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Disturbed Follicular Architecture in B Cell A Disintegrin and Metalloproteinase (ADAM)10 Knockouts Is Mediated by Compensatory Increases in ADAM17 and TNF-α Shedding

Lauren Folgosa,*† Hannah B. Zellner,† Mohey Eldin El Shikh,‡,1 and Daniel H. Conrad†,1

B cell A disintegrin and metalloproteinase 10 (ADAM10) is required for the development and maintenance of proper secondary lymphoid tissue architecture; however, the underlying mechanism remains unclear. In this study, we show disturbances in naive lymph node architecture from B cell–specific ADAM10-deficient mice (ADAM10B−/−) including loss of B lymphocyte/T lymphocyte compartmentalization, attenuation of follicular dendritic cell reticula, excessive collagen deposition, and increased high endothelial venule formation. Because TNF-α signaling is critical for secondary lymphoid tissue architecture, we examined compensatory changes in ADAM17 and TNF-α in ADAM10B−/− B cells. Surprisingly, defective follicular development in these mice was associated with increased rather than decreased TNF-α expression. In this article, we describe an increase in TNF-α message, mRNA stability, soluble protein release, and membrane expression in ADAM10B−/− B cells compared with wild type (WT), which coincides with increased ADAM17 message and protein. To assess the mechanistic contribution of excessive TNF-α to abnormal lymphoid architecture in ADAM10B−/− mice, we performed a bone marrow reconstitution study. Rectification of WT architecture was noted only in irradiated WT mice reconstituted with ADAM10B−/− + TNF knockout bone marrow because of normalization of TNF-α levels not seen in ADAM10B−/− alone. We conclude that ADAM17 overcompensation causes excessive TNF-α shedding and further upregulation of TNF-α expression, creating an aberrant signaling environment within B cell cortical regions of ADAM10B−/− lymph nodes, highlighting a key interplay between B cell ADAM10 and ADAM17 with respect to TNF-α homeostasis. The Journal of Immunology, 2013, 191: 000–000.

A disintegrin and metalloproteinases (ADAMs) are a family of zinc-dependent proteinases known to be involved in ectodomain cleavage and regulated intramembrane proteolysis of transmembrane proteins. Of all of the ADAMs, ADAM10 and ADAM17, commonly referred to as TNF-α converting enzyme, are known to be most closely related with regard to structure and share many overlapping substrate specificities (1, 2). Classically, ADAM17 is thought to orchestrate inflammatory responses as the principle, physiologic sheddase of pro-TNF-α; however, ADAM10 can also cleave membrane TNF-α (mTNF-α) when ADAM17 is not present (3). In addition, ADAM10 is crucial for functional and phenotypic maturation of the immune system.

We have shown it is critical in Notch2-mediated marginal zone B cell development and CD23-mediated regulation of allergic diseases (4, 5). Lastly, although we have previously reported that B cell ADAM10 is required for maintenance of proper secondary lymphoid tissue architecture, formation of germinal centers, as well as optimal class-switched Ab (Ig) production, the underlying mechanism was unclear (6).

TNF-α is a key proinflammatory cytokine, which exists as a 26-kDa transmembrane protein (mTNF-α) before it is shed from the surface as a 17-kDa soluble molecule (sTNF-α) (7). Tristetraprolin (TTP), also known as ZFP36, is a low-abundance cytosolic zinc finger protein induced by LPS and is critical for mRNA degradation of multiple mRNA targets including TNF-α (8, 9). TTP-deficient mouse models portray the downstream consequences of increased TNF-α mRNA stability including inflammatory arthritis, autoimmunity, and cachexia (10, 11). In addition, B cell–TNF-α has been implicated in the functional decline of aging B cells where increased TNF-α production is inversely correlated with response to stimulation in vitro by LPS. Interestingly, aging B cells additionally exhibit increased TTP, which causes reduced optimal class-switched Ab production by downregulating E47 and activation-induced cytidine deaminase. The paradoxical increase of both TTP and TNF-α in unstimulated B cells from old mice may reflect increased TNF-α transcription by these B cells to overcome elevated TTP, thus placing them in a preactivated state that is less susceptible to subsequent stimulation (12).

