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IL-17 augments B cell activation in ocular surface autoimmunity

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Abstract

Accumulating evidence shows that IL-17 is critically involved in diverse autoimmune diseases. However, its effect on the induction and progression of the humoral immune response is not fully understood. Using a preclinical model of IL-17-mediated dry eye disease (DED), we demonstrate that upon encountering both the B cell receptor and a secondary T cell signal, IL-17 can enhance B cell proliferation and germinal center formation in DED mice, suggesting that a stable antigen-dependent T-B cell interaction is required. In addition, IL-17 also promotes the differentiation of B cells into isotype-switched B cells and plasma cells. Furthermore, we show that Th17 cells are more effective than Th1 cells to provide B cell help. Reduced B cell response correlates with significant reduction in clinical disease after in vivo IL-17A neutralization. In conclusion, our findings demonstrate a new role of IL-17 in promoting autoimmunity in part through directly enhancing B cell proliferation, differentiation and plasma cell generation.

Keywords

B cell; IL-17; autoimmunity

Introduction

Both T-helper, Th1 and Th17 subsets, and their secreted signature cytokines, interferon (IFN)-γ and interleukin (IL)-17, respectively, are critically involved in the pathogenesis of autoimmune diseases (1, 2). In addition, increasing evidence indicates that effector T helper cells can also support B cell response which may in turn also be involved in disease pathogenesis. Th1 and Th2 cells are known to support B cell responses, and their signature cytokines, IL-4 and IFN-γ, induce isotype-switching in B cells (3–7). In contrast, very few studies have investigated the role of Th17/IL-17 in the induction and maintenance of the humoral immune response (8–11); recent studies with lupus-and arthritis-prone BXD2 mice have shown that IL-17 plays a critical role in autoimmunity by promoting germinal center development (9, 11). However, to date, the effect of IL-17 on B cell proliferation has

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remained unclear (8, 9) and its distinct role in promoting B cell differentiation into isotype-switched B cells and plasma cells is not known.

Experimental desiccating stress-induced dry eye disease (DED) is a well-recognized preclinical model for ocular surface autoimmunity (12, 13). DED is a T-cell mediated autoimmune disease, and Th1 and Th17 cells have been shown to be crucial in the pathogenesis of dry eye disease (12–16). A recent study by Stern et al. has demonstrated the role of autoantibodies in dry eye pathogenesis (17). However, the role of Th17/IL-17 to influence the humoral immune response is unknown. Here, we utilized the dry eye mouse model to determine the potential of IL-17 and IFN-γ to induce and progress humoral immune responses.

In our study, we demonstrate that Th17 cells are significantly more effective than Th1 cells in inducing B cell proliferation in vitro. *In vitro* IL-17 acts directly on IL-17RA+ B cells promoting B cell proliferation and inducing differentiation of activated B cells and plasma cell formation. Further, we show that in vivo blockade of IL-17 reduces the frequencies of germinal center B cells and $IgM^{-}IgD^{-}$ (antigen-experienced) memory B cells. These data provide evidence that IL-17 has a direct effect on the induction and progression of the humoral immune responses in dry eye disease, one of the most common ophthalmic conditions.

Materials and Methods

Mice

Female 6–8-week-old C57BL/6mice (Charles River Laboratories, Wilmington, MA) and B6.129S7-Rag1tm1Mom/J mice (The Jackson Laboratory, Bar Harbor, ME) were used for this study. All experiments were performed in accordance with the guidelines described by the Institutional Animal Care and Use Committee. The animals were treated according to the ARVO Statement for the use of animals in Ophthalmic and Vision Research.

Dry eye induction

Dry eye disease (DED) was induced in mice as previously described with some modifications (Chauhan et al., 2009). Briefly, mice were placed in the controlled environmental chamber with a relative humidity of < 15%, and airflow rate of 15L/min for 3 weeks. In addition, to increase the ocular surface dryness, scopolamine hydrobromide (anti-cholinergic) (Sigma-Aldrich Corp, St. Louis, MO) was injected subcutaneously (0.5 mg/0.1 mL) three times a day. Age- and sex- matched mice kept in a standard environment were used as controls.

Cell separation

Draining cervical lymph nodes (LNs) were harvested from DED and naïve mice, and single cell suspensions were prepared. Mouse B cells and T effectors (CD4+CD25−) were isolated via magnetic sorting using mouse B and T cell isolation kits (Miltenyi Biotec Inc, Cambridge, MA), respectively, according to the manufacturer's instructions. The purity of sorted cells was routinely ≥95%. For isolating IL-17-depleted CD4+CD25-cells and IFN-γ-

depleted CD4+CD25− cells, CD4+CD25− cells were magnetically sorted followed by FACS sorting with a mouse IL-17 secretion assay and mouse IFN-γ secretion assay kit (Miltenyi Biotech), respectively.

