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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.

Keywords

MZ B cell; cGVH; CD1d; GC; MHC II

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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-cell via CD1d influenced the B cells' 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 might 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 provide intrinsic feedback inhibition on a TLR-driven inflammatory response by coopting the IFNAR-STAT1 cassette to upregulate 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 afterwards. 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 μ g of biotin-labeled anti-IL-4 or anti-INF γ in 200 μ l 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 μ l of 2 μ g/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 μ l 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-nitrophenylphosphate (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 μ g/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 hr 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 1:20000 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 CaCl₂ at 3×10^6 cells/ml. Base line 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: PE-B220, PE-CD1d, FITC-MAdCAM, and PNA (Sigma-Aldrich) for GC. Images were acquired using an Olympus BX60 fluorescence microscope equipped with camera (Center Valley, PA).

2.8. Total phosphotyrosine levels

B cells were isolated as previously described and incubated in PBS without protein for 15 minutes at 37°C, followed by 10 min incubation at room temperature. Cells were then incubated with 5 µg/ml biotin labeled anti-mouse MHC-II at room temperature for 5 min and cross-linked with 10 µg/ml streptavidin for an additional 20 min. As a positive control, 10 µg/ml of LPS and ionomycin/PMA (500 nM and 5 ng/ml, respectively) were used. Total phosphotyrosine were stained with rabbit anti-mouse phosphotyrosine (BD Biosciences) according to manufacturer's instructions. FITC labeled donkey anti-rabbit secondary antibody was used to detect the signal.

2.9. Statistical analysis

Data are presented as means ± SD. Statistical significance was determined using Student's *t* test. Asterisks: **p*<0.05, ** *P*<0.01.

3. Results

3.1. Intrinsic role of B-cell defects in the development of cGVH from *Mertk*^{-/-} mice

We previously showed B-cell unresponsiveness upon induction of cGVH in *Mertk*^{-/-} hosts [14]. The observed intrinsic B-cell defects could be due to a direct requirement of *Mertk* by B cells when engaged in T-cell mediated cognate activation; yet it could also reflect exposure of the B cells to other cells lacking *Mertk* expression. In other words, it was formally possible that B cells raised in a *Mertk*-deficient environment might acquire a deficiency in class II-mediated activation. To examine this question, we reconstituted

C57BL/6 background RAG-KO mice with bone marrow cells from C20 mice (a allotype) plus an equal number of bone marrow cells from either *Mertk*^{-/-} or wild-type C57BL/6 mice (b allotype). In this experiment, B cells from *Mertk*^{-/-} mice matured in the presence of *Mertk*^{+/+} cells, including other B cells. Three months later, chimerism was confirmed by flow cytometry (data not shown), and GVH disease was induced with purified CD4⁺ T cells from bm12 mice. As shown in fig. 1A, B cells from C20 mice were activated as expected and produced a high titer of 'a' allotype anti-dsDNA autoantibodies, regardless of whether wild-type or *Mertk*^{-/-} B cells were present in the same mice. In contrast, b allotype anti-dsDNA was produced in the chimeras that had received bone marrow from C57BL/6 WT mice (Fig. 1B, white bar) but not from *Mertk*^{-/-} mice (Fig. 1B, black bar). Thus, the *Mertk*^{-/-} B-cell defect in this system is truly B-cell intrinsic and is not influenced by the presence or absence of *Mertk* on other cell types.

3.2. Unresponsiveness to cGVH in *Mertk*^{-/-} mice is Gas6-independent

Two ligands (ProS and Gas6) have been shown to bind to *Mertk* and act as bridge molecules in *Mertk*-mediated phagocytosis. Recently, Caberoy et al presented evidence for two additional TAM receptor ligands, Tubby and tubby-like protein 1 (Tulp 1) [21]. While ProS deficient mice are lethal at the embryonic stage, *Gas6*^{-/-} mice are viable with some alterations in thrombosis [22]. *Gas6*^{-/-} mice are reported to be protected from hepatic GVH disease induced by T cells prepared from FVB mice [23]. We, therefore, examined the role of Gas6 in bm12 T-cell induced GVH. Fig. 2 shows that, as expected, there was no response in *Mertk*^{-/-} hosts. In contrast, *Gas6*^{-/-} hosts produced levels of anti-dsDNA autoantibodies comparable to WT mice. Thus, Gas6 signaling through *Mertk* is not mandatory for autoantibodies in this system; we conclude that ProS, or possibly one of the more recently identified potential *Mertk* ligands, is sufficient for *Mertk*-dependent B-cell activation.

