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Intrinsic unresponsiveness of Mertk$^{-/-}$ B cells to chronic graft-versus-host disease is associated with unmodulated CD1d expression

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ABSTRACT

Activation and migration of marginal zone B (MZB) cells into follicular (FO) regions of the spleen has been proposed as one of the mechanisms that regulate the development of autoreactive B cells. The mer receptor tyrosine kinase (Mertk) mediates apoptotic cell clearance and regulates activation and cytokine secretion. In the well-studied class II chronic GVH model of bm12 cells into B6 hosts, we observed that Mertk deficient B6 mice did not generate autoantibodies in response to this allogeneic stimulus. We posited that Mertk is important in MHC-II-mediated B cell signaling. In the present study, we show that B cells from Mertk$^{-/-}$ mice but not WT B6 mice exhibited decreased calcium mobilization and tyrosine phosphorylation when stimulated by MHC-II cross-linking. The finding that Mertk was important for class II signaling in B cells was further supported by the preponderance of a-allotype autoantibodies in cGVH in RAG-KO mice reconstituted with a mixture of bone marrow from Mertk$^{-/-}$ mice (b-allotype) and C20 mice (a-allotype). MZB cells from Mertk$^{-/-}$ mice were unable to down regulate surface CD1d expression and subsequent inclusion in the MZ, associated with significantly lower germinal center responses compared to MZB cells from WT. Moreover, Mertk$^{-/-}$ mice treated with an anti-CD1d down regulating antibody responded significantly to bm12 cells, while no response was observed in Mertk$^{-/-}$ mice treated with control antibodies. Taken together, these findings extend the role of Mertk to include CD1d down regulation on MZB cells, a potential mechanism limiting B cell activation in cGVH.

1. Introduction

Marginal zone B cells (MZB) are long-lived and non-circulating B cells residing in the MZ sinuses of the spleen. They are thought to constitute a first line of host defense to blood-borne microorganisms and viruses. MZB cells are well-equipped to deliver immune complexes to dendritic cells (DCs) and to prime or directly activate CD4$^+$ T cells. They respond to a wide range of antigens and migrate into the splenic follicle and differentiate into plasma cells within the germinal center (GC) [1]. MZB cells also express high levels of CD1d. A correlation of CD1d down regulation and MZ B cell migration was established [2]. A large number of autoreactive clones are reported to originate from the MZB cells. Expansion of the MZB cell compartment has been implicated in the NZB × NZW F1 murine model of lupus [2,3]. Sequestration of autoreactive B cells in the MZ area has also been proposed as a mechanism to prevent autoimmunity. Accordingly, the lupus-resistant variant (TAN) of the NZM2410-derived B6.NZM2410-*sle1.sle2.sle3* (B6.TC) showed an enlarged population of CD5^{hi} nonfunctional MZB cells, which, in contrast to MZB cells in the lupus-susceptible B6.TC substrain, failed to migrate into the follicles [4]. Interestingly, Wermeling et al. reported recently that the interaction of iNKT cells with MZB cells via CD1d influenced the B cell activation and migration into GC, and thus provided an important tolerance checkpoint [5].

The mer receptor tyrosine kinase (Mertk) belongs to the TAM (Tyro-3, Axl, and Mertk) family of receptor tyrosine kinases. It plays a central role in the immune system by clearing apoptotic debris, which otherwise may accumulate and provide chronic inflammatory stimuli. Autoimmunity occurs spontaneously in Mertk single deficient [6] and more strikingly TAM triple deficient mice [7,8]. Mertk-mediated engulfment of apoptotic cells requires the opsonizing molecules growth-arrest specific protein 6 (Gas6) or Protein S (ProS) [9]. Rothlin et al. revealed that the TAM receptors can

Abbreviations: Mertk, mer receptor tyrosine kinase; TAM, Tyro-3 Axl and Mertk; cGVH, chronic graft-versus-host; Gas 6, growth-arrest specific protein 6; ProS, Protein S; MZB, marginal zone B cell; FOB, follicular B cell; GC, germinal center.

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provide intrinsic feedback inhibition on a TLR-driven inflammatory response by coopting the IFNAR-STAT1 cassette to up regulate the suppressors of cytokine signaling, SOCS1 and SOCS3 [10]. Williams et al. found an increased number of all immune cell types in the peritoneal cavity of Mertk$^{-/-}$ mice [11]. The role of Mertk in regulating central tolerance was demonstrated in the NOD.Mertk$^{-/-}$ mice (nonobese diabetic mice lacking the expression of Mertk), in which the absence of Mertk leads to enhanced thymocyte negative selection and protection from diabetes [12]. Mertk also has a key role in mediating apoptotic cell-induced inhibition of DC activation/maturation [13].

We recently reported that B6 congenic Mertk$^{-/-}$ mice were unresponsive in chronic GVH disease induced by allogenic T cells from bm12 mice [14]. This defect was found to be B cell intrinsic, as we showed further that allostimulated mature B cells from Mertk$^{-/-}$ mice failed to produce autoantibodies in RAG-KO mice. An increased number of MZB cells has also been observed in naïve and immunized Mertk$^{-/-}$ mice [15,16]. In the present report, we explore further the mechanisms underlying the resistance of Mertk$^{-/-}$ B cells to allostimulation. We have induced cGVHD in Mertk deficient mice and mixed bone marrow chimeric mice to study the ability of Mertk deficient B cells to differentiate into antibody secreting cells. We demonstrate that these B cells exhibit an autonomous defect that is characterized by an abnormal calcium response to activation through MHC class II and failure to down regulate CD1d and migrate into follicles and form the GC that are associated with autoantibody production.