B cell stimulation and proliferation

To assess B cell proliferation 1×10^5 purified B cells were stimulated or unstimulated as indicated with 1μg/ml $F(ab')_2$ goat-anti-mouse IgM, μ chain specific (Jackson ImmunoResearch, West Grove, PA), 1μg/ml Hamster anti-mouse CD40 Ab (HM40-3, BD Pharmingen) for 48hrs in complete RPMI 1640 medium (Invitrogen) with 1mM sodium pyruvate, 2mM L-glutamine, 10mM HEPES, 10% FBS, 50μM 2-mercaptoethanol in the presence and absence of 1 ng/ml of recombinant mouse IL-17A (Peprotech, Rocky Hill, NJ) or 10μg/ml of neutralizing anti–mouse IL-17 (R&D Systems, Minneapolis, MN). In selected experiments, B and T cells were cocultured (ratio 1:1) and stimulated with 1μg/ml of anti-mouse CD3 antibody (eBioscience, San Diego, CA). In Supplement Figure S3, B cells were stimulated in the presence or absence of 1 ng/ml recombinant mouse IL-17, IFN-γ, or IL-4 (Peprotech, Rocky Hill, NJ).

Flow cytometry analysis of B cell population

Phenotypic analyses of B cells were performed using flurochrome-conjugated anti-mouse antibodies and flow cytometry. After Fc blocking with anti-CD16/32 (eBioscience), cells were stained with BV421, AF488, AF647- conjugated anti-CD19 (6D5), anti-B220 (RA3-6B2), anti-GL7 (GL7), anti-IgD (11-26c.2a), anti-CD138 (281-2) (all from BioLegend, San Diego, CA), anti-IgM (Southern Biotech, Birmingham, AL), anti-FAS (15A7, eBioscience), or isotype control antibodies.

For Ki67 staining, cells were stained for their surface markers CD19 (BV421) and IL-17RA (AF488; R&D Systems) and fixed in 2% paraformaldehyde. Subsequently, cells were permeabilized in 0.7% Tween20 (SigmaAldrich) and stained with Ki67-AF647 (16A8, BioLegend) diluted in perm buffer. Cells were acquired on the LSRII (BD Bioscience) and data were analyzed using Flow Jo software (Tree Star).

Immunohistochemical staining for lymphoid follicle

Draining cervical lymph nodes were harvested 21 days after DED induction and snap-frozen in optimum cutting temperature (OCT) medium and stored at −80°C until sectioning. 8 mm sections were cut and fixed in ice-cold acetone for 10 min and air dried for 30 min. Sections were blocked with 10% FBS and Fc block for 1 hour at room temperature (RT) and then stained for 1 hour at RT with anti-IgM TRITC (1021-03, SouthernBiotech), anti-Ki67 FITC (ab15580, Abcam), and anti-IL-17RA APC (PAJ-17R, eBioscience). Subsequently, stained sections were mounted using vector shield fluorescent mounting medium. All images were captured using a DMI6000 Inverted Microscope (Leica Microsystems, Buffalo Grove, IL).

In vivo IL-17A neutralization

LEAF™ Purified anti-mouse IL-17A Antibody (TC11-18H10.1, Biolegend) was administered to mice by intraperitoneal injections at day 12, 14, 16, and 18 (200μg/mouse) after placing them into the controlled-environment chamber. We chose these time points for

injections because it is after we see a B cell response in dry eye mice (data not shown). Corneal fluorescein staining was performed daily to assess disease severity. At day 20, mice were euthanized, and draining lymph nodes were either frozen in OCT medium for immunohistological analysis or DLNs were analyzed using flow cytometric analysis.

B cell adoptive transfer

B6.129S7-Rag1tm1Mom/J (RAG−/−) mice received 5×10^6 B cells intravenously before placing them in the controlled environmental chamber. Corneal fluorescein staining was performed daily to assess disease severity. On day 7 after transfer, mice were euthanized. Blood was drawn by cardiac puncture and draining lymph nodes were either frozen in OCT medium for immunohistological analysis or DLNs were analyzed using flow cytometric analysis.

