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Integrin-Mediated Interactions between B Cells and Follicular Dendritic Cells Influence Germinal Center B Cell Fitness

Xiaoming Wang, Lauren B. Rodda, Oliver Bannard, and Jason G. Cyster

Integrin–ligand interactions between germinal center (GC) B cells and Ag-presenting follicular dendritic cells (FDCs) have been suggested to play central roles during GC responses, but their *in vivo* requirement has not been directly tested. In this study, we show that, whereas integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$ are highly expressed and functional on mouse GC B cells, removal of single integrins or their ligands had little effect on B cell participation in the GC response. Combined β_2 integrin deficiency and α_4 integrin blockade also did not affect the GC response against a particulate Ag. However, the combined integrin deficiency did cause B cells to be outcompeted in splenic GC responses against a soluble protein Ag and in mesenteric lymph node GC responses against gut-derived Ags. Similar findings were made for β_2 -deficient B cells in mice lacking VCAM1 on FDCs. The reduced fitness of the GC B cells did not appear to be due to decreased Ag acquisition, proliferation rates, or pAKT levels. In summary, our findings provide evidence that $\alpha_L\beta_2$ and $\alpha_4\beta_1$ play overlapping and context-dependent roles in supporting interactions with FDCs that can augment the fitness of responding GC B cells. We also find that mouse GC B cells upregulate $\alpha_v\beta_3$ and adhere to vitronectin and milk-fat globule epidermal growth factor VIII protein. Integrin β_3 -deficient B cells contributed in a slightly exaggerated manner to GC responses, suggesting this integrin has a regulatory function in GC B cells. *The Journal of Immunology*, 2014, 192: 4601–4609.

Several *in vitro* studies over the last 25 years have highlighted the ability of germinal center (GC) B cells to undergo integrin $\alpha_L\beta_2$ (LFA1)- and $\alpha_4\beta_1$ -mediated adhesive interactions with follicular dendritic cells (FDCs) (1–5). $\alpha_L\beta_2$ and $\alpha_4\beta_1$ on the GC B cell bind cell adhesion molecules ICAM1 and VCAM1, respectively, that are upregulated on GC FDCs (5, 6). Mucosal addressin cell adhesion molecule 1 (MADCAM1), a ligand for both of the α_4 -containing integrins, $\alpha_4\beta_7$ and $\alpha_4\beta_1$, has also been detected on FDCs (4). As well as promoting cell–cell adhesion, both β_1 - and β_2 -containing integrins are able to mediate outside-in signaling in cells via tyrosine kinases, PI3Ks, and small G proteins (7–9). In short-term tissue culture, B cells that are associated with FDCs show enhanced survival, and this trophic effect is reduced when $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin function is blocked (3, 10–13). Integrins have been shown to increase cell viability in a number of contexts (7), and this can occur via activation of AKT-dependent prosurvival pathways (9), but whether integrin signaling is required for GC B cell survival *in vivo* has not been directly examined. In mice in which inhibitor of κ B kinase β

(IKK2) was ablated from FDCs, there was a loss of ICAM1 and VCAM1 expression and GC responses were diminished (14). However, this study could not rule out important roles for additional IKK2-dependent molecules in FDCs. Another study associated lower ICAM1 induction on FDCs under conditions of TLR4 blockade with a reduced GC response, but again the conclusion was correlative as TLR4 signaling influences many cell types (15).

GC B cells must efficiently acquire, process, and present Ag to receive positive selection signals from T follicular helper cells (16, 17). Much of the Ag present in GCs is displayed on the surface of FDCs in the light zone (6, 18). *In vitro* studies have shown for non-GC B cells that acquisition of surface-bound Ags from lipid bilayers can be augmented by $\alpha_L\beta_2$ - and $\alpha_4\beta_1$ -ligand interactions (19–21). Whether such interactions are important for Ag capture by GC B cells *in vivo* has not been determined.

In addition to cell adhesion molecules, a second group of integrin ligands are the extracellular matrix components. Although the GC is relatively devoid of collagens, laminin and fibronectin, studies in human tissue show the GC light zone contains the 70-kDa glycoprotein vitronectin (VN) (6). VN binds a number of integrins, including $\alpha_v\beta_3$ (22). Another secreted protein that is abundant in GCs is milk-fat globule epidermal growth factor VIII (MFGE8), a phosphatidylserine-binding protein that promotes clearance of apoptotic cells by engaging $\alpha_v\beta_3$ on macrophages (23, 24). MFGE8 is made by FDCs (25), and deficiency in MFGE8 is associated with development of lupus-like autoantibodies (26). However, whether GC B cells undergo integrin-mediated interactions with MFGE8 is unknown.

In this study, we report that neutralization of β_2 - and α_4 -containing integrin function has varying impacts on GC B cells depending on the type of response being mounted. During the polyclonal response to SRBCs, cells without β_2 and α_4 integrin function were able to participate efficiently in the GC response, indicating that these integrins are not universally required for Ag

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Abbreviations used in this article: BM, bone marrow; DEL, duck egg lysozyme; FDC, follicular dendritic cell; GC, germinal center; HEL, hen egg lysozyme; IKK2, inhibitor of κ B kinase β ; LN, lymph node; MADCAM1, mucosal addressin cell adhesion molecule 1; MFGE8, milk-fat globule epidermal growth factor VIII; SIP, sphingosine-1-phosphate; VN, vitronectin.

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capture or GC B cell survival. Importantly, however, during the response of B cells to a soluble protein Ag, β_2 and α_4 integrin deficiency compromised participation in the GC. This compromise did not involve obvious effects on affinity maturation, cell turnover, or induction of pAKT, suggesting that integrin-mediated adhesion to FDCs augments GC B cell fitness through additional pathways. We also find that GC B cells express elevated levels of $\alpha_v\beta_3$ integrin and bind both VN and MFGE8, ligands that are abundant within the GC.