The role of TNF-α in maintaining proper secondary lymphoid tissue architecture is indisputable, and ADAM10 also seems to be involved in this maintenance. Both B cell–specific ADAM10−/− mice (ADAM10B−/−) and global TNF-α−/− deficient mice exhibit disorganized follicular dendritic cell (FDC) networks, aberrant germinal centers, and lack of splenic B cell follicles (13). Furthermore, using B cells that express a noncleavable form of

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Abbreviations used in this article: ADAM, A disintegrin and metalloproteinase; ADAM10B−/−, B cell–specific ADAM10-deficient mouse; BrT, B lymphocyte/ T lymphocyte; FDC, follicular dendritic cell; HEV, high endothelial venule; KO, knockout; LN, lymph node; LT, lymphotixin; MMP, matrix metalloproteinase; mTNF-α, membrane TNF-α; qPCR, real-time quantitative PCR; sTNF-α, soluble molecule; TTP, tristetraprolin; WT, wild type.

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mTNF-α showed that adequate levels of B cell–produced sTNF-α was critical for maintaining secondary architecture in the lymph node (LN), spleen, and Peyer’s patches, and for IgG production against T-dependent Ags (14). Although it is clear that regulation of B cell TNF-α is required for proper follicular architecture and B cell function, the role of TNF-α cleaving enzymes (ADAM10 and ADAM17) has yet to be explored.

In this article, we investigate the hypothesis that compensatory overexpression of ADAM17 after B cell–specific ADAM10 deletion mediates excessive TNF-α levels in ADAM10−/− mice, ultimately providing the mechanism underpinning the aberrant secondary lymphoid tissue architecture in these mice.

Materials and Methods

Mice

All mice were housed in the Virginia Commonwealth University Molecular Medicine Research Building Facility in accordance with institutional and National Institutes of Health guidelines. All animal care and experimental protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. C57BL/6 ADAM10−/− (CD19−/−) mice were generated as previously described and compared with littermate wild type (WT) controls (CD19−/−) (4). TNF-α knockout (TNF-α−/−) mice were purchased from Jackson Laboratory (no. 005540, B6.129S1-Tnfa−/−). B cells isolated by positive selection (B220+) using magnetic beads and following manufacturer’s protocol (Miltenyi). B cells were grown in complete RPMI 1640 medium containing 10% heat-inactivated (56°C, 30 min) FBS (Gemini Bio-Products, West Sacramento, CA), 2 mM l-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-ME, 1× nonessential amino acids, and 20 mM HEPES buffer (all from Invitrogen, Carlsbad, CA), and stimulated in vitro for 1, 3, or 5 d with 1000 U IL-4 and either 50 μg/ml LPS (LPSs from Escherichia coli 0111:B4; Sigma) or 1.25 μg/ml purified anti-mouse CD40 (no. 102902; Biologend). Proliferation was assessed after 72 h of growth, and a 24-h pulse of [3H]thymidine, 1 mCi/well (Perkin Elmer), was used. Plates were then harvested using a Filtermate cell harvester onto UniFilter-96 GF/C microplates (Perkin Elmer) and analyzed using Topcount Plate Counter (Perkin Elmer).

RT-PCR, quantitative PCR, and Western blotting

Total RNA was extracted from naive and stimulated total B cells using TRIzol reagent (Invitrogen) according to manufacturer’s protocol and RNA concentration quantified by a ND-100 NanoDrop spectrophotometer. RNA (400 ng/μl) was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR (qPCR) was performed with a real-time PCR machine (iQ5; Bio-Rad Laboratories). Primers and probes used for TaqMan qPCR assay (all from Applied Biosystems) were the following: TNF-α (Mm00443258), ADAM10 (Mm00545742), ADAM17 (Mm00456428), TTP/Zfp36 (Mm00457144), matrix metalloproteinase 13 (MMP13; Mm00439491), and 18s (Mm03928990). Fold variation was assessed after 72 h of growth, and a 24-h pulse of [3H]thymidine, 1 mCi/well (Perkin Elmer). Soluble TNF-α (CD19−/−) was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR (qPCR) was performed with a real-time PCR machine (iQ5; Bio-Rad Laboratories). Primers and probes used for TaqMan qPCR assay (all from Applied Biosystems) were the following: TNF-α (Mm00443258), ADAM10 (Mm00545742), ADAM17 (Mm00456428), TTP/Zfp36 (Mm00457144), matrix metalloproteinase 13 (MMP13; Mm00439491), and 18s (Mm03928990). Fold variation was determined using the ΔΔCt method of analysis (15).

Protein lysates were made according to the manufacturer’s protocol using Cell Lysis Buffer (no. 9803; Cell Signaling). Equal amounts of protein were loaded onto Novex NuPAGE 10% Bis-Tris gels (Invitrogen), run for 35 min at 200 V, transferred to nitrocellulose membrane, and equal transfer verified by Ponceau S (Sigma) staining. Blots were probed with primary anti-mouse Abs: anti-β-actin peroxidase (no. A3854; Sigma) or anti-ADAM17 (no. 20936; Molecular Probes). Goat anti-rabbit IgG, HRP conjugate secondary Ab was used for ADAM17 blots and signal detected with SuperSignal West Pico Chemiluminescent Substrate (no. 34080; Thermo Scientific).

Flow cytometry with tyramide signal amplification

Single-cell suspensions of naive or stimulated B cells were stained using tyramide signal amplification (TSA) Kit #26 with HRP streptavidin (no. T20936; Molecular Probes) and one of the following Ig: anti-mouse FITC-conjugated B220 or PE-conjugated B220 (Biolegend). Kit reagents were prepared according to manufacturer’s protocol, and tyramide amplification using the “Peroxidase Labeling assay” was performed with the following modifications: cells were incubated with blocking reagent (10 μg anti-mouse unlabeled CD16/32 [2.4G2]) for 15 min; stained with biotin anti-mouse TNF-α primary Ab (Biolegend); and after tyramide labeling, cells were washed twice and stained with anti-mouse B220 (see earlier) for 30 min and examined on a BD Canto Flow analyzer; data analysis was with FCS Express, v. 4.

Bone marrow reconstitution

Bone marrow cells were isolated as previously described with the following modifications (16). In brief, two femurs and two tibias from each mouse (WT [CD45.2], ADAM10−/− [CD45.2], or TNF-αKO) were centrifuged, RBCs lysed with ACK Lysing Buffer (Quality Biological), bone marrow cells counted, and 5 million cells were i.v. injected. For 50/50 mixtures, such as ADAM10−/− + TNFKO, 100 μl2.5 million cells from each were used to prepare the final injection mixture.

B6-Ly5.2/Cr (CD45.1) congenic mice from National Cancer Institute/National Institutes of Health were pretreated 5 d before irradiation with 100 mg/ml (concentration 0.01%) enrofloxacin (Baytril) in sterile water. CD45.1 mice were anesthetized using a 100 μl i.p. injection of ketamine/xylazine in PBS at a dose of 80 and 8 mg/kg, respectively. This was followed by two doses of 550 μg i.v. irradiation, separated by a 2-h rest period, using an MDS Nordion Gammaxcel 40 research irradiator with a [137Cs] source. After irradiation, mice were reconstituted by i.v. injection of the indicated bone marrow cells as described earlier. After 6 wk of reconstitution, mice were footpad immunized in two ipsilateral paws with 10 μg 4-hydroxy-3-nitrophenylacetyl coupled to keyhole limpet hemocyanin at a ratio of 27:1 (Biosearch Technologies) in 4 mg alum. Draining and

FIGURE 1. Naive ADAM10−/− LNs display abnormal follicular architecture. Compared with WT, ADAM10−/− mice (A) lack well-developed FDC reticula (red, CR1/2) in the B cell follicle (blue, B220), (B) lack B cell/T cell node (red, Thy1.2) segregation with more HEVs (green, peripheral node addressin [pNADI]) in the LN cortex, (C) display more collagen (green) deposition in the B cell follicle, and (D) express higher levels of TNF-α (green) than WT mice. Scale bar, 50 mm. Micrographs are representatives of at least three LNs.
nondraining popliteal and axillary LNs were dissected at 14 d postimmunization and analyzed by immunohistochemistry.

**Immunohistochemistry, tyramide amplification, and confocal microscopy**

Ten-micrometer-thick frozen sections were cut from the excised mouse LNs, fixed in absolute acetone, air-dried, and blocked with serum-free protein block (X0909; Dako). The sections were dual- and triple-labeled for FDCs (PE anti-mouse CD21/CD35; 123410; Biolegend), B cells (Alexa Fluor 647 anti-mouse/human CD45R/B220; 103226; Biolegend), high endothelial venules (HEVs; anti-mouse/human peripheral node addressin; 120804; Biolegend), T cells (rat anti-mouse CD90.2/Thy-1.2-PE; 1750-09L; Southern Biotech), collagen type 1 (ab21286; Abcam), and TNF-\(\alpha\) (ab34674; Abcam). The Ig concentrations ranged between 5 and 10 mg/ml.

Sections were mounted with antifade mounting medium, Vectashield (Vector Laboratories), coverslipped, and examined with a Leica TCS-SP2 AOBS confocal laser-scanning microscope. Three lasers were used: argon (488 nm), HeNe (543 nm), and HeNe (633 nm; far red emission is shown as pseudoblue). Parameters were adjusted to scan at 1024 \(\times\) 1024 pixel density and 8-bit pixel depth. Emissions were recorded in two or three separate channels, and digital images were captured and processed with Leica Confocal, LCS Lite software, and ImageJ for color separation and quantitative assessment of immunohistochemistry.

TNF-\(\alpha\) labeling was enhanced using fluorescein-TSA (TSA Plus Fluorescein System; NEL74001KT; Perkin Elmer). In brief, after quenching endogenous peroxidase using 1% \(\text{H}_2\text{O}_2\), anti-TNF-\(\alpha\) Ig was applied for 2 h, washed, then HRP-conjugated secondary Ig was added for 1 h. After washing, HRP was allowed to catalyze the deposition of fluorescein-labeled tyramide for 10 min, then was washed, mounted, and examined.

**Statistical analysis**

The \(p\) values were calculated using unpaired two-tailed Student \(t\) tests in GraphPad Prism. Error bars represent the SEM between samples. A \(p\) value \(<0.05\) is considered significant.

**Results**

C57BL/6 ADAM10\(^{−/−}\) mice exhibit abnormal LN architecture and excessive TNF-\(\alpha\) in B cell regions

Our laboratory previously described secondary lymphoid tissue architectural defects in immunized and naive LNs isolated from C57BL/6 ADAM10\(^{−/−}\) mice. These defects included improper localization of B and T cells, reduced germinal center formation, and a decrease in FDC networks (6). These architecture aberrancies bore some similarity with secondary lymphoid tissue defects.

