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The Mertk Receptor Tyrosine Kinase Promotes T-B interaction Stimulated by IgD B-cell Receptor Cross-linking

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Abstract

The Mertk receptor tyrosine kinase facilitates macrophage and DC apoptotic-cell clearance and regulates immune tolerance. Mertk may also contribute to B-cell activation, because Mertk-KO mice fail to develop autoantibodies when allo-activated by T cells. We investigated this possibility with a well-characterized model in which injection of mice with goat anti-IgD antibody causes membrane IgD cross-linking that induces T-independent B cell activation and antigen presentation to T cells. Goat anti-mouse IgD antibody-injected C57BL/6 Mertk-KO mice had normal initial B cell activation and proliferation, but significantly lower T cell activation and proliferation, as well as lower IgE and IgG anti-goat IgG responses, as compared to C57BL/6 WT controls. B cell antigen processing, analyzed by evaluating B cell fluorescence following injection of monoclonal anti-IgD antibody labeled with biotin or FITC, was comparable between Mertk-KO mice and WT mice. IgD Ab primed B cells from Mertk-KO mice exhibited significantly lower ability in activating memory T cells isolated from WT mice injected with the same antigen 10 days before. These observations suggest that Mertk expression is required for optimal B-cell antigen presentation, which is, in turn, required in this model for optimal T cell activation and subsequent T cell-dependent B cell differentiation.

Keywords

Mertk; Ag presentation; IgD cross-linking

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1. Introduction

Goat IgG anti-mouse IgD antibody (Ab) (GamD) binds to B-cell membrane IgD and cross-links this B cell receptor (BCR), inducing T cell-independent B-cell activation, GamD internalization and processing, and presentation of Class II MHC-associated GamD-derived peptides to CD4⁺ T cells [1, 2]. The goat IgG-specific CD4⁺ T cells primed or activated by these B cells then secrete cytokines, including IL-4, and express surface molecules that activate newly generated B cells to proliferate and differentiate into Ab secreting cells [3]. Most initially activated B cells undergo apoptosis 2–5 days after injection [4]. Major findings that have been reported with this well-characterized model are summarized in figure 1 [5–7]. The IL-4 produced by T cells in this response is required for Ig isotype switching to IgE and suppresses complement-fixing isotypes (IgG2a and IgG3) [8, 9]. Studies with univalent F(ab') fragments of anti-IgD Ab and with mAbs that poorly crosslink IgD demonstrate that T-independent B cell activation is required to stimulate T cell activation and B cell antibody production [10]. Indeed, failure to activate B cells by this mechanism can induce Ag specific T-cell tolerance [11].

Mertk belongs to the TAM (Tyro-3, Axl, and Mertk) subfamily of receptor tyrosine kinases [12]. Mertk mediates the engulfment of apoptotic cells through the bridging molecules (growth-arrest-specific gene 6 (Gas6) or Protein S (ProS)), and promotes maturation of macrophages to a non-inflammatory phenotype [13–15]. Without Mertk, mice develop lupus-like autoimmune manifestations and retinal degeneration-associated blindness [13, 16]. Mertk is expressed on subpopulations of macrophages and dendritic cells [17] and is also found on certain B cells, where it is tightly regulated [18]. Mertk-deficient B cells are unresponsive to T-cell help in a chronic graft-versus-host (cGVHD) model [18, 19], yet the mechanisms whereby Mertk regulates B-cell function remain obscure.

To understand better how Mertk regulates B-cell responses, we chose the anti-IgD model because it allows in vivo evaluation of T-independent B cell activation by membrane IgD cross-linking, B cell Ag processing, B cell-dependent T cell activation, and T cell-dependent Ag production. We cross-linked B-cell IgD receptors with GamD and studied the subsequent B-cell activation-induced immune responses. We report here that B cells lacking Mertk have a defect in activating T cells, and are associated with lower Ab production compared to B cells from WT mice.