Materials and Methods

Mice

C57BL/6 and C57BL/6 CD45.1⁺ mice were from the National Cancer Institute or Jackson ImmunoResearch Laboratories. Integrin *Itgb2*^{-/-} mice (27) were backcrossed to C57BL/6J for six generations. Hy10 mice with knock-in expression of Ig specific for hen egg lysozyme were from an internal colony (28). Mice expressing the CD21Cre transgene (B6.Tg[Cr2-Cre]3Cgn) (29) were from K. Rajewsky (CBR Institute for Biomedical Research and Harvard Medical School). *Itgb1*^{fl/fl} mice (30), VCAM1^{fl/fl} mice (31), and *Itgb3*^{-/-} mice (32) were from Jackson ImmunoResearch Laboratories (004605, 007665, and 008819, respectively). Animals were housed in a specific pathogen-free environment in the Laboratory Animal Research Center at the University of California San Francisco, and all experiments conformed to ethical principles and guidelines approved by the Institutional Animal Care and Use Committee of the University of California San Francisco.

Adoptive transfer, immunization, and treatment

Bone marrow (BM) chimeras were generated by mixing equal amounts of the indicated BM types and injecting $2\text{--}5 \times 10^6$ BM cells into lethally irradiated recipients, as described (33). Mice were reconstituted for 6–12 wk before immunization or analysis. SRBC immunizations were i.p. with 2×10^8 cells (Colorado Serum). For adoptive transfers, 5×10^4 each of integrin *Itgb2*^{+/+} (CD45.1/2 GFP⁺, CD45.1/2⁺, or CD45.1⁺) and *Itgb2*^{-/-} (CD45.2⁺GFP⁺, CD45.1⁺, or CD45.1/2⁺) Hy10 B cells were transferred into CD45.2⁺ hosts together with 5×10^4 OT-II T cells. To avoid rejection issues, all the hosts used as transfer recipients were C57BL/6J wild-type or VCAM1^{fl/fl} CD21Cre mice that had been lethally irradiated and reconstituted with 90% C57BL/6J and 10% integrin *Itgb2*^{+/+} BM cells in which the latter were from *Itgb2*^{+/+} intercrossed mice. The ratio of *Itgb2*^{-/-} and *Itgb2*^{+/+} Hy10 B cells in control GCs varied between experiments, probably because of slight inaccuracies in cell counts in the input mixtures. Due to the low number of B cells transferred, it was not possible to determine the ratio of engrafted *Itgb2*^{-/-} and *Itgb2*^{+/+} Hy10 B cells in the follicular compartment. Mice were immunized i.p. with 50 μ g duck egg lysozyme (DEL)-OVA in the Sigma-Aldrich adjuvant system. Some of the mice were i.v. injected with 200 μ g integrin α_4 Ab (PS/2; Bio X Cell) or MADCAM1 Ab (MECA367; Bio X Cell) at the indicated time. Some of the mice were also injected with 50 μ g anti-DEC205-OVA together with anti- α_4 , as indicated. The E α -GFP construct was provided by M. Jenkins (University of Minnesota), and the protein was produced, chemically conjugated to hen egg lysozyme (HEL), and purified, as described (34). Mice were immunized i.v. with 20 μ g HEL-E α -GFP 4 h prior to analysis.

Flow cytometry

Spleen and lymph nodes (LNs) were isolated and mashed into media containing 2% FCS. For analysis of GC B cells, cells were stained with anti-B220 (RA3-6B2; BD Biosciences or BioLegend); anti-CD19 (6D5; BD Biosciences); anti-Fas (Jo2; BD Biosciences); anti-IgD (11-26c.2a; BD Biosciences or BioLegend); anti-CD45.1 (A20; BD Biosciences or BioLegend); anti-CD45.2 (104; BD Biosciences or BioLegend); anti-IgG2b (RMG2b-1; BD Biosciences); homemade Alexa647-conjugated DEL; Ab to T cell and B cell activation Ag (GL7; BD Biosciences); anti-mouse E α 52-68 peptide bound to I-A^b (Y-Ae; eBioscience); anti-integrin β_1 (MB1.2; Chemicon); anti-integrin β_2 (C71/16; BD Biosciences); anti-integrin β_3 (2C9.G2; BioLegend); anti-integrin β_7 (M293; BD Biosciences); anti-integrin α_4 (PS/2; Bio X Cell); anti-integrin α_L (M17/4; Bio X Cell); anti-integrin α_v (RMV-7; BD Biosciences); rat IgG2a isotype control (2A3; Bio X Cell); rat IgG2b isotype control (LTF-2; Bio X Cell); or rat IgG1 isotype control (R3-34; BD Biosciences). BrdU staining was done using BrdU flow kit (BD Biosciences) following manufacturer's instructions. For intracellular staining of phosphorylated AKT at Ser⁴⁷³ (pAKT), cells were instantly fixed and stained, as described (35). Anti-pAKT (9271; Cell Signaling Technology) was used.

Immunofluorescence

Cryosections 7 μ m in thickness were cut and fixed in cold acetone. Sections were stained with anti-ICAM1 (3E2; BD Biosciences); anti-VCAM1 (429; BD Biosciences); anti-MADCAM1 (MECA367; Bio X Cell); anti-IgD (11-26c.2a; BioLegend); anti-VN (347317; R&D Systems); rat IgG2a isotype control (2A3; Bio X Cell); or anti-CD35 (8C12; BD Biosciences), using described protocols (36).

Adhesion assay

Adhesion assay was done, as described (37). Plates were coated with 10 μ g/ml ICAM1, VCAM1, MADCAM1, VN, or MFGE8. VN was from Abcam. All of the other reagents were from R&D Systems.

Statistics

Statistical analysis was performed using two-tailed Student *t* tests.