**FIGURE 2.** Increased TNF-\(\alpha\) surface expression and production in ADAM10\(^{−/−}\) B cells (A10KO). Naive (A) and 5-d-stimulated with LPS + IL-4 (B). WT (black thin line) and A10KO (black bold line) live B cells were analyzed for coexpression of TNF-\(\alpha\) using TSA. Black bars (overlay plots) indicate B cells staining high in mTNF-\(\alpha\), represented in bar graphs (A, B). \(n = 9\) per group, 3 independent studies. (C and D) Supernatants were harvested on day 1, 3, or 5 from WT (white) or A10KO (black) B cell cultures stimulated with LPS + IL-4 (C) or anti-CD40 + IL-4 (D) and sTNF-\(\alpha\) determined by ELISA. \(n = 9\) per group, three independent studies. *\(p < 0.05\), **\(p < 0.005\).
noted in early studies of global TNF-α-deficient mice. Specifically, lack of splenic B cell follicles, disorganized FDC networks, and aberrant germinal centers were noted (13). Recent studies using B cells only capable of expressing mTNF-α showed that sTNF-α produced by B cells is required for maintaining secondary architecture in LN, spleen, Peyer’s patches, and for IgG production against T-dependent Ags (14). Taken together, ADAM10 and TNF-α seem to reciprocally interact to maintain proper secondary lymphoid architecture and permit class-switched Ab production. Fig. 1 shows immunohistochemistry analysis of LNs from naive (nonimmunized) WT and ADAM10B−/− mice. Although our initial studies had indicated a relatively normal architecture in naive ADAM10B−/− nodes (6), additional analysis revealed that not only are LN FDC networks largely absent and B lymphocyte/T lymphocyte (B/T) boundaries intermingled (Fig. 1A, 1B), but other abnormalities can also be seen. These changes include excessive collagen deposition, as well as an increase in HEVs, especially within B cell cortical regions (Fig. 1B, 1C). Most striking, however, was the dramatic increase in TNF-α within the B cell regions of ADAM10B−/− nodes (Fig. 1D). Given the data in Fig. 1 and previous reports of secondary lymphoid tissue architecture abnormalities in TNF-α KO mice and those capable of expressing only mTNF-α, it appears that not only subnormal but also excessive TNF-α levels in the LN cortices may lead to disruption of normal follicular architecture.

**B cells from C57BL/6 ADAM10B−/− mice exhibit increased expression, stability, and production of TNF-α**

Given the excessive TNF-α staining in B cell regions of C57BL/6 ADAM10B−/− LNs (Fig. 1), we further analyzed differences in TNF-α expression and production in B cells purified from both ADAM10B−/− and WT mice as well. As can be seen in Fig. 2A and 2B, flow analysis for mTNF-α revealed that both naive and stimulated ADAM10B−/− B cells exhibit increased mTNF-α. Furthermore, ELISA analysis of supernatants from ADAM10B−/− B cells cultured with LPS/IL-4 (Fig. 2C) or anti-CD40/IL-4 (Fig. 2D) for 1, 3, or 5 d all showed significantly higher sTNF-α production compared with WT.

Relative (Fig. 3A) and absolute (Fig. 3B) message analysis by qPCR indicated a strong increase in TNF-α message in ADAM10B−/− compared with WT B cells poststimulation in vitro. Furthermore, TTP is known to promote TNF-α mRNA degradation (8, 9). We, therefore, analyzed TTP message expression in both WT and ADAM10B−/− B cells and found it to be 2-fold higher in WT B cells, which also produce less sTNF-α and mTNF-α (Figs. 2C, 2D). The combined results of increased TNF-α message and soluble protein suggest a possible feedback mechanism in which increased TNF-α shedding upregulates further TNF-α production. In any case, it is clear that TNF-α is increased at both the message and protein level in ADAM10B−/− B cells.

**ADAM10B−/− B cells exhibit higher ADAM17 gene and protein expression**

ADAM17 is known to be the principle sheddase of the membrane-expressed pro–TNF-α. Because ADAM10B−/− B cells exhibit higher expression and production of TNF-α, we next examined ADAM17 expression and function by analyzing ADAM17 message (Fig. 4A, 4B) and protein levels (Fig. 4C, 4D) in WT compared with ADAM10B−/− B cells. Although both naive and stimulated ADAM10B−/− B cells express significantly more ADAM17 message (Fig. 4A, 4B), relative gene expression analysis showed that in the naive state, ADAM10B−/− B cells express two times higher ADAM17 compared with WT, which increased to 4-fold higher expression upon stimulation (Fig. 4A). Similarly, absolute RNA quantification revealed that naive ADAM10B−/− B cells exhibit significantly increased ADAM17 RNA expression, which increases further upon stimulation (Fig. 4B). Western blot analysis, furthermore, showed a 2.3-fold increase in ADAM17 protein levels in naive ADAM10B−/− B cells over WT, which also increased upon stimulation to 5-fold (Fig. 4C, 4D). Upon overexposure of the blots, both the precursor and glycosylated forms of ADAM17 were seen (data not shown).

