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Distinct roles for Blimp-1 in autoreactive CD4 T cells during priming and effector phase of autoimmune encephalomyelitis

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Abstract

B lymphocyte-induced maturation protein (Blimp-1) is a transcription factor that regulates effector/memory B cells and CD8 T cells. Here we show that Blimp-1 is expressed in both Th1 and Th17 cells *in vitro* and highly expressed in effector/memory myelin-specific CD4 T cells in experimental autoimmune encephalomyelitis (EAE) mice. The immunized Blimp-1 conditional knockout mice have a significantly delayed disease onset but enhanced disease severity during the effector phase compared to their wild-type littermates, suggesting that Blimp-1 is a unique

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⁸Authors' contributions

Y.Y. designed research, analyzed the results and wrote the paper. S.A., M.C.G., P.K.N-G., performed the experiments. W.P. provided support of mouse studies. Y.L. provided general support. A.L.R. and M.K.R. helped with data discussion and manuscript review. All authors read and approved the final manuscript.

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⁶Ethics Statement

The protocols used for these experiments received prior approval by the OSU Institutional Animal Care and Use Committee and were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

⁹Competing interests

The authors declare that they have no competing interests with the contents in this paper.

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transcription factor with distinct roles in the regulation of myelin-specific CD4 T cells during priming and effector phase of EAE.

Keywords

Multiple Sclerosis (MS); experimental autoimmune encephalomyelitis (EAE); B lymphocyte-induced maturation protein (Blimp-1); Th1; Th17

1. Introduction

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS), where myelin-specific CD4 T cells play a critical role in the formation of acute CNS lesions and disease progression in MS and experimental autoimmune encephalomyelitis (EAE), a well-defined murine model of MS (Frohman et al. , 2006). Myelin-specific IFN- γ -producing Th1 and IL-17 producing Th17 subsets of CD4 T effector cells are highly encephalitogenic when transferred into WT recipient mice (Ando et al. , 1989, Langrish et al. , 2005, Pettinelli and McFarlin, 1981, Waldburger et al. , 1996, Yang et al. , 2009). In MS, the effector/memory CD4 T cells that are reactive to myelin-antigens likely contribute to the progressive disease course. Although the precursor frequency of myelin-reactive T cells in peripheral blood is similar between MS patients and healthy controls (HCs) (Jingwu et al. , 1992, Joshi et al. , 1993), myelin-specific T cells in MS patients have a memory phenotype as their activation is independent of costimulatory signals, while those in HCs have a naive phenotype, which requires costimulatory signals for their activation (Burns et al. , 1999, Lovett-Racke et al. , 1998, Markovic-Plese et al. , 2001, Scholz et al. , 1998). Transcription factors control the differentiation and function of CD4 T cells. The critical role of transcription factors T-bet and ROR γ t as lineage-driving transcription factors inducing Th1 and Th17 differentiation respectively has been well-established. However, so far little is known about the molecular mechanisms that regulate the effector function of myelin-specific CD4 T effector/memory cells.

B lymphocyte-induced maturation protein (Blimp-1) is a transcription factor that is critical for the regulation of effector and memory development of B cells and CD8⁺ T cells (Kallies et al. , 2006, Kallies et al. , 2009, Martins et al. , 2006, Minnich et al. , 2016, Rutishauser et al. , 2009, Shapiro-Shelef et al. , 2003, Shapiro-Shelef et al. , 2005). Blimp-1 is required for development of immunoglobulin-secreting B cells and for maintenance of long-lived plasma cells (Martins and Calame, 2008). Furthermore, Blimp-1 regulates the quantity and quality of memory CD8 T cells generated in response to viral infection (Kallies, Xin, 2009, Rutishauser, Martins, 2009); suggesting Blimp-1 might play a role in the regulation of CD4 T effector/memory cells. Furthermore, IL-23, a cytokine that is not only critical for encephalitogenic Th1 and Th17 development as well as EAE development (Cua et al. , 2003, Langrish, Chen, 2005, Langrish et al. , 2004, Lee et al. , 2017, McGeachy et al. , 2009), but also important for CD4 T memory responses (Haines et al. , 2013, Oh et al. , 2011, Siegel et al. , 2011), induces Blimp-1 expression in CD4 T cells (Jain et al. , 2015), suggesting Blimp-1 might play a role in the regulation of myelin-specific CD4 T effector/memory cells and EAE development. However, previous studies on the role of Blimp-1 in the regulation of

Th1 and Th17 subsets of CD4 T effector cells as well as EAE development are controversial (Heinemann et al. , 2014, Jain, Chen, 2015, Lin et al. , 2014). Therefore, in this study, we have characterized the expression of Blimp-1 in myelin-specific CD4 T effector/memory cells differentiated *in vitro* and in EAE mice *in vivo*, and evaluated the functional consequences of Blimp-1 deficiency in T cells in the regulation of effector function of myelin-specific CD4 T cells during priming phase and effector phase of EAE development.