Results

β_2 and α_4 integrin and integrin–ligand expression in the GC

By flow cytometric analysis, splenic GC B cells had elevated α_L and β_2 integrin expression compared with follicular B cells (Fig. 1A). Integrin β_1 levels were slightly elevated, whereas β_7 was reduced (Fig. 1A), suggesting GC B cells shift from a mixture of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ heterodimers to predominantly expressing $\alpha_4\beta_1$. Immunofluorescence analysis of serial sections from SRBC-immunized spleens showed abundant expression of ICAM1, VCAM1, and MADCAM1 on the GC FDC network (Fig. 1B), as expected (4, 38, 39). In a static adhesion assay, GC B cells adhered more strongly than follicular B cells to ICAM1 (Fig. 1C), consistent with the higher expression of $\alpha_L\beta_2$ in GC B cells. Both cell types adhered strongly to VCAM1 and weakly to MADCAM1 (Fig. 1C).

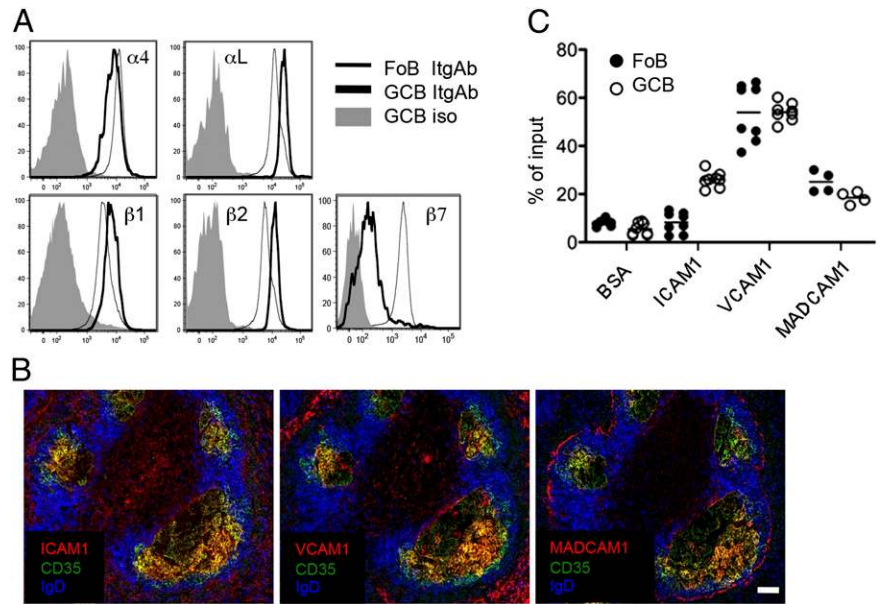
Intact GC response to complex particulate Ag

To test whether β_1 integrin-mediated interaction with FDC-expressed VCAM1 was important during the GC response, we generated mixed chimeras by reconstituting irradiated wild-type mice with *Itgb1*^{fl/fl} or *Itgb1*^{fl/+} Mb1Cre⁺ CD45.2⁺ BM and wild-type CD45.1/2⁺ BM. Eight days following SRBC immunization, there was an underrepresentation of CD45.2⁺ GC B cells compared with follicular B cells in both groups that most likely reflects the impact of Mb1 heterozygosity in the Mb1Cre⁺ cells (40). However, there was no significant reduction in GC representation of the *Itgb1*^{-/-} B cells compared with their heterozygote controls (Fig. 2B). We confirmed that there was efficient ablation of integrin β_1 from *Itgb1*^{fl/fl} Mb1Cre⁺ GC B cells (Fig. 2B). A similar mixed BM chimera analysis using *Itgb2*^{-/-} and littermate control donors showed that this integrin was also not essential for B cell participation in the GC response (Fig. 2C). To test for possible redundancy between $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins in GC B cells, we induced GCs in *Itgb2*^{-/-}:wild-type mixed BM chimeras and then treated them with anti- α_4 blocking Ab from days 7 to 14 after SRBC immunization. Analysis of GC B cells at the time of isolation confirmed that their α_4 integrins were saturated with the Ab (Fig. 2D). Even under these conditions, in which $\alpha_L\beta_2$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ integrins are absent or blocked from binding FDC-expressed ICAM1, VCAM1, and MADCAM1, we observed no impact on B cell participation in the SRBC-induced GC response (Fig. 2D).

Role for $\alpha_L\beta_2$ and $\alpha_4\beta_1$ in response against soluble protein Ag

We considered it likely that the contribution of integrins to the GC response may vary depending on the form of Ag. To test whether integrin function is required during the response to a soluble protein Ag, we intercrossed lysozyme-specific IgH knock-in and L chain transgenic (Hy10) mice (28) to the *Itgb2*^{-/-} background. B cells from these mice were cotransferred with congenically marked wild-type Hy10 B cells and wild-type OTII T cells to syngeneic

FIGURE 1. $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_L\beta_2$ integrin and ligand expression in the GC. **(A)** C57BL/6 mice were immunized with SRBCs, and on day 8 expression of integrins on B220⁺IgD⁺Fas⁻ follicular B cells (FoB) and B220⁺IgD⁻Fas⁺ GL7⁺ GC B cells (GCB) was analyzed by flow cytometry with chain-specific Abs (ItgAb). **(B)** Serial sections of a SRBC-immunized spleen stained with the indicated integrin ligand Abs (red), CD35 (green), and IgD (blue). Scale bar, 100 μ m. Data in (A) and (B) are representative of more than three mice. **(C)** Splenocytes from SRBC-immunized mice were allowed to adhere to plastic coated with ICAM1, VCAM1, or MADCAM1 and adherent subsets enumerated by flow cytometry. Data are shown as percentage of input cells that remained adherent and are representative of at least two experiments.



recipients that were then immunized with a conjugate of DEL and OVA (OVA; DEL-OVA) in monophosphoryl lipid A-based adjuvant (28). In a first experiment, the impact of α_4 integrin blockade during the early phase of the response was tested. Treating with Ab on days 1, 3, and 5, followed by analysis on day 7, showed the GC response was unaffected by α_4 -Ab treatment (Fig. 3B). These data suggested that β_2 and α_4 integrin function on B cells was not crucial for entry into the GC response against a soluble Ag. We next tested the impact of blocking integrin function during the GC response. Transfers were performed as before, and mice were left untreated until day 7; they were then treated with α_4 -neutralizing Ab on days 7, 9, 11, and 13, or only on day 12, and analyzed on day 14. Under these conditions, the β_2 -deficient GC B cells were underrepresented compared with the WT GC B cells. This effect was more striking with the longer period of treatment (Fig. 3C).

