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/content/172/7/4646.2.full.pdf
Cutting Edge: BAFF Regulates CD21/35 and CD23 Expression Independent of Its B Cell Survival Function

Leonid Gorelik,1* Anne H. Cutler,1,2* Greg Thill,† Steven D. Miklasz, * Dianna E. Shea, * Christine Ambrose,‡ Sarah A. Bixler,‡ Lihe Su,§ Martin L. Scott, * and Susan L. Kalled3*

We demonstrate that B cell-activating factor of the TNF family (BAFF), a B cell survival factor, also regulates CD21/35 and CD23 expression. BAFF blockade in wild-type mice down-modulates CD21/35 and CD23 on B cells while survival remains intact, and BAFF exposure causes elevated CD21/35 and CD23 expression. Similar down-modulation is observed in bcl-2-transgenic mice treated with a BAFF inhibitor. This is the first evidence that BAFF has a function independent of B cell survival. Reports using CD21/35 and CD23 expression to assess splenic B cell subsets in BAFF-null mice concluded a lack of B cells beyond the immature stage. Since CD21/35 and CD23 are inadequate for delineating B cell subpopulations in BAFF-null mice, we used expression of BAFF-R and several B cell markers to identify more mature splenic B cells in these mice. These data broaden our understanding of BAFF function and correct the view that BAFF-null mice lack mature B cells. The Journal of Immunology, 2004, 172: 762–766.

Although the developmental stages of B cell maturation from bone marrow (BM)4 precursors to mature peripheral B cells are known, the survival and differentiation factors required along this pathway have remained elusive. B cell-activating factor of the TNF family (BAFF), a TNF family ligand, (also known as BLyS, THANK, TALL-1, and zTNF4) (1) was identified as a B cell survival factor and shown to be required for B cell survival since BAFF knockout (KO) mice exhibit >90% loss of peripheral B cells (2, 3). Conversely, BAFF-transgenic (Tg) mice, which overexpress BAFF, have markedly elevated numbers of peripheral B cells and develop autoimmunity (4–6). Thus, the function of BAFF has significance in B cell biology as well as disease. BAFF has three known receptors, BAFF-R (also called BR3), BCMA, and TACI, and while BAFF-R binds only to BAFF, TACI and BCMA also bind APRIL, a related protein (1).

The earliest emigrants from BM to the spleen are transitional type 1 (T1) B cells. These cells develop into transitional type 2 (T2), mature follicular, and marginal zone (MZ) B cells. From analysis of BAFF KO mice, it was concluded that B cell development was blocked at the T1 stage (2, 3). However, it was shown that while the humoral responses to T-dependent Ags were severely impaired in BAFF KO mice, Ag-specific, class-switched Ab was still produced (2, 3). Recently, we showed that BAFF KO mice form germinal centers (GC) with intact somatic hypermutation after Ag challenge (7). These findings suggest that BAFF KO mice possess more differentiated, mature B cells than originally believed. Therefore, we re-examined the nature of splenic B cells in BAFF KO mice. During this investigation, we found CD21/35 and CD23 expression to be regulated by BAFF.

A common method to delineate B cell subsets in the spleen employs surface markers CD21/35 and CD23 since B cells can be defined as T1, CD21/35CD23−, T2, CD21/35highCD23+, mature, CD21/35lowCD23+; and MZ, CD21/35highCD23− (8). We and others previously concentrated on CD21/35 and CD23 expression to conclude that B cell development in BAFF KO mice was blocked at the T1 stage (2, 3). However, B cell subsets can also be defined by IgM and IgD expression: T1, IgMhighIgDlow/MZ, IgMhighIgDhigh; mature, IgMlowIgDhigh; and MZ, IgMhighIgDlow(9). Herein we show that BAFF-R, the only BAFF receptor to be expressed on all peripheral B cells, exhibits a differentially regulated pattern of expression on maturing B cells and can be used to identify B cell subsets. Therefore, using expression of IgM, IgD, BAFF-R, and other B cell markers, we show that BAFF KO mice have all splenic B cell subsets, but that CD21/35 and CD23 are markedly reduced on mature, T2, and MZ B cells.

These data led us to hypothesize that BAFF regulates CD21/35 and CD23 expression independently of its survival function. To test this, we examined B cell CD21/35 and CD23 expression under conditions of BAFF deficiency and excess in vivo and in vitro. We present data that support our hypothesis.
and discuss the implications of diminished CD21/35 and CD23 expression on B cell biology and immunity.