2. Materials and methods

2.1. Mice

Six- to 8-wk-old mice wild-type (WT) C57BL/6J (B6: H-2^{b}, Igh^{b}), B6.C20 (C20: H-2^{b}, Igh^{a}), and B6.C-H-2^{bm12} (bm12: H-2^{bm12}, Igh^{b}) were originally purchased from the Jackson Laboratory (Bar Harbor, ME). Mertk$^{-/-}$ and Gas6$^{-/-}$ mice were on the B6 background [14]. Mice were bred in our facility in pathogen-free conditions. Animals were handled in accordance with the guidelines of the Temple University Institutional Animal Care and Use Committee.

2.2. Induction of cGVH

cGVH disease was induced as previously described [14]. Briefly, recipient mice were injected i.p. with 1×10^8 bm12 splenocytes or 1×10^7 purified CD4 T cells in single-cell suspensions. Serum samples were prepared from peripheral blood of experimental mice and non-graft-versus-host control animals at the day of injection and on a weekly basis afterward. Mouse sera were stored at −20 °C for later analysis. In the anti-CD1d antibody treatment experiment, Mertk-KO mice treated with 0.5 mg of anti-CD1d (clone 1.3.7, FD. Finkelman lab, unpublished data) or isotype control antibody (2 days apart for 14 days) were subjected to the same induction of cGVH as described.

2.3. Reconstitution of bone marrow mix-chimeras

Single-cell suspensions of bone marrow were obtained from the tibias and femurs of B6, C20, and Mertk$^{-/-}$ mice, respectively. RAG-KO mice were reconstituted intravenously with 1×10^7 bone marrow cells from C20 mice in combination with equal numbers of marrow cells from either B6 or Mertk$^{-/-}$ mice. Three months after bone marrow transfer, recipients were checked for chimerism by flow cytometry of their peripheral blood lymphocytes with anti-IgM^{a} and anti-IgM^{b} and resulted in 1.7% for a-allotype and 1.5% for b-allotype [17].

2.4. Measurement of cytokines in vivo

In vivo IL-4 and INF-γ were measured according to the IVCCA (*in vivo* cytokine capture assay) protocol originally developed by Finkelman and colleagues [18]. Briefly, mice were intraperitoneally injected with 10 μg of biotin-labeled anti-IL-4 or anti-INF-γ in 200 μl of 1% autologous mouse serum in PBS. Twenty-four hours later, blood was collected and serum samples were prepared. Ninety-six-well Costar high-binding plates were coated with 50 μl of 2 μg/ml antibody specific for either IL-4 or INF-γ, respectively, in TBS (0.15 M NaCl, 0.025 M Tris-base, pH7.2) and incubated at 4 °C overnight. Twenty-five μl of diluted serum sample or standard was added into each well after 4 washes. The plate was then incubated at room temperature for 30 min, followed by 7 washes. One to 2000 diluted Streptavidin conjugated alkaline phosphatase was added and incubated 20 min at room temperature. The plate was finally washed 10 times and p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) was added and optical density was read at 405 nm.

2.5. ELISA of anti-dsDNA autoantibodies

Serum titers of anti-dsDNA were measured as previous described [14,19]. Briefly, plates were first coated with poly-L-lysine (1 μg/ml) (Sigma-Aldrich, St. Louis, MO) overnight at 4 °C. The plates were then washed with BBS and blocked with BBST (BBS, 0.4% Tween 80, 0.5% BSA) for 2 h at room temperature. Serum samples, diluted 1/250 in BBST, were added in duplicate and incubated overnight at 4 °C. Plates were washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc' specific; Jackson ImmunoResearch Laboratories, West Grove, PA) 2 h at room temperature. The plates were washed again, and p-nitrophenyl phosphate substrate (1 mg/ml) was added. Serum samples from MRL/lpr mice were used as standards. The plates were read at various time points with an automated ELISA reader (Molecular Devices, Sunnyvale, CA). To detect allotype-specific autoantibodies, the goat anti-mouse IgG step was replaced with rabbit anti-mouse preabsorbed allotype reagents (anti-IgG2a^{a} or anti-IgG2a^{b}; Accurate Chemical and Scientific, Westbury, NY) and detected with alkaline phosphatase-conjugated anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories) [20]. For mouse total IgG ELISA, serum samples were diluted at 1:20,000 ratio.

2.6. Calcium flux

Splenic B cells were prepared using the magnetic beads based anti-CD43-negative selection kit from Miltenyi Biotec, as described [14]. Purified B cells were then washed and resuspended in 1 × PBS containing 0.1% BSA at 3×10^6/ml. Fura-2 solution (2 μg/ml, Life Technology, Carlsbad, CA) was added and incubated at 37 °C for 30 min in the dark. Cells were washed once and incubated with 10 μg/ml of biotinylated anti-MHC-II (eBiosciences, San Diego, CA) for 15 min at room temperature. B cells were washed again and suspended in HBSS (Hanks' Balanced Salt Solution, Life Technology) containing 1 mM $CaCl_2$ at 3×10^6 cells/ml. Baseline calcium signals were first recorded. Calcium influx was induced with 10 μg/ml streptavidin and recorded with the QuantaMaster 80 spectrofluorometer (Photon Technology International, NJ).

2.7. Immunofluorescent staining

Spleens were snap-frozen in liquid nitrogen. Sections (4 μm) were air-dried for 10 min, followed by 30 min incubation with 1% BSA in PBS. Sample sections were then blocked with 5% BSA plus 0.1% Tween in TBS buffer. Sections were then incubated with 1:100 dilutions of various anti-mouse antibodies from BD Biosciences: