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Spontaneous loss of tolerance of autoreactive B cell in *Act1*-deficient AM14 Tg rheumatoid factor (RF) mice

Natalia V. Giltiay^{*,1}, Yi Lu^{*}, Jaime L. Cullen[‡], Trine N. Jørgensen^{*}, Mark J. Shlomchik[‡], and Xiaoxia Li^{*}

^{*}Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA

[‡]Section of Immunology and Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT 06520

Abstract

Self-reactive B cells in BALB/c AM14 transgenic (AM14 Tg) rheumatoid factor (RF) mice are not subject to central or peripheral tolerization. Instead, they remain at a stage of “clonal ignorance”, i.e. they do not proliferate and differentiate into Ab-producing cells. However, the immunoregulatory mechanisms that prevent autoantibody production in these mice remain unclear. In this study, we show that crossing AM14 Tg mice to a mouse strain deficient in *Act1*, a molecule involved in the regulation of BAFF-R and CD40-signaling in B cells, results in spontaneous activation of AM14 Tg B cells and production of AM14-specific antibodies. Three to five-month old AM14 Tg *Act1*^{-/-} mice showed significant expansion of AM14 Tg B cells, including a 2–3 fold increase in the spleen and cLNs compared to AM14 Tg *Act1*^{+/+} mice. Furthermore, in the presence of endogenous self-Ag (IgH^a congenic background), AM14 Tg *Act1*^{-/-} B cells were spontaneously activated and differentiated into antibody forming cells (AFC). In contrast with previous studies using AM14 Tg MLR.Fas^{lpr} mice, we found that a significant number of AM14 Tg cells AM14 Tg *Act1*^{-/-} mice displayed phenotypic characteristics of GC B cells. Anti-CD40L treatment significantly limited the expansion and activation of AM14 Tg *Act1*^{-/-} B cells, suggesting that CD40L-mediated signals are required for the retention of these cells. Our results support the important role of *Act1* in the regulation of self-reactive B cells and reveal how *Act1* functions to prevent the production of autoantibodies.

INTRODUCTION

Systemic autoimmunity is characterized by the loss of tolerance to self-antigens and the production of autoantibodies by activated B cells. The origins of pathogenic B cells in autoimmune diseases and the mechanisms involved in their regulation are not fully understood (1–3). B cells recognizing high avidity self-antigens are eliminated in the bone marrow through mechanisms of receptor editing, anergy, and clonal deletion (4–8). By contrast, autoreactive B cells that recognize soluble self-antigens with low-to-moderate affinity are not subject to central tolerization in the bone marrow, but instead colonize the peripheral lymphoid organs (5). An additional selection of autoreactive B cells occurs at the

Address correspondence and reprint requests to Dr. Xiaoxia Li or Dr. Trine N. Jørgensen, Department of Immunology, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44915, Tel: (216) 445-8706, Fax: (216) 444-9329, lix@ccf.org (X.L.); Tel: (216) 444-7454, Fax: (216) 444-9329, jorgent@ccf.org (T.N.J).

¹Current address: Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195

DISCLOSURES

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transitional checkpoint, as immature B cells develop into more mature follicular (Fo) or marginal zone (MZ) B cells (9, 10).

Signaling through the B cell antigen receptor (BCR) is necessary but not sufficient for the full activation of autoreactive B cells. Recent studies suggested that dual engagement of the B-cell receptor (BCR) and intracellular TLRs by endogenous nucleic acid-containing immune complexes can promote the activation of autoreactive B cells and drive the production of pathogenic antibodies (11–13). In addition, alterations of the strength of signaling via the CD40 and BAFF receptors were shown to disrupt the normal selection of autoreactive B cells and to promote their survival and activation (14–16).

Transgenic (Tg) mice expressing BCRs designed to specifically recognize self-antigens are valuable tools in identifying the critical checkpoints of B cell development involved in the regulation of self-reactive cells (5, 17–24). AM14 H chain Tg mice, originally derived from the MRL.Fas^{lpr} mouse strain, harbor an increased frequency of B cells with low to moderate affinity to self protein, in this case IgG2a (RF-specificity) (25, 26). RFs are a dominant class of autoantibodies commonly found in patients with autoimmune diseases, including Rheumatoid Arthritis (RA), Sjögren's Syndrome (SjS), and Systemic Lupus Erythematosus (SLE) (27, 28). In the presence of self-Ag (Igh^a-congenic background), AM14 Tg B cells are not subject to tolerization in the BM, and are not anergized in the periphery. Instead, they develop into B cells with phenotypic and functional characteristics of pre-immune follicular B cells (26). Under normal conditions, such cells remain at a stage of “clonal ignorance”, i.e. they do not proliferate and differentiate into plasmablasts. The mechanisms required for the maintenance of this “ignorant” state are still unclear.

When crossed onto the MRL.Fas^{lpr} lupus-prone background, AM14 Tg mice develop RF-secreting cells as they age (22). The activation of AM14 Tg cells in MRL.Fas^{lpr} mice takes place at extrafollicular sites in the spleen and, upon proper stimulation, can occur even in the absence of Ag-specific T cell help (29–31). The ability of AM14 Tg B cells to become spontaneously activated and to differentiate into AFC in other autoimmune-prone backgrounds has not been studied.

In previous work, we have identified the molecule Act1, an inhibitor that functions downstream of BAFF-R- and CD40-signaling pathways, as an important regulator of peripheral B cell homeostasis (32–34). Deficiency of Act1 results in B cell hyperplasia, and is associated with splenomegaly and lymphadenopathy. *Act1*^{-/-} mice showed increased numbers of Fo, MZ and transitional (TR) B cell subsets in the spleen (32, 34). Histological analyses of the spleen and cervical lymph nodes from *Act1*^{-/-} mice indicated increased GC formation and accumulation of large numbers of CD138⁺ plasma cells. Consistent with these findings, *Act1*^{-/-} mice developed hypergammaglobulinemia, characterized by increased levels of serum IgG1, IgG2a/c, and IgG2b (34). The development of systemic autoimmune disease in the *Act1*^{-/-} mice was also associated with increased titers of circulating RF and the appearance of anti-histone, anti-ssDNA and anti-dsDNA IgG autoantibodies (33, 34). As early as 8 weeks of age, *Act1*^{-/-} BALB/c mice developed symptoms of Sjögren's Syndrome (SjS), characterized by eye and mouth dryness, lymphoid infiltration of the lacrimal and salivary gland, and the production of anti-SS-A/Ro and anti-SS-B/La autoantibodies, which are two characteristic autoantibodies for SjS (33). Interestingly *Act1*^{-/-} BAFF^{-/-} double deficient mice produced detectable levels of anti-SS-A/Ro and anti-SS-B/La autoantibodies. In contrast, no autoantibody production was measurable in *Act1*^{-/-} CD40^{-/-} double knockout mice, suggesting that CD40-mediated activation plays a key role in the activation and differentiation of autoantigen-specific AFCs (33).

In the current study, we tested whether Act1 deficiency can promote the activation of RF-specific autoreactive B cells by generating AM14 Tg *Act1*^{-/-} BALB/c mice.

MATERIALS AND METHODS

Mice

AM14 Tg BALB/c (IgH^a) or AM14 Tg CB.17 (IgH^b) mice were crossed to the *Act1*^{-/-} BALB/c mice. AM14 Tg *Act1*^{+/-} mice were used to generate AM14 Tg *Act1*^{-/-} and AM14 Tg *Act1*^{+/+} control mice on IgH^a or IgH^b backgrounds. Animals were maintained under specific pathogen-free conditions at the Biological Resource Unit of the Cleveland Clinic. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

FACS analysis

FACS analysis was performed as described (32). Antibodies used for FACS analysis included: FITC-conjugated anti-IgM (121-15F9), PE-conjugated anti-CD93 (AA4.1), PE-conjugated anti-CD22 (Cy-34.1), APC-conjugated anti-CD44 (IM7), FITC-conjugated anti-CD69 (H1.2F3), Biotin-conjugated anti-GL7 (GL-7) and Streptavidin-PerCP-Cy5.5 (e-Biosciences, San Diego, CA); FITC-conjugated and PE-conjugated anti-CD21/35 (7G6), FITC- and PerCP conjugated anti-B220 (RA3-6B2) (BD Biosciences, Franklin Lakes, NJ); APC-conjugated anti-CD23 (Caltag Laboratories, Carlsbad, CA). Anti-Id (4-44) Ab, which recognizes the AM14 H chain only in combination with L chain of V κ 8-19 or V κ 8-28, joined to J κ 4 or J κ 5, combinations that encode anti-IgG2a specificity, was described previously (22). All samples were analyzed on a BD FACS Calibur or LSRII (BD Biosciences). All data were analyzed using FlowJo 7.5 software (TreeStar, Ashland, OR).