Results

Th17 cells are more effective than Th1 cells in inducing B cell proliferation in a contact-dependent fashion

To determine the effect of T cells on the proliferation of B cells, purified B cells from DED mice were co-cultured with either naïve T cells or DED T cells. We assessed the proliferation of B cells by Ki67 (Fig. 1 A) and CFSE staining (Fig. S1). DED T effector cells significantly enhanced B cell proliferation compared to naïve T effector cells (P=0.028; Fig. 1 A). Next, we investigated whether B cell proliferation is T cell contact-dependent, and found that IgM-stimulated B cells proliferated only when in cell-to-cell contact with T cells, but not when cultured in transwells (Fig. 1 B). To determine which subset (Th1 or Th17) principally provides B cell help, IFN-γ - or IL-17-depleted T effector (CD4+CD25−) cells were cocultured with B cells. Neither IFN-γ- nor IL-17-depleted T effector cells were as efficient as total T effector cells in inducing B cell proliferation *in vitro* (P= 0.002; total Teff vs. IFN-γ-depleted Teff, P=0.0004; total Teff vs. IL-17-depleted Teff). However, B cell proliferation was significantly more dependent on IL-17+ than on IFN-γ+ T effector cells (P= 0.0142; Fig. 1 C), suggesting that Th17 cells are more functionally relevant in triggering B cell than Th1 cells.

IL-17 directly induces B cell proliferation

To determine whether the T cell-derived soluble factor IL-17 has a direct effect on B cell proliferation, CD3-stimulated T effector cells from DED mice were cocultured with IgM/CD40-stimulated B cells using a transwell insert with or without neutralizing anti-IL-17A antibody. B cell proliferation was significantly reduced after blocking IL-17 signaling *in vitro* (P=0.002; Fig. 2 A). To verify the direct effect of IL-17A on B cell proliferation, we induced purified B cell proliferation with anti-IgM and anti-CD40 alone or in addition with recombinant IL-17A. Addition of recombinant IL-17A increased proliferation of anti-IgM and anti-CD40-stimulated B cells from DED mice, but not in B cells from naïve mice (Fig. 2 B). We also show that IL-17 can only increase B cell proliferation when B cells are stimulated with both anti-IgM and anti-CD40 (Fig. S2), suggesting that IL-17 enhances the stimulatory effects of anti-IgM and anti-CD40 resulting in increased proliferation.

To assess the effect of crucial T helper cell cytokines on B cell proliferation, we cultured naïve and DED B cells with anti-IgM and anti-CD40 in the presence or absence of IL-17, IFN-γ and IL-4. Only IL-17 and IFN-γ significantly increased B cell proliferation, whereas IL-4 had no effect on B cell proliferation. Culturing B cells with both IL-17 and IFN-γ had no additional effect on their proliferation compared to IL-17 alone (Fig. S3).

Activation of B cells leads to increased IL-17R expression

To confirm that increased B cell proliferation upon IL-17A administration was due to its ligation of its receptor on DED B cells, we first determined the expression of IL-17 receptor A (IL-17RA) in the lymph nodes (LNs) of naïve and DED mice using immunohistochemistry and flow cytometry (Fig. 3). Immunohistochemistry revealed that more IL-17RA+ cells localized in the B cell zone in DED mice compared to naïve mice, and that these cells coexpressed IgM+ and Ki67+ in the B cell follicle (Fig. 3 A). To determine whether CD40 stimulation is necessary for the expression of IL-17RA on B cells, purified B cells were cultured with medium alone or with anti-IgM, with anti-IgM/anti-CD40, or anti-IgM/anti-CD40/IL-17A. We found that IL-17R is upregulated on naïve and DED B cells after in vitro stimulation with anti-IgM and anti-CD40; however, addition of IL-17A does not further induce IL-17R in these B cells (Fig. 3 B).

B cells from autoimmune DED mice affect ocular surface damage in immune deficient mice

To determine the pathogenicity of B cells from DED mice, we adoptively transferred B cells from naïve and DED mice to immune deficient RAG knock-out mice and challenged these mice with desiccating environmental stress. To determine disease severity, we measured corneal epithelial integrity using corneal fluorescein staining. A schematic diagram of the experimental design is shown in Fig. 4 A. RAG knock out mice that had received DED B cells showed a significantly increased disease score compared to RAG knock-out mice that had received naïve B cells (P= <0.001; Fig. 4 B) suggesting that the DED B cells is a dominant pathogenic factor.