2. Materials and Methods

2.1. Mice and reagents

C57BL/6J (B6; 6–8 weeks old; WT controls) and C57BL/6J Mertk deficient (Mertk-KO; 6–8 weeks old) mice were bred and maintained in our mouse colony. Experimental mice were sex and age matched. All mouse procedures followed the guidelines for the use of animals in research and were approved by the Institutional Animal Care and Use Committee of Temple University. Affinity-purified goat Ab specific for mouse IgD (GamD) and monoclonal mouse IgG1 anti-mouse IgD (clone 1.3.7) were produced and tested as previously described [20]. All other antibodies were from BD Biosciences (San Jose, CA) unless otherwise specified.

2.2. Immunization of mice with GamD antisera

Age- and sex-matched individual mice were injected intraperitoneally with 200 μ l of antiserum produced in goats (GamD) [20]. Blood was collected through tail vein bleeding at day 0, 4, 8, 10, and day 14 and sera stored at -20°C .

2.3. Measurement of serum Ig

Total serum IgG levels were measured by ELISA as previously described [19]. In brief, 96-well PVDF plates were coated with anti-mouse IgG at 10 μ g/ml in 1x PBS. Serum samples were added to plates blocked with 1% BSA in 1x PBS. Plates were then washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The plates were read with an automated ELISA reader (Molecular Devices, Sunnyvale, CA). Total IgE levels were determined with the BD OptEIA mouse IgE ELISA Set (BD Biosciences, San Jose, CA). For isotype-specific anti-goat Ab levels, plates were coated with goat IgG (10 μ g/ml) and binding detected with AP-conjugated anti-mouse immunoglobulin isotype Abs.

2.4. BrdU proliferation assay

Mice were injected intraperitoneally with 1.0 mg of BrdU (Sigma-Aldrich, St. Louis, MO) every 12 hours for 3 times. Cells from the spleen were subsequently harvested and stained for intracellular BrdU incorporation (BD Biosciences).

2.5. Anti-mouse IgD antibody labeling with FITC and biotin

Monoclonal anti-mouse IgD (clone 1.3.7) was first dialyzed in 1x PBS over night at 4°C . 10 mg of the mAb (in 1ml of 0.1M NaHCO_3 , pH8.0) was incubated with 1 mg of the biotin reagent (EZ-Link Sulfo-NHS-LC-Biotin, Fisher Scientific, Pittsburgh, PA) for 2 hours at room temperature. Labeled proteins were then dialyzed against normal saline. For the FITC labeling, 0.5 ml of mAb was prepared in 1xPBS containing 40 μ l of the borate buffer (0.67M) and added to the FITC reagent (Pierce FITC Antibody Labeling Kit, Thermo Scientific, Rockford, IL). The reaction mixture was then incubated at room temperature for 60 minutes protected from light. Labeled proteins were purified with the purification resin provided with the kits.

2.6. B-cell Ag processing assay

B cells from the spleen of Mertk-KO and WT mice were isolated with the anti-CD43-negative selection kit from Miltenyi Biotec (Auburn, CA) as previously described. Single-cell suspensions were then plated in 96-well plates (2×10^6 cells/well in 200 μ l of RPMI-1640 complete medium). Biotin- or FITC-labeled monoclonal anti-mouse IgD antibodies (40 μ g/ml) were added into each well. B cells were then harvested at different time points and processed for FACS analysis, as indicated in the figure legends.

2.7. Ex vivo T cell stimulation with in vivo GamD-stimulated B cells

Total T cells were isolated from the spleens of WT or Mertk-KO mice 10 days after GamD injection using the CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA). T cells were then labeled with CFSE (Life Technologies Co., Grand Island, NY) and co-cultured with B

cells (stimulators) obtained from the spleens of WT or Mertk-KO mice after 4-hr priming with the same Ag (GamD) *in vivo*. Intensity of CFSE on T cells was assessed by FACS analysis after 72 hrs.

2.8. Statistical analysis

Data are presented as the mean $\pm$ SD (Standard Deviation). Statistical significance was determined using Student's *t* test. Asterisks: * $p < 0.05$, ** $p < 0.01$.