Materials and Methods

Mice
Female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), BAFF KO, and wild-type (WT) littermates (2) were housed in the Biogen Animal Facility under barrier conditions. Protocols were approved by the Biogen Idec Institutional Animal Care and Use Committee.

BAFF inhibitor reagents and treatment
Human BCMA-Fc (11) and BAFF-R:Fc (12) were described previously. Mice received i.p. 250 μg of BAFF-R:Fc, BCMA-Fc, or human IgG (hIgG; Novartis, Basel, Switzerland) as indicated.

Flow cytometric analysis
Splenocytes were stained with mAbs (BD PharMingen, San Diego, CA) directed against various markers: IgM-allophycocyanin (11/41), CD21/35-FITC (7G6), CD23-biotin (B3B4), B220-FITC (RA3-6B2), CD43-PE (S7), CD22-biotin (Cy34.1), CD24-FITC (M1/69), CD40-FITC (HM40-3), CD19-FITC (1D3), IgD-PE (11-26; Southern Biotechnology Associates, Birmingham, AL). Anti-BAFF-R-biotin (P1B8 and B9C11) was generated at Biogen. FITC (1D3), IgD-PE (11-26; Southern Biotechnology Associates, Birmingham, AL). Anti-BAFF-R-biotin (P1B8 and B9C11) was generated at Biogen.

Results

Statistical analysis was performed using Student’s t test.

Immunohistochemistry

Frozen spleen sections were prepared as described previously (13). Anti-mucosal addressin cell adhesion molecule 1 (MadCAM-1) (Southern Biotechnology Associates) followed by streptavidin-alkaline phosphatase and 3,3′-diaminobenzidine (Vector Laboratories, West Grove, PA) followed by streptavidin-alkaline phosphatase and 3,3′-diaminobenzidine (Vector Laboratories, West Grove, PA) followed by streptavidin-alkaline phosphatase and 3,3′-diaminobenzidine (Vector Laboratories, West Grove, PA) followed by streptavidin-alkaline phosphatase and 3,3′-diaminobenzidine (Vector Laboratories, West Grove, PA) followed by streptavidin-alkaline phosphatase and 3,3′-diaminobenzidine (Vector Laboratories, West Grove, PA).

Statistical analysis

Statistical analysis was performed using Student’s t test.

Results

IgM and IgD expression identifies mature B cells in BAFF KO mice
Using four-color flow cytometric analysis with mAbs specific for IgM, IgD, CD21/35, and CD23, we found that by the IgD vs IgM profile, like age-matched WT controls, 12-wk-old BAFF KO mice appear to possess all splenic B cell subsets (Fig. 1A). Furthermore, when comparing the frequencies in the context of total splenic IgM$^+$ B cells, mature B cells represent the largest population in BAFF KO and WT mice (33.8 ± 6.7% and 53.5 ± 2.1%, respectively) followed by IgM$^+$IgD$^+$ cells (KO = 29.8 ± 6%, WT = 21.9 ± 3.2%) which include MZ and IgD$^+$ T1 B cells (referred to as MZ/T1). The frequencies of T2 B cells in BAFF KO (6.9 ± 0.3%) and WT mice (9.7 ± 0.2%) do not differ greatly, while the frequency of T1 B cells is elevated in BAFF KO mice compared with WT controls (KO = 12.7 ± 1.8%, WT = 4.5 ± 0.1%).

Previous CD21/35 and CD23 expression analyses of splenic B cells from BAFF KO mice concluded that mature B cells were absent. Results using IgM/IgD to define B cells in BAFF KO mice are in conflict with this conclusion; therefore, IgD vs IgM gated cells were further examined for CD21/35 and CD23 expression. As expected, CD21/35 and CD23 expression was minimal or nonexistent on T2 and mature B cells (Fig. 1B). Although by IgM/IgD gating MZ B cells appear to exist at similar frequencies (MZ/T1 gate), as described above, by examination of CD21/35, CD21/
FIGURE 2. BAFF-R expression on B cell subsets. A, FACS analysis of BM from WT mice. Gates were set to identifying pre-B, pro-B, immature, and mature B cell populations before analysis of BAFF-R expression (solid line). Dashed line indicates isotype control. B, FACS analysis of splenocytes from BAFF KO and WT mice. Gates were set on T1, T2, MZ/T1, and mature (M) B cells based on IgM/IgD. The data in A and B are representative of three to four mice assessed in each group.