IL-17A signaling is required for GC formation and isotype-class switching

To determine whether IL-17A signaling is necessary for GC formation and isotype-class switching in DED mice, we blocked IL-17A signaling in vivo. Because GC formation and isotype class switching usually occurs between day 14 and 21 after DED induction (unpublished data), we injected an anti-IL-17A antibody from day 12 to day 18, and then analyzed GC formation and isotype class switching. A schematic diagram of the experimental design is shown in Fig. 5 A. The clinical disease score significantly decreased after injecting anti-IL-17A antibody (P= <0.001; Fig. 5 B). On day 20, we observed an increase in the frequencies of GC B cells (GL7+FAS+B220+) in DED mice compared to naïve mice (P=0.0004), which was IL-17 dependent (Fig. 5 C). The frequencies of $IgM^{-}IgD^{-}$ isotype-switched B cells or antigen-experienced memory B cells also increased on day 20 after DED induction compared to naïve mice (P=0.0007; Fig. 5 D). Blockade of IL-17 signaling significantly decreased B cell class switching compared to untreated (P= <0.0001) and naïve mice (P=0.0059; Fig. 5 D). Thus, these data clearly demonstrate that neutralizing IL-17 decreases the frequency of GC B cells and prevents B cell class switching.

IL-17 acts directly on B cells to induce B cell differentiation

To analyze whether IL-17 or IFN-γ neutralization inhibits B cell class switching directly or indirectly, we treated purified IgM-stimulated B cells with recombinant IL-17 or IFN-γ. Both IL-17 and IFN-γ significantly increased B cell class switching comparable (P=0.0007; B+ IgM vs. B+ IgM/IFN-γ, P=0.0002; B+ IgM vs. B+ IgM/IL-17, Fig. 6A). Next, we analyzed the effect of IL-17 and IFN-γ on the expression of syndecan-1, a plasma cell marker. Before cytokine stimulation only very few $IgM^{-}IgD^{-}B220+$ B cells expressed syndecan-1. Addition of IL-17 and IFN-γ significantly increased the frequencies of plasma cells compared to B cells stimulated with IgM only (P=0.0024; B+ IgM vs. B+ IgM/IL-17, P=0.023; B+ IgM vs. B+ IgM/IFN-γ, Fig. 6 B), the effect was more pronounced in the IL-17-treated group (P=0.023; B+ IgM/IFN-γ vs. B+ IgM/IL-17, Fig. 6. B). These data indicate that IL-17 and IFN-γ directly induces both isotype class switching and plasma cell differentiation in vitro.

Discussion

Our study provides novel evidence supporting a critical role for IL-17 in humoral immune responses; we found that IL-17 directly, and more effectively than IFN-γ, promotes B cell proliferation and induces isotype class switching and plasma cell differentiation in an experimental model of ocular surface autoimmunity.

Proinflammatory Th17 cells cause chronic inflammation and tissue damage predominantly by secreting IL-17 (1). IL-17 binds to its receptor on epithelial, endothelial and stromal cells where it induces expression of other proinflammatory mediators, such as TNFα, IL-1, G-CSF, and various chemokines (18). Limited studies on Th17 cells have shown that they can provide B cell help via the secretion of IL-21, a cytokine known to promote B cell activation (8, 19, 20). However, the role of IL-17 alone on B cell activation has not been elucidated (8, 9, 21). Our data show that T effector cells isolated from mice with dry eye disease induce B cell proliferation in vitro and IFN-γ-depleted T effector cells are significantly more effective in inducing B cell proliferation than IL-17-depleted T effectors. We further show that B cells cultured in the presence of IL-17 display a significantly increased proliferation. In addition, neutralizing IL-17 in an *in vitro* coculture with dry eye-specific B cells and T effector cells significantly reduces B cell proliferation. A previous study using autoimmune BXD2 mice has reported that IL-17 does not induce B cell proliferation (9). This lack of consistency may be due to different stimulation protocols, where other investigators have stimulated B cells with anti-IgM or anti-CD40 or LPS in addition to IL-17, which may not be sufficient to alter the kinetics of B cell proliferation. However, this may not reflect the physiological process, where B cell receptor (BCR) cross linking followed by CD40-CD40L interaction is necessary for efficient B cell activation. Thus, our data demonstrate that only after encountering both BCR and secondary T cell signal at the same time, IL-17 can act as a co-stimulatory signal to further enhance B cell proliferation suggesting that a stable antigen-dependent T- B cell interaction is crucial to boost the effect of IL-17A on B cell proliferation. Complimenting this finding, IL-17 receptor (IL-17R) expression increases after IgM/CD40 stimulation and only DED B cells respond to IL-17A stimulation and enhance their proliferation. Consistent with a previous study in BXD2 mice, IL-17RA+ B

cells are increased in GCs of LNs of autoimmune mice, whereas few IL-17RA+ B cells are seen in naive C57BL/6 mice (9).