To confirm that the α_4 -blocking Ab was acting by inhibiting GC B cell interaction with FDC-expressed VCAM1, we generated mice lacking VCAM1 on FDCs by intercrossing VCAM1^{fl/fl} mice (31) with CD21cre mice (14). Immunofluorescence analysis confirmed the efficient and selective ablation of VCAM1 from the FDC networks (Fig. 3D). Using these mice as recipients for transferred Itgb2^{-/-} Hy10 B cells showed that, compared with littermate control hosts, there was a significant reduction in GC participation on day 14 (Fig. 3E). Additionally, blocking MADCAM1 by treatment with a neutralizing Ab had only a mild effect in further reducing the response. Because the integrin ligands are most abundant in the GC light zone, we examined whether the proportion of light zone GC B cells was affected by β_2 integrin and VCAM1 deficiency. Analysis of the fraction of GC B cells with a light zone (CXCR4^{low}CD86^{high}) phenotype showed comparable frequencies for integrin function-deficient and control cells (Fig. 3F). We attempted to perform experiments to confirm that the B cell β_2 integrin requirement was for interaction with FDC-expressed ICAM1 by using as recipients chimeric ICAM1^{-/-} mice that had been reconstituted with wild-type BM. However, in the course of these experiments, we observed that ICAM1^{-/-} mice retained measurable ICAM1 on FDCs, most likely due to alternative splicing around the targeted exon (exon 4) (41) prohibiting us from performing this analysis. Finally, we asked whether the endogenous GC response that takes place in mesenteric LNs against commensal flora-derived Ags was influenced by integrin deficiency. Analysis of

Itgb2^{-/-}:wild-type mixed BM chimeras 8–12 wk after reconstitution showed that β_2 deficiency alone had no effect on GC participation, but, when combined with VCAM1 deficiency from FDCs, the β_2 -deficient GC B cells became underrepresented (Fig. 3G). In summary, these data provide *in vivo* evidence that GC B cell participation in the splenic response against a soluble Ag and in the mucosal response against gut-derived Ags is augmented by $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrin-mediated interactions with ligands in the GC, including VCAM1 and most likely ICAM1 on FDCs.

Affinity maturation, turnover rate, and pAKT levels are unaffected

To test the hypothesis that GC B cell–integrin–mediated adhesion promotes Ag capture and presentation to T cells and thus selection of high-affinity cells, we used the Hy10 B cell transfer system and examined the cells for improved binding of the DEL immunogen (16) (Fig. 4A). Surprisingly, even though the cells were being outcompeted from the GC, the cells that remained had an equivalent improvement in DEL binding by day 14 of the response to that occurring in the wild-type controls (Fig. 4A). They also exhibited normal extents of isotype switching to the favored isotype for this response, IgG2b (Fig. 4A). Despite the lack of evidence for a defect in affinity maturation, it seemed possible that the integrin-deficient cells were capturing less Ag and being outcompeted due to insufficient T cell help (16, 17). To test this possibility, we treated mice with DEC205-OVA to target a surplus of Ag to the B cells irrespective of the efficiency of Ag encounter via the BCR (42). The DEC205-OVA Ab was effective at delivering Ag *in vivo*, as demonstrated by its ability to promote a vigorous OVA-specific CD8 (OT-1) T cell response (Fig. 4B). However, even when the cells were provided with this surplus Ag, there was no measurable rescue of Itgb2^{-/-} cell participation in the GC response (Fig. 4B). As an approach to test the efficiency of Ag acquisition, mice were injected with HEL-E α -GFP at the peak of the GC response and then examined for the amount of GFP capture and E α -peptide display, the latter being detected using the I-A^b-E α -specific Ab Y-Ae (34). When analyzed 4 h after HEL-E α -GFP injection, Itgb2^{-/-} and control Hy10 B cells showed equivalent amounts of Ag capture and MHC class II–peptide display (Fig. 4C). BrdU-labeling experiments showed the integrin-