CD21/35 and CD23 expression on B cells from BAFF Tg mice

Since B cells in a BAFF-deficient environment exhibit reduced levels of CD21/35 and CD23, we speculated that the converse would be true in BAFF Tg mice. Therefore, we examined B cells from BAFF Tg mice for CD21/35 and CD23 expression. Consistent with the notion of CD21/35 regulation by BAFF, mature, MZ, and T2 B cells from BAFF Tg mice have a markedly increased MFI for CD21/35 when compared with WT controls (Fig. 3, upper panel). No increase in CD23 expression was observed in BAFF Tg mice (Fig. 3, lower panel).

B cells from BAFF KO mice up-regulate CD21/35 and CD23 when exposed to BAFF

To determine a direct relationship between BAFF and CD21/35 and CD23 expression, we asked whether BAFF could stimulate CD21/35 and CD23 expression to WT levels on T2 and mature B cells from BAFF KO mice. Splenocytes from WT and BAFF KO mice were cultured with or without sBAFF for 24, 48, and 72 h. At each time point, cells were collected and subjected to four-color flow cytometric analysis to examine the level of CD21/35 and CD23 expression on T2 and mature B cells defined by IgM and IgD. The MZ/T1 gate was not examined since we could not rule out increasing CD21/35 and CD23 expression due to maturation of T1 B cells. Compared with freshly prepared (time = 0 h) BAFF KO splenocytes, the expression level of CD21/35 increased with time, with ~3.5-fold increase on mature and T2 BAFF KO B cells by 72 h when cultured with sBAFF (Fig. 4, left panel). WT mature and T2 B cells also increased CD21/35 expression when cultured with sBAFF, with a 2.3- and 2-fold increase, respectively, observed at 72 h. BAFF KO and WT B cells in medium-only cultures exhibited no or <2-fold increase in CD21/35 expression (Fig. 4, left panel). sBAFF also mediated increased CD23 expression with the maximum expression observed at 72 h. At this time point, cultures of BAFF KO splenocytes plus sBAFF had the greatest increase in CD23 expression compared with freshly prepared cells (3.8-fold vs 1.6-fold medium alone; Fig. 4, right panel). Mature B cells from BAFF KO mice exhibited a 1.8-fold increase in CD23 expression.
BAFF-R:Fc or hIgG as a control, and spleens were harvested 48 h later for flow cytometric analysis. Forty-eight hours posttreatment no difference in the number of total splenic B cells was observed (Fig. 5A). Mature, MZ and T2 B cells were gated based on IgM/IgD. CD23 (Fig. 5B) and CD21/35 (Fig. 5C) were significantly reduced on all B cell subsets examined when compared with hIgG-treated mice. CD21/35 expression was also found to be significantly diminished after only 24 h post-BAFF-R:Fc treatment, while no change was observed for CD23 (data not shown).

A second system utilized bcl-2 Tg mice for in vivo blockade with BCMA-Fc. bcl-2 Tg mice contain the human bcl-2 gene under the control of the Eμ promoter, allowing B cell-specific expression. bcl-2, an antiapoptotic factor, was shown to enhance B cell survival when overexpressed (15). bcl-2 Tg mice were treated with a single dose of 250 μg BCMA-Fc or hIgG and splenic B cells were examined for CD21/35 and CD23 expression 2 days later. A separate cohort received 250 μg BCMA-Fc or hIgG on days 0 and 4, with splenic B cell analysis on day 8. On day 8, BCMA-Fc and hIgG-treated mice had similar numbers of splenic B220+ B cells (Table II). However, diminished levels of CD21/35 and CD23 were observed on mature B cells in BCMA-Fc-treated mice, when compared with controls, as early as 2 days after a single dose (Table I).

Discussion
Although the role of BAFF in B cell survival is well established, it is unclear whether BAFF is required for B cell differentiation and/or function. Despite a presumed dominance of T1 B cells, BAFF KO mice mount class-switched, Ag-specific immune responses, albeit at reduced levels (2). Furthermore, WT mice treated with a BAFF inhibitor produce high-affinity Ab, although the quantity is diminished (16, 17). Recently we showed that BAFF KO and BCMA-Fc-treated WT mice form GCs with intact somatic hypermutation (7). Together, these data suggest the existence of mature B cells in BAFF KO mice. Our expression data of IgM, IgD, and other B cell markers to define mature B cells is in agreement with this notion. The differentially regulated expression of CD21/35 and CD23 was found to be coincident with surface IgM, provided an additional means to define B cell subsets and allowed us to show that BAFF KO mice have mature B cells, but with diminished CD21/35 and CD23 levels. The finding that BM B cell precursors lack BAFF-R expression likely explains the BAFF-independent nature of these cells (2, 3).