ELISA

Serum AM14 levels were determined by ELISA as previously described (25). Briefly, ELISA plates were pre-coated with 10 μ g/ml goat anti-mouse IgM (Chemicon International, Inc., Temecula, CA) overnight in PBS, washed and blocked. Serum samples were incubated in duplicates for 2 hours and plates were washed and incubated for 1 hour with biotinylated anti-idiotypic Ab 4-44 (0.25 μ g/ml in 1% BSA/PBS). After washing, plates were incubated with streptavidin-alkaline phosphatase (R&D Systems, Inc., Minneapolis, MN), and, finally, development with p-nitrophenylphosphate. The OD at 405 nm was read on a Perkin Elmer plate reader (Wellesley, MA).

ELISpot analysis

96-well MultiScreenTM-IP plates (Millipore, Billerica, MA) were pre-coated with 10 μ g/ml goat anti-mouse IgM (Chemicon International) and incubated overnight at 4°C. Cells were suspended in RPMI medium with 2.5% FBS at concentrations 1.5×10^7 cells/ml. Cells were plated in duplicate and then serially diluted by a factor of 3. ELISpot plates were centrifuged and incubated at 37°C, 5% CO₂ for 12-14 hours. After washing, plates were incubated with biotin-conjugated anti-AM14 (4-44) Ab (1:500 in 1% BSA). Immune reaction was detected using an ELISpot Blue Color Module, (RnD Systems, Minneapolis, MN). Immune spots were analyzed using an ImmunoSpot[®]-S1 Analyzer and the ImmunoSpot[®]-4.0 Software (Cellular Technologies Ltd., Cleveland, OH). Final values were calculated as number spots per 1×10^6 cells.

Histology

Tissue samples were snap-frozen in OCT medium and stored at -80°C. Sections were prepared and stained with Abs as described previously (35). Abs used include 4-44-biotin,

anti-CD4 Alexa 647 (GK1.5), anti-CD19 Alexa 647 (1D3.2), anti-CD90.2 Alexa 647 (30H12), PNA-FITC (Vector Laboratories, Inc., Burlingame, CA), anti-CD45R Alexa 488 (6B2), anti-F4/80 Alexa 647 (BM8), streptavidin Alexa 555 (Molecular Probes), streptavidin Brilliant Violet 421 (Biolegend), anti-Ki67 rabbit IgG supernatant (SP6), and anti-rabbit IgG Alexa 568 (Molecular Probes). A 5 × 5 array of tiled images at 100x power was captured for each sample at a resolution of 1600 × 1200 pixels per image. Images were captured with a Nikon Eclipse Ti-U microscope and processed using Nikon Software and composited and cropped using Adobe Photoshop.

CD40L Blocking

AM14 Tg *Act1*^{-/-} mice with comparable levels of serum AM14 (OD reading between 0.4 and 0.55) were selected for this experiment. Mice were injected i.p on days 1 and 3 with 250 µg sterile anti-CD40L mAb (MR1) or control hamster IgG (BD Biosciences), diluted in sterile PBS. Mice were sacrificed on day 7 days after the first injection and spleens and cLNs were analyzed for the presence of CD22^{hi} 4-44⁺, CD22^{lo} 4-44⁺, and GL7⁺4-44⁺ B220⁺ B cells by FACS.

Statistical analysis

Differences between various groups were determined using unpaired two-tailed student *t* test. All analyses were performed using GraphPad Prism 5.0 Software (La Jolla, CA). Values are reported as mean ± SD; P values < 0.05 were considered significant.

RESULTS

Development of AM14 Tg B cells on *Act1*^{-/-} background

To study the effect of *Act1* deficiency on the development of RF-specific (AM14 Tg) B cells in the presence or absence of endogenous Ag, we generated AM14 Tg *Act1*^{-/-} mice on two different backgrounds: AM14 Tg *Act1*^{-/-} BALB/c (IgH^a) and AM14 Tg *Act1*^{-/-} CB.17 (IgH^b). At 8–10 weeks of age, AM14 Tg *Act1*^{-/-} mice on both backgrounds had visibly enlarged spleens and cervical LNs when compared to their AM14 Tg *Act1*^{+/+} littermates (Fig. 1A). FACS analysis confirmed that lymphoid organ enlargement was associated with an increase in the number of IgM⁺B220⁺ B cells (data not shown). To evaluate if the B cell expansion also involved AM14 Tg B cells, we performed flow cytometry of spleen and cLN cells using anti-Id (4-44) Ab, which recognizes the 2–3% of B cells in AM14 H Tg mice that have an L chain that confers BCR specificity for IgG2a. Results from these analyses revealed a 2-fold (spleen) and 2.3-fold (combined cLNs) increase in the number of 4-44⁺ cells in AM14 Tg *Act1*^{-/-} IgH^a compared to AM14 Tg *Act1*^{+/+} IgH^a. Similar increases were observed in spleens and cLNs of AM14 Tg *Act1*^{-/-} IgH^b mice as compared to AM14 Tg *Act1*^{+/+} IgH^b mice (Fig. 1B).

Previous analyses of peripheral B cell subsets in *Act1*^{-/-} mice revealed defective selection of immature B cells as they passed the T1-to-T2 transitional checkpoint, as well as an increase in the number of T2, FO and MZ B cells within the spleen (32). To address whether the lack of *Act1* similarly affected the development of 4-44⁺ B cells, we examined their representation within different B cell subsets in the spleen, including within T1 (B220⁺AA4.1⁺CD23⁻IgM⁺), T2 (B220⁺AA4.1⁺CD23⁺IgM^{hi}), Fo (B220⁺AA4.1⁻CD23⁺IgM⁺) and MZ (B220⁺AA4.1⁻CD23⁻IgM^{hi}) B cells. We found no significant differences in the percentage of 4-44⁺ B cells between AM14 Tg IgH^a and AM14 Tg *Act1*^{-/-} IgH^a mice within any of these B cell subsets. As previously reported (22, 35), we observed that the majority of 4-44⁺ B cells in the spleens of both AM14 Tg *Act1*^{+/+} and AM14 Tg *Act1*^{-/-} mice (age 3–4.5 months) displayed a phenotype of either Fo (72–80% of 4-44⁺ cells in the spleen) or T2 B cells (4–16% of 4-44⁺ cells in the spleen)

(Supplementary Fig. 1). Thus, the 2-fold increase in the number of AM14 Tg B cells in AM14 Tg *Act1*^{-/-} mice was proportional to the overall increase in the numbers of Fo and T2 B cells in these mice.

In summary, we observed a significant increase in the number of AM14 Tg B cells in the spleen and cLN of AM14 Tg *Act1*^{-/-} mice, with no obvious skew in their development. In agreement with previous studies in AM14 Tg MRL.Fas^{lpr} mice, we did not find evidence of tolerization or elimination of 4-44⁺ B cells in the periphery.

Spontaneous, Ag-driven activation of AM14 Tg B cells in AM14 Tg *Act1*^{-/-} mice

Previous studies have demonstrated that 4-44⁺ B cells on the autoimmune-prone MRL.Fas^{lpr} background become spontaneously activated and differentiate into Ab-secreting plasmablasts (26). Such activation requires the presence of endogenous Ag and occurs only in IgH^a expressing mice, but not in IgH^b expressing mice.