In addition, Vandenbroucke et al. (17) recently established that MMP13 also cleaves TNF-α at least in intestinal epithelium. qPCR analysis of MMP13 in naive B cells, however, showed there was no difference between WT and ADAM10B−/− B cells (ΔΔCt = 1.04; fold change ADAM10B−/− over WT). Western blot, furthermore, failed to show MMP13 protein in naive WT and ADAM10B−/− B cells compared with positive control, RAW 264.7 macrophages (data not shown). This finding is in agreement with the report that significant levels of MMP13 are not found in B cells (18). Taken together, Figs. 1–4 demonstrate that ADAM10 deletion from B cells results in a compensatory increase in ADAM17 expression and activity, leading to excessive TNF-α cleavage. The aberrant signaling environment created by this compensatory effect is an excellent candidate to explain the abnormal lymphoid architecture in ADAM10 KO mice.
tissue architecture in our ADAM10^−/− model and was thus further explored.

Reconstitution of irradiated C57BL/6 WT with combination ADAM10^−/− + TNFKO bone marrow rectifies LN follicular abnormalities

To assess whether TNF-α is involved in the mechanism underlying LN tissue abnormalities in ADAM10^−/− mice, we performed a bone marrow chimera experiment in which irradiated CD45.1 WT C57BL/6 mice were reconstituted with one of the following bone marrow combinations: 1) WT (CD45.2) alone; 2) ADAM10^−/− (CD45.2) alone; 3) TNF-α-deficient (TNFKO) alone; 4) 50/50 mix ADAM10^−/− + WT (CD45.1); or 5) 50/50 mix ADAM10^−/− + TNFKO. After 6 wk of reconstitution, mice were bled and analyzed for successful reconstitution, and CD45.2 cells predominated (Supplemental Fig. 1). The mice were then footpad immunized with a T-dependent Ag, 4-hydroxy-3 nitrophenylacetyl coupled to keyhole limpet hemocyanin, and draining LNs were assessed by immunohistochemistry 14 d postimmunization (Fig. 5). As expected, LNs from WT mice reconstituted with WT (CD45.2) bone marrow exhibited normal LN architecture (Fig. 5D) and TNF-α levels (Fig. 5G), which was comparable with nonirradiated WT nodes (Fig. 5A, 5G). Those reconstituted with ADAM10^−/− bone marrow (Fig. 5B, 5G), however, had a similar phenotype to those of our ADAM10^−/− mice (Fig. 1); loss of B cell/T cell segregation, decreased FDC networks, and increased TNF-α. Those reconstituted with TNFKO bone marrow alone (Fig. 5C) exhibit no TNF-α staining but do still demonstrate FDC networks because these are resistant to irradiation. Furthermore, reconstitution with 50/50 mix ADAM10^−/− + WT CD45.1 still yielded abnormal LN architecture (Fig. 5E) and high TNF-α staining (Fig. 5G) similar to ADAM10^−/− mice. Thus, the amount of TNF-α made by B cells from this combination is still too high. Interestingly, when WT mice were reconstituted with combination ADAM10^−/− + TNFKO bone marrow (Fig. 5F, 5G), LN tissue architecture and TNF-α staining returned to WT levels. TNF-α staining is further compared and quantified by mean gray values in multiple equal areas (at least six) in Fig. 5H. Mice reconstituted with ADAM10^−/− alone as well as ADAM10^−/− + WT CD45.1 exhibit significantly more TNF-α staining compared with WT and ADAM10^−/− + TNFKO (Fig. 5H). We reason that although B cells from ADAM10^−/− mice produce excessive TNF-α, those from the TNFKO mice produce none, thus averaging to a normal, WT range allowing proper TNF-α signaling to occur.