2. Material and methods

2.1 Animals

C57/B6, Blimp1 reporter mice (YFP/Blimp-1), Blimp1^{flox/flox} mice and CD4^{Cre} mice were purchased from the Jackson Laboratory and bred in a specific pathogen-free animal facility at Ohio State University (OSU) Wexner Medical Center. Blimp1^{flox/flox} mice were mated to CD4^{Cre} mice to generate T cell conditional Blimp-1 knockout mice (CKO). TCR transgenic mice (2D2) specific for the MOG 35–55 peptide were also purchased from the Jackson Laboratory and bred in a specific pathogen-free animal facility at Ohio State University (OSU) Wexner Medical Center. Blimp1^{flox/flox} mice, CD4^{Cre} and 2D2 mice were mated to generate CKO/2D2 mice. All animal protocols were approved by the OSU Institutional Animal Care and Use Committee.

2.2 *In vitro* culture of splenocytes from 2D2 mice

Splenocytes were prepared from naive 5–10-wk-old WT/2D2 or CKO/2D2 mice and cultured in 24-well plates at 2×10^6 cells/well with irradiated B6 splenocytes (6×10^6 cells/well). Cells were activated with MOG 35–55 (10 μ g/ml) and different combination of cytokines or neutralizing antibodies for cytokines to differentiate effector T helper cells. Cytokines and antibody concentrations were as follows: 0.5 ng/ml IL-12, 25 ng/ml IL-6, 1 ng/ml TGF β 1, 2 μ g/ml α IFN γ , 1 μ g/ml α IL-12, 2 μ g/ml α IL-4, and 0.35 μ g/ml α TGF β (Yang, Weiner, 2009).

2.3 EAE induction

Immunization: 8–10 week old naïve YFP/Blimp-1 mice, CKO mice or WT littermates of CKO mice were subcutaneously injected (s.c.) over four sites in the flank with 200 μ g MOG 35–55 peptide (C S bio) in an emulsion with CFA (Difco). 200ng pertussis toxin (List) per mouse in PBS was injected i.p. at the time of immunization and 48 h later. The mice were evaluated daily for clinical signs of EAE. Mice were scored on scale of 0 to 6: 0, no clinical disease; 1, limp/flaccid tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5 quadriplegia or premoribund state; and 6, death.

2.4 ELISA

ELISA was performed to detect the expression of IL-17, IFN γ , GM-CSF and IL-10 in supernatant. Purified anti-mouse IL-17, GM-CSF and IL-10 primary antibody (BD bioscience) was diluted in 0.1 M NaHCO₃ (pH 8.2) at 2 μ g/ml while purified anti-mouse IFN γ primary antibody was diluted in 0.1M NaHCO₃ (pH 9.5) at 2 μ g/ml. Immunolon II plates (Dynatech Laboratories) were coated with 50 μ l of primary antibodies per well and incubated overnight at 4°C. The plates were washed twice with PBS/0.05% Tween 20. The

plates were blocked with 200 μ l of 1% BSA in PBS per well for 2 h. The plates were washed twice with PBS/0.05% Tween 20, and 100 μ l of supernatants were added in duplicate. The plates were incubated over-night at 4°C and washed four times with PBS/0.05% Tween 20. Biotinylated rat anti-mouse secondary antibody (BD bioscience) were diluted in PBS/1% BSA, 100 μ l of 1 μ g/ml biotinylated antibody was added to each well, and plates were incubated at room temperature for 1 h. The plates were washed six times with PBS/0.05% Tween 20, and 100 μ l avidin-peroxidase was added at 2.5 μ g/ml and incubated for 30 min. The plates were washed eight times with PBS/0.05% Tween 20, and 100 μ l ABTS substrate containing 0.03% H₂O₂ (for IL-17, GM-CSF) or TMB substrate (for IFN γ and IL-10) was added to each well. The plate was monitored for 10–20 min for color development and read at A 405. A standard curve was generated from cytokine standard, and the cytokine concentration in the samples was calculated.

2.5 Intracellular staining and flow cytometric analysis

Flow cytometric analysis was performed to evaluate the expression of surface markers and cytokines (IL-17 and IFN γ) in CD4 T cells, as previously described (Yang, Weiner, 2009). Briefly, splenocytes were activated with myelin-antigen or α CD3/CD28 for 3–7 days. Cells were then collected, washed, and resuspended in staining buffer (1% BSA in PBS). The cells were incubated with mAbs to the cell-surface markers for 30 min at 4°C. After washing twice with staining buffer, cells were fixed and permeabilized using Cytotfix/Cytoperm solution (BD Bioscience) for 20 min at 4°C. Cells were stained for intracellular cytokines (IL-17 or IFN γ) for 30 min at 4°C. 80,000–100,000 live cell events were acquired on a FACSCantoII (BD) and analyzed using FlowJo software (Tree Star, Inc.). PE- α PD-L1, PerCP- α CD4, Pacific Blue- α CD44, PerCP-Cy5.5- α PSGL1, PE- α PSGL1, PE- α IL-17 and APC- α IFN γ were purchased from BD. APC- α Ly6C was purchased from ebioscience. FITC- α CXCR5, PE- α PD-1 and PE-Cy7- α IL-7R α were purchased from Biolegend Biotechnology, Inc.

2.6 Statistical analysis

GraphPad software (GraphPad Prism Software, Inc., San Diego, CA, USA) was utilized for statistical analysis. A statistically significant difference in EAE clinical scores was considered to be $P < 0.05$, as determined by Mann-Whitney U-test. The Mann-Whitney U-test is non-parametric, and therefore accounts for the fact that EAE scores are ordinal and not interval-scaled. ELISA and quantitated flow data comparisons were performed using two-tailed unpaired Student's t-tests. The quantitated flow data comparisons with three groups (Fig 6C) were performed using one-way ANOVA. Differences with $P < 0.05$ were considered significant.