To test whether *Act1* deficiency was sufficient to mediate spontaneous activation of RF-producing B cells, we measured the concentration of AM14 Abs (RF) in the serum of BALB/c (wild type), AM14 Tg *Act1*^{+/+} IgH^a, AM14 Tg *Act1*^{+/+} IgH^b, AM14 Tg *Act1*^{-/-} IgH^a and AM14 Tg *Act1*^{-/-} IgH^b mice. ELISA results showed a significant increase of AM14 Ab titers in the serum of AM14 Tg *Act1*^{-/-} IgH^a mice, while no significant levels of AM14 Ab were detected in AM14 Tg *Act1*^{+/+} IgH^a, AM14 Tg *Act1*^{+/+} IgH^b and AM14 Tg *Act1*^{-/-} IgH^b mice (Fig. 2).

The presence of AFCs in AM14 Tg *Act1*^{-/-} IgH^a mice was further confirmed in ELISpot assays (Fig. 3). Splenocytes from BALB/c AM14 Tg *Act1*^{+/+} IgH^a, AM14 Tg *Act1*^{-/-} IgH^a and AM14 Tg *Act1*^{-/-} IgH^b mice (6 mice per group) had a 5-to-6-fold increase in the number of AM14 (4-44⁺) AFCs in the AM14 Tg *Act1*^{-/-} IgH^a mice as compared to AM14 Tg *Act1*^{+/+} IgH^a mice (Fig. 3A). Only low frequencies of AFCs were observed in AM14 Tg *Act1*^{-/-} IgH^b mice, supporting a requirement for endogenous Ag for the activation of AM14 Tg *Act1*^{-/-} B cells. Interestingly, high numbers of AM14-specific AFC cells were also detected in cervical LNs isolated from AM14 Tg *Act1*^{-/-} IgH^a mice (Fig. 3B), suggesting that these LNs serve as an additional location for the accumulation and, possibly, for the activation of RF-specific cells in AM14 Tg *Act1*^{-/-} mice.

Studies using AM14 Tg MRL.Fas^{lpr} mice identified two populations of B cells involved in the RF response: 4-44⁺CD22^{hi}CD44^{hi} cells, which do not secrete autoantibodies but instead appear to be immediate precursors of a second population, and 4-44⁺CD22^{lo}CD44^{brigt} cells, which display features of differentiated plasmablasts capable of producing AM14 Ab.

We used FACS analyses to identify these two cell populations and compared them between *Act1*^{-/-} and *Act1*^{+/+} AM14 Tg mice (on IgH^a or IgH^b backgrounds). Consistent with the elevated levels of serum AM14 Ab in the AM14 Tg *Act1*^{-/-} IgH^a mice, we found a significant increase in the frequency of 4-44⁺CD22^{lo} B cells in the spleens of AM14 Tg *Act1*^{-/-} IgH^a mice (0.48 ± 0.11 % versus 0.08 ± 0.01 and 0.08 ± 0.04 % in AM14 Tg *Act1*^{+/+} and AM14 Tg *Act1*^{-/-} IgH^b, respectively (Fig. 4A, upper panel and Fig. 4B). 4-44⁺CD22^{lo} Tg B cells were also clearly detectable in the cervical LNs of AM14 Tg *Act1*^{-/-} IgH^a mice (Fig. 4A, lower panel and Fig. 4C).

The frequency of 4-44⁺CD22^{lo} Tg B cells in the periphery was variable between individual AM14 Tg *Act1*^{-/-} IgH^a mice. Still, their frequency in the spleen positively correlated with the increased serum levels of AM14 Ab (R²=0.47, p=0.015, data not shown). Consistent with data from AM14 Tg MRL.Fas^{lpr} mice (26), the frequency of 4-44⁺CD22^{lo} Tg B cells increased in AM14 Tg *Act1*^{-/-} IgH^a mice as they aged (data not shown).

Further analyses of 4-44⁺CD22^{hi} B cells in the spleen of AM14 Tg *Act1*^{-/-} IgH^a mice and control mice showed a significant increase in the percentage of cells expressing CD44 and CD69 markers on their surface (Fig. 4D), suggesting that these cells were also undergoing Ag-driven activation. As expected, the majority of 4-44⁺CD22^{lo} cells in AM14 Tg *Act1*^{-/-} IgH^a mice expressed high levels of CD44 and CD69. In contrast, we did not observe significant accumulation of CD44⁺ or CD69⁺ 4-44⁺CD22^{lo} cells in AM14 Tg *Act1*^{-/-} IgH^b mice (Fig. 4D).

Due to the low frequency of 4-44⁺CD22^{lo} cells in AM14 Tg *Act1*^{+/+} IgH^a and AM14 Tg *Act1*^{-/-} IgH^b mice, we were unable to determine the levels of CD44 and CD69 expression in these cells (Fig. 4D). Based on these observations, we concluded that *Act1* deficiency in 4-44⁺ B cells (IgH^a background) leads to the spontaneous activation and differentiation of these cells from ignorant follicular B cells into functional AFCs.

Development of 4-44⁺ germinal center B cells in spleens and cLNs of AM14 Tg *Act1*^{-/-} mice

Previously, we found an extensive and presumably spontaneous formation of germinal centers (GC) in the cLNs and spleens of *Act1*^{-/-} mice (34). The exact mechanisms triggering this GC formation are still under investigation, although we suspect a role for T cell-dependent CD40 ligation, as both T-cell deficient *Act1*^{-/-} mice (36) and *Act1*^{-/-}CD40^{-/-} double deficient mice show a complete abrogation of GC formation (Y. Qian, N. Giltiay, unpublished data). Activated 4-44⁺ B cells in AM14 Tg MLR.Fas^{lpr} autoimmune mice displayed a phenotype of short-lived plasmablasts and were found to actively proliferate and to undergo somatic hypermutations outside of the GC-follicles (30, 37).

To determine the location of expanded 4-44⁺ B cells in AM14 Tg *Act1*^{-/-} mice, we analyzed splenic and cLNs sections. In agreement with our FACS and ELISpot data, consistent with an extrafollicular response 4-44⁺ splenic cells from AM14 Tg *Act1*^{-/-} mice were observed to cluster outside of the B cell follicles (Fig. 5A). The expansion of 4-44⁺ B cells was more apparent in the cLN of AM14 Tg *Act1*^{-/-} mice, where 4-44⁺ B cells showed both follicular and extrafollicular localization (Fig. 5B). We found that a significant percentage of AM14 Tg *Act1*^{-/-} mice (~60%) developed 4-44⁺ positive GCs in their cLNs (Fig. 5B, right panel). This result was supported by flow cytometry analysis that showed a significant increase in the percentage of GL7⁺ 4-44⁺ B cells in cLNs from AM14 Tg *Act1*^{-/-} mice cells as compared to AM14 Tg *Act1*^{+/+} controls (Fig. 5C-D).

Immunohistological analyses of cLNs from *Act1*^{-/-} AM14Tg IgH^a using Ki67 revealed the presence of proliferating 4-44⁺ B cells both in the GC as well as at extrafollicular sites (Supplementary Figure 2).

Dependence of 4-44⁺ *Act1*^{-/-} B cells on CD40-mediated signaling

In our previous studies we found that intact CD40/CD40L interaction is required for the formation of disease-associated autoantibodies in the *Act1*^{-/-} mice (33). In *ex vivo* settings, we observed that *Act1*^{-/-} mature B cells responded more vigorously to CD40-ligation (anti-CD40 Ab) as compared to WT B cells, and differentiated much faster into proliferating blast-like cells (Supplementary Figure 3). Previously we also showed that agonistic anti-CD40Ab treatment was associated with a more significant up-regulation of the Bcl_{xL} protein in *Act1*^{-/-} B cells as compared to WT B cells (34).

