2 T cells were purified from mice with different genotypes and were injected into each RAG-$1^{-/-}$ mice i.v. at a dose of 1×10^6/mouse. The recipient mice were immunized with MOG/CFA/pertussis toxin and EAE development were examined daily.

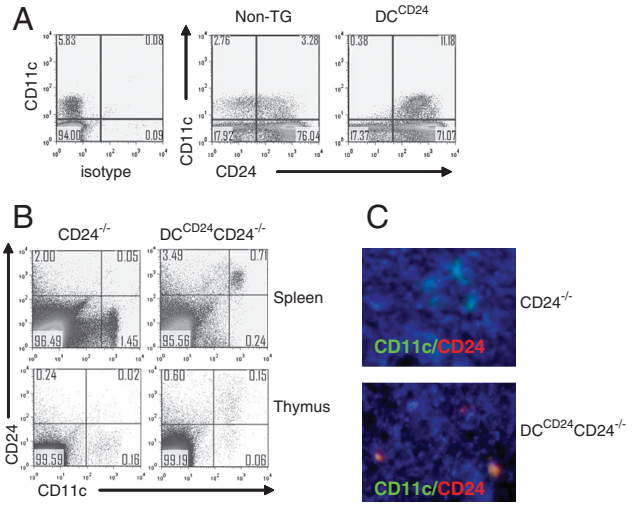

Figure 7. Generation of $DC^{CD24}CD24^{-/-}$ mice. (A) Flow cytometry analysis of CD24 expression on DCs in CD24 transgenic mice (DC^{CD24}). (B) DC^{CD24} mice were crossed with $CD24^{-/-}$ mice and $DC^{CD24}CD24^{-/-}$ mice were generated. Flow cytometry analysis showed that CD24 expression was limited to the DC lineage in $DC^{CD24}CD24^{-/-}$ mice. Data shown are representative of three experiments involving 6 pairs of mice with similar results. (C) Immunostaining of tissue sections of thymus revealed that CD24 was expressed on DCs in the thymus but not the spleen. Alexa Fluor 488-labeled anti-CD11c (HL-3), Biotin-labeled anti-CD24 (M1/69) and Texas Red-labeled Avidin were used for the staining. Original magnification: 400×. Data shown are representative of three experiments involving four pairs of mice with similar results.

performing real-time PCR analysis, we found that CD24 may mediate expression of antigen genes in the thymic stroma (Fig. 6A and B). However, this effect is mild and since $2D2^{+}CD24^{-/-}$ mouse T cells are depleted by antigen, it is unlikely that CD24-mediated antigen expression plays a significant role in negative selection.

In addition to mTEC, considerable evidence exists that peripheral antigens can come from the blood, captured and presented by DCs to immature thymocytes [8, 9]. In addition, DCs can also acquire tissue antigens from mTEC and cross-present them to thymocytes to mediate negative selection [36]. Thus, it was conceivable to speculate that CD24 on DCs can also rescue immature SP thymocytes from negative selection. Indeed, in this study we found that restoration of CD24 expression on DCs alone, fully restored 2D2 T-cell generation and function in about 50% $2D2^{+}CD24^{-/-}$ mice. Interestingly, we found that 50% $2D2^{+}CD24^{-/-}$ mice that had corrected phenotype were mostly male mice. It remains undetermined if CD24 transgene expression in DCs is higher in male mice or alternatively, male thymocytes are more susceptible to CD24 protection. In contrast to the protective effects of CD24 on DCs, restoration of CD24 in thymocytes alone, was insufficient to rescue 2D2 T-cell generation, as we previously demonstrated [27]. Since DC expression of CD24 only rescued T-cell generation in 50% of $2D2^{+}CD24^{-/-}$ mice, we propose that both mTEC and DCs are required to express CD24 in order for the full generation of autoreactive T cells.

The collective observation that CD24-deficient mice failed to generate 2D2 T cells, their dependence on the presence of cognate antigen/Aire and the role of CD24 on DCs in 2D2 T-cell generation can be explained by at least two mechanisms. First, since CD24 on DCs negatively regulates DC response to tissue injury [37, 38] and since thymus is a site of massive cell death, it is possible that CD24-deficient DCs may be more potent in inducing T-cell activation. However, as the comparison for expression of activation markers on thymic DCs did not reveal a difference between WT and $CD24^{-/-}$ mice (not shown), this mechanism is unlikely. Second, it is possible that CD24 on thymic APCs transmits an inhibitory signal to immature thymocytes to prevent myelin antigen-induced deletion of immature thymocytes. In this regard, it remains to be determined what receptor(s) on thymocytes are involved.