3. Results

3.1 Blimp-1 is expressed at a minimal level during primary stimulation but highly expressed in Th1 and Th17 cells after secondary stimulation.

To determine whether Blimp-1 is a transcription factor that regulates the early phase or late phase of CD4 T cell activation, we compared Blimp-1 expression in different subsets of CD4 T cells during primary stimulation and secondary stimulation *in vitro*. Different subsets

of CD4 T effector cells have distinct encephalitogenic potential. We and others have previously demonstrated that Th17 cells differentiated with IL-6 plus TGF β 1 or TGF β 3 are not encephalitogenic, while Th17 cells differentiated with IL-6 in the presence of neutralizing antibodies (α IFN γ , α IL-12 and α IL-4) to block Th1 and Th2 signaling are highly encephalitogenic following adoptive transfer (Das et al. , 2009, Ghoreschi et al. , 2010, Lee et al. , 2015, Yang, Weiner, 2009). Thus, we compared Blimp-1 expression in five subsets of CD4 T effector cells that have different encephalitogenic potential during primary and secondary stimulation *in vitro*. Splenocytes from YFP/Blimp-1 mice were activated with α CD3/CD28 in the presence of no exogenous cytokines (ThN), IL-12 (Th1), IL-6/ α IFN γ / α IL-12/ α IL-4 (encephalitogenic Th17 condition), IL-6/TGF β 1 (non-encephalitogenic Th17 condition) or IL-6/TGF β 3 (non-encephalitogenic Th17 condition) for 3 days. As shown in Fig 1A, Blimp-1 expressing cells were very low among all five CD4 T effector subsets at 24h and 48h after primary stimulation. At 72h after primary activation, Blimp-1 expressing cells slightly increased in Th1 and Th17 cells differentiated with IL-6/ α IFN γ / α IL-12/ α IL-4. Then the cells were rested for 5 days followed by reactivation with α CD3. After secondary stimulation with α CD3, Blimp-1 expressing population was notably increased in ThN, Th1 cells and Th17 cells differentiated with IL-6/ α IFN γ / α IL-12/ α IL-4, while remained low in Th17 cells differentiated with IL-6/TGF β 1 or IL-6/TGF β 3 cells (Fig 1B). The lack of Blimp-1 expression in Th17 cells differentiated in the presence of TGF β 1 or TGF β 3 suggests that TGF β 1 and TGF β 3 suppress Blimp-1 expression in CD4 T cells, which is consistent with one previous study showing TGF β 1 suppresses Blimp-1 expression (Salehi et al. , 2012). These data suggest that Blimp-1 is a transcription factor induced by the conditions inducing encephalitogenic T cells and highly expressed in the later phase of CD4 T cell activation, suggesting it may play a role in the regulation of effector function of encephalitogenic CD4 T effector cells during the later phase of T cell activation.

3.2 Blimp-1 is highly expressed in myelin-specific CD4 T cells with an effector/memory phenotype in EAE mice.

To understand the roles of Blimp-1 during EAE development *in vivo*, we determined Blimp-1 expression in myelin-specific CD4 T cells in EAE mice. EAE was induced in YFP/Blimp-1 mice by immunization with MOG35-55 and draining lymph node (dLN) cells were analyzed. Blimp-1 expressing cells were notably higher in CD44⁺CD62L⁺ central memory population and CD44⁺CD62L⁻ effector/memory population, compared to those in CD44⁻CD62L⁺ naïve CD4 T cells (Fig 2A). Furthermore, the majority of Blimp-1 expressing cells in central memory and effector/memory populations express IL-7R α (CD127), a surface marker that is important for memory responses. As recent studies have shown that memory CD4 T cells preferentially reside and rest in the bone marrow (BM) (Oh, Yu, 2011, Tokoyoda et al. , 2009a, Tokoyoda et al. , 2009b), we determined whether Blimp-1 is expressed in BM CD4 T cells in EAE mice. As shown in Fig 2B, BM CD4 T cells have a notably larger CD44⁺CD62L⁺ central memory population compared to those in dLNs or spleen, suggesting bone marrow is an important location for CD4 T memory cells. Moreover, the Blimp-1 expressing population was considerably higher in BM compared to those in dLNs and spleens, suggesting Blimp-1 is highly expressed in CD4 T memory cells residing in bone marrow of EAE mice. Together, these data indicated that Blimp-1 is highly expressed in myelin-specific CD4 T cells with an effector/memory phenotype in EAE mice,

suggesting a possible role of Blimp-1 in the regulation of effector/memory myelin-specific CD4 T cells during effector phase of EAE.