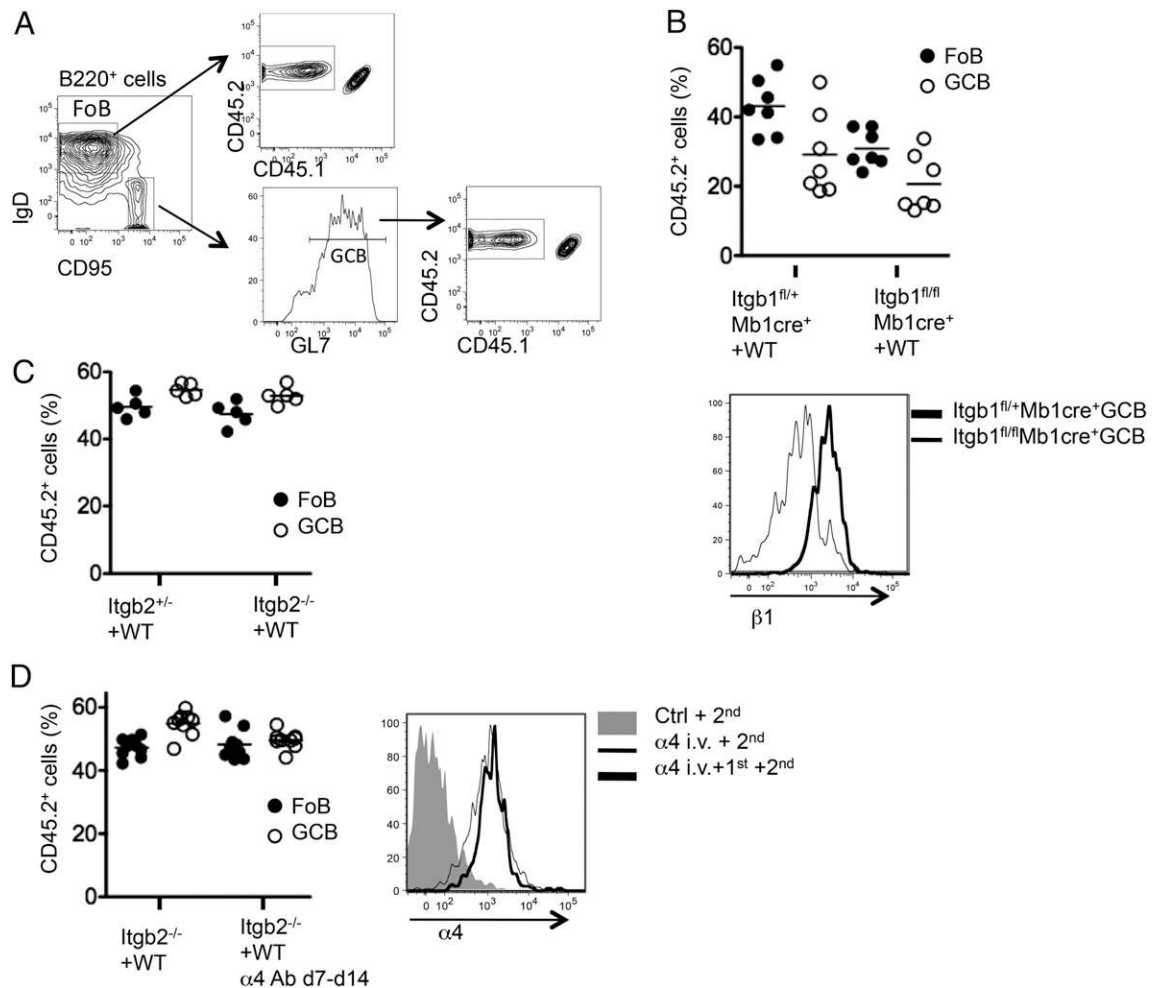


FIGURE 2. β_1 and β_2 integrin-deficient B cells mount intact GC response to SRBCs. **(A)** Flow cytometric gating strategy to identify GC and follicular B cells in mixed BM chimeric mice. **(B)** Flow cytometry of mixed wild-type (CD45.1/2⁺) and Itgb1^{fl/fl}Mb1cre⁺ or Itgb1^{fl/fl}Mb1cre⁺ (CD45.2⁺) BM chimeras, immunized i.p. with SRBCs 8 d before analysis. Contribution of CD45.2⁺ cells to follicular and GC B cells was plotted. Each dot represents one mouse. *Bottom panel*, Integrin β_1 staining of GC B cells from Itgb1^{fl/fl}Mb1cre⁺ and Itgb1^{fl/fl}Mb1cre⁺ mice. **(C)** Contribution of CD45.2⁺ integrin Itgb2^{+/-} or Itgb2^{-/-} cells to follicular and GC B cells in mixed integrin Itgb2^{+/-} or Itgb2^{-/-} plus wild-type (WT, CD45.1/2⁺) BM chimeras, immunized i.p. with SRBCs 8 d before analysis. Each dot represents one mouse. Data in (B) and (C) were pooled from two independent experiments. **(D)** Mixed integrin Itgb2^{-/-} (CD45.2⁺) plus wild-type (WT, CD45.1/2⁺) BM chimeras were immunized i.p. with SRBCs, treated with control (PBS or isotype control) or integrin α_4 Ab every other day from day 7 after immunization, and analyzed on day 14. Contribution of CD45.2⁺ cells to follicular and GC B cells was plotted. *Right panel*, GC B cells from α_4 Ab-injected mice were stained with anti-rat secondary Ab alone (α_4 i.v. + 2nd), α_4 Ab plus anti-rat secondary Ab (α_4 i.v. + 1st + 2nd), or an isotype control Ab and anti-rat secondary (Ctrl + 2nd). Each dot represents one mouse. Data are representative of three independent experiments.

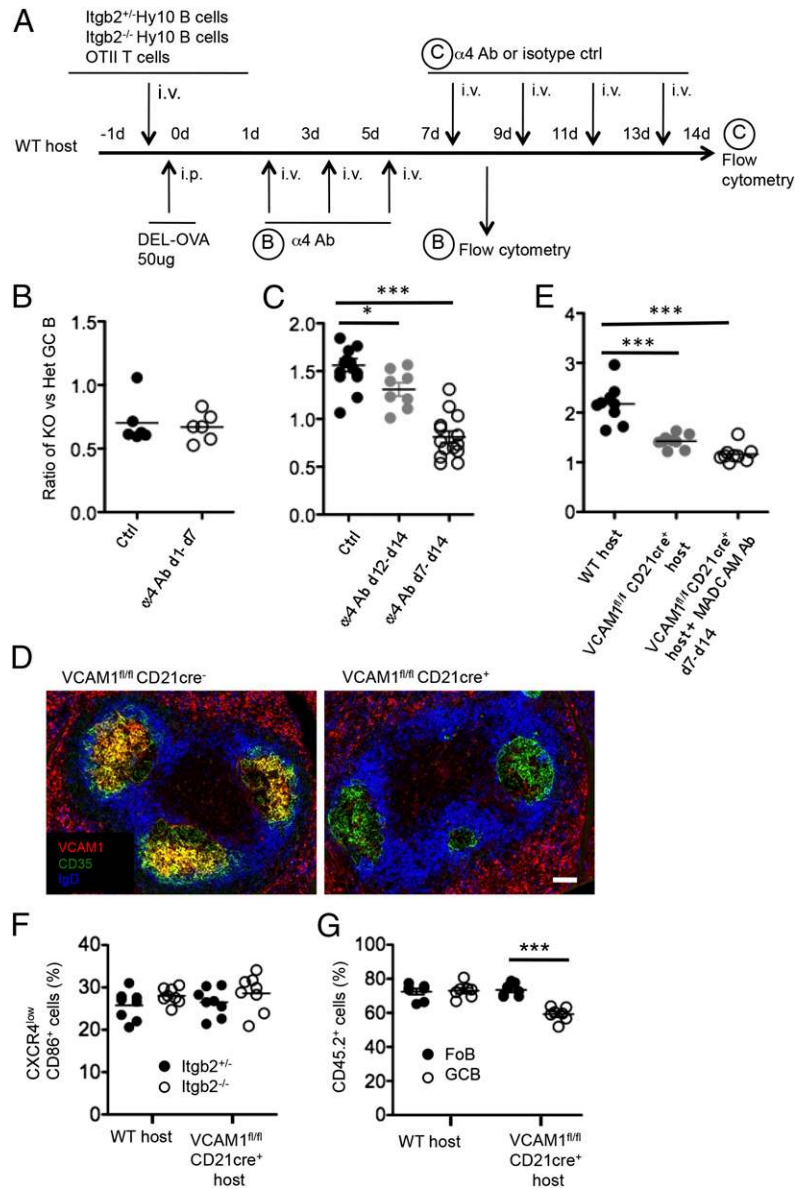
deficient GC B cells were proliferating at a comparable rate to control cells in the same animals as well as in matched control chimeras (Fig. 4D). Finally, we examined pAKT levels in GC B cells using a procedure in which the cells are fixed with paraformaldehyde during isolation in an attempt to maintain their signaling molecules in the same status as within the GC (35). Signaling via β_2 - and β_1 -containing integrins has been reported to increase pAKT levels in cells directly (43, 44) or by augmenting BCR signaling (9, 45). GC B cells had elevated pAKT levels, as determined by comparison with cells treated with calf intestinal phosphatase that serve as a background control. However, integrin-deficient GC B cells did not show any alteration in pAKT levels compared with the matched control cells (Fig. 4E).