Recently, it has been shown that passive transfer of IgG autoantibodies from dry eye mice facilitates inflammation and tissue damage in nude recipient mice (17). In accord, our data show increased disease severity after adoptive transfer of dry eye B cells indicating the relevance of B cells in the pathogenesis of ocular surface autoimmunity. B cell pathogenicity involves different cellular functions, including the well-established secretion of autoantibodies. Although the role of IL-17 on autoantibody production has been documented in several autoimmune models (8, 9, 21), the ability of IL-17 to promote B cell differentiation to memory B cells and plasma cells has remained unknown. In our autoimmune model, we found that IL-17 blockade significantly decreases disease severity as well as GC formation and B cell class switching. Previously, Hsu et al. have shown that germinal center formation is impaired in IL-17R−/− mice on a BXD2 mice (9). Because IL-17RA binds also IL-17F and IL-25 (22), impaired GC formation in BXD2 mice may not be solely due to IL-17A. In our study we blocked IL-17A in vivo, and thus providing direct evidence that GC formation is dependent on IL-17A signaling in ocular autoimmunity. Reduced B cell isotype class switching upon blockade of IL-17A could be direct, or a consequence of decreased GC B cells. Our data suggests that IL-17A does indeed have a direct effect on B cell differentiation since we used purified B cells for the in vitro experiments.

The role of Th1-derived IFN-γ to promote B cell differentiation and to increase secretion of IgG autoantibodies is well known (3, 6). Addition of IL-17 and IFN-γ to IgM-stimulated B cells in vitro enhances B cell class switching to a comparable degree, while addition of IL-17 seems to be more prominent than IFN-γ in enhancing plasma cell differentiation, suggesting that in DED Th17 cell are likely to enhance B cell proliferation and plasma cell development due to their vicinity of B cells and developing plasmablasts in vivo (Fig. 3A). Our data demonstrate that IL-17 directly induces antigen-experienced memory B cell and plasma cell differentiation without secondary T cell signals, suggesting that IL-17 can induce rapid B cell activation upon re-exposure to the same antigen, resulting in an accelerated B cell response. Further investigations are necessary to elucidate the precise mechanisms underlying the effect of IL-17 on B cell maturation and plasma cell differentiation.

In conclusion, this study provides compelling new evidence indicating the critical role of IL-17 in promoting B cell differentiation and plasma cell generation providing further evidence for the previously undefined role of this cytokine in B cell biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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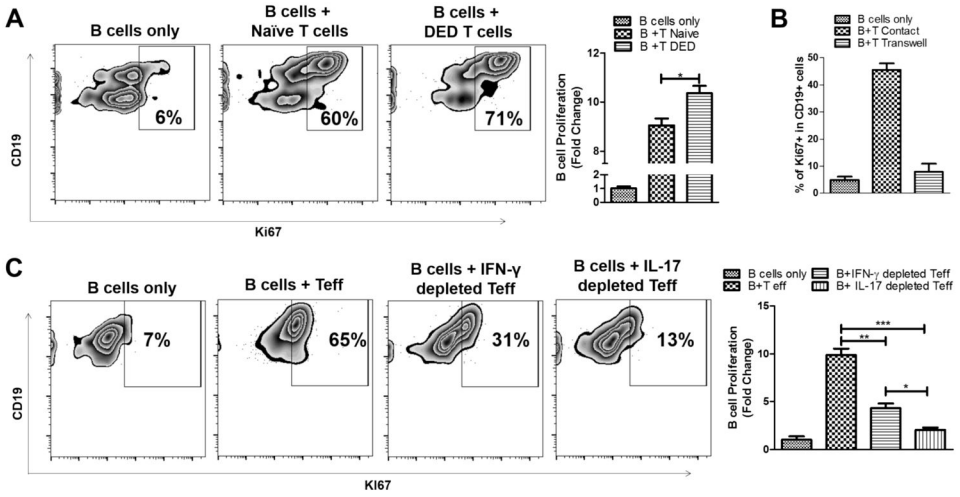

Figure 1. Th17 cells are more effective than Th1 cells to induce B cell proliferation in a contact dependent fashion

(A) B cells from the draining lymph nodes (LNs) of dry eye (DED) mice were stimulated with anti-IgM alone or cocultured with anti-CD3 stimulated purified (CD4+CD25−) T effector cells from DED or naïve mice for 48 hrs. The frequencies of proliferating $CD19^+$ B cells were determined by Ki67 staining using flow cytometry (mean± SEM; n=4 mice/group; triplicate cultures). (B) Flow cytometric analysis of Ki67 staining of purified $CD19^+$ B cells from DED mice that were stimulated with anti-IgM alone or cocultured with T cells directly or in transwell inserts with 0.4-μm pores (mean± SEM; n=4 mice/group; triplicate cultures). (C) CD3-stimulated (CD4+CD25−) T effector cell (Teff), IL-17–depleted Teff cells, or IFN-γ–depleted Teff cells were cocultured with IgM-stimulated B cells for 48 hrs. B cell proliferation was assessed with KI67 staining using flow cytomtery. (mean ± SEM; n=6 mice/group; quadruplicate cultures). B cell proliferation is shown as fold change (B cell only [B cell+ anti-IgM] = 1). Data are representative of at least two or more independent experiments. Student's t test. *, $P<0.05$; **, $P<0.01$.

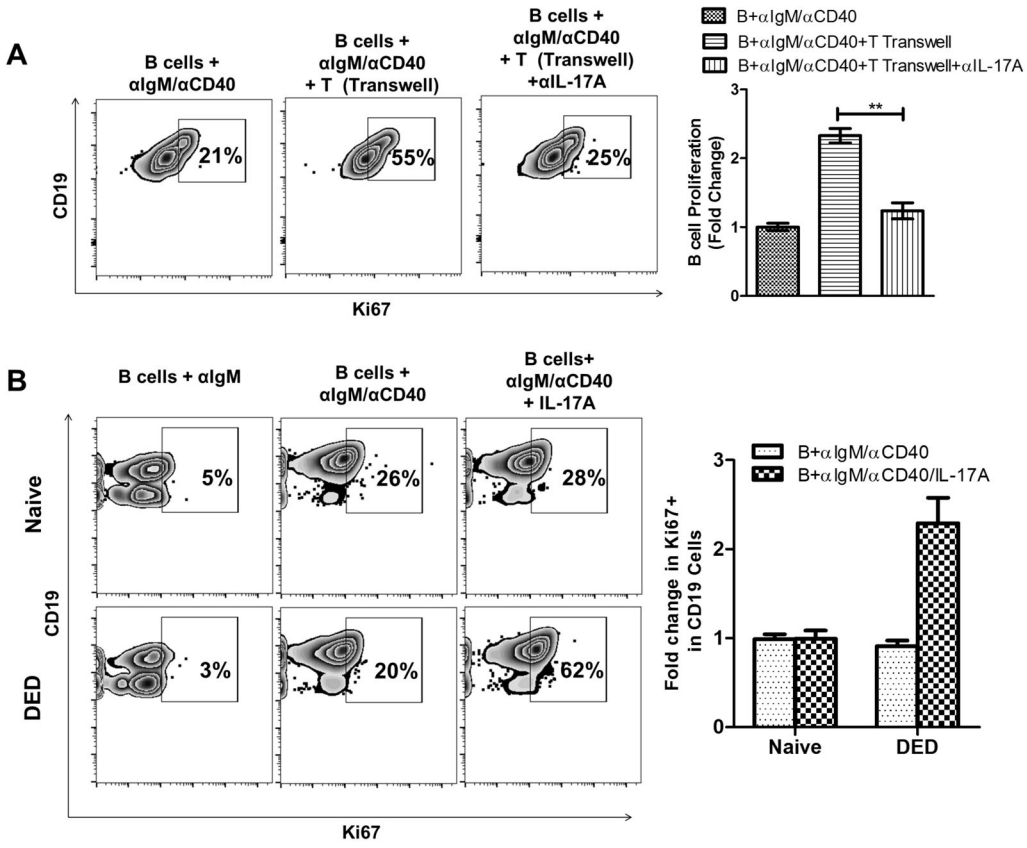

Figure 2. B cell proliferation induced by IgM/CD40 stimulation is enhanced by co-stimulation with IL-17 only in autoimmune B cells

(A) anti-IgM/anti-CD40–stimulated B cells were cultured at the bottom of the transwell, and anti-CD3-stimulated T effector cells were cultured in the insert (top) in the presence or absence of anti-IL-17A antibody. Flow cytometric analysis showing Ki67 staining of purified B cells from dry eye (DED) mice. (B) Naive or DED B cells were stimulated with anti-IgM/anti-CD40 and with or without recombinant IL-17A. Flow cytometric analysis showing Ki67 staining of purified B cells from DED and naïve mice after 48 hrs stimulation. B cell proliferation is shown as fold change (B cell+ anti-IgM and anti-CD40 =1). All results are representative of three independent experiments. (mean ± SEM; n=4–6 mice/group; triplicate cultures). Student's t test **, P<0.01.