3.3 Blimp-1 is highly expressed in encephalitogenic Th1 and Th17 cells in the CNS of EAE mice.

A previous study has shown that Blimp-1 is a Th17 specific transcription factor during EAE development (Jain, Chen, 2015), but our data show that Blimp-1 is highly expressed in Th1 and Th17 cells differentiated with IL-6/ α IFN γ / α IL-12/ α IL-4 *in vitro* (Fig 1). Therefore, we compared Blimp-1 expression in encephalitogenic Th1 and Th17 cells in the CNS of immunized YFP/Blimp-1 mice. CNS infiltrating cells were isolated from immunized YFP/Blimp-1 mice during the effector phase of EAE (Day 21 post immunization). The infiltrating CD4 T cells in the CNS of EAE mice consist of three cytokine producing populations, IL-17 producing cells, IFN γ producing cells and the cells producing both IL-17 and IFN γ , which have been suggested to be highly encephalitogenic. As shown in Fig 2C, there is a large population of YFP/Blimp-1 expressing cells in all three cytokine producing populations, while CD4 T cells that do not produce cytokines have a very small Blimp-1 expressing population. Similar Blimp-1 expression pattern was observed in dLNs and spleens (data not shown). These data suggest that Blimp-1 is highly expressed in encephalitogenic myelin-specific Th1 and Th17 cells in the CNS of EAE mice and may play a role in the regulation of effector function of encephalitogenic Th1 and Th17 cells during EAE development.

3.4 Blimp-1 deficiency leads to increased IL-17 production in myelin-specific Th17 cells differentiated *in vitro*.

To understand whether Blimp-1 plays a role in the regulation of IL-17 production in myelin-specific Th17 cells, we compared IL-17 production in myelin-specific Th17 cells differentiated *in vitro* from CD4 T cells from T cell conditional Blimp-1 knockout mice (CKO) mice and WT littermates. The CKO mice were crossed to the 2D2 mice which express a MOG35-55-specific T cell receptor to generate MOG 35-55-specific Blimp-1 deficient CD4 T cells (CKO/2D2). Splenocytes from naive CKO/2D2 mice and WT/2D2 littermates were activated with MOG 35-55, MOG 35-55 plus IL-12 (Th1), MOG 35-55 plus IL-6/ α IFN γ / α IL-12/ α IL-4 (encephalitogenic Th17), IL-6/TGF β 1 (non-encephalitogenic Th17) or IL-6/TGF β 3 (non-encephalitogenic Th17) for 3 days. IL-17 and IFN γ production was determined by flow cytometric analysis. Our data show that there are notably more IL-17 producing cells in Blimp-1 deficient myelin-specific Th17 cells differentiated with IL-6/ α IFN γ / α IL-12/ α IL-4, IL-6/TGF β 1 or IL-6/TGF β 3 (Fig 3B), compared to WT myelin-specific Th17 cells differentiated under same conditions (Fig 3A), suggesting Blimp-1 inhibits IL-17 production in myelin-specific Th17 cells differentiated *in vitro*. However, our data did not show any significant effects of Blimp-deficiency on IFN γ production in Th1 cells.

3.5 Regulation of T effector function of myelin-specific CD4 T cells and EAE development by Blimp-1

The role of Blimp-1 in the regulation of EAE development is controversial (Heinemann, Heink, 2014, Jain, Chen, 2015, Lin, Yeh, 2014). Therefore, we compared EAE development in CKO mice and WT littermates immunized with MOG 35-55. As shown in Fig 4A and

Table 1, the EAE disease course was significantly altered in immunized CKO mice compared to WT littermates. EAE onset in immunized CKO mice is significantly delayed compared to immunized WT littermates (Fig 4A and Table 1). However, disease severity during effector phase of EAE (After day 20 post immunization) is significantly higher in CKO mice compared to WT littermates (Fig 4A and table 1). To understand the possible mechanisms regulating the altered EAE disease course in CKO mice, we compared the expression of activation marker CD44 and production of proinflammatory cytokines, IL-17, IFN γ and GM-CSF in myelin-specific CD4 T cells from immunized CKO mice and their WT littermates, during priming phase of EAE (day 7-10 post immunization) and effector phase of EAE (day 25-30 post immunization). During the priming phase of EAE, although the activated CD44⁺CD4⁺ T cells were at similar levels in CKO mice and WT littermates (Fig 4B), the production of IL-17, IFN γ and GM-CSF was significantly lower in dLN cells from CKO mice compared to those from WT littermates (Fig 4C), which correlates with an delayed EAE onset in immunized CKO mice. However, during the effector phase of EAE, the activated CD44⁺CD4⁺ T cells in CKO mice were dramatically increased to a level that was significantly higher than those in WT littermates (Fig 4D). Correspondingly, the production of IL-17, IFN γ and GM-CSF in dLNs and spleens from CKO mice was significantly higher compared to those from WT littermates (Fig 4E and 4F), suggesting enhanced effector function of myelin-specific CD4 T cells in CKO mice during effector phase of EAE compared to WT littermates. As Blimp-1 has been previously shown to be essential for IL-10 expression by Th1 cells during *T. gondii* infection (Neumann et al. , 2014), we also determined IL-10 in supernatants of myelin-specific CD4 T cells from immunized CKO mice and their WT littermates, during priming phase of EAE and effector phase of EAE by ELISA. Our ELISA data show that IL-10 was undetectable in both WT and CKO groups (data not shown); suggesting IL-10 may not be a major contributor to the phenotype we observed in our experimental settings. Together, these data suggest that Blimp-1 deficiency in T cells leads to decreased production of proinflammatory cytokines in myelin-specific CD4 T cells during priming phase of EAE, which may contribute to delayed EAE onset. However, during effector phase of EAE, Blimp-1 deficiency leads to increased activation of myelin-specific CD4 T cells and enhanced production of proinflammatory cytokines, which may contribute to increased EAE severity during effector phase and suggest that Blimp-1 is a transcription factor with distinct roles during priming and effector phase of EAE.