Integrin $\alpha_v\beta_3$ expression and function in GC B cells

By genome-wide gene expression analysis, mouse GC and follicular B cells were found to have a 2- to 3-fold increase in Itgb3 transcripts and a slight increase in Itgav transcripts (www.Immgen.org and data not shown). Flow cytometric analysis confirmed that

both β_3 and α_v integrin chains are upregulated on GC versus follicular B cells (Fig. 5A). The secreted proteins VN and MFGE8 are ligands for $\alpha_v\beta_3$ (22, 23). VN expression has been reported in human GCs but has not been studied in the mouse (46–49). Analysis of mouse spleen and mesenteric LN tissue sections demonstrated that VN expression is a conserved property of the GC light zone (Fig. 5B). Using static adhesion assays, GC B cells adhered strongly to VN and less strongly to MFGE8, whereas follicular B cells showed only weak adhesion to VN (Fig. 5C). Adhesion of GC B cells to both VN and MFGE8 was β_3 dependent (Fig. 5C). To test whether $\alpha_v\beta_3$ played a role in B cells during the GC response, mixed Itgb3^{-/-}:wild-type BM chimeras were generated and immunized with SRBCs. This analysis revealed that, compared with their representation in the follicular compartment, Itgb3^{-/-} cells were slightly overrepresented in the splenic GC compartment (Fig. 5D). This was also seen for the chronic GC responses within mesenteric LNs that are induced by commensal flora (Fig. 5D). Finally, we asked whether the response to soluble protein Ag was affected. Itgb3^{-/-} Hy10 B cells and

FIGURE 3. Role of integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$ in the GC response against soluble Ag. **(A)** Schematic diagram of experimental procedures used for **(B)** and **(C)**. Wild-type mice were adoptively transferred with congenically marked lysozyme-specific integrin $Itgb2^{+/-}$ and $Itgb2^{-/-}$ Hy10 B cells plus OVA-specific OT-II T cells, immunized with DEL-OVA, and treated with Abs, as indicated. **(B)** Mice were treated with saline (Ctrl) or α_4 blocking Ab from day 1 for every other day and analyzed on day 7. Ratio of integrin $Itgb2^{-/-}$ (KO) versus $Itgb2^{+/-}$ (Het) Hy10 GC B cells was plotted. Each dot represents one mouse. Data were pooled from two independent experiments. **(C)** Mice were treated with saline (Ctrl) or α_4 blocking Ab from day 7 or 12 for every other day and analyzed on day 14. Data are representative of at least three experiments ($*p < 0.05$, $***p < 0.001$). The ratio of $Itgb2^{-/-}$ and $Itgb2^{+/-}$ Hy10 B cells in control GCs varied between experiments, most likely because of slight inaccuracies in cell counts in the input mixtures. **(D)** VCAM1 staining of sections from VCAM1^{fl/fl} CD21cre⁻ or VCAM1^{fl/fl} CD21cre⁺ mice that were transferred with Hy10 B cells plus OT-II T cells and DEL-OVA immunized 14 d before. Scale bar, 100 μ m. **(E)** Transfers were performed as in **(A)**, except, rather than wild-type mice, VCAM1^{fl/fl} CD21cre⁺ mice were used as hosts. Some of the mice were further treated with MADCAM1 blocking Ab from day 7 for every other day. Analysis was on day 14. Data are representative of at least three independent experiments ($***p < 0.001$). **(F)** Flow cytometric analysis of GC light and dark zone cells from mice in **(E)**. Frequency of CD86⁺CXCR4^{low} light zone cells was plotted. Data are representative of at least two independent experiments. **(G)** Flow cytometry of mesenteric LNs from wild-type or VCAM1^{fl/fl} CD21cre⁺ mice reconstituted with mixed wild-type (CD45.1/2⁺) and integrin $Itgb2^{-/-}$ (CD45.2⁺) BM (ratio ~25:75). Each dot represents one mouse. Data were pooled from two independent experiments ($***p < 0.001$).



control Hy10 B cell and OTII T cells were transferred to syngeneic recipients, and the mice were analyzed 14 d after immunization with DEL-OVA in monophosphoryl lipid A–based adjuvant. Under these immunization conditions, there was no impact of β_3 integrin deficiency on Hy10 B cell participation in the splenic GC response (Fig. 5E). These data suggest that distinct integrin–ligand interactions can have quite different influences on the GC response.