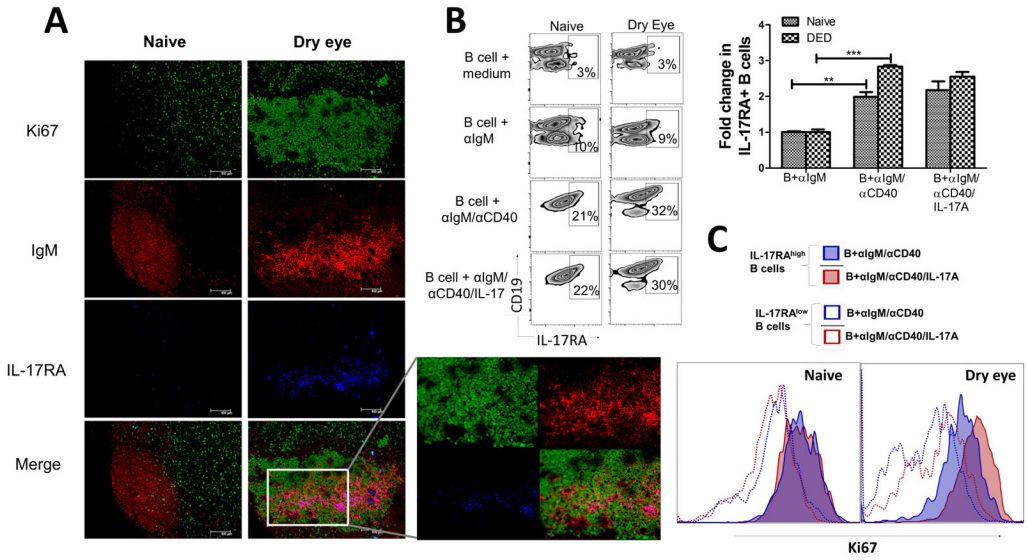

Figure 3. Activated B cells show enhanced IL-17 receptor expression

(A) Immunohistochemistry was performed on submandibular lymph nodes of naïve and dry eye (DED) mice (21 days after DED induction) to visualize Ki67 (proliferating cells, green), IgM (B cells, red), and IL-17RA (receptor expression, blue) expression. Data are representative of 4–6 mice examined. Images are 10x magnification and 20x magnification for magnified view of areas in white box. (B) Flow cytomteric analysis showing IL-17RA+ CD19+ cells after stimulating purified B cells with medium alone or in the presence or absence of anti-IgM, anti-CD40, and IL-17A for 48 hrs. (mean ± SEM; n=4 mice/group; triplicate cultures). Two-way ANOVA. **, P<0.01, ***, P<0.001.

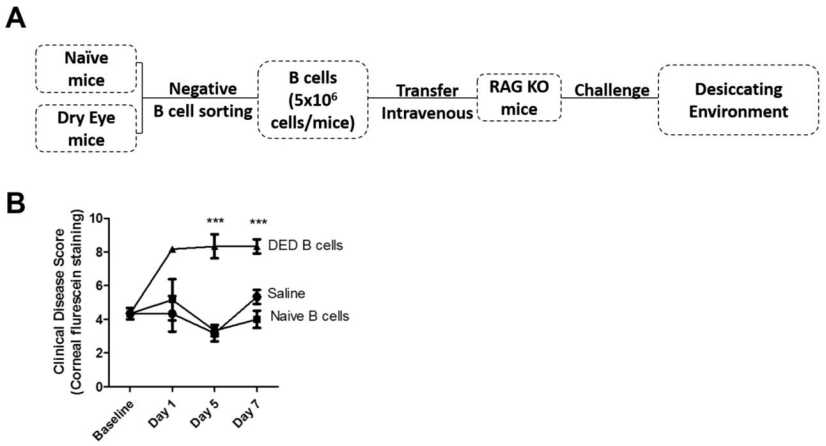

Figure 4. B cells from dry eye mice severely damage corneal epithelial integrity in immune deficient mice

(A) Schematic diagram showing the experimental design to study the effect of adoptive transfer of B cells into RAG knock-out mice. (B) Disease scores (Corneal fluorescein staining) after adoptive transfer of naïve B cells, dry eye (DED) B cells or saline from day 0 (baseline) till day 7 is shown (mean ± SEM; n=8 eyes/group). Two-way ANOVA. ***, $P<0.001$.