To test if blocking CD40 ligation *in vivo* had an effect on the activation of 4-44⁺ B cells and the formation of plasmablasts and GCs in AM14 Tg *Act1*^{-/-} mice, we treated mice with anti-gp39 blocking Ab (clone MR-1). Spleens and cLNs of injected mice were harvested on day

7 after the initial injection and were analyzed by FACS. Results from this analysis showed that blocking of CD40 resulted in significantly decreased frequencies of 4–44⁺ CD22^{hi} and 4–44⁺ CD22^{lo} B cells in the spleen and cLN of AM14 Tg *Act1*^{-/-} mice (Fig. 6 A and B). No significant decrease in total B cells was observed (not shown). MR-1 treatment also led to decrease in the frequency of 4–44⁺ GL7⁺ cells in the cLNs of AM14 Tg *Act1*^{-/-} mice (Fig. 6 A and B).

Finally, we detected a reduction in serum AM14 Ab titers in anti-CD40L Ab-treated mice (Fig. 6 C), indicating that this serum Ab derives from short-lived AFCs, presumably plasmablasts, rather than from long-lived plasma cells, which would not be affected directly by MR-1 Ab treatment, nor substantially even indirectly via GC inhibition, over the space of a week (38). Thus, it appears that CD40 signaling is important for the optimal activation and differentiation of 4–44⁺ B cells in AM14 Tg *Act1*^{-/-} mice *in vivo*. Based on these findings, we propose a model in which the lack of *Act1* leads to dysregulation of CD40 signaling and results in spontaneous activation of 4–44⁺ B cells.

DISCUSSION

Loss of B cell tolerance plays a key role in the development of systemic autoimmunity. We previously reported that deficiency of *Act1* in mice leads to dysregulation of B cell function in the periphery and results in the development of SJS- and SLE-like autoimmune diseases (33, 34).

In this study, we show that crossing of AM14 Tg mice to *Act1*^{-/-} mice leads to the activation of AM14 Tg B cells. We found that AM14 Tg *Act1*^{-/-} mice developed enlarged spleens and cLNs, and displayed expansions of RF-specific autoreactive B cells as compared to AM14 Tg *Act1*^{+/+} mice. Furthermore, our data indicated that in the presence of endogenous self-Ag (BALB/c background), AM14 Tg *Act1*^{-/-} B cells became activated and differentiated into functional AFCs. Notably, we found that a significant number of activated AM14 Tg (4–44⁺) B cells accumulated in the cLN of AM14 Tg *Act1*^{-/-} mice, where they differentiated into GC B cells as well as AFCs.

The exact mechanisms by which *Act1* regulates B cell function in the periphery are not fully understood. In our previous studies, we found that *Act1* interacts with TRAF family members and functions as an inhibitor of BAFF-R- and CD40-signaling pathways by regulating the activation of the NF- κ B1/ NF- κ B2 pathways and MAPK signaling (34). In addition to its regulatory function in BAFF-R and CD40 signaling in B cells, the *Act1* molecule was identified as a key signaling component of IL-17R signaling in fibroblasts, endothelial and epithelial cells, where it plays an opposite function in promoting NF- κ B1 activation (39–41).

Under normal conditions, several mechanisms function to prevent the activation of autoreactive B cells (2, 3). Alterations that enhance BCR signaling have been associated with a breakdown in B cell tolerance and the development of autoimmunity. For example, mutations in the Bruton's tyrosine kinase (*Btk*) gene and tyrosine phosphatase nonreceptor type 22 (*PTPN22*) gene, two molecules that function downstream of the BCR, have been implicated in defective selection of autoreactive B cells at the central tolerance checkpoint in humans (42). Mice that lack the expression of *Lyn*, a Src family kinase that activates the inhibitory receptors CD22 and *Fc γ RIIb*, which are involved in the negative regulation of BCR signaling, develop autoimmunity associated with production of autoantibodies to nuclear Ags (43–47).

In addition to BCR-hyper-responsiveness, alterations in the strength of B cell pro-survival signals can disrupt the negative selection of autoreactive B cells. For example, overexpression of B cell survival factor BAFF in BAFF Tg mice has been shown to breach the peripheral tolerance checkpoint and to promote the expansion of T2-MZ precursors and MZ B cells, resulting in the development of SLE- and SjS-like diseases (15, 48, 49).

In previous studies, we have shown that Act1 deficiency resulted in dysregulation of BAFF signaling during transitional B cell maturation and promotes the maturation of HEL-specific autoreactive B cells (32). Here, we found that Act1 deficiency did not affect the development of AM14 Tg B cells in the periphery, as we did not find significant differences in the percentages of 4-44⁺ cells within T1, T2, Fo B cells subset in the AM14 Tg Act1^{-/-} as compared to AM14 Tg Act1^{+/+} littermates. In addition, while previous analysis of Act1^{-/-} mice showed an increase in MZ B cells (32, 34) we did not find a significant percentage of 4-44⁺ B cells expressing a MZ phenotype in the AM14 Tg Act1^{-/-} mice. Thus, our data are in agreement with previously published studies in AM14 Tg mice, where 4-44⁺ B cells progress normally through immature stages and accumulate in the B cell follicles, but not in MZs (22, 35). The lack of impact on AM14 Tg B cell development by Act1 deficiency may reflect relatively weak self-reactivity, as well as the fact that AM14 Tg B cells are clonally ignorant.

The appearance of 4-44⁺ CD22^{lo} cells in AM14 Tg Act1^{-/-} mice suggests that Act1 deficiency leads to spontaneous activation of AM14 Tg B cells, and drives the differentiation of AM14 Tg B cells into AFCs. Similar to previous studies (26, 31) using the AM14 Tg MLR.Fas^{lpr} mouse model, we found that the spontaneous activation of 4-44⁺ B cells was associated with up-regulation of CD69 and CD44 markers, and occurred only in the presence of endogenous autoantigen (i.e. on the IgH^a BALB/c background). The extent of AFC development was variable between individual AM14 Tg Act1^{-/-} IgH^a mice and increased with age, suggesting the stochastic nature of their activation.

Interestingly, we found a significant accumulation of AM14 Tg B cells in the cLNs, which were visibly enlarged. The exact mechanism driving the accumulation of activated autoreactive 4-44⁺ B cells in the cLN of AM14 Tg Act1^{-/-} remains unclear, however it might be related to the fact that Act1^{-/-} BALB/c mice develop symptoms of SjS, associated with infiltration of activated autoreactive B cells in the salivary and lacrimal glands (33).

The activation of AM14 Tg cells in previously studied AM14 Tg MLR.Fas^{lpr} mice was found to occur mainly outside of the GC in the spleen resulting in the generation of short-lived plasmablasts (50). However, AM14 B cells did form GCs in the LNs of both BALB/c and MRL.Fas^{lpr} mice, but only in the presence of self-Ag (51). The observation that 4-44⁺ B cells form GCs in AM14 Tg Act1^{-/-} mice suggests a parallel mechanism of action of Act1 in the regulation of autoreactive B cells.

It is well accepted that T-cells express CD40L, along with other co-stimulatory molecules and cytokines, and thus provide help to B cells, resulting in clonal expansion, enhanced Ab production, class-switching, and somatic hypermutation (52). The requirements of T-cell help for the spontaneous activation of AM14 cells was extensively studied in AM14 Tg MLR.Fas^{lpr} mice (31), as well as in AM14 BALB/c mice challenged with chromatin-containing immune complexes (29). These studies demonstrated that exposure of AM14 Tg cells to IgG2a anti-chromatin complexes can sustain the activation of AM14 Tg B cells even *in the absence* of antigen-specific T cell help. Following up on this observation, a number of recent studies have suggested an important role for TLRs in driving T-cell independent activation of autoreactive B cells (42, 53). Still, for isotype-switched responses, T cell help

had a substantial effect on the magnitude of the response (4–5-fold), indicating that T cells can and do participate in generating extrafollicular plasmablasts (31).