Discussion

Based on a series of in vitro studies, the concept emerged that $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins play an essential role in GC responses. Their role was thought to include provision of necessary trophic signals and augmentation of Ag capture. Our findings above contrast with the simplest version of this model by showing that B cells lacking $\alpha_L\beta_2$ and $\alpha_4\beta_1$ function continue to be able to mount GC responses against a particulate Ag. However, these integrins did participate in the GC B cell response against a soluble protein Ag and undefined gut-derived Ags. During the protein Ag response, we were not able to observe defects in Ag acquisition, cell turnover rate, or pAKT levels in the integrin-deficient cells. These data

suggest that integrin-mediated adhesion to FDCs augments GC B cell fitness through additional pathways that are still to be defined. Finally, we found that GC B cells express functional $\alpha_V\beta_3$, and we show that VN is a conserved component of the GC light zone. β_3 integrin deficiency had a mild augmenting effect on the GC response to particulate and commensal Ags. Taken together, our findings suggest a highly contextual involvement of integrin–ligand interactions in the GC response, with their contributions most likely depending heavily on the type of Ag driving the response and the amounts and types of costimulatory inputs available.

Following from an early study that showed GC B cell lines could adhere to GCs on sections in an $\alpha_4\beta_1$ –VCAM1–dependent manner (1), several in vitro experiments showed $\alpha_4\beta_1$ - and $\alpha_L\beta_2$ -mediated adhesion between isolated FDCs and B cells (3, 10, 11, 13). Blocking these adhesive interactions led to reduced survival of the isolated B cells (3, 10, 11, 13). In contrast to these observations, our studies show in vivo that $\alpha_L\beta_2$ and $\alpha_4\beta_1$ are not essential for GC B cell survival. An explanation for this discrepancy most likely lies in the greater availability of trophic factors in vivo than are provided in the in vitro cultures. In addition, whereas integrin–

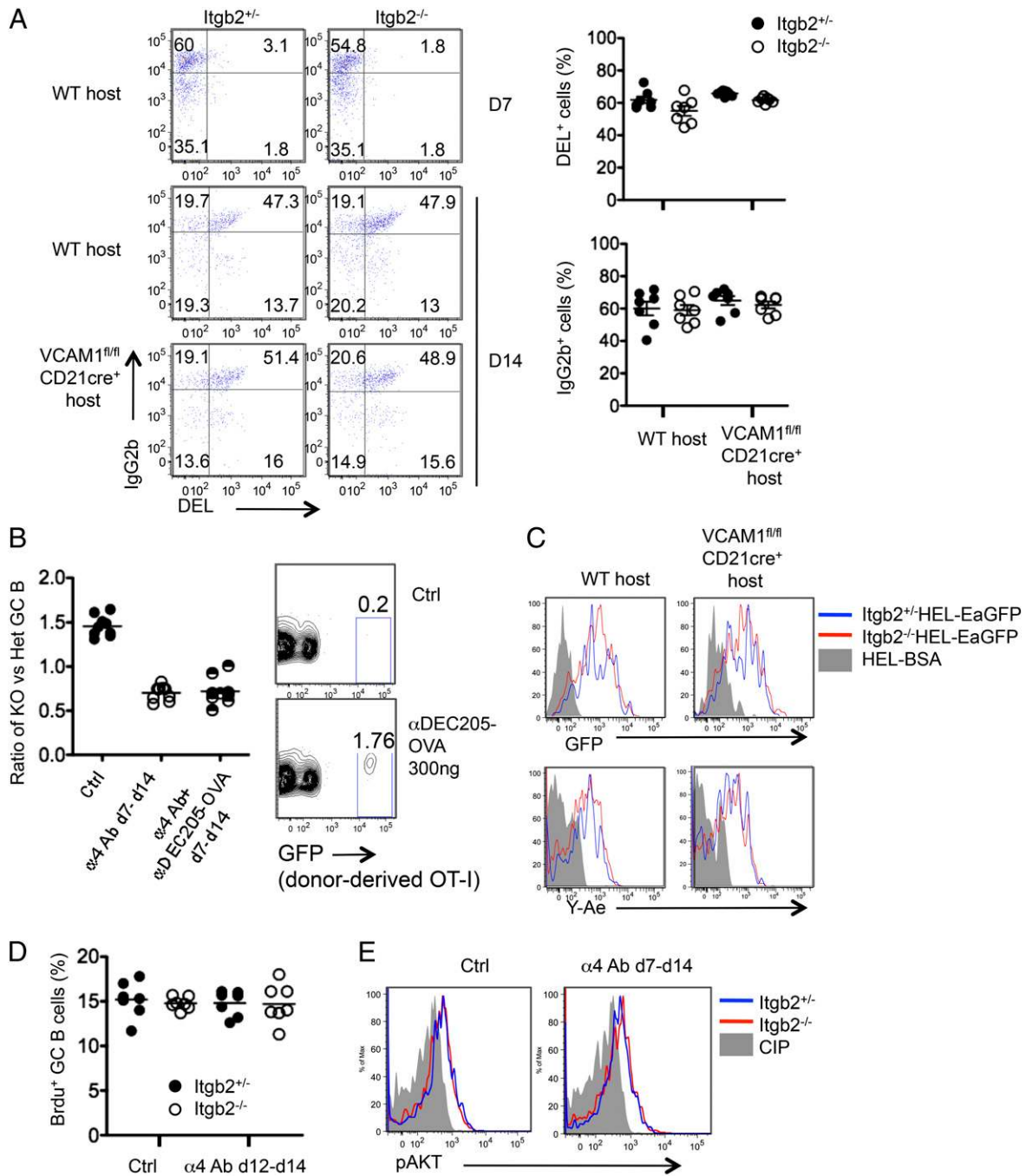
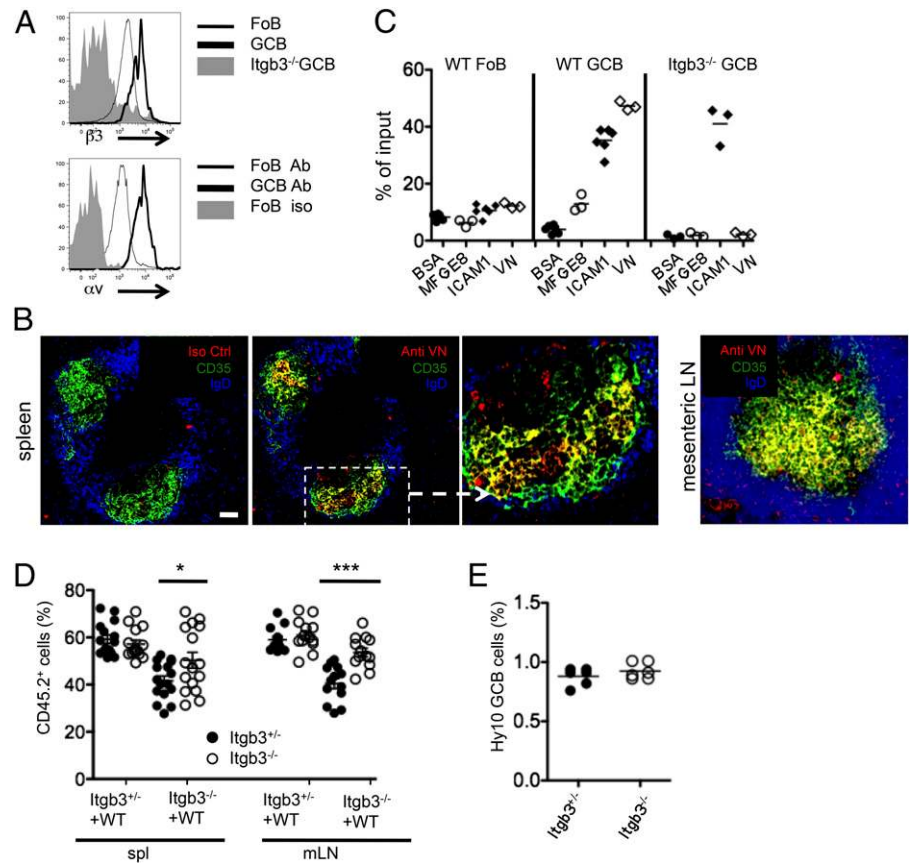