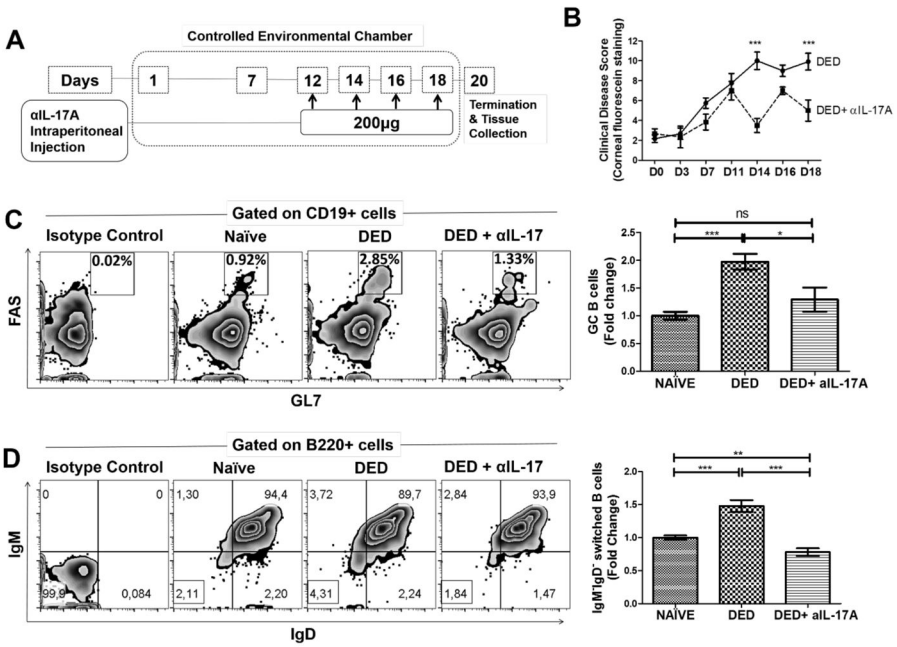

Figure 5. IL-17 blockade decreases germinal center formation and B cell class switching in dry eye mice

(A) Schematic diagram showing the experimental design to study the effect of anti-IL-17A treatment in dry eye (DED) mice. (B) Disease severity scores of DED mice with or without treatment of anti-IL-17A antibody (mean ± SEM; n=8 eyes/group). ***, P<0.001 (C) Flow cytometric analysis of germinal center (GL7+FAS+ B220+) B cells in the lymph nodes of naïve, DED, and anti-IL-17A treated mice. (D) Flow cytometric analysis of IgD and IgM expression on CD19+ B cells in the lymph nodes of naïve, DED, and anti-IL-17A treated mice. Data are representative of two independent experiments (mean ± SEM; n=4 mice/group; triplicate cultures). Two-way ANOVA (B), student's t test (C and D) *, P<0.05; **, P<0.01, ***, P<0.001, ns; not significant.

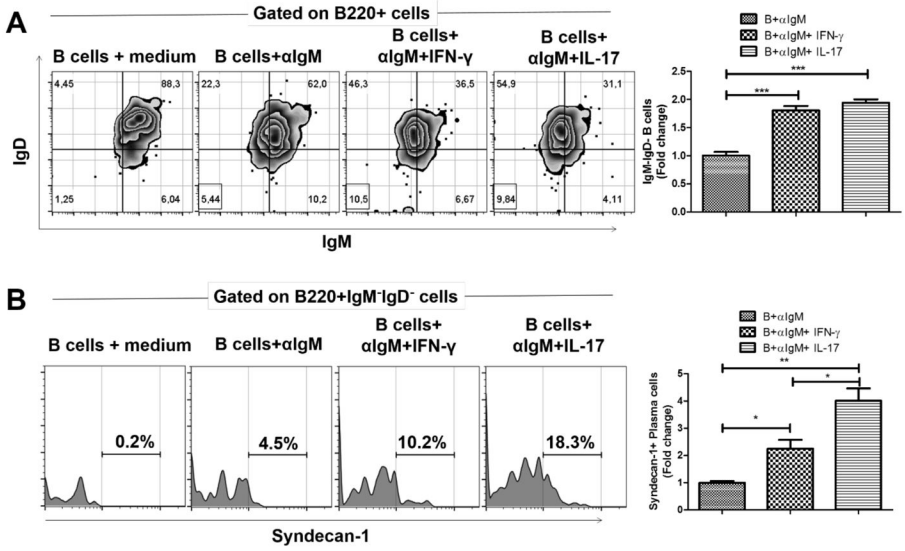

Figure 6. IL-17 directly promotes B cell class switching and plasma cell formation

(A) Purified B cells from dry eye (DED) mice were cultured in medium or with anti-IgM, IFN-γ, or IL-17 for 48 hrs and then analyzed for their IgM/IgD expression. (B) Flow cytometric analysis showing B cells cultured as described in (A) were analyzed for their expression of syndecan-1 (CD138, a plasma cell marker) (mean ± SEM; n=6 mice/group; triplicate cultures). Data are representative of two independent experiments. Student's t test *, P<0.05; **, P<0.01, ***, P<0.001.