3.6 Regulation of effector/memory phenotype of myelin-specific CD4 T cells by Blimp-1

Naïve CD4 T cells differentiate into effector subsets with different potential of forming memory cells. PSGL1^{hi}Ly6C^{lo} CD4 T effector cells have been shown to have increased survival during the contraction phase and proliferate more upon secondary infection compared to PSGL1^{hi}Ly6C^{hi} CD4 T effector cells (Marshall et al. , 2011). Moreover, the transcriptional profile of PSGL1^{hi}Ly6C^{lo} CD4 T effector cells resembles memory CD4 T cells, suggesting memory precursor CD4 T cells may be present in PSGL1^{hi}Ly6C^{lo} population (Marshall, Chande, 2011). To understand whether Blimp-1 deficiency affects PSGL1^{hi}Ly6C^{lo} CD4 T population, we analyzed the expression of PSGL1 and Ly6C in the spleens of immunized CKO mice and their WT littermates during effector phase of EAE. PSGL1^{hi}Ly6C^{lo} CD4 T effector population is significantly higher in CKO mice compared to

their WT littermates (Fig 4G), suggesting Blimp-1 deficiency in T cells leads to the enhanced development of myelin-specific CD4 T effector cells that have a greater capacity to persist and respond to secondary antigen stimulation, which may contribute to enhanced cytokine production and more severe EAE during effector phase of EAE.

3.7 Regulation of immune checkpoint PD-1/PD-L1 axis by Blimp-1

The PD-1 is an important immune checkpoint role that negatively regulates T effector function and EAE development (Carter et al. , 2007, Nuro-Gyina et al. , 2016). Therefore we compared the expression of PD-1 and its ligand, PD-L1, in myelin-specific CD4 T effector cells from immunized CKO mice and WT littermates during priming phase and effector phase of EAE. There were significantly more PD-1 expressing CD44⁺CD4⁺ T effector cells in immunized CKO mice compared to those in WT littermates during both priming and effector phases of EAE (Fig 4H); suggesting Blimp-1 is a transcription factor that inhibits PD-1 expression in myelin-specific CD4 T cells, regardless the different phases of EAE. However, PD-L1 expressing myelin-specific CD44⁺CD4⁺ T effector cells are at similar levels between CKO mice and WT littermates (Fig 4H). Similar to PD-1 expression, there is no difference of PD-L1 expression in Blimp-1 deficient myelin-specific CD4 T cells during priming phase and effector phase of EAE.

3.8 Blimp-1 is dispensable for the IL-23 induced IL-17 production in myelin-specific CD4 T cells in EAE mice.

IL-23 induces Blimp-1 expression in CD4 T cells (Jain, Chen, 2015), but it is not clear whether Blimp-1 is required for the IL-23 induced IL-17 production in myelin-specific CD4 T cells during EAE development. To determine whether Blimp-1 is required for IL-23 signaling in myelin-specific CD4 T cells, we activate dLN cells isolated from immunized CKO mice or WT littermates during effector phase (day 20-25 post immunization) with MOG 35-55 in the presence or absence of IL-23 for 3 days. Surprisingly, as shown in Fig 5, exogenous IL-23 significantly increases IL-17 production in myelin-specific CD4 T cells from both Blimp-1 deficient CKO mice and WT littermates, suggesting that IL-23 enhances IL-17 production in myelin-specific CD4 T cells independent of Blimp-1 and therefore the Blimp-1 is not required for the IL-23 induced Th17 development.

4. Discussion

The role of Blimp-1 in the regulation of CD4 T effector function and EAE development is controversial (Cimmino et al. , 2008, Heinemann, Heink, 2014, Jain, Chen, 2015, Lin, Yeh, 2014, Martins et al. , 2008, Salehi, Bankoti, 2012). Although Blimp-1 was previously characterized as an essential transcription factor to activate the Th17 program, along with the Th17 signature transcription factor ROR γ t (Jain, Chen, 2015), our data show that Blimp-1 is not a Th17 lineage-specific transcription factor that drives Th17 differentiation. First, both Th1 and Th17 differentiating cytokines, IL-12 and IL-6, induce Blimp-1 expression in myelin-specific CD4 T cells *in vitro* (Fig 1). Second, Blimp-1 is highly expressed in encephalitogenic Th1 and Th17 cells in CNS of EAE mice (Fig 2). Furthermore, our EAE studies show that the production of all three inflammatory cytokines, IFN γ , IL-17 and GM-CSF was altered in immunized CKO mice compared to WT mice (Fig

4). Although IL-23 expands Teff population with enhanced Blimp-1 expression, IL-23 increases IL-17 production in myelin-specific CD4 T cells from both immunized CKO mice and WT littermates (Fig 5), suggesting Blimp-1 is dispensable for IL-23 mediated IL-17 production. Together, our data suggest that Blimp-1 is not a Th17 lineage-specific transcription factor and may play important roles in the regulation of multiple subsets of myelin-specific CD4 T cells during EAE development.