Discussion

This study provides evidence that B cell TNF-α and lymphoid tissue architecture are regulated by the orchestrated interplay between ADAM10 and ADAM17. Mezyk-Kopeć et al. (3) previously described, in mouse embryonic fibroblasts, a compensatory relationship between ADAM10 and ADAM17 with regard to TNF-α cleavage. In that study, ADAM10-deficient mouse embryonic fibroblasts still exhibited TNF-α cleavage because of a compensatory increase in ADAM10 (3). In this study, we show, in a B cell–targeted model, that in the absence of B cell ADAM10, ADAM17 is overexpressed more than WT levels (Fig. 4). This compensatory change in B cell ADAM17 results in increased TNF-α shedding, gene expression, mRNA stability, and surface expression, and thus skews the cytokine environment within B cell follicles (Figs. 2, 3). Although the absence of B cell sTNF-α is known to cause LN architecture defects, we demonstrate that excessive sTNF-α also dramatically disrupts LN follicular architecture (14).

In addition to B cells, T cells, FDCs, and tingible body macrophages contribute to TNF-α expression in secondary lymphoid tissues (19–21). Cooperation between these sources is needed to maintain TNF-α homeostasis, which is essential for lymphoid tissue architectural maintenance and ultimately B cell humoral responses. Tumanov et al. (14) clearly demonstrated this concept, showing impaired LN organization and B cell Ab production postimmunization when either B or T cell TNF-α was inhibited.

FIGURE 4. Increased ADAM17 message and protein levels in ADAM10^−/− B cells (A10KO). Naïve [(A) checkerboard, (B) left, (C) center] or 5-d-stimulated with LPS + IL-4 [(A) stripe, (B) right, (D) center] B cells were analyzed by qPCR and Western blotting. [(A) Relative ADAM17 expression normalized to 18s presented as fold change of A10KO over WT using the ΔΔCt method of analysis. (B) Absolute quantification of ADAM17 RNA normalized to 18s. n = 9 mice per group, 3 independent studies. Band densitometry of naive (C) and stimulated (D) B cells represents ADAM17 (∼93 kDa, mature form) normalized to actin (∼42 kDa). n = 6 per group total, four independent studies. *p < 0.05, **p < 0.005, ***p < 0.0005.
Furthermore, therapeutic inhibition of TNF-α with etanercept inhibited the maintenance of FDC networks, resulting in decreased germinal center responses and Ab production (22). Although insufficient TNF-α levels lend to disorganization and stunted B cell function, an example of pathologic overexpression of B cell TNF-α is seen in B cell chronic lymphocytic leukemia. Leukemic B cells overexpress TNF-α, which then acts in an autocrine fashion to upregulate its own mRNA and protein expression (23). Enhanced leukemic cell TNF-α signaling leads to upregulation of MMP9, a key protease involved in proangiogenic pathways needed for cancer metastasis (24).

Possible candidates responsible for defects characterizing ADAM10<sup>b<sup>−/−</sup> LNs are TNF-α and lymphotoxin (LTα, LTβ). These structurally homologous and genetically linked cytokines have been studied individually and as double-deficient mouse models, in an attempt to tease out the contributions of each to secondary lymphoid tissue microarchitecture development and maintenance (25). LTα-deficient mice lack LNs and Peyer’s patches, and exhibit abnormal splenic architecture including loss of B/T segregation and a complete absence of FDC networks, germinal center formation, and marginal zone B cells (26, 27). LTα as a soluble homotrimer (LTα3) is also known to play an integral role in
lymphoid organization including B/T segregation by binding to the TNF-α receptor, p55TNFR-1, suggesting that blockade of this interaction would result in a similar phenotype to ADAM10-deficient LNs (28). LTβ-deficient mice, however, experience more mild disruption as they retain mesenteric and cervical LN development and maintain B/T segregation, FDC networks, and germinal center formation in spleen (29). More similar to LTα-deficient mice, TNF-α KOs lack FDC networks in B cell follicles and fail to form germinal centers postimmunization (13, 30, 31). Furthermore, it is known that without TNF-α or its receptor p55TNFR-1, B cell follicles and FDC networks fail to form in peripheral LNs, Peyer’s patches, and spleen; however, the effects of B cell TNF-α overexpression are unreported (13, 32).