Our recent studies showed that lack of T-cells in B6.*Act1*^{-/-}*TCRβ*^{-/-}*TCRδ*^{-/-} mice resulted in an almost complete inhibition of anti-nuclear IgG Abs in the serum, suggesting that T-cell help is required for the development of self-reactive antibodies in the B6.*Act1*^{-/-} mouse (36). Likewise, we have previously shown that *CD40*^{-/-} *Act1*^{-/-} BALB/c mice fail to develop serum auto-Abs and/or SjS- and SLE-like disease (33, 34). Based on these observations, we predicted that stimulation through CD40 plays a key role in the activation of 4–44⁺ B cells in AM14 Tg *Act1*^{-/-} mice and in the selection of 4–44⁺ cells to the GCs. Indeed, blocking CD40L by MR-1 Ab treatment led to a reduction in the percentage of 4–44⁺ B cells, decreased AM14 Ab serum titers, and reduced percentages and numbers of 4–44⁺ GL7⁺ cells. Together, these findings strongly support a model in which CD40-mediated signaling plays a key role in the activation of 4–44⁺ B cells in AM14 Tg *Act1*^{-/-} mice. The development of Ag-driven GCs in AM14 Tg *Act1*^{-/-} mice suggests the generation of cognate T cell help at the target LNs. The mechanisms by which the lack of *Act1* can promote breakdown of T cell tolerance remain unknown. One possibility is that activated AM14 Tg *Act1*^{-/-} B cells present Ags to self-reactive T cells, which express CD40L along with other cytokines and costimulatory molecules. Activation of B cells via CD40L-CD40 can promote the activation and differentiation of CD4⁺ T cells to T follicular helper cells, for example by ICOSL-ICOS interactions (54), thus mediating the formation of GCs.

Recent studies have implicated a role for Th17-derived cytokines IL-17 in promoting Ab-production by B cells and the formation of GC (55). Despite the fact that *Act1*^{-/-} mice show an increase in the production of IL-17 (56), it is unlikely that IL-17-mediated signals contribute directly to the expansion of AM14 Tg and GC formation in the AM14 Tg *Act1*^{-/-} mice due to their inability to mount IL-17 responses (41). Consistent with its role in IL-17 mediated inflammation, we and others have shown that *Act1* is essential for the development of EAE and CIA (41, 57). Recent studies revealed a novel role of *Act1* in controlling the activation of Th17 cells and showed an increased production of IL-22 by Th17 cells in *Act1*^{-/-} mice (56). However, since Th17 cells can make other cytokines, most notably IL-21, they might contribute to the activation of AM14 Tg B cells (58, 59). Interestingly, lack of *Act1* was recently shown to protect *FcγRIIb*^{-/-} mice from development of lethal glomerulonephritis by preventing the influx of inflammatory myeloid-derived cells into the kidneys, without affecting the production of anti-nuclear Abs (60). Thus, the role of *Act1* molecule in SLE pathology can be multi-faceted. Despite the lack of IL-17R signaling, *Act1*^{-/-} BALB/c mice spontaneously develop autoimmune pathology, associated with GC formation and production of autoantibodies (33, 34), underscoring the important, cell-intrinsic role of *Act1* in B cell regulation. Previous studies using *Act1*^{-/-} mice have supported a role for *Act1* in the selection of autoreactive B cells at the transitional checkpoint (32, 36). Results presented in this study further suggest that *Act1* plays an important function in the suppression of AFC and GC formation by autoreactive B cells. This function of *Act1* is most likely mediated via the regulation of CD40 responsiveness of B cells. Our findings support the requirement for a strict regulation CD40-mediated signaling in the toleralization of low affinity autoreactive B cells and suggest how we might interfere with pathogenic B cell responses in autoimmune diseases such as SLE and SjS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AFC	Antibody Forming cells
BAFF	B cell-activating factor belonging to the TNF family
BCR	B cell antigen receptor
BM	bone marrow
cLN	cervical lymph node
FM	follicular mature
MZ	marginal zone
RF	rheumatoid factor
SjS	Sjögren's Syndrome
SLE	Systemic Lupus Erythematosus

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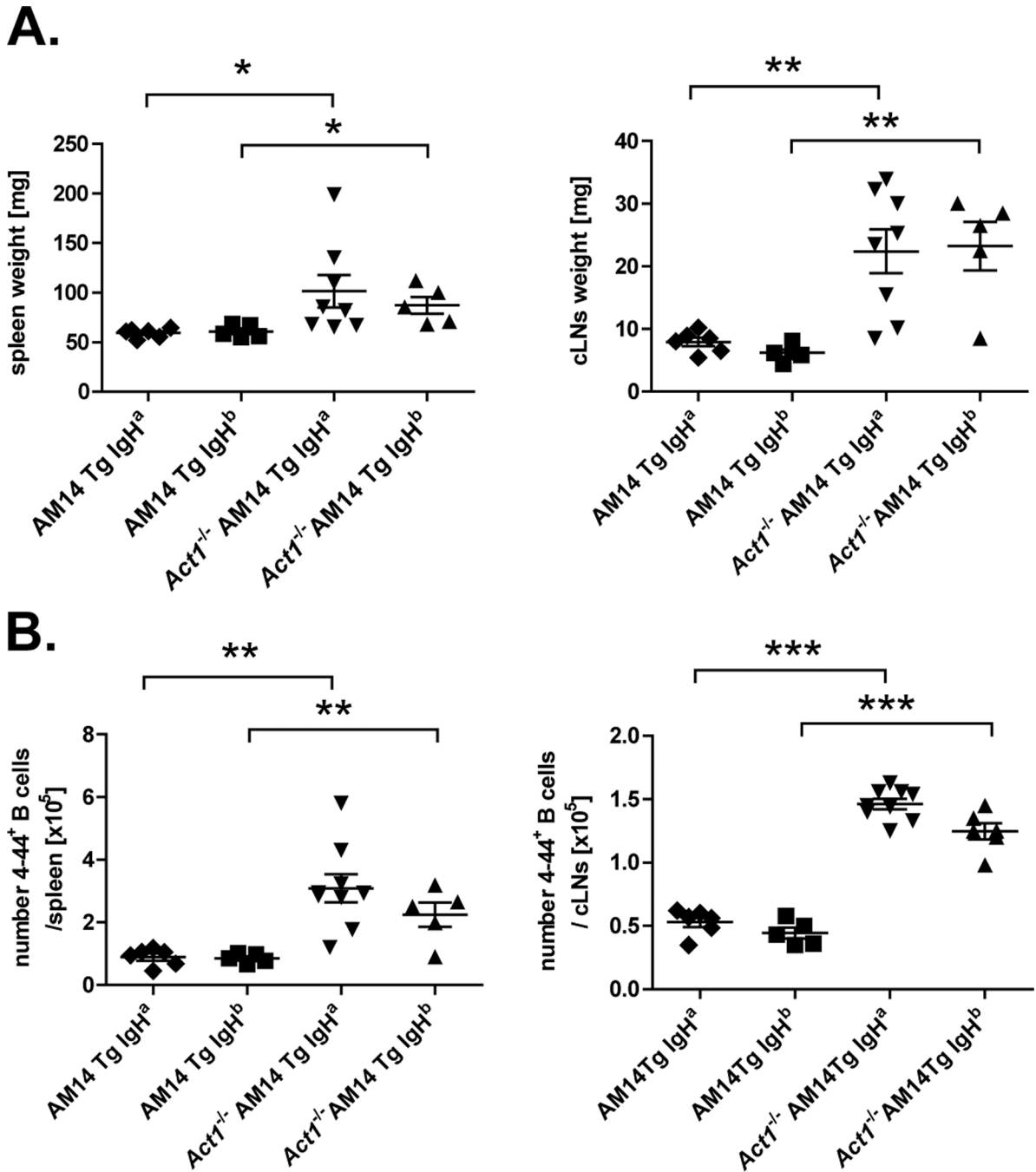


Figure 1. Expansion of AM14 Tg (4-44⁺) B cells in spleen and cLNs of AM14 Tg *Act1*^{-/-} mice associated with splenomegaly and lymphadenopathy. A. Aged-matched AM14 Tg or AM14 Tg *Act1*^{-/-} (IgH^a and IgH^b backgrounds) were analyzed for the development of splenomegaly and lymphadenopathy. Data are presented as mg tissue (spleen or combined superficial cervical LNs), for each individual mouse. B. Summary of FACS data showing the number of 4-44⁺ cells per spleen or combined cLN. The percentage of 4-44⁺ of total cells was determined by FACS and the cell numbers were calculated based on the total cell count for each individual mouse. ** p < 0.01, *** p < 0.001.