3.3. B cells from *Mertk*^{-/-} mice failed to respond to activation by non-cognate T-cell help

In the model of bm12 induced chronic GVH, all B cells in normal hosts are generally activated as indicated by the upregulation of activation markers and total IgG shows a global increase. We thus asked whether the observed defects in autoantibody production in *Mertk*^{-/-} mice were accompanied by a defect in global activation of B cells. As shown in Fig. 3A, *Mertk*^{-/-} mice failed to respond to bm12 T-cell stimulation with the marked polyclonal increase in IgG seen in WT mice. Correspondingly, in the absence of *Mertk*, host *in vivo* cytokine production, measured by IVCCA, showed a significant decrease in secretion of a Th2 cytokine (IL-4) and a trend towards an increase in secretion of IFN- γ . Data shown here indicate a general unresponsiveness of B cells from *Mertk*^{-/-} mice to the induction of cGVH at early (Fig. 3B) and later stages (Fig. 1).

3.4. Significant reduced response of *Mertk*^{-/-} B cells to MHC class II cross-linking

In human and murine B cells, ligation of MHC-II by Abs triggers downstream signals that activate B cells [24, 25]. In the model of bm12-induced cGVH, B-cell activation is dependent on cognate recognition of MHC-II by disparate T cells [17]. To test MHC-II signaling in *Mertk* deficient B cells, we first examined direct effects of MHC ligation on phosphotyrosine levels. Pure B cells were isolated and incubated with biotinylated anti-MHC II, followed by 20 min cross-linking with streptavidin. Cells were then fixed, permeabilized, and incubated with anti-phosphotyrosine antibody. As shown in fig. 4A, ligation of MHC-II induced a modest increase in tyrosine phosphorylation level in B cells from *Mertk*^{-/-} mice. In contrast, WT B-cell phosphotyrosine levels increases were substantially higher than those seen for *Mertk*^{-/-} B cells. No activity was detected following incubation with the secondary Ab (donkey anti-rabbit) alone (data not shown). Calcium influx was also measured following cross-linking of MHC-II with biotin labeled anti-MHC followed by streptavidin. The baseline calcium signal was similar between *Mertk*^{-/-} and WT

mice (Fig. 4B). In WT mice, cross-linking of MHC-II on the surface of B cells caused a rapid influx of calcium, with a gradual return to baseline levels. In contrast, peak calcium influx was delayed and decreased in *Mertk*^{-/-} mice, along with a delayed return to baseline. This suggests that both B cell signaling and desensitization are retarded in the absence of *Mertk* (Fig. 4B). The observed difference in calcium response was not due to different expression levels of MHC-II on *Mertk*^{-/-} mice (Fig. 4B insertion). Taken together, data shown here indicate significant lower responses of B cells from *Mertk*^{-/-} mice to various stimulations when assessed at different points of the B-cell signal cascade, compared to B cells from WT B6 mice (Fig. 4).

3.5. B cells from *Mertk*^{-/-} do not down regulate surface CD1d and migrate into the FO region

The MZB-cell compartment plays an important role in autoreactive B-cell generation and expansion. Increased numbers of MZB cells are seen in several models of lupus-proven mice, including NZB/NZW, before the occurrence of disease [3, 26, 27]. Similarly, we consistently observed an elevated number of MZB cells in our *Mertk*^{-/-} mice with or without cGVH, as shown by both immunofluorescent staining and FACS analysis (Fig. 5 and 6B).

