Journal of Autoimmunity 34 (2010) 349–355

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Evidence that *Yaa*-induced loss of marginal zone B cells is a result of dendritic cell-mediated enhanced activation

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ARTICLE INFO

Article history:
Received 9 December 2009
Received in revised form
5 January 2010
Accepted 7 January 2010

Keywords:
Systemic lupus erythematosus
Marginal zone B cell
Dendritic cell
The *Yaa* mutation

ABSTRACT

The development of systemic lupus is accelerated by the *Yaa* (*Y-linked autoimmune acceleration*) mutation, which is the consequence of a translocation of the telomeric end containing the *Tlr7* gene from the X chromosome onto the Y chromosome. However, the loss of marginal zone (MZ) B cells, one of the *Yaa*-linked cellular abnormalities, has previously been shown to be unrelated to the *Tlr7* gene duplication, and the present study therefore aimed to investigate the mechanism responsible for MZ B-cell loss. Analyses of *Yaa* and non-*Yaa* C57BL/6 male mice expressing an MD4 anti-HEL IgM transgene or those deficient in fms-like tyrosine kinase 3 ligand (FL) revealed that the proportion of MZ B cells in these *Yaa* mice was comparable to that of the respective non-*Yaa* control mice. Notably, the activation of MZ B cells was compromised in both of these transgenic model systems, due to the absence of cognate antigens or the impaired development of dendritic cells, respectively. These results contrasted with the loss of MZ B cells in non-*Yaa* mice treated with FL and the lack of accumulation of MZ B cells in *Yaa* mice treated with a B-cell survival factor, BAFF. Taken together, our results suggest that the persistent and enhanced activation of *Yaa*-bearing hyperactive MZ B cells by dendritic cells is responsible for the loss of this B-cell subset in *Yaa* mice.

1. Introduction

The BXSB strain of mice spontaneously develops an autoimmune syndrome with features of systemic lupus erythematosus (SLE) that affects males much earlier than females [1]. The accelerated development of SLE in BXSB male mice results from the genetic abnormality, *Yaa* (*Y-linked autoimmune acceleration*), present on the BXSB Y chromosome [2]. Studies of *Yaa* and non-*Yaa* double bone marrow chimeric mice showed that anti-DNA autoantibodies are selectively produced by B cells bearing the *Yaa* mutation, and that T cells from both *Yaa* and non-*Yaa* origin efficiently promote anti-DNA autoantibody responses [3,4]. Based on these results, it has been speculated that the *Yaa* defect may decrease the threshold for BCR-mediated signaling, thereby triggering and excessively stimulating autoreactive B cells. More recently, the *Yaa* mutation was identified to be a consequence of a translocation of the telomeric end containing the gene encoding TLR7 from the X chromosome onto the Y chromosome [5,6]. Since the synergistic engagement of TLR7 and BCR in response to nuclear antigens could induce the activation of autoreactive B cells in SLE [7,8], the *Tlr7* gene duplication has been proposed to be the etiologic basis for the *Yaa*-mediated enhancement of disease [5,6,9,10].

Newly generated B cells in the bone marrow emigrate to the spleen, where they further differentiate into follicular or marginal zone (MZ) B cells. Follicular B cells are $IgM^{int}IgD^{hi}CD21^{int}CD23^{hi}$, and MZ B cells located at the junction of white and red pulps are $IgM^{hi}IgD^{lo}CD21^{hi}CD23^{neg/lo}$ [11–13]. While follicular B cells respond to T-dependent antigens, MZ B cells play a critical role in host defense against T-independent blood-borne pathogens [14,15]. Significantly, mice bearing the *Yaa* mutation display a marked reduction of the MZ B-cell subset, which results from a defect intrinsic to *Yaa*-bearing B cells, independently of the development of SLE [16]. Although the *Tlr7* gene duplication was proposed as a cause of this cellular defect [9], we observed that the extent of the MZ B-cell loss in C57BL/6 mice bearing the *Yaa* mutation (B6.*Yaa*) was essentially unchanged by the introduction of the *Tlr7* null mutation on the X chromosome which

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doi:10.1016/j.jaut.2010.01.001

left only a single "*Yaa*-copy" of the *Tlr7* gene expressed [17]. These results indicated that the loss of MZ B cells occurring in *Yaa*-bearing mice cannot be explained by the *Tlr7* gene duplication alone and suggested a critical contribution of other duplicated genes in the translocated X chromosomal end. Since the loss of MZ B cells in *Yaa* mice can be related to the hyperactive phenotype of *Yaa*-bearing B cells, which contributed to the accelerated development of murine SLE, it remains important to define the mechanism responsible for this *Yaa*-linked MZ B-cell loss.

Studies on several different genetically manipulated mice revealed that the reduction of MZ B cells could be related to hypersensitive BCR signaling [18–21]. It has been hypothesized that the strength of the signal elicited via the BCR may regulate the lineage commitment of mature B cells into follicular vs. MZ B cells, with hypersensitive BCR signaling favoring an accelerated maturation towards follicular B cells [22]. Thus, a hyperactive phenotype of *Yaa*-bearing B cells could contribute to the enhanced maturation towards follicular B cells and the decrease in MZ B cells [16]. An alternative possibility could be that the reduction of *Yaa*-bearing MZ B cells is due to their constant and enhanced activation followed by subsequent emigration from the MZ into the red pulp or B-cell follicles [23–26]. In the present study, we provide evidence that enhanced activation by dendritic cells (DC) is likely to be responsible for the loss of MZ B cells in B6.*Yaa* mice.