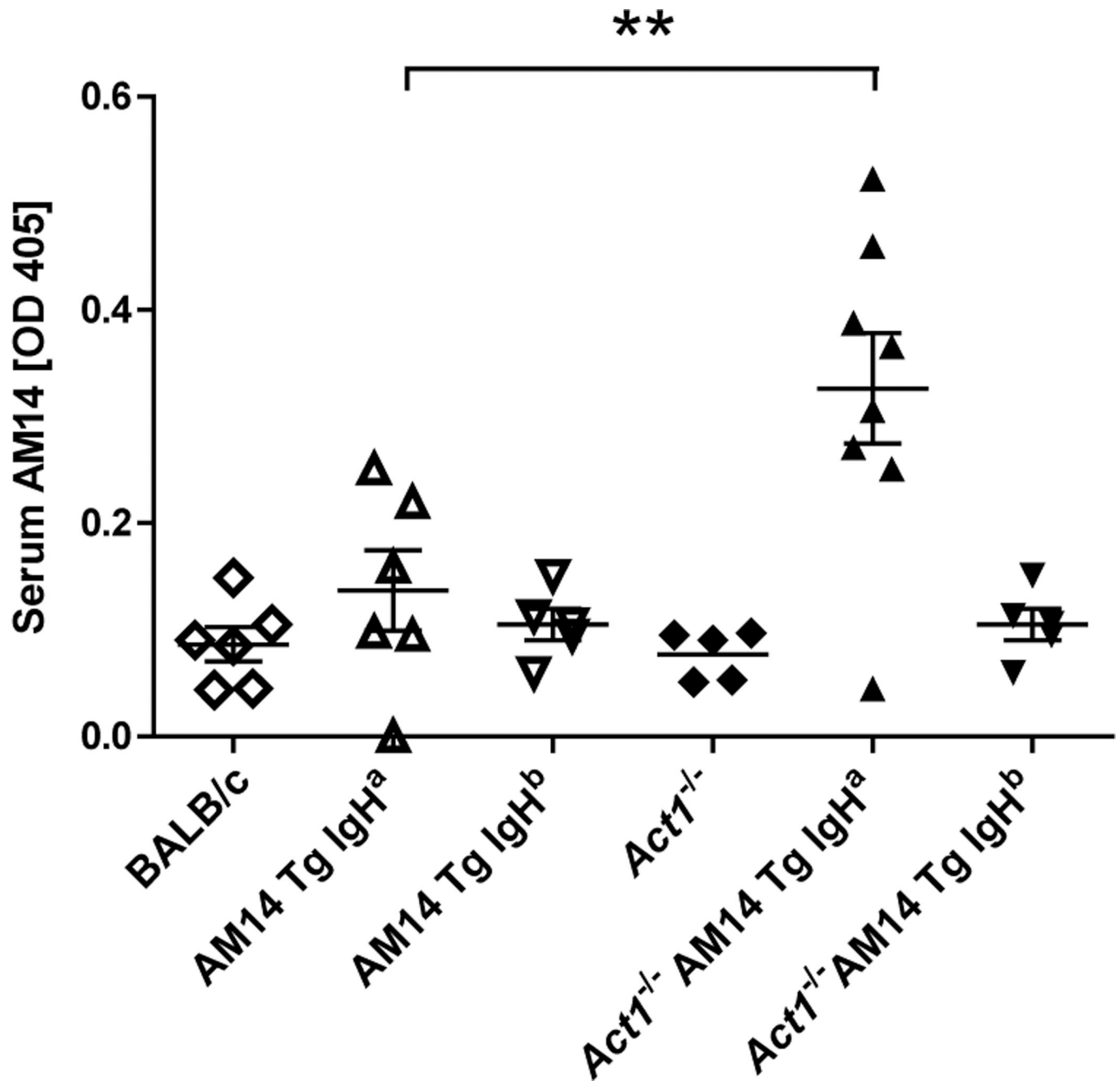
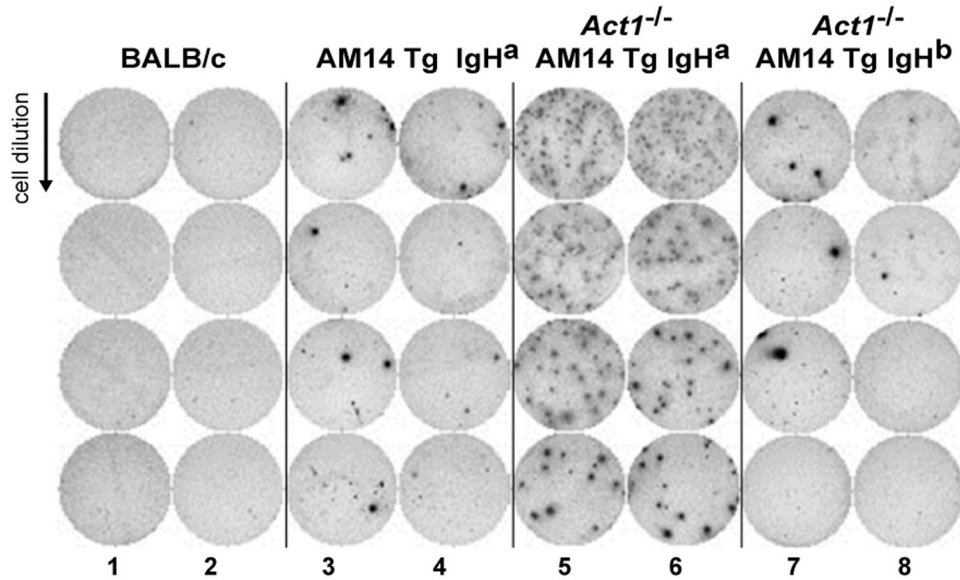
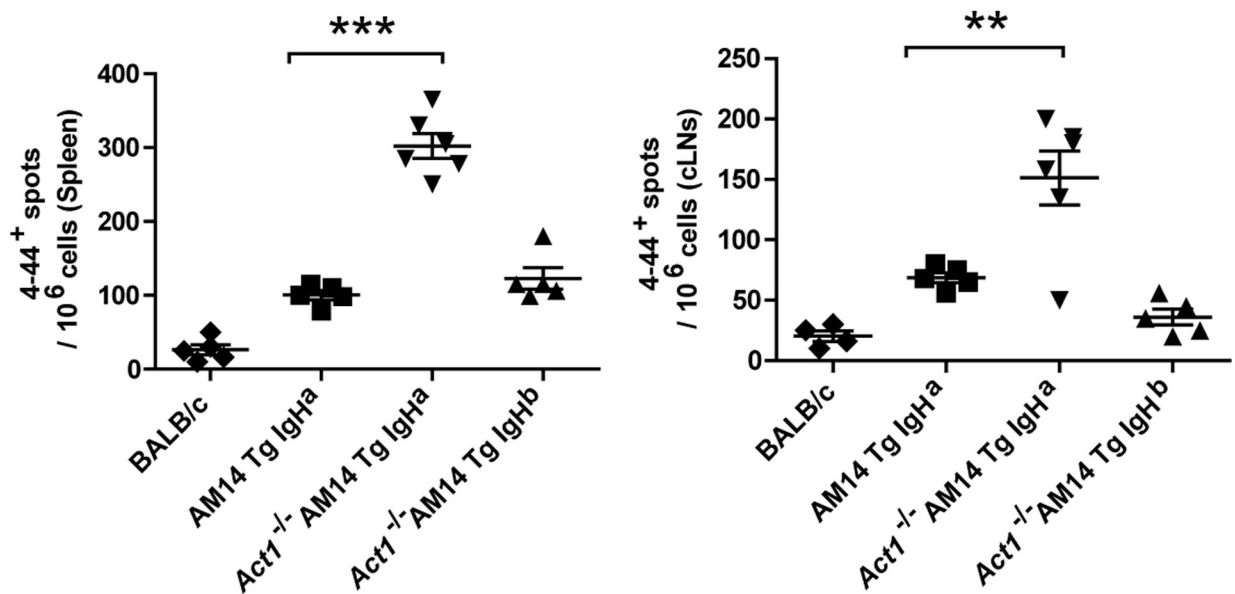


Figure 2.