Because p55TNFR-1 binds both TNF-α and LTα3, it is reasonable to conclude that excessive TNF-α could outcompete LTα3 for binding to this receptor, resulting in noted defects in B/T segregation. Furthermore, it has been shown that local cytokine factors such as TNF-α contribute to HEV neogenesis (33). Excessive B cell TNF-α could, therefore, explain the induction of increased cortical HEV neogenesis resulting in increased T cell recruitment via CCL21/CCR7 interactions and ultimate aberrations in B/T segregation (Fig. 1). Alternatively, Blobel et al. (34) recently demonstrated that a conditional KO model where ADAM17 was selectively deleted from endothelial cells and pericytes had significantly inhibited pathologic neovascularization. Therefore, ADAM17 overexpression by ADAM10-deficient B cells may be directly involved in increased follicular HEVs.

In addition, we reason that defective follicular remodeling resulted in excessive collagen deposition associated with the lack of FDC reticula. Bajenoff and Germain (35) elegantly showed that FDC development replaces fibroblastic reticular cells during B cell follicle maturation. Therefore, lack of FDC development in ADAM10-deficient follicles could have resulted in the persistence of collagen-producing fibroblasts. Furthermore, in a Notch-dependent pathway, ADAM17 overactivation has been implicated in fibroblast activation, excessive collagen formation, and fibrosis (36). Increased ADAM17 in ADAM10-deficient B cells could lead to aberrant Notch signaling and increased fibrosis within the LN (Fig. 1). Given the profibrotic properties of ADAM17, our result that ADAM17 overcompensates for ADAM10 deficiency must be well considered before attempting ADAM10 therapeutic neutralization.

The most conclusive data furthering the mechanistic contribution of TNF-α over other ligands, however, is the rectification of WT architecture in irradiated WT mice reconstituted with ADAM10-deficient TNFKO bone marrow (Fig. 5F, 5G). Compared with those reconstituted with ADAM10-deficient bone marrow alone (Fig. 5B, 5G) or a 50/50 mix of ADAM10-deficient and WT CD45.1+ (Fig. 5E, 5G), these produce an appropriate level of TNF-α considering ADAM10 deficiency. B cells make too much and TNFKO B cells make none, thus further supporting the mechanistic contribution of TNF-α. With the combination ADAM10+/- + TNFKO model, it is important to note that all cells in the TNFKO do not make TNF-α, which may contribute to the effect seen.

To conclude, this study first reveals compensatory changes in B cell Adam17 in the absence of Adam10. This finding has substantial implications in therapeutic design where specific targeting of one ADAM may lead to changes in other closely related ADAMs. In this scenario, compensation by ADAM17 would lead to excessive cleavage of TNF-α or other ligands resulting in foreseeable albeit undesirable side effects. It is important to study the interaction between ADAM10 and ADAM17 on other cell types to further elucidate other potential complications. Second, in pathologic conditions where TNF-α is directly implicated in pathology such as insulin resistance and rheumatoid arthritis, this study suggests that ADAM10 and ADAM17 levels should be monitored as a direct indication of disease susceptibility and severity. Lastly, in this article, we demonstrate that compensatory increases in ADAM17 occur in ADAM10-deficient B cells leading to increased production of TNF-α, which ultimately underlies architectural aberrancies noted in ADAM10-deficient LNs. This finding lends new insight to the discussion regarding how B cell TNF-α helps regulate secondary lymphoid tissue organization and how a proper ADAM10/ADAM17 ratio is needed to ultimately control TNF-α signaling.

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Supplemental Fig 1. Confirmation of successful bone marrow reconstitution. Irradiated CD45.1 WT mice were reconstituted with CD45.2 WT (A) or ADAM10<sup>−/−</sup> (CD45.2) (B) bone marrow and peripheral blood sample collected at 6 weeks post reconstitution. Percentage of CD45.2<sup>+</sup> live peripheral blood lymphocytes is shown.