2. Materials and methods

2.1. Mice

B6 mice expressing an MD4 anti-hen egg lysozyme (HEL) IgM transgene [27] were created by backcross procedures at the 8th generation. Mice deficient in C3 [28], kindly provided by Dr M. Carroll, Harvard Medical School, Boston, were backcrossed for 7 generations with B6 mice. B6 mice deficient in fms-like tyrosine kinase 3 ligand (FL), generated by gene targeting in B6-derived ES cells [29], were purchased from Taconic Farms, Inc., Germantown, NY. The *Yaa* mutation was introduced into these different transgenic mice by crossing with B6.*Yaa* mice [30]. Animal studies described in the present study have been approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine, University of Geneva.

2.2. Flow cytometric analysis

Flow cytometry was performed using three- or four-color staining of spleen cells, and analyzed with a FACSCalibur (Becton Dickinson, Mountain View, CA). The following antibodies were used: anti-CD21 (7G6), anti-CD23 (B3B4), anti-B220 (RA3-6B2), anti-CD11c (N418), anti-MHC class II I-A (Y3P) and anti-BAFF-receptor (BAFF-R; 9B9) [31]. For the analysis of splenic DC, spleen cells were prepared according to the method described by Kamath et al. [32]. Briefly, spleen fragments were digested with a mixture of 1 mg/ml Liberase (Roche Diagnostics, Mannheim, Germany) and 1 mg/ml DNAse I (Sigma–Aldrich, St Louis, MO) for 20 min at room temperature and treated with 10 mM EDTA to disrupt T cell-DC complexes. Cells were then filtered with a cell strainer (70 μm), resuspended and stained in PBS containing 1% BSA, 2 mM EDTA and a saturating concentration of 2.4G2 anti-FcγRII/III mAb.

2.3. Immunohistochemistry

Spleens were embedded in Tissue-Tek O.C.T. compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Four μm frozen sections were stained with PE-labeled anti-IgM (1B4B1; Southern Biotechnology, Birmingham, AL) and FITC-labeled MOMA-1 (Serotec, Kidlington, England) mAb in the presence of 2.4G2 anti-FcγRII/III mAb.

2.4. Treatment with recombinant FL or BAFF in vivo and in vitro

Mice were subcutaneously injected daily for 9 days with 1 μg of mouse serum albumin (Sigma-Aldrich) plus 10 μg of human recombinant FL (a kind gift of Amgen Inc., Thousand Oaks, CA) or with mouse serum albumin alone. One day after the last injection of FL, the proportion of MZ B cells in spleens was analyzed by flow cytometric and immunohistochemical analysis. The in vivo effect of BAFF was determined in mice injected i.v. on days 0 and 14 with 100 μg of human recombinant BAFF, prepared as described previously [31]. Seven days after the second injection of BAFF, the size of MZ B-cell compartments was assessed. To determine the survival of splenic B cells in vitro, B cells were purified from spleen cells using mouse B-cell enrichment kit (StemCell Technologies, Grenoble, France). The purity of B cells, as documented by flow cytometric analysis, was superior to 95%. Purified B cells were cultured in IMDM containing 5×10^{-5} 2-ME, 0.03% primatone (Quest International, Naarden, The Netherlands) and 5% FCS at 5×10^5 cells/ml in the presence or absence of 500 ng/ml of recombinant BAFF. At various time points viable cells were counted by the trypan blue exclusion test. Serum levels of BAFF were quantified by using BAFF, Soluble (mouse) ELISA kit (Apotech Corporation, Epalinges, Switzerland).

2.5. Quantitative RT-PCR

RNA from spleen cells was purified with TRIzol reagent (Invitrogen AG, Basel, Switzerland). The abundance of *Flt3l* mRNA was quantified by real-time RT-PCR with cDNA prepared from RNA. *Flt3l* cDNA was amplified using a forward primer (5′-TTCAGCCA CAGTCCCATCTC-3′) and reverse primer (5′-CCTGCCACAGTCTTC AGTTG-3′). PCR was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Philadelphia, PA) and iQ SYBR green Supermix (Bio-Rad). Results were quantified relative to a standard curve generated with serial dilutions of a reference cDNA preparation from spleen cells and normalized using TATA-binding protein mRNA.

2.6. Statistical analysis

Statistical analyses were performed using the Mann–Whitney *U*-test. Probability values >5% were considered insignificant.

3. Results

3.1. No reduction of MZ B cells in MD4 anti-HEL IgM transgenic B6.Yaa male mice

We have previously shown that the proportion of MZ B cells in lupus-prone BXSB *Yaa* males expressing an Sp6 anti-TNP/DNA transgenic IgM was substantially increased, as compared with non-transgenic littermates, reaching a level nearly identical to that of non-*Yaa* BXSB transgenic males [16]. The increase in the size of the MZ B-cell compartment in the Sp6 transgenic BXSB mice could be related to the findings that autoreactive B cells tend to be accumulated in the MZ [33,34]. However, larger MZ B-cell compartments have also been reported in non-autoreactive IgM transgenic mice such as MD4 anti-HEL transgenic mice [27]. Therefore, we determined whether B cells expressing an MD4 anti-HEL IgM transgene similarly accumulated in the MZ of B6.*Yaa* male mice. As was the case of Sp6 transgenic mice, the proportion of MZ B cells in MD4 transgenic B6.*Yaa* males (means ± SD: 15.6 ± 2.4%) was as