Increase of AM14 protein levels in AM14 Tg *Act1*^{-/-} IgH^a mice. Serum levels of AM14 protein were measured by ELISA as described in Materials and Methods. Samples were collected from 3 to 4.5-months-old mice. Each symbol represents one individual mouse. ** $p < 0.01$.

A.**B.****Figure 3.**

Accumulation of AM14 Tg AFCs in spleens and cLNs of AM14 Tg Act1^{-/-} mice. A. Representative ELISpot analysis used to quantify splenic 4-44⁺ AFCs in AM14 Tg Act1^{+/+} or AM14 Tg Act1^{-/-} mice (on IgH^a and IgH^b backgrounds). B. Number of 4-44⁺ AFCs detected by ELISpot in spleens or cLNs from individual AM14 Tg or AM14 Tg Act1^{-/-} mice. Data is presented as number of spots per 10⁶ cells. ** p < 0.01, *** p < 0.001.

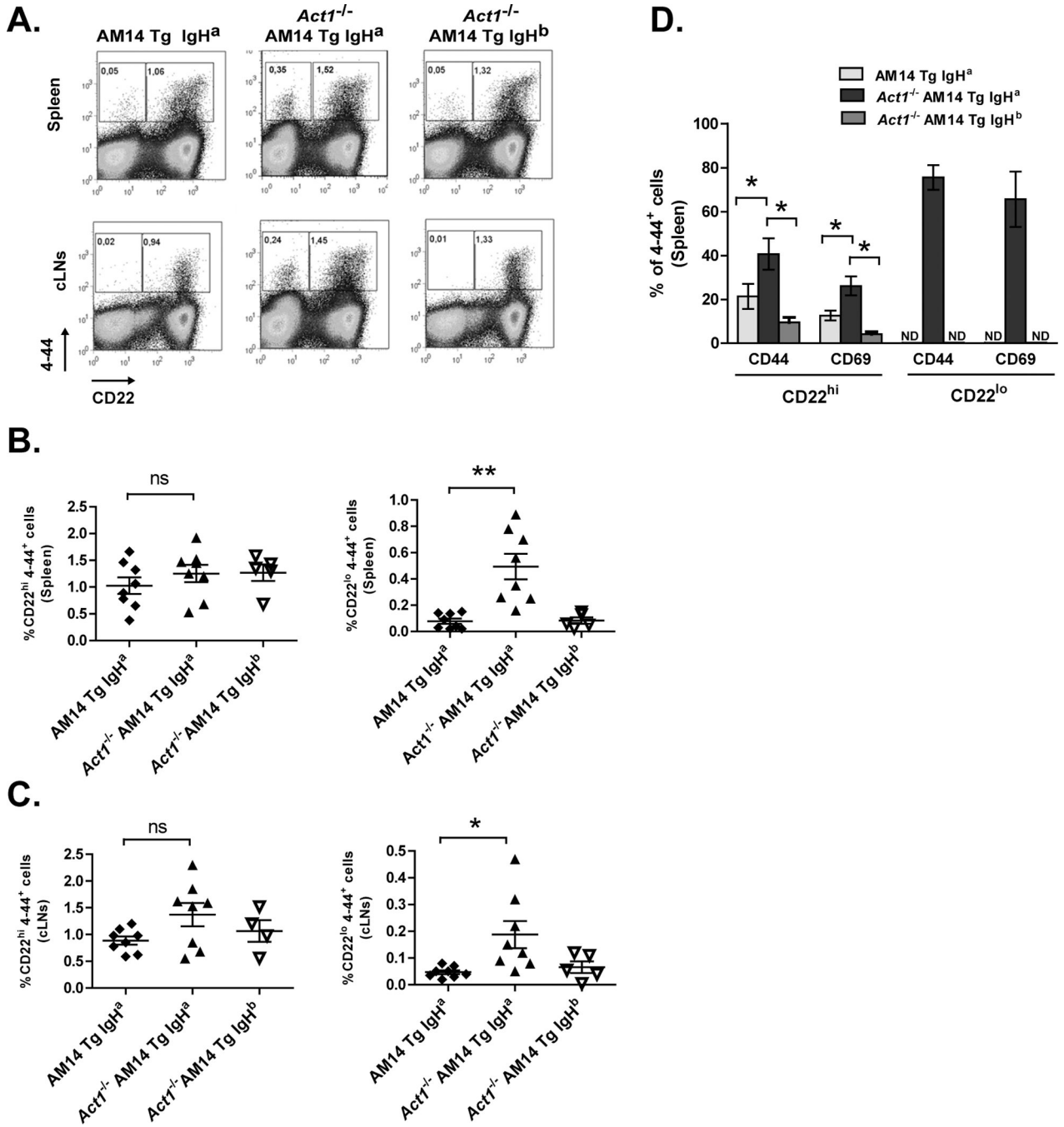


Figure 4.

Detection of AM14 Tg plasmablast in AM14 Tg *Act1*^{-/-} mice. A. Representative FACS plots of cells from AM14 Tg *Act1*^{+/+}, AM14 Tg *Act1*^{-/-} IgH^b and AM14 Tg *Act1*^{-/-} IgH^a mice. Gates identify 4-44+ CD22^{hi} AM14 Tg B cells and 4-44+ CD22^{lo} AM14 Tg cells. B and C. Summary of FACS data, showing the percentage of 4-44+ CD22^{hi} and 4-44+ CD22^{lo} B cells of total live cells in spleens and cLNs from individual AM14 Tg IgH^a, AM14 Tg *Act1*^{-/-} IgH^a and AM14 Tg *Act1*^{-/-} IgH^b mice. D. Summary of FACS data of the percentage of 4-44+ CD22^{hi} and 4-44+ CD22^{lo}, expressing CD44 and CD69 activation markers. No results available for 4-44+ CD22^{lo} in AM14 Tg mice due to low cell count. AM14 Tg IgH^a

mice (n=5), AM14 Tg *Act1*^{-/-} IgH^a mice (n=6) and AM14 Tg *Act1*^{-/-} IgH^b mice (n=3) * p < 0.05, ** p < 0.01, *** p < 0.001.