Most importantly, our data provide the first evidence that BAFF has a function in addition to and independent of B cell survival. Although others demonstrated that T1 B cells in culture with BAFF acquire CD21/35 and CD23, it was presumed that expression of these molecules was related to differentiation of immature B cells (18, 19). Although this may be true, our in vitro results show that BAFF can directly mediate CD21/35 and CD23 expression on more mature B cells. The elevated level of CD21/35 on B

expression with no increase seen in the medium-alone culture (Fig. 4, right panel).

These data show that BAFF can increase expression of CD21/35 and CD23 on B cells from WT and BAFF KO mice. Although the significance of lower CD21/35 expression on B cells from BAFF KO mice in culture is unclear, B cells from BAFF KO mice still can reach the level observed on freshly isolated WT cells.

BAFF regulates CD21/35 and CD23 expression independent of B cell survival function
To determine whether BAFF regulates CD21/35 and CD23 expression independent of its survival function, we used two in vivo settings. The first utilized WT mice and a receptor-Fc decoy to block BAFF for a brief period so that B cell survival remained intact. BALB/c mice were given a single i.p. dose of 250 μg BAFF-R:Fc or hIgG as a control, and spleens were harvested 48 h after 24, 48, and 72 h, cells were harvested, B cell subsets were gated based on IgM/IgD, and assessed for CD21/35 and CD23 expression. The data are representative of two experiments.

FIGURE 4. BAFF stimulates CD21/35 and CD23 expression on B cells from BAFF KO mice. Splenocytes from BAFF KO (○, △) and WT control (●, ▲) were prepared and cultured with (○, ●) or without (△, ▲) sBAFF. After 24, 48, and 72 h, cells were harvested, B cell subsets were gated based on IgM/IgD, and assessed for CD21/35 and CD23 expression. The data are representative of two separate experiments.

FIGURE 5. Brief BAFF blockade results in intact B cell survival but reduced CD21/35 and CD23 expression. BALB/c mice (n = 3/group) received BAFF-R:Fc or hIgG 48 h before FACS analysis of splenic B cells. A, Number of splenic B cells. Expression levels of CD23 (B) and CD21/35 (C) on B cell subsets (■, hIgG; □, BAFF-R:Fc). * p < 0.05. The data are representative of two separate experiments.

Table II. BAFF/APRIL blockade in bcl-2 Tg mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>B cells (×10^6)</th>
<th>CD21/35 (MFI)</th>
<th>CD23 (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCMA-Fc</td>
<td>4.0</td>
<td>13.7</td>
<td>145</td>
</tr>
<tr>
<td>BCMA-Fc</td>
<td>4.3</td>
<td>13.0</td>
<td>136</td>
</tr>
<tr>
<td>BCMA-Fc</td>
<td>6.1</td>
<td>12.1</td>
<td>174</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.2 ± 1.0</td>
<td>12.9 ± 0.7</td>
<td>151.7 ± 16</td>
</tr>
<tr>
<td>hIgG</td>
<td>5.7</td>
<td>16.9</td>
<td>256</td>
</tr>
<tr>
<td>hIgG</td>
<td>5.7</td>
<td>19.2</td>
<td>279</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.3 ± 0.8</td>
<td>18.1 ± 1.2</td>
<td>268 ± 12</td>
</tr>
</tbody>
</table>

* Assessed on day 8 after two doses of BCMA-Fc.
* Assessed 2 days after a single dose of BCMA-Fc.
cells from BAFF Tg mouse correlates with these data. That BAFF Tg mice do not express elevated CD23 on B cells suggests an intrinsic factor in vivo that may prohibit excessive CD23 surface expression or cause down-regulation when CD23 is overexpressed. BAFF-R-Fc treatment of WT mice and BCMA-Fc treatment of bcl-2 Tg mice confirmed the disassociation between BAFF-regulated CD21/35 and CD23 expression and B cell survival. Although BCMA-Fc also blocks APRIL, the results with BAFF-R-Fc treatment of WT mice demonstrate that BAFF alone can regulate CD21/35 and CD23 expression.

It may be argued that BAFF-mediated regulation of CD21/35 and CD23, markers used to distinguish differentiated B cells, constitutes BAFF as a differentiation factor. From a strict phenotypic definition, this may be accurate. However, IgM<sub>low</sub>IgD<sub>high</sub>CD23<sup>-</sup> CD23<sup>-</sup> B cells in BAFF KO mice are functional and can be activated to form GCs, somatically mutate, and produce high-affinity Ab (2, 3, 7), and our recent experiments indicate that Ag-specific immune responses in (bcl-2 Tg × BAFF KO)F<sub>1</sub> mice, possessing mature CD21/35 CD23<sup>-</sup> B cells, are normalized. It is possible these cells represent a unique BAFF-independent B cell population, or lineage, and are too sparse to detect in normal mice. However, some specific functions, as discussed below, may be impaired similar to CD21/35 KO and CD23 KO mice, and this remains to be tested.

The Cr2 locus encodes complement receptors CD21/35 which are expressed on B cells and follicular DCs (FDCs). Through the generation of Cr2 KO mice, the importance of complement and complement receptors in adaptive immunity was established. CD21/35, CD19, and CD81 comprise a signaling complex on B cells that along with the B cell receptor lowers the threshold for activation and, unlike WT B cells, Cr2-null B cells with low affinity to Ag are not maintained in the B cell follicle. Furthermore, it was found that B cells require Cr2 for sufficient signaling to survive in GCs (14). That is of particular interest since GCs formed in a BAFF-deficient environment are unstable, and a mature FDC reticulum fails to form in these GCs (7). This may be due to the loss of CD21/35 on B cells and an impaired cosignal. Interestingly, expression of the CD35 isoform of Cr2 remains intact on FDCs in a BAFF-deficient environment (7).

CD23, the low-affinity IgE receptor, is expressed on B cells, FDCs, and a subset of T cells. Data suggest that B cell CD23 is involved in Ag presentation and that CD23 KO mice exhibit enhanced IgE responses (20). Interestingly, human CD21 is a ligand for human CD23. Therefore, loss of B cell-expressed CD23 and/or CD21/35 may contribute to compromised humoral immunity in a BAFF-deficient environment. Given the importance of B cell-FDC interaction in humoral immunity, the expression of CD21/35 and CD23 on B cells, and our current understanding of the functions of these proteins, independent from survival activity, BAFF may be important for B cell function through regulation of CD21/35 and CD23.

The exact nature of the BAFF-R<sup>-</sup>IgM<sub>low</sub>IgD<sub>high</sub>CD21/35 CD23<sup>-</sup> mature B cells in BAFF KO mice is unclear, and additional studies are required to fully elucidate the survival pathway and functional capacity of these cells in BAFF KO mice and to determine the existence of such cells in WT animals. Nevertheless, our data correct a misperception that B cells fail to develop beyond the T1 stage in a BAFF-deficient environment and broaden what was a narrow view of the role of BAFF in B cell biology to include regulation of important B cell surface proteins, CD21/35 and CD23.

Acknowledgments
We thank Y.-M. Hsu, D. Gong, T. Cachero, F. Qian, K. Mikatkowski, K. Strauch, and C. Mullen for BAFF-R-Fc, BCMA-Fc, and sBAFF reagents; the FACS Laboratory for technical assistance; and S. Rao, M. Dobles, F. Mackay, and T. Novobrantseva for critical reading of this manuscript.

References
CORRECTIONS


In Materials and Methods, under the heading RT-PCR analysis, the primer sequences for CCR7 are incorrect. The other data on the probes (accession number and positions of the primers) are correct as published. The correct primer sequences are shown below.

sense: 5’-GCTCCAGGCACGCAACTTT-3’
antisense: 5’-ACCACGACCACACGATGA-3’
probe (FAM): 5’-AGCGCAACGACGATGAAGGGTG-3’


In Results, there were errors in Table II. The legend and conclusion are correct as published. The revised table is shown below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>B Cells(^{a}) ((\times 10^7))</th>
<th>CD21/35(^{b}) (MFI)</th>
<th>CD23(^{b}) (MFI)</th>
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</thead>
<tbody>
<tr>
<td>BCMA-Fc</td>
<td>15.8</td>
<td>13.7</td>
<td>145</td>
</tr>
<tr>
<td>BCMA-Fc</td>
<td>16.0</td>
<td>13.0</td>
<td>136</td>
</tr>
<tr>
<td>BCMA-Fc</td>
<td>24.3</td>
<td>12.1</td>
<td>174</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>18.7 ± 4.9</td>
<td>12.9 ± 0.8</td>
<td>151.7 ± 19.9</td>
</tr>
<tr>
<td>hIgG</td>
<td>21.8</td>
<td>16.9</td>
<td>256</td>
</tr>
<tr>
<td>Mean</td>
<td>21.3</td>
<td>18.1</td>
<td>268</td>
</tr>
</tbody>
</table>

\(^{a}\) Assessed on day 8 after 2 doses of BCMA-Fc.

\(^{b}\) Assessed 2 days after a single dose of BCMA-Fc.

In Results, the flow cytometry histogram labels MFI and M1 are incorrect in three of the four panels in Figure 6A. The revised figure is shown below.