Taken together, our data suggest that CD24 inhibits deletion of autoreactive T cells. The significance of this function of CD24

deserves consideration. As most tumor antigens are self antigens [39], this mechanism may allow preservation of some autoreactive T cells to participate in tumor immune surveillance. It is of note that targeted mutation of CD24 significantly reduced mice susceptibility to EAE. Although priming of MOG-reactive T cells have been demonstrated, our data suggest that high-affinity autoreactive T cells likely have been deleted in the CD24-deficient mice. Thus, in addition to its role in T-cell activation in the CNS [40], CD24 may regulate autoimmune diseases by regulating the repertoire of autoreactive T cells. Given the impacts of CD24 polymorphism on the susceptibility to multiple sclerosis [41–43], systemic lupus erythematosus [42], and rheumatoid arthritis [44], the mechanism of CD24-mediated regulation of negative selection may contribute to our understanding of the pathogenesis of human autoimmune diseases.

Materials and methods

Mice

C57BL6 mice and Aire$^{-/-}$ mice in the C57BL6 background were purchased from the Jackson Laboratory. 2D2 TCR transgenic mice [45] were described before [27]. CD24$^{-/-}$ mice in the C57BL6 background have been described [40, 46]. Transgenic mice with CD24 expression exclusively on T cells have been described [26, 47]. C57BL6-deficient mice for myelin antigen MOG (MOG$^{-/-}$C57BL6) have been described [28]. 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.

Production of 2D2^{+}CD24$^{-/-}$MOG$^{-/-}$ mice

Male 2D2^{+}CD24$^{-/-}$ mice were crossed with female MOG$^{-/-}$ C57BL6 mice. The F1 2D2^{+}CD24$^{+/-}$MOG$^{+/-}$ mice were crossed with CD24$^{+/-}$MOG$^{+/-}$ mice and the resulting offspring were screened for 2D2^{+}CD24$^{-/-}$MOG$^{-/-}$ genotypes. Briefly, we stained peripheral blood cells for Vα3.2 and used flow cytometry to identify 2D2 TCR transgene positive mice. We isolated DNA from tail biopsy samples of offspring mice and used PCR to identify their genotypes. We have designed the following PCR primers to amplify WT alleles of MOG gene and CD24 gene: mMOG.F: 5′-GTG GTA ATG CTT ACA TGG AGG TTG-3′ and mMOG.R: 5′-CTA GGG CTA CAG ATA GAG CTT AGT-3′. CD24.F: 5′-TTG CTG CTT CTG GCA CTG CTC CTA-3′ and CD24.R: 5′-CTA AGA GAG GAA GCG TCT GTG GAC-3′. CD24-deficiency was also verified by staining of peripheral blood cells for CD24 and B220 markers followed by flow cytometry analysis. CD24$^{-/-}$ mice have no CD24 expression on B220^{+} cells.

Generation of 2D2^{+}CD24$^{-/-}$Aire$^{-/-}$ mice

Aire$^{-/-}$ C57BL6 mice were bred with 2D2^{+}CD24$^{-/-}$ mice for two generations to generate 2D2^{+}CD24$^{-/-}$Aire$^{-/-}$ mice. The expres-