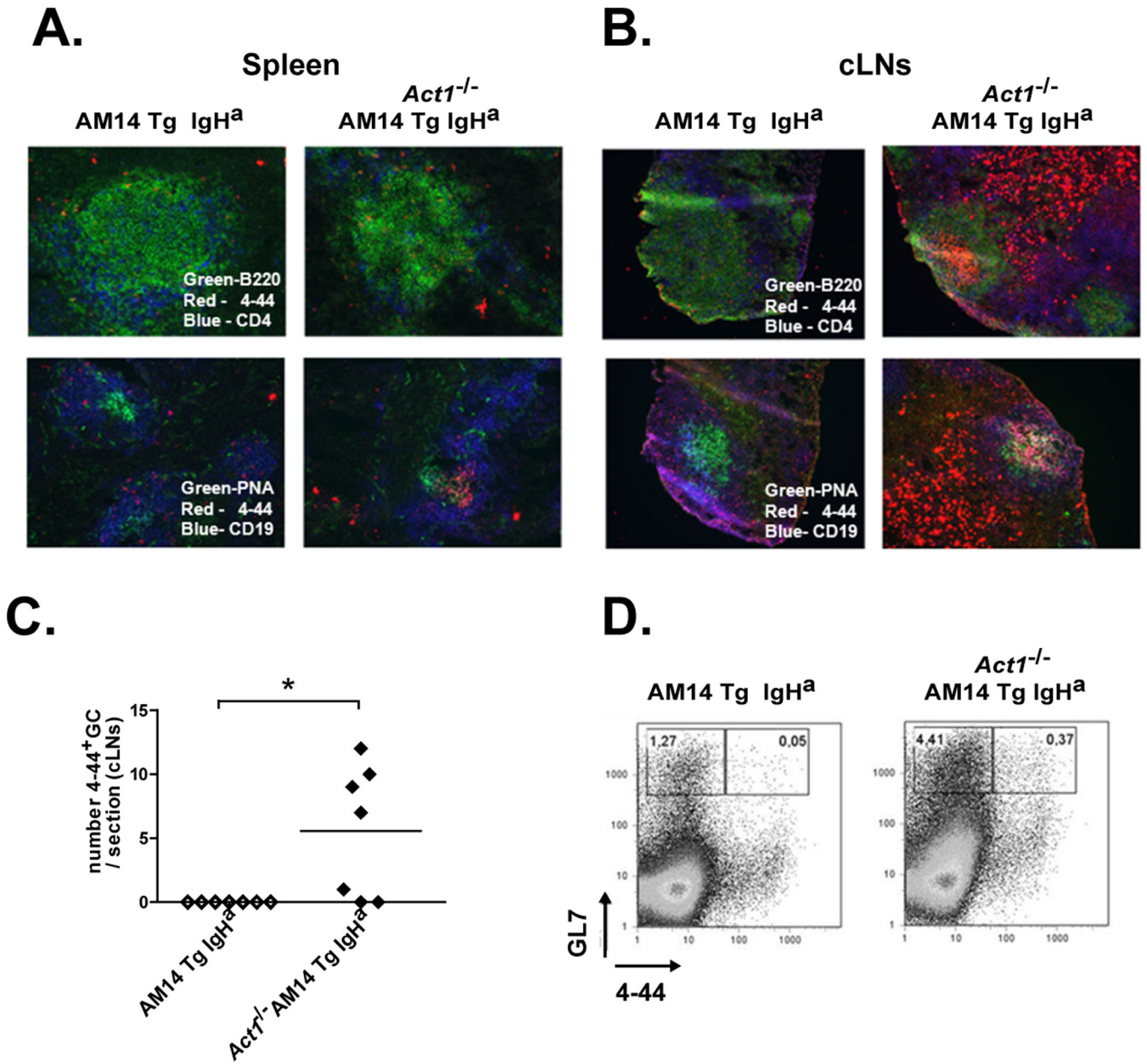


Figure 5. AM14 Tg B cells in the AM14 Tg *Act1*^{-/-} mice undergo activation at extra-follicular sites and form GCs. A and B. Micrographs of immunofluorescent staining of splenic (A) and cLN (B) sections from AM14 Tg *Act1*^{+/+} IgH^a and AM14 Tg *Act1*^{-/-} IgH^a mice showing: B220⁺ B cells (green), CD4⁺ T cells (blue) and 4-44⁺ AM14 Tg cells (red) – upper panel; and PNA⁺ GC cell (green), CD19⁺ B cells (blue) and 4-44⁺ AM14 Tg cells (red) – lower panel. C. Graph showing the number of GC per section of cLN from AM14 Tg *Act1*^{+/+} and AM14 Tg *Act1*^{-/-} mice. D. Representative FACS plots showing the percentage of CD44⁺ GL7⁺ in cLNs from AM14 Tg and AM14 Tg *Act1*^{-/-} mice. * p < 0.05.

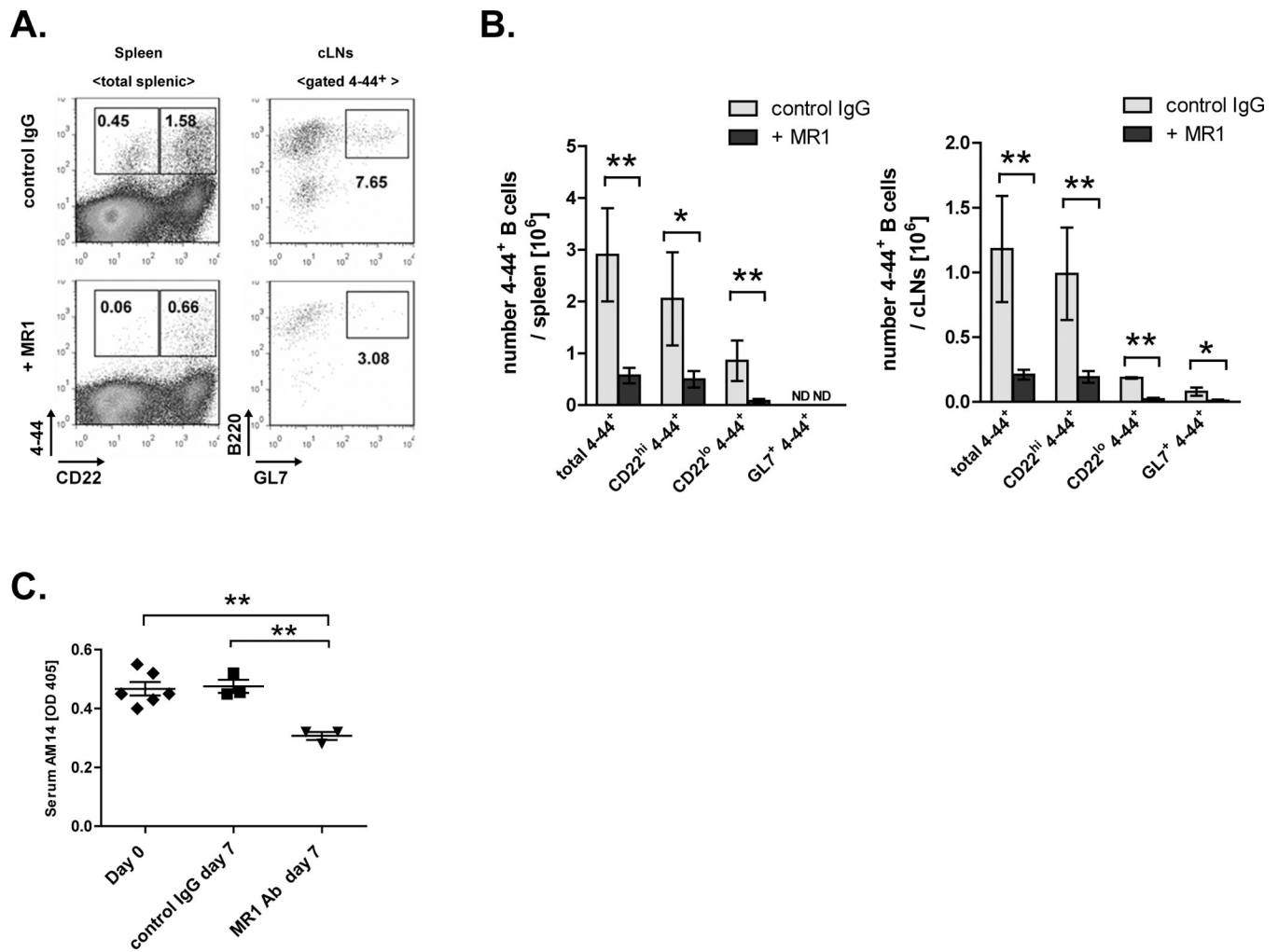


Figure 6. AM14 Tg *Act1*^{-/-} B cells are susceptible to anti-CD40L treatment. A. Representative FACS plot showing the percentage CD44⁺CD22^{hi} and CD44⁺CD22^{lo} cells of splenic cells from AM14 Tg *Act1*^{-/-} mice treated with control IgG (left top panel) or anti-CD40L Ab MR1 (left bottom panel). Right panels show the percentage of B220⁺GL7⁺ of gated CD44⁺ from cLN from mice, treated with control IgG (top panel) or MR1Ab (bottom panel). B. Numbers of total 4-44⁺, 4-44⁺CD22^{hi}, 4-44⁺ CD22^{lo} and 4-44⁺ GL7⁺ in spleen and cLN of control and MR1-injected mice analyzed at day 7. C. Levels of AM14 protein measured by ELISA in serums from AM14 Tg *Act1*^{-/-} mice at day 0 and day 7 after the IgG or MR1 administration. * p < 0.05, ** p < 0.01.