Recent studies point to a CD1d-associated checkpoint for MZB activation and subsequent migration into the follicular area to form GC [2, 5]. Abnormalities in MZ/CD1d⁺ B cells are associated with autoimmune pathology in a mouse model of lupus [2]. We found that the constitutive expression level of surface CD1d was similar between *Mertk*^{-/-} and WT mice (Fig. 6A and 6B). During the course of cGVH development, WT MZB cells down-regulated CD1d as early as day 3 (Fig. 6B). Accordingly, decreased numbers of MZB cells were found in WT mice spleens (Fig. 5 and 6A). In contrast, in *Mertk*^{-/-} mice, B cells bearing high levels of CD1d remained in the MZ (Fig. 5 and 6A). We further treated *Mertk*^{-/-} mice with anti-CD1d downregulating antibody and induced cGVH, they then started to produce anti-dsDNA autoantibodies. While age and sex-matched *Mertk*^{-/-} mice treated with the control antibody remained unresponsive to cGVH induction (Fig. 6C). Taken together, in the absence of *Mertk*, MZB cells with constitutively high levels of CD1d were trapped in the marginal zone, indicating an impaired CD1d checkpoint.

3.6. Decreased GC response in *Mertk*^{-/-} mice in response to the induction of cGVH

Defects in CD1d are associated with decreased B-cell activation and GC formation [5, 28]. We stained *Mertk*^{-/-} spleen sections with PNA to quantitate GCs in comparison with spleens from WT hosts also undergoing cGVH. Fig. 7A (left panel) shows few GCs and low intensity PNA staining in sections from *Mertk*^{-/-} mice 26 days after bm12 splenocyte injection. Quantitative analysis (Fig. 7A, right panel) revealed a significant decrease in GCs per 10 follicles in *Mertk*^{-/-} mice. No staining for CD1d in GCs was seen in spleens from WT mice (Fig. 7B). By comparison, in *Mertk*^{-/-} mice, CD1d⁺ B cells in the follicular area did not show PNA staining, suggesting that CD1d⁺ B cells in the follicle area do not form GC in *Mertk*^{-/-} mice. These data support the notion that consistent high expression of CD1d on MZB cells is associated with a low GC response.

4. Discussion

This report adds to our knowledge of B-cell abnormalities occur in mice lacking the *Mertk*. Within the immune system, this molecule is most strongly expressed on macrophages and DCs, yet B cells from *Mertk*^{-/-} mice subjected to class II disparate chronic GVH fail to generate autoantibodies [14]. The present data implicate a true intrinsic defect of B cells from *Mertk*^{-/-} mice in response to class II allostimulation, as this deficiency could not be rectified for *Mertk* deficient B cells maturing in the presence of a wild type immune system.

The deficiency in *Mertk*^{-/-} B cells is reflected in deficient signaling, as stimulation by antibody-mediated cross linking of surface class II MHC resulted in decreased signals (phosphotyrosine level, IgG secretion, and calcium influx) at different points in the signaling pathway. Unlike the unresponsiveness observed in *Mertk*^{-/-} mice to cGVH, B cells from *Mertk*^{-/-} mice responded to all stimuli, yet to a much lesser degree compared to B cells from WT mice. Our data indicate an important new function for *Mertk* in shaping B-cell immune responses by participating in cell signal regulation. These *in vitro* findings must be explored more deeply *in vivo*, where there may be synergistic effects from different types of cells and a more complex signaling network. The mechanism whereby *Mertk* participates in MHC-II signaling remains to be elucidated, and may operate through the transient *Mertk* expression (detectable on day 5 and fell after day 14) on MHC II-activated B cells which we reported previously [14].

Our previous work showed that lupus-like autoimmunity developed in older *Mertk*^{-/-} mice, presumably caused by chronic defect in apoptotic cell clearance [6]. Autoreactive B cells found in the old *Mertk*^{-/-} mice may come from the peritoneal B1-B cells being primed by numbers of T cells, DCs, and macrophages. Those immune cells were found to have migrated and accumulated in the peritoneal cavity [11]. Over time, defects in apoptotic cell-clearance may favor an autoreactive environment in the peritoneal cavity. In cGVH, however, conventional B cells are responsible for the autoreactive B cells [29]. We then examined old *Mertk*^{-/-} mice with higher titer of autoantibodies for MZ B-cell numbers and CD1d expression, and found CD1d bearing B cells also accumulated in the MZ (data not shown), suggesting a disassociation of MZ B-cell phenotype from spontaneous autoimmunity in *Mertk*-KO mice. The activation of *Mertk* and the other TAM kinases requires cross-linking with the ligands ProS, Gas6, or possibly two newly implicated molecules, *tubby* and *tulp1*. We therefore wondered about the relative roles of the different ligands in *Mertk* activation in cGVH. Our data show that cGVH proceeds normally even in the absence of Gas6, indicating that ProS or other ligands can activate *Mertk*. It is not possible to do the reciprocal experiment with ProS knockout animals due to the embryonic lethality of this defect, and the roles of *tubby* or *tulp1* remain to be tested (20). It also seems possible that *Mertk* has multiple functions and that the defects in MHC-II mediated activation in cGVHD are not related to any of the known *Mertk* ligands or to phagocytosis of apoptotic cells. A requirement of *Mertk* intracellular portion for scavenger receptor A (SR-A) was demonstrated by Todt et al [30]. In the case of MHC-II activation, *Mertk* may help in modulating the signal strength.

The present studies of B-cell subpopulations (notably MZ B cells) in *Mertk*^{-/-} mice undergoing cGVH shed light on the role of *Mertk* in B-cell function. MZ B cells exhibit unique activation, proliferation and immunoglobulin secretory responses [31]. They are positioned to capture antigen more efficiently, and generate effector cells more rapidly than FO B cells [32]. CD1d interaction with iNKT cells was proposed as a peripheral checkpoint for MZ B-cell activation by Karlsson and colleagues [5]. Remarkably, MZ B cells from *Mertk*^{-/-} mice bore constant high levels of surface CD1d upon allogeneic T-cell activation through MHC-II. Subsequent impaired migration and GC formation occurred (Fig. 6 and 7). We propose that CD1d down regulation is dependent on *Mertk*, and that in the absence of this molecule, the failure of CD1d bright cells to migrate into follicular areas leads to impaired activation and GC formation. These data are consistent with our observation of increased numbers of MZ B cells in *Mertk*^{-/-} mice [15, 16]. More strikingly, when treated with anti-CD1d antibody, *Mertk*^{-/-} mice which had been given with bm12 splenocytes started to produce autoantibodies. Although, the anti-dsDNA titers did not reach WT levels, they were significantly higher than those seen in *Mertk*^{-/-} mice treated with isotype control antibodies. Therefore, *Mertk*-mediated CD1d down-regulation is one of the mechanisms accounting for the intrinsic B-cell unresponsiveness to bm12-cGVH.

Neutralizing surface CD1d may also target other CD1d-bearing cells (DCs, epithelial cells), yet it is unlikely that those cells augment the disease because cGVH is mediated through donor CD4⁺ T cells and host B cells [29]. It remains unclear whether FO B cells also contribute to the autoantibody production. Adoptive transfer experiments using isolated MZ or FO B cells from *Mertk*^{-/-} mice given to different RAG-KO mice, followed by cGVH induction, should clarify this issue. Clearly, further studies to ascertain how *Mertk* signals interacting with CD1d signals are warranted and are likely to uncover novel targets for autoimmune regulation.

Abnormalities of MZB cells have also been observed in several autoimmune models. For example, NZB×NZW F1 mice have a substantial increased number of MZ B cells before the onset of disease [33]. The relationship of such MZ B cell abnormalities to the TAM receptor system in murine and human autoimmunity is unexplored, and understanding of their role in the complex B cell abnormalities in disease may yield important insights.

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Abbreviations

Mertk	mer receptor tyrosine kinase
TAM	<u>T</u> yro-3, <u>A</u> xl, and <u>M</u> ertk
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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Highlights

B cells from Mer deficient mice were intrinsically unresponsive to bm12-induced cGVH.