One major controversy is on the role of Blimp-1 in regulating EAE development. Previous studies have shown completely opposite EAE clinical courses in immunized CKO mice, making the role of Blimp-1 in the regulation of EAE development unclear (Heinemann, Heink, 2014, Jain, Chen, 2015, Lin, Yeh, 2014). While Lin et al showed that Blimp-1 deficiency exacerbated EAE (Lin, Yeh, 2014); Jain et al showed that Blimp-1 CKO mice had reduced EAE development (Jain, Chen, 2015). However, in Jain's study, the observation of EAE disease course ended on day 16 post immunization, analyzing only the priming phase of EAE. Our observation of EAE development during priming phase of EAE confirmed delayed EAE onset, which is similar to what has been observed by Jain et al (Jain, Chen, 2015). However, during effector phase of EAE (after day 20 post immunization), the disease severity in CKO mice continued to increase and became significantly more severe than WT littermates from day 20 to 40. Together, our clinical data showed a significantly delayed onset, but significantly enhanced disease severity during effector phase of EAE in immunized CKO mice compared to WT littermates (Fig 4A and Table 1). The delayed onset in CKO mice is associated with decreased production of proinflammatory cytokines by myelin-specific CD4 T cells while the enhanced disease severity during the effector phase is associated with increased number of effector CD4 T cells and production of proinflammatory cytokines in CKO mice.

One of the differences among these EAE studies of Blimp-1 deficient mice is the use of different Cre systems. In our study and the study from Lin et al (Lin, Yeh, 2014), EAE was induced by immunization of CKO mice where Blimp-1 deletion was induced by conventional *Cd4^{Cre}* (*Cd4^{Cre}* x *Blimp1^{flox/flox}*), which may involve reduced thymocyte numbers and defective Th1, Th2, and Treg cell populations. The study from Jain et al (Jain, Chen, 2015) used a distal *Lck-Cre* system that promotes deletion of genes during the late single positive thymic development stage, which does not alter the number of thymocytes. Furthermore, in another study by Heinemann et al (Heinemann, Heink, 2014), immunized lethally irradiated mice that were reconstituted with bone marrow from CKO mice (*Cd4^{Cre}* x *Blimp1^{flox/flox}*) also showed more severe EAE compared to those reconstituted with bone marrow from control WT mice. Therefore, whether the altered EAE clinical course observed in CKO mice (*Cd4^{Cre}* x *Blimp1^{flox/flox}*) is complicated by the defects of other immune cells in CKO mice requires further investigation.

The molecular mechanisms that govern the differentiation, function, and maintenance of effector/memory CD4 T cells are not well-elucidated. In CD8 T cells, there are two distinct populations of antigen-experienced CD8 T cells, short-lived effector cells (SLECs) and memory-precursor cells (MPCs), generated during infection. The deficiency of transcription factor T-bet, Blimp-1 or Id2 leads to severe impairments in SLEC differentiation, suggesting these transcription factors promote terminal differentiation of CD8 T effector cells.

Combined deficiency in Blimp-1 and T-bet further impair effector function of CD8 Teff cells, resulting in an inability to control systemic viral infection and severe immune pathology, suggesting these T-bet and Blimp-1 promote the terminal differentiation in a collaborative and partially redundant manner (Xin et al. , 2016). Our data suggest that Blimp-1 is involved in the regulation of effector/memory myelin-specific CD4 T cells during EAE development. However, it is not clear if T-bet and Blimp-1 promote effector T cell differentiation in a collaborative and partially redundant manner in myelin-specific CD4 T cells or other autoreactive CD4 T cells during autoimmunity. Further studies of EAE development in T-bet and Blimp-1 double knockout mice may reveal the effects of these two transcription factors in the regulation of effector/memory myelin-specific CD4 T cells.

Blimp-1 and Bcl-6 regulatory axis are critical regulators of effector and memory differentiation (Crotty et al. , 2010, Martins and Calame, 2008). Blimp-1 is critical for the terminal differentiation of B cells and the formation of plasma cells (Shapiro-Shelef, Lin, 2003, Shapiro-Shelef, Lin, 2005); suggesting Blimp-1 may have a similar role in regulating effector/memory phenotype of myelin-specific CD4 T cells during EAE development. So far, there are still no good markers for precursor memory CD4 T cells, but memory precursor CD4 T cells were recently proposed to be in a PSGL1^{hi}Ly6C^{lo} population. There are two different CD4 T effector cell populations identified during infection *in vivo* (Marshall, Chande, 2011). The PSGL1^{hi}Ly6C^{lo} CD4 T cells show greater longevity and proliferation to secondary infection but having lower effector function with decreased T-bet expression, which is required for maximal CD4 T cell expansion. Contrarily, Ly6C^{hi} effector CD4⁺ T cells represent more terminally differentiated Th1 cells with higher expression of T-bet, CD122, IFN γ , and GzmB. Our data show the PSGL1^{hi}Ly6C^{lo} CD4 T cells are significantly higher in immunized CKO mice than WT littermates, suggesting that Blimp-1 deficiency leads to increased numbers of CD4 T cells with greater longevity and increased survival but lower effector function, which may contribute to the decreased cytokine production and delayed EAE onset during induction phase of EAE. However, the PSGL1^{hi}Ly6C^{lo} CD4 T cells survive better during contraction phase and respond robustly to secondary encounter of antigen, which may contribute to the increased number of CD44⁺CD4 T cells and enhanced cytokine production during effector phase of EAE. However, further investigation is needed to determine the detailed mechanisms by which Blimp-1 and/or other transcription factors regulate the terminal differentiation and/or memory formation of myelin-specific CD4 T cells during EAE development.