3. Results

3.1. Mertk-KO mice exhibit significantly reduced responses to goat anti-mouse IgD cross-linking

We previously reported an intrinsic B-cell unresponsiveness to bm12 induced chronic GVHD (graft-versus-host disease) from Mertk-KO mice [18, 19]. To further explore the function of Mertk on B cells, we injected Mertk-KO mice with goat anti mouse IgD antibody (GamD) and measured immunoglobulin levels compared to WT mice undergoing the same treatment. We therefore measured the serum level of total IgG with untreated mice serum as control. As expected, WT mice showed a dramatic increase of total IgG in the serum 10 days after GamD injection. Mertk-KO mice also responded with elevated serum IgG, but to a significantly lower level as compared to the WT mice (Figure 2A). Serum IgE reached peak levels 8 days after anti-IgD injection in WT mice, at which time they were increased ~5-fold above baseline. In contrast, serum IgE increases were significantly less in Mertk-KO mice that received anti-IgD (Figure 2A, right panel). We further measured antigen-specific IgG isotype responses in WT and Mertk-KO mice against goat IgG. Serum IgG1 and IgG3 levels increased substantially in WT mice treated with GamD, but significantly less in Mertk-KO mice subjected to the same treatment (Figure 2B). Thus, Mertk-KO mice are able to make IgE and IgG responses to anti-IgD Ab, but these are modest and considerably lower than what is seen in WT mice, suggesting that Mertk is important in B-cell mediated cellular or molecular signals in response to surface IgD cross-linking.

3.2. IgD cross-linking leads to Mertk-KO B-cell activation and proliferation

To evaluate whether the results in figure 2 reflected a direct effect on B-cell responses in Mertk deficient mice, we used *in vivo* BrdU incorporation to measure B cell proliferation 2 days after GamD injection. Results (Figure 3A) showed that the percentage of BrdU⁺ B cells from Mertk-KO mice was comparable to that observed in WT mice. B-cell activation was also measured through up-regulation of surface markers: CD80, CD86, CD95 (Fas), and MHC class II. Compared to naïve B cells, Mertk-KO B cells were activated and upregulated most surface activation markers to the same level seen for WT B cells (Figure 3B). These results demonstrated that Mertk-null IgD-bearing B cells underwent initial anti-immunoglobulin-activation to the same degree as WT B cells.

3.3. T cells from Mertk-KO mice display significantly less activation and reduced proliferation

Stringent cross-linking of B-cell membrane IgD induces them to present Ag to naïve T cells in a stimulatory rather than a tolerogenic fashion (Morris SC, JI, 1994). We asked whether T cells from Mertk-KO mice injected with GamD became activated and proliferated to the same extent as in WT mice. T-cell activation and proliferation were quantitated 4 days after GamD injection by measuring BrdU incorporation. As shown in figure 4A, over 50% of T cells from WT mice proliferated, while only 19% of T cells from Mertk-KO mice proliferated. FACS analysis of T-cell activation markers (up-regulation of CD44 and down-regulation of CD62L) revealed that a relatively small percentage of T cells from Mertk-KO was activated by Ag-presenting B cells in response to IgD cross-linking (Figure 4B). Thus, there was a significant decrease in T-cell activation and subsequent proliferation in the Mertk-KO mice.

3.4. Mertk-KO B cells process goat Ag as efficiently as WT B cells

Cross-linking of IgD results both in B-cell Ag processing and presentation associated with activation, yet Ag presentation appears to be the major function of those B cells that are initially activated by cross-linking of their membrane IgD [3]. Initial B-cell activation and Ag presentation also facilitate T-cell activation. Data from figure 2 suggest that Mertk-KO B cells were activated and proliferated normally. We wondered if decreased T-cell activation and proliferation was due to inefficient Ag processing by B cells from Mertk-KO mice. To test B-cell Ag processing, we labeled a monoclonal mouse anti-IgD antibody with biotin or FITC, then used it to stimulate splenocytes from naïve WT and Mertk-KO mice. The decrease of MFI (mean fluorescent intensity) for the biotin-anti-IgD-stained cells indicated internalization of membrane IgD. FITC is pH sensitive and the decrease in MFI for the FITC-anti-IgD-stained cells indicated phagosomal maturation (namely acidification), a separation between the end of internalization, beginning of acidification and subsequent phagosome fusion to endosomes/lysosomes. As shown in figure 5, increasing incubation time led to decreases in the fluorescence intensity of WT B cells, indicating Ag processing. Mertk-KO B cells internalized anti-IgD to the same extent and with the same speed as WT B cells (Figure 5). This suggests that Mertk receptor tyrosine kinase does not influence B-cell Ag processing.