Mer-KO B cells displayed decreased calcium and phosphorylation when stimulated through MHC-II cross-linking.

CD1d is highly expressed on MZ B cells and has been shown as a checkpoint for MZ autoreactive B cells. Failure of CD1d down regulation was shown to be associated with MZ B-cell unresponsiveness in Mer-KO mice.

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Fig. 1. RAG-KO mice recipients of Mertk^{-/-} bone marrows fail to produce allotype-specific autoantibody

RAG-KO mice were injected with 1×10^7 bone marrow cells from B6.C20 mice mixed with same number of bone marrow cells from Mertk^{-/-} or WT mice, respectively. cGVH was induced 3 month later with bm12 T cells. Mice were bled and serum levels of allotype specific anti-dsDNA were determined. A. a allotype (produced by B6.C20 donor); B. b allotype (produced by C57BL/6 WT or Mertk^{-/-} donor). Means and STDEV are shown. Data are representative of three repeats.

Fig. 2. Gas6^{-/-} mice develop a cGVH response

WT, Mertk^{-/-}, and Gas6^{-/-} mice were injected i.p. with 100 M bm12 splenocytes. Serum levels of anti-dsDNA were determined by ELISA. This experiment was repeated three times with similar results.

Fig 3. MERTK^{-/-} mice undergoing cGVH have deficient IgG and cytokine responses
 cGVH was induced as previously described [14] in MERTK^{-/-} and WT mice. A. Serum levels of total IgG were measured by ELISA. B. In vivo cytokines were determined by i.v. injection of biotin-labeled anti-IL-4 and anti-IFN γ at day 5. Mice were then bled 1 day later and IL-4 and IFN γ secretion were determined as described in the methods. This ELISA was repeated in four experiments with similar results. Representative data are shown.

Fig 4. Reduced signals in B cells from *Mertk*^{-/-} mice

A. B cells from naïve B6 and *Mertk*^{-/-} mice were purified and rested for 30 min, followed by 5 min incubation with biotinylated antibodies against MHC-II. Tyrosine phosphorylation was stimulated by streptavidin-mediated cross-linking, and measured by intracellular FACS staining. B. Purified B cells were loaded with Fura-2. Calcium mobilization was stimulated through biotin-streptavidin mediated MHC-II cross-linking. Insertion, expression level of MHC-II on B cells from B6 and *Mertk*^{-/-} mice determined by FACS analysis. All experiments were repeated at least twice.

Fig. 5. Consistent high numbers of MZB cells in Mertk^{-/-} mice during the course of cGVH induction

Splenocytes were first gated for CD19 positivity. Percentage of MZB (CD21-high, CD23-low) and FOB (CD21-low, CD23-high) cells was then determined by flow cytometry. Four mice from each time point were analyzed and data are representative of two experiments.

A.

B.

C.

Fig. 6. *Mertk*^{-/-} MZB cells bear constant levels of CD1d

A. Spleen samples from *Mertk*^{-/-} and WT mice were snap-frozen in liquid nitrogen and sectioned at 4 μ m at various time during the course of cGVH-induction as indicated. MZ sinus was highlighted by FITC-MAdCAM staining. CD1d expression levels were visualized with PE conjugated anti-mouse CD1d antibody. B. FACS analysis of CD1d expression on MZ B cells from mice under normal conditions or 3 days after GVH induction. C. *Mertk*^{-/-} mice treated with 0.5 mg anti-CD1d or control antibodies were injected with 10⁷ bm12 splenocytes at day 0, and antibody was given every two days up to 14 days. Mice sera were collected through tail vein bleeding and anti-dsDNA autoantibodies were measured by ELISA as described [14]. This antibody treatment experiment was repeated twice.

Fig. 7. Decreased GC responses in the spleen of Mertk^{-/-} mice

A. Left, representative GC numbers in the spleen sections of Mertk^{-/-} and B6 mice 26 days after cGVH induction. Right, GC numbers were quantitated in every 10 follicles. B. Counter staining of spleen sections with PE-CD1d and FITC-PNA 26 days after bm12 injection.