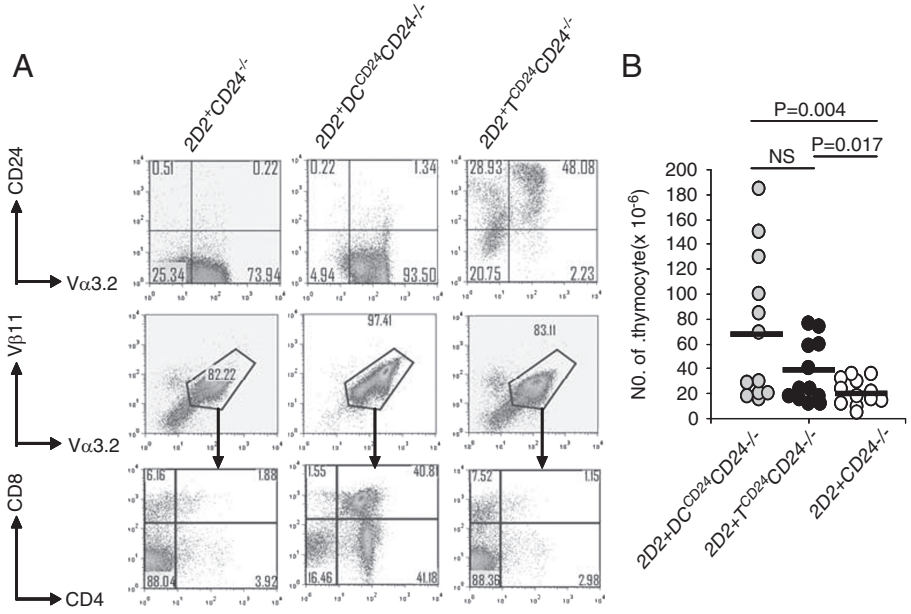

Figure 8. Impact of DC expression of CD24 on thymic cellularity in 2D2^{+}CD24$^{-/-}$ mice. (A) Cell surface markers of thymocytes from mice of three different genotypes. Data from one representative mouse of each genotype are shown. (B) Numbers of thymocytes from mice of three different genotypes. Each circle in (B) represents data from one single mouse. Student's *t*-test was used for the comparison. Bars represent medians. Data shown in (B) were accumulated from three experiments with similar results.

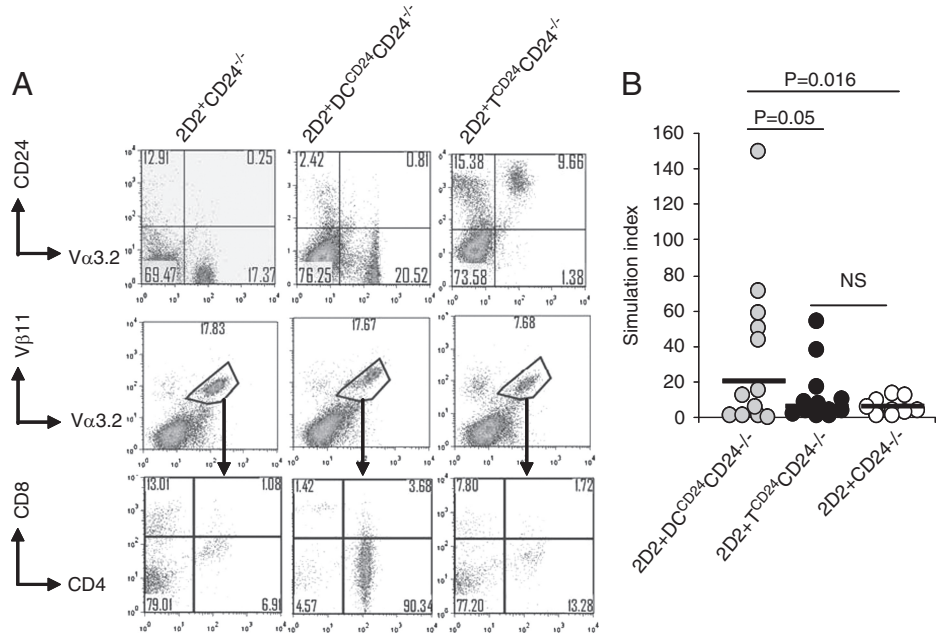

Figure 9. Phenotypic analysis of the splenocytes in 2D2^{+}DC^{CD24}CD24$^{-/-}$ mice and controls. (A) Cell surface markers of splenocytes from mice with different genotypes. (B) Summary of proliferation assay. Each circle represents stimulation index from a single mouse. Student's t-test was used for the comparison. Bars represent medians. Data shown in (B) were accumulated from three experiments with similar results.

sion of 2D2 TCR and CD24 was determined as described before [27]. For genotyping of Aire-deficiency, PCR was used to amplify genomic DNA from mice to detect a 750 bp band (mutated) using the following primers: Aire.F: 5′-GTC ATG TTG ACG GAT CCA GGG TAG AAA GT-3′ and Aire.R: 5′-AGA CTA GGT GTT CCC TCC CAA CCT CAG-3′. Once the 2D2^{+}CD24$^{-/-}$ Aire$^{-/-}$ mice were generated, thymocytes and splenocytes were stained with anti-CD4, anti-CD8, anti-Vα3.2 and anti-Vβ11 antibodies and were analyzed by flow cytometry. Spleen cells were tested for proliferative response to MOG35-55 as we described before [27].

Generation and characterization of DC^{CD24} mice

To investigate the role of CD24 on DCs in T-cell development, we have generated CD24 transgenic mice in which CD24 expression is under the control of CD11c promotor (designated as DC^{CD24} mice). A CD11c transgenic construct [48, 49] was kindly provided by Dr. Jin Wang (Baylor College of Medicine). The transgenic vector contains a 5.5-kb mouse CD11c promotor, a chicken β-globulin intron sequence and polyadenylation signal. CD24 cDNA (coding region) was inserted into the EcoR I site flanked by chicken β-globulin intron sequences. A transgenic DNA fragment was released from the construct by digestion with Not 1 and Xho I restriction enzymes and was used for microinjection of fertilized eggs. C57BL6 oocytes were injected using conventional microinjection technology by the transgenic core-facilities of the Ohio State University. PCR was used to identify transgene positive mice. Tail DNA samples were amplified for the transgene using the following primers: mCD24.F: 5′-CAG GAT CCG AAC ATC TAG AGA GTC GCG CCG-3′ and PCD11c.R: 5′-TCC CAT ATG TCC TTC CGA GTG AGA-3′.

Abs and flow cytometry

The following Abs were used in the experiments according to manufacturer's recommendations: unlabeled, FITC-, PE-, PerCp-, allophycocyanin- or biotin-labeled anti-CD4 (GK1.4), -CD5 (53-7.3), -CD8α (53-6.7), -CD11c (HL3), -CD24 (M1/69), -CD25 (7D4), -CD44 (IM7), -CD69 (H1.2F3), -Vα3.2 (RR3-16), -Vβ11 (RR3-15). These antibodies were purchased from BD Biosciences (San Diego, CA, USA) or eBioscience (West San Diego, CA, USA). For flow cytometry analysis, cells were incubated with antibodies on ice for 30 min followed by extensive washing. Cells were collected on an FACScalibur cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed using the Flowjo software (Treestar).

Immunofluorescence microscopy

The antibodies used for immunofluorescence staining of brain and thymus sections were the following: biotinylated anti-CD24 (M1/69; BD Biosciences); Alexa-488-anti-CD24 (Biolegend); Alexa-488-anti-CD11c (Biolegend); biotinylated rat anti-MOG (R&D systems) and Texas Red-avidin (Thermo

Scientific). Brain and thymus were harvested and frozen in Tissue-Tek OCT media (Sakura Finetek), and 10-μm-thick slices were prepared. Tissue sections were fixed in ice-cold acetone for 30 s and were then stained with the corresponding fluorescent antibodies for another 2 h at 24°C. After washing with phosphate-buffered saline (PBS), slides were mounted with DAPI-containing vectashield mounting medium (Vector Laboratories) and were examined and photographed on an inverted three-color fluorescence microscope system (Nikon Ti-U).

Proliferation assay

Splenocytes (1×10^6/mL) from various strains of 2D2 TCR transgenic mice with or without CD24-deficiency were stimulated with titrated MOG 35-55 peptide in 96-well U-bottomed plates. ^{3}H-Thymidine was added into the culture at 48 h and harvested 12 h later. ^{3}H-incorporation was measured with a scintillation counter. MOG 35–55 (MEVGWYRSPFSRVVHLYRNGK) was purchased from Genemed Synthesis (South San Francisco, CA, USA).

Real-time PCR

The thymi from 6–8-week-old mice were separated and thymocytes were removed by cutting each thymic lobe into multiple pieces and applying pressure with the head of a syringe. After washing the remnants three times with serum-free RPMI (Invitrogen, Carlsbad, CA, USA), total RNA was prepared from the remnants using the Trizol method followed by cDNA synthesis. Real-time PCR was performed to detect the expression of MOG or NF-M genes in thymic stromas as described previously [27]. The following primers were used: MOG.F: 5′-CCG GTA ACC ATA AAG ATG GC-3′; MOG.R: 5′-GGG ATA CCC TGG TCC TAT CA-3′. NF-M.F: 5′-TCG TCA TTT GCG AGA ATA CC-3′; NF-M.R: 5′-TGT CGG TGT GTG TAC AGA GG-3′. HPRT.F: 5′-AGC CTA AGA TGA GCG CAA GT-3′; HPRT.R: 5′-TTA CTA GGC AGA TGG CCA CA-3′.

Adoptive transfer of 2D2 T cells and induction of EAE

2D2 T cells were purified from spleens and lymph nodes of $2D2^+CD24^{-/-}$, $2D2^+CD24^{-/-}MOG^{-/-}$ and $2D2^+CD24^{-/-}Aire^{-/-}$ mice by negative selection as we described [27]. For induction of EAE in RAG-$1^{-/-}$ mice, mice of 8–12 wks of age first received purified 2D2 T cells (1×10^6/mouse) i.v. Twenty four hours later, 2D2 T-cell recipients were immunized s.c. with 200 μg MOG 35–55 in CFA (containing 400 μg of Mycobacterium tuberculosis) in a total volume of 100 μL. Mice received 100 ng of pertussis toxin (List Biological, Campbell, California, USA) in 200 μL PBS in the tail vein immediately after the immunization and again 48 h later. The mice were observed every day and were scored on a scale of 0–5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, moribund; and 5, death.

Acknowledgements: This study was supported by a grant from National Institutes of Health (R21 AI078286 to XFB). X. Z. is a visiting scholar who is partially supported by China National Natural Science Foundation (Grant numbers 30870562 and 30770432).

Conflict of interest: The authors have no financial and commercial conflict of interest.

References

1 **Sprent, J. and Kishimoto, H.,** The thymus and negative selection. *Immunol. Rev.* 2002. **185**: 126–135.

2 **von Boehmer, H. and Kisielow, P.,** Negative selection of the T-cell repertoire: where and when does it occur? *Immunol. Rev.* 2006. **209**: 284–289.

3 **Hanahan, D.,** Peripheral-antigen-expressing cells in thymic medulla: Factors in self-tolerance and autoimmunity. *Curr. Opin. Immunol.* 1998. **10**: 656–662.

4 **Derbinski, J., Schulte, A., Kyewski, B. and Klein, L.,** Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 2001. **2**: 1032–1039.

5 **Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C.,** Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* 2003. **4**: 350–354.

6 **Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H. et al.,** Projection of an immunological self shadow within the thymus by the aire protein. *Science* 2002. **298**: 1395–1401.

7 **Anderson, M. S., Venanzi, E. S., Chen, Z., Berzins, S. P., Benoist, C. and Mathis, D.,** The cellular mechanism of Aire control of T-cell tolerance. *Immunity* 2005. **23**: 227–239.

8 **Throsby, M., Homo-Delarche, F., Chevenne, D., Goya, R., Dardenne, M. and Pleau, J. M.,** Pancreatic hormone expression in the murine thymus: Localization in dendritic cells and macrophages. *Endocrinology* 1998. **139**: 2399–2406.

9 **Pugliese, A., Brown, D., Garza, D., Murchison, D., Zeller, M., Redondo, M. J., Diez, J. et al.,** Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J. Clin. Invest.* 2001. **107**: 555–564.

10 **Rincon, M., Whitmarsh, A., Yang, D. D., Weiss, L., Derijard, B., Jayaraj, P., Davis, R. J. et al.,** The JNK pathway regulates the In vivo deletion of immature CD4(+)CD8(+) thymocytes. *J. Exp. Med.* 1998. **188**: 1817–1830.

11 **Sabapathy, K., Kallunki, T., David, J. P., Graef, I., Karin, M. and Wagner, E. F.,** c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T-cell apoptosis and proliferation. *J. Exp. Med.* 2001. **193**: 317–328.

12 **Behrens, A., Sabapathy, K., Graef, I., Cleary, M., Crabtree, G. R. and Wagner, E. F.,** Jun N-terminal kinase 2 modulates thymocyte apoptosis

and T-cell activation through c-Jun and nuclear factor of activated T-cell (NF-AT). *Proc. Natl. Acad. Sci. USA* 2001. **98**: 1769–1774.

13 **Finnish-German APECED Consortium,** An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat. Genet.* 1997. **17**: 399–403.

14 **Nagamine, K., Peterson, P., Scott, H. S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K. J. et al.,** Positional cloning of the APECED gene. *Nat. Genet.* 1997. **17**: 393–398.

15 **Sun, J. B., Olsson, T., Wang, W. Z., Xiao, B. G., Kostulas, V., Fredrikson, S., Ekre, H. P. et al.,** Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls. *Eur. J. Immunol.* 1991. **21**: 1461–1468.

16 **Liblau, R., Tournier-Lasserve, E., Maciazek, J., Dumas, G., Siffert, O., Hashim, G. and Bach, M. A.,** T-cell response to myelin basic protein epitopes in multiple sclerosis patients and healthy subjects. *Eur. J. Immunol.* 1991. **21**: 1391–1395.

17 **Ota, K., Matsui, M., Milford, E. L., Mackin, G. A., Weiner, H. L. and Hafler, D. A.,** T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* 1990. **346**: 183–187.

18 **Kuchroo, V. K., Anderson, A. C., Waldner, H., Munder, M., Bettelli, E. and Nicholson, L. B.,** T-cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T-cell repertoire. *Annu. Rev. Immunol.* 2002. **20**: 101–123.

19 **Klein, L., Klugmann, M., Nave, K. A., Tuohy, V. K. and Kyewski, B.,** Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat. Med.* 2000. **6**: 56-61.

20 **Anderson, A. C., Nicholson, L. B., Legge, K. L., Turchin, V., Zaghouani, H. and Kuchroo, V. K.,** High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. *J. Exp. Med.* 2000. **191**: 761–770.

21 **Sarma, S., Guo, Y., Guilloux, Y., Lee, C., Bai, X. F. and Liu, Y.,** Cytotoxic T lymphocytes to an unmutated tumor rejection antigen P1A: normal development but restrained effector function in vivo. *J. Exp. Med.* 1999. **189**: 811–820.

22 **Williams, L. A., McLellan, A. D., Summers, K. L., Sorg, R. V., Fearnley, D. B. and Hart, D. N.,** Identification of a novel dendritic cell surface antigen defined by carbohydrate specific CD24 antibody cross-reactivity. *Immunology* 1996. **89**: 120–125.

23 **Kay, R., Rosten, P. M. and Humphries, R. K.,** 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.

24 **Jung, K. C., Park, W. S., Kim, H. J., Choi, E. Y., Kook, M. C., Lee, H. W. and Bae, Y.,** TCR-independent and caspase-independent apoptosis of murine thymocytes by CD24 cross-linking. *J. Immunol.* 2004. **172**: 795–802.

25 **Hough, M. R., Takei, F., Humphries, R. K. and Kay, R.,** Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. *J. Exp. Med.* 1994. **179**: 177–184.

26 **Zhou, Q., Wu, Y., Nielsen, P. J. 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.

27 **Carl, J. W., Jr., Liu, J. Q., Joshi, P. S., El-Omrani, H. Y., Yin, L., Zheng, X., Whitacre, C. C. et al.,** Autoreactive T cells escape clonal deletion in the thymus by a CD24-dependent pathway. *J. Immunol.* 2008. **181**: 320–328.

28 **Linares, D., Mana, P., Goodyear, M., Chow, A. M., Clavarino, C., Huntington, N. D., Barnett, L. et al.,** The magnitude and encephalogenic potential of autoimmune response to MOG is enhanced in MOG deficient mice. *J. Autoimmun.* 2003. **21**: 339–351.

29 **Tarakhovsky, A., Kanner, S. B., Hombach, J., Ledbetter, J. A., Muller, W., Killeen, N. and Rajewsky, K.,** A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* 1995. **269**: 535–537.

30 **Azzam, H. S., Grinberg, A., Lui, K., Shen, H., Shores, E. W. and Love, P. E.,** CD5 expression is developmentally regulated by T-cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* 1998. **188**: 2301–2311.

31 **Delarasse, C., Daubas, P., Mars, L. T., Vizler, C., Litzenburger, T., Iglesias, A., Bauer, J. et al.,** Myelin/oligodendrocyte glycoprotein-deficient (MOG-deficient) mice reveal lack of immune tolerance to MOG in wild-type mice. *J. Clin. Invest.* 2003. **112**: 544–553.

32 **Fazilleau, N., Delarasse, C., Sweenie, C. H., Anderton, S. M., Fillatreau, S., Lemonnier, F. A., Pham-Dinh, D. et al.,** Persistence of autoreactive myelin oligodendrocyte glycoprotein (MOG)-specific T-cell repertoires in MOG-expressing mice. *Eur. J. Immunol.* 2006. **36**: 533–543.

33 **Fazilleau, N., Delarasse, C., Motta, I., Fillatreau, S., Gougeon, M. L., Kourilsky, P., Pham-Dinh, D. et al.,** T-cell repertoire diversity is required for relapses in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis. *J. Immunol.* 2007. **178**: 4865–4875.

34 **Krishnamoorthy, G., Saxena, A., Mars, L. T., Domingues, H. S., Mentele, R., Ben-Nun, A., Lassmann, H. et al.,** Myelin-specific T cells also recognize neuronal autoantigen in a transgenic mouse model of multiple sclerosis. *Nat. Med.* 2009. **15**: 626–632.

35 **Marx, A., Wilisch, A., Schultz, A., Greiner, A., Magi, B., Pallini, V., Schalke, B. et al.,** Expression of neurofilaments and of a titin epitope in thymic epithelial tumors. Implications for the pathogenesis of myasthenia gravis. *Am. J. Pathol.* 1996. **148**: 1839–1850.

36 **Gallegos, A. M. and Bevan, M. J.,** Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 2004. **200**: 1039–1049.

37 **Chen, G. Y., Tang, J., Zheng, P. and Liu, Y.,** CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. *Science* 2009. **323**: 1722–1725.

38 **Liu, Y., Chen, G. Y. and Zheng, P.,** CD24-Siglec G/10 discriminates danger-from pathogen-associated molecular patterns. *Trends Immunol.* 2009. **30**: 557–561.

39 **Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P. and Van Pel, A.,** Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* 1994. **12**: 337–365.

40 **Bai, X. F., Li, O., Zhou, Q., Zhang, H., Joshi, P. S., Zheng, X., Liu, Y. et al.,** 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.

41 **Zhou, Q., Rammohan, K., Lin, S., Robinson, N., Li, O., Liu, X., Bai, X. F. et al.,** CD24 is a genetic modifier for risk and progression of multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 15041–15046.

42 **Wang, L., Lin, S., Rammohan, K. W., Liu, Z., Liu, J. Q., Liu, R. H., Guinther, N. et al.,** A dinucleotide deletion in CD24 confers protection against autoimmune diseases. *PLoS Genet.* 2007. **3**: e49.

43 **Liu, Y. and Zheng, P.,** CD24: a genetic checkpoint in T-cell homeostasis and autoimmune diseases. *Trends Immunol.* 2007. **28**: 315–320.

44 **Sanchez, E., Fernandez-Gutierrez, B., Gonzalez-Gay, M. A., Balsa, A., Garcia, A., Rodriguez, L., Pascual-Salcedo, D. et al.,** Investigating the role of CD24 gene polymorphisms in rheumatoid arthritis. *Ann. Rheum. Dis.* 2008. **67**: 1197–1198.

45 **Bettelli, E., Pagany, M., Weiner, H. L., Linington, C., Sobel, R. A. and Kuchroo, V. K.,** Myelin oligodendrocyte glycoprotein-specific T-cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J. Exp. Med.* 2003. **197**: 1073–1081.

46 **Bai, X. F., Liu, J. Q., Liu, X., Guo, Y., Cox, K., Wen, J., Zheng, P. et al.,** The heat-stable antigen determines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. *J. Clin. Invest.* 2000. **105**: 1227–1232.

47 **Zhou, Q., Guo, Y. and Liu, Y.,** Regulation of the stability of heat-stable antigen mRNA by interplay between two novel cis elements in the 3′ untranslated region. *Mol. Cell. Biol.* 1998. **18**: 815–826.

48 **Brocker, T., Riedinger, M. and Karjalainen, K.,** Driving gene expression specifically in dendritic cells. *Adv. Exp. Med. Biol.* 1997. **417**: 55–57.

49 **Chen, M., Wang, Y. H., Wang, Y., Huang, L., Sandoval, H., Liu, Y. J. and Wang, J.,** Dendritic cell apoptosis in the maintenance of immune tolerance. *Science* 2006. **311**: 1160–1164.

Abbreviations: **Aire:** autoimmune regulator · **DN:** double negative · **DP:** double positive · **MOG:** myelin oligodendrocyte glycoprotein · **mTEC:** medulla thymic epithelial cell · **SP:** single positive

Full correspondence: Dr. Xue-Feng Bai, Department of Pathology and Comprehensive Cancer Center, Ohio State University Medical Center, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, USA
Fax: +1-614-292-7072
e-mail: Xue-Feng.Bai@osumc.edu

Received: 11/8/2011
Revised: 18/11/2011
Accepted: 2/12/2011
Accepted article online: 23/12/2011