Blimp-1 is a transcription factor with multifunctional roles in several different subsets of CD4 T cells, including CD4 Teff/Tmem cells, Tfh cells and Tregs. In CKO mice, Blimp-1 is deficient in all T cells. Therefore, the EAE phenotype observed in CKO mice might reflect combined effects of direct regulation of Teff differentiation and memory formation of CD4 T effector cells caused by Blimp-1 deficiency in Teff cells, as well as indirect impact on Teff cells by Tfh cells and/or Tregs caused by Blimp-1 deficiency in those cells. We didn't detect significant differences in the number of Tregs in CKO mice or WT mice (data not shown), but it is not clear whether Blimp-1 deficiency causes any defects in IL-10 production, the stability and/or suppression function of Tregs. Further investigations are needed to elucidate the specific effects of Blimp-1 deficiency in other subsets of T cells in EAE and potentially in other autoimmune diseases.

B cells play an important role in MS pathogenesis although the detailed mechanisms are not well-elucidated (Bar-Or et al. , 2008, Hauser et al. , 2008, Hawker et al. , 2009, Kappos et al. , 2011, Magliozzi et al. , 2007). T follicular helper (Tfh) cells are a subset of CD4 T helper cells that are critical for the generation of B cell mediated immune responses, including class switch recombination, germinal center differentiation, and affinity maturation. The development of Tfh cells is mediated by transcriptional factor Bcl-6 (Nurieva et al. , 2009). Blimp-1 and Bcl-6 are reciprocal and antagonistic regulators of Tfh differentiation. Blimp-1 is an antagonist of Bcl-6 and inhibits Tfh differentiation, thereby preventing B cell germinal center and antibody responses (Johnston et al. , 2009). CNS infiltrating Tfh cells are a key cell type that contributes to an inflammatory B cell response in EAE induced by adoptive transfer of myelin-specific Th17 cells (Quinn et al. , 2018), suggesting Blimp-1 may regulate Tfh development and inflammatory B cells response during EAE development. However, the effects of Blimp-1 on Tfh cell development and inflammatory B cell response during EAE are not fully appreciated in C57BL/6 mice immunized with MOG 35-55, which is mainly B cell independent without strong humoral responses. EAE induced by immunization with recombinant human MOG (rhMOG) protein is both T cell and B cell dependent (Lyons et al. , 2002, Marta et al. , 2005, Oliver et al. , 2003). Further studies of Tfh cells and inflammatory B cell response in the CNS of CKO mice or their WT littermates immunized with rhMOG protein will elucidate the effects of Blimp-1 on Tfh cell development as well as inflammatory B cell response during EAE development.

The expression of inhibitory receptor PD-1 on T cells represents another layer of regulation of T cells effector function, which has been well-established in T cell exhaustion during chronic viral infection and observed in tumor microenvironment. Our data show that PD-1 expressing effector CD4 T cells are significantly higher in immunized CKO mice than WT littermates in both priming phase and effector phase, suggesting Blimp-1 suppresses PD-1 expression in myelin-specific CD4 T cells. However, PD-L1 expression is compatible between CKO mice and littermates, which decreased the potential suppressive effects of PD-1 signaling on T effector function in CKO mice. Inhibitory receptors usually act synergistically to prevent autoimmunity (Okazaki et al. , 2011). T cell exhaustion observed in chronic infection and tumor microenvironment involves the overexpression of multiple inhibitory receptors, including PD-1, Lag-3, CTLA-4 et al. We characterized PD-1 expression in CKO mice as PD-1 is considered the major inhibitory receptor regulating T cell exhaustion and has been targeted therapeutically. However, further investigation is needed to determine the expression levels of other inhibitory receptors as well as the detailed mechanisms regulating multiple inhibitor receptors in myelin-specific CD4 T cells. PD-L1 is the major ligand that interacts with PD-1 to regulate the severity of EAE (Carter, Leach, 2007). However, the molecular mechanisms that regulate the expression of PD-1 and PD-L1 are still largely unknown. Previous studies show that both Blimp-1 and T-bet repress PD-1 expression in CD8 T cells (Kao et al. , 2011, Lu et al., 2014). T-bet directly represses transcription of the gene encoding PD-1 and results in lower expression of other inhibitory receptors (Kao, Oestreich, 2011). Blimp-1 represses PD-1 through a feed-forward repressive circuit by regulating PD-1 directly and by repressing NFATc1 expression, an activator of PD-1 expression (Lu, Youngblood, 2014). Different from cytokine production, PD-1 is

elevated in CKO mice regardless of disease phases, suggesting the downstream signaling pathway through which Blimp-1 regulates PD-1 expression might be different from the molecular mechanisms by which Blimp-1 regulates cytokine production in myelin-specific CD4 T cells.

5. Conclusions

In this study, we have demonstrated that Blimp-1 is a transcription factor expressed in both Th1 and Th17 subsets of CD4 T effector cells *in vitro* and in EAE mice *in vivo*, and has multiple function in the regulation of myelin-specific CD4 T cells during EAE development. More importantly, Blimp-1 plays distinct roles in the regulation of disease development during priming and effector phases of EAE. Further studies elucidating the detailed mechanisms will greatly broaden our understanding of the transcriptional regulation of CD4 T cell development in normal and pathogenic conditions.

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Highlights:

- Blimp-1 is highly expressed in both Th1 and Th17 cells *in vitro* and in EAE mice *in vivo*.
- Blimp-1 is highly expressed in effector/memory autoreactive CD4 T cells in EAE mice.
- Blimp-1 knockout mice have delayed EAE onset but enhanced EAE severity than WT mice.
- More PSGL1^{hi}Ly6C^{lo} CD4 Teff cells in Blimp-1 knockout mice than WT littermates.
- Blimp-1 is dispensable for IL-23 induced IL-17 production in autoreactive CD4 T cells.

Figure 1. Blimp-1 expression in CD4 T effector cells differentiated *in vitro*.

Splenocytes from naive YFP/Blimp-1 mice were stimulated with αCD3/CD28 and different cytokines (as indicated) for 3 days. **(A)** Blimp-1 expression in CD4 T cells was represented by YFP and was determined by flow cytometry at 24h, 48h and 72h after activation. The cells were then rested for 5 days and reactivated with αCD3 for 24h and 48h **(B)**. Cells were gated on CD4⁺ cells. Data represent two independent experiments.

Figure 2. Blimp-1 is highly expressed in myelin-specific CD4 T cells with an effector/memory phenotype in EAE mice.

Naive YFP/Blimp-1 mice were immunized with MOG 35-55. **(A)** dLNs were isolated on day 8 after immunization and activated with MOG 35-55 for 7 days. **(B)** dLNs, splenocytes and bone marrow cells were isolated on day 21 after immunization and activated with MOG 35-55 for 7 days. **(C)** CNS infiltrating cells were isolated on day 21 after immunization and activated with MOG 35-55 overnight. The expression of CD62L, CD44, CD127 (IL-7R α) and YFP/Blimp-1 was determined by flow cytometric analysis. IL-17 and IFN γ was determined by intracellular staining. Cells were gated on CD4⁺ cells. Data represent two independent experiments.

Figure 3. Regulation of cytokine production in myelin-specific CD4 T cells by Blimp-1. Splenocytes from naive WT/2D2 (A) or CKO/2D2 (B) mice were activated with MOG 35-55 in the presence of different cytokines (as indicated) for 3 days. IL-17 and IFN γ production was determined by intracellular staining. Cells were gated on CD4⁺CD44⁺ cells. Data represent two independent experiments.

Figure 4. Regulation of T effector function of myelin-specific CD4 T cells and EAE development by Blimp-1.

(A) Naïve WT and CKO mice were immunized with MOG 35-55 and EAE development was monitored. Disease incidence (sick mice/total mice) is indicated in parentheses. Data are representative of three independent experiments. (B-C) dLN cells from immunized WT or CKO mice between day 6-10 post immunization (priming phase of EAE) were activated with MOG 35-55 for 3 days. (B) CD44 expression was determined by flow cytometric analysis. Cells were gated on CD4⁺ cells. The group means were calculated and compared. (C) IL-17, IFN γ and GM-CSF in supernatant were determined by ELISA. (D-H) dLN cells and splenocytes from immunized WT or CKO mice between 20-25 days (effector phase of EAE) were activated with MOG 35-55 for 3 days. (D) CD44 expression was determined by

flow cytometric analysis. Cells were gated on CD4⁺ cells. The group means were calculated and compared. **(E-F)** IL-17, IFN γ and GM-CSF in supernatant were determined by ELISA. **(G-H)** Ly6C and PSGL1 in CD4⁺ cells from spleens of immunized WT or CKO mice between day 20-25 after immunization was determined by flow cytometry (G) and summarized in (H). Cells were gated on CD4⁺ cells. **(I-J)** PD-1 or PD-L1 in CD44⁺CD4⁺ T cells from immunized WT or CKO mice (between day 8-21 post immunization) was determined by flow cytometry (I) and summarized in (J). Cells were gated on CD44⁺CD4⁺ T cells. The group means were calculated and compared. All flow data are representative of at least three independent experiments. Error bars denote s.e.m. *P<0.05.

Figure 5. Blimp-1 is dispensable for the IL-23 induced IL-17 production in myelin-specific CD4 T cells.

dLN cells from immunized WT or CKO mice between day 20-25 post immunization were activated with MOG 35-55 or MOG 35-55 plus IL-23 for 3 days. **(A)** IL-17 and IFN γ in CD44⁺CD4⁺ T cells from immunized CKO mice was determined by intracellular staining. **(B)** IL-17 and IFN γ in supernatant was determined by ELISA. The group means were calculated and compared. One to four mice from each group were analyzed in each independent experiment. Data are representative of three independent experiments.

Table 1.

EAE development in CKO mice and WT littermates immunized with MOG 35-55

Groups	Number of mice	Mean day of onset of EAE	Mean peak clinical score
WT	40	12.85 ± 0.7^a	3.375 ± 0.2^b
CKO	30	17.57 ± 1^a	4.233 ± 0.2^b

^aMean day of onset of EAE: WT/B6 vs CKO/B6 (p=0.0002).^bMean peak clinical score: WT/B6 vs CKO/B6 (p=0.0012).