3.5. Memory T cells proliferated less when stimulated by Mertk-KO B cells

To test the ability of Mertk-KO B cells to stimulate memory T cells, we purified T cells from WT mice 10 days after GamD injection, labeled them with CFSE, and co-incubated them for 4 hours with B cells from GamD-primed WT or Mertk-KO mice. Three days later, we gated on CD4⁺ T cells and analyzed CFSE intensity by FACS. Figure 6 shows that approximately 10% of memory T cells had divided once and 10% had divided twice when cultured with equal numbers of wild-type (WT) Ag-primed WT B cells, while stimulation with Ag primed B cells from Mertk-KO mice induced considerably less T cell proliferation (Fig. 6). Comparable differences were observed with a 1:2 ratio of T:B cells. Taken together, the data suggest that Mertk deficiency results in defective B-cell mediated T-cell activation and proliferation.

4. Discussion

Studies on the function of Mertk receptor tyrosine kinase have focused on apoptotic cell clearance and immune regulation through Mertk-bearing macrophages and DCs [12, 13, 21–24]. We have noted expression and function of Mertk on certain B cells [18, 19]. In this report, we describe deficient antigen presentation by B cells from Mertk deficient mice. Similar to B cells from WT mice, B cells from Mertk-KO mice were activated and proliferated normally in response to membrane IgD cross-linking. Those B cells then internalized the goat antibodies and processed/presented them to T cells. Surprisingly, we found that T cells from Mertk-KO mice showed a significantly decreased degree of activation and proliferation compared to those from WT mice. Because Mertk is not expressed on mouse T cells, we suspected that the deficient T-cell activation was due to a deficiency in B cells from the Mertk deficient mice.

We measured membrane IgD⁺ B cells numbers and surface IgD intensity and found no difference between WT and Mertk-KO B cells (data not shown). Previous studies showed that Mertk did not increase the amount or rate of binding of apoptotic cells to phagocytes [25], but rather facilitated the process of engulfment by initiating the subsequent cytoskeletal rearrangements needed for phagocytosis. Because Ag internalization and processing also engage cellular compartment movement facilitated by cytoskeleton rearrangements, we asked if Mertk might act through an influence on cytoskeletal rearrangement to facilitate antigen processing. We labeled anti-IgD with biotin and FITC respectively to examine the rate of Ag internalization and processing initiated by IgD cross-linking. To our surprise, we did not observe any difference of anti-IgD Ag internalization and processing (Figure 5). Thus, the impaired antigen presentation by Mertk deficient B cells could not be ascribed to their ability to process antigen, although we cannot rule out the possibility that Mertk-deficient B cells have a defect in the complexing of Ag-derived peptides with MHC class II or in the migration of these complexes to the cell membrane.

To understand how Mertk facilitates B cell Ag presentation, further and more defined studies are needed to identify the stage at which Mertk contributes to this process. We previously reported decreased calcium mobilization and a delayed desensitization phase of calcium signal transduction in Mertk-KO B cells stimulated by MHC-II cross-linking [26]. Finkelman et al reported a requirement for sustained cognate T-B interaction in the large antibody responses in the GamD model [1]. Over the last decade, live cell imaging has revealed a specialized contact between T cells and antigen presenting cells (APCs), called the immunological synapse, which is characterized by accumulation of TCR in the center of the contact zone with a surrounding ring of adhesion molecules. During this process, microclusters formed in the periphery are transported to the center of the synapse in an actin-dependent process. Similarly, the interaction between phagocytes and apoptotic cells is also orchestrated by the formation of a phagocytic synapse. Mertk was shown to directionally and functionally link to the integrin pathway, to stabilize the existence of phagocytic synapse [27]. Thus, it is likely that Mertk is involved in the modulatory dynamics of the TCR during the immunological synapse, which is a key regulatory mechanism in Ag presentation and T-cell activation.

Injection of mice with GamD stimulates a large, T cell-dependent, polyclonal increase in serum levels of total IgG [2], 5–20% of which is goat IgG-specific IgG1 [2]. Both the polyclonal and goat IgG-specific responses were suppressed in Mertk-KO mice. This is most likely a result of the considerable decrease in T-cell activation in Mertk-KO mice, which, in turn, likely results from an abnormal T-B interaction in these mice. When memory T cells generated in WT mice were stimulated with same Ag-primed Mertk-KO B cells, they proliferated significantly less than those stimulated by WT B cells. Adoptive transfer studies comparing antibody responses by GamD-immunized RAG-deficient mice reconstituted with WT B cells and WT or Mertk-KO T cells should help to identify the role of Mertk-KO T cells in this model.

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Abbreviations

Mertk	Mer receptor tyrosine kinase
Gas6	growth-arrest-specific protein 6
GamD	goat anti-mouse IgD antisera
B6	C57BL/6
APC	antigen presenting cell

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Highlights

Mertk-KO mice exhibit reduced IgG and IgE responses to anti-mouse IgD.

Mertk expression is required for optimal T-B interaction after B-cell IgD crosslinking.

Mertk may modulate the immunological synapse during T-B interaction.

Figure 1. Immune responses to B-cell membrane IgD cross-linking in mice

Shortly after GamD BCR cross-linking, B cells undergo activation and proliferation, while internalizing, processing, and presenting antigens to T cells. T cells then become activated and present peptides to naive B cells. Initially activated B cells undergo apoptosis starting at day 2. T-cell activated-B cells will eventually become antibody-secreting cells (ASC). Cytokines are also known to play an essential role in this model.

Figure 2. Decreased immune responses to GαmD in Mertk-KO mice

Mice were injected with 200 μ l of goat anti-mouse IgD serum at day 0. Serum samples were collected at day 0, 8, and 14. A. Total serum levels of IgG (day 10) and IgE (day 8 and 14) were measured by ELISA. B. Isotype-specific anti-goat antibodies were measured by ELISA as described in the methods. This experiment was repeated three times and representative data are shown here.

Figure 3. Comparable B-cell activation and proliferation from Mertk-KO mice after GamD injection

WT and Mertk-KO mice were injected with 200 μ l of goat anti-mouse IgD serum. A. One mg of BrdU was given intraperitoneally 1 day later (3 times at 12-hr interval). Spleen single cell suspensions were prepared and cell proliferation was determined by FACS analysis gated on B220⁺/BrdU⁺. B. B-cell activation was measured with surface markers at day 2. The data shown here are representative of two independent experiments.

Figure 4. Reduced T-cell activation and lower proliferation in Mertk-KO mice injected with G α mD

WT and Mertk-KO mice were injected with 200 μ l of goat anti-mouse IgD antiserum. A. T-cell proliferation was analyzed as mentioned in the B-cell assay except for one-day delay of BrdU injection. B. T-cell activation was investigated at day 4 after antiserum injection by FACS analysis as reflected by surface markers.

Figure 5. Goat anti-IgD antigen processing by Mertk-KO B cells
 B cells were incubated with FITC- (A) or biotin-labeled (B) G α mD (40 μ g/ml) for various time points as indicated in the figures. B cell antigen processing of goat anti-IgD was analyzed by flow cytometry. Data are expressed as MFI (mean fluorescent intensity).

Figure 6. Mertk-KO B cells provoke less activation of memory T cells

T cells were isolated from WT and Mertk-KO mice 10 days after GamD injection. B cells were also prepared from these mice. CFSE-labeled memory T cells were then incubated with Ag-primed B cells for 3 days at various ratios. Cell proliferation is shown by CFSE intensity (A). Percentages of cells undergoing division were shown (B).