FIGURE 4. Integrin-deficient GC B cell Ag acquisition, affinity maturation, and turnover. **(A)** Integrin $\beta_2^{-/-}$ and $\beta_2^{-/-}$ Hy10 B cells plus OT-II T cells were transferred into wild-type or VCAM1^{fl/fl} CD21cre⁺ mice, which were then immunized i.p. with DEL-OVA. At day 7 or 14, splenic GCB cells were stained for class switch to IgG2b and affinity maturation (DEL-binding). Mice analyzed at day 7 had no measurable affinity maturation. Plots are gated on transferred B cells, and numbers indicate percentage of cells in each quadrant. Graphs on right show percentage of DEL-binding or IgG2b⁺ cells on day 14. Data are representative of at least three independent experiments. **(B)** Wild-type mice were adoptively transferred with integrin Itgb2^{+/-} and Itgb2^{-/-} Hy10 B cells plus OT-II T cells, immunized with DEL-OVA, treated with α_4 Ab or α_4 Ab plus anti-DEC205-OVA (50 μ g) for every other day, and analyzed on day 14. Each dot represents one mouse. Data are representative of two independent experiments. Right plots show frequency of OVA-specific OT-I CD8 T cells in spleens of recipient mice 3 d after injection of saline (Ctrl) or anti-DEC205 OVA (300 ng). **(C)** Mice of the type in (A) were injected with 20 μ g HEL-Ea-GFP or HEL-BSA on day 14. Four hours later, they were analyzed for GC B cell GFP level and Ea peptide display using Y-Ae Ab. Data are representative of two independent experiments. **(D)** Mice of the type in (B) were injected with 2 mg BrdU on day 14 and analyzed 30 min later for BrdU incorporation by GC B cells. Data are representative of three independent experiments. **(E)** Cells of the type in (B) were fixed at the time of isolation, left untreated, or treated with calf intestinal phosphatase (CIP) and stained for intracellular pAKT. Data are representative of three independent experiments.

ligand interactions are probably essential for the B cells to maintain any association with isolated FDCs in a tissue culture dish, this may not be the case in the animal. In the intact tissue, there are many cues promoting GC B cell clustering around FDCs. For example, sphingosine-1-phosphate (S1P) receptor 2 responding to a

repulsive gradient of S1P promotes GC B cell clustering over the FDC network (50). When GC B cells lack S1P receptor 2, they are less well confined to the GC (50). Analysis of tissue sections from mice harboring integrin-deficient GC B cells did not show any major alteration in GC B cell distribution (data not shown). The

FIGURE 5. Integrin $\alpha_v\beta_3$ expression and function in GC B cells. **(A)** C57BL/6 mice were immunized with SRBCs and after 8 d examined for expression of integrin $\alpha_v\beta_3$ on follicular and GC B cells by flow cytometry. *Itgb3*^{-/-} mice or isotype-matched Abs were used as staining controls. Data are representative of two independent experiments. **(B)** Spleen and mesenteric sections from a SRBC-immunized mouse were stained with anti-VN or isotype-matched control. Scale bar, 100 μ m. Data are representative of tissue from more than three mice. **(C)** Splenocytes from SRBC-immunized mice were allowed to adhere to plastic coated with ICAM1, VN, or MFGE8, and adherent follicular and GC B cells were enumerated by flow cytometry. **(D)** Flow cytometry of mixed BM chimeras generated with integrin *Itgb3*^{+/-} or *Itgb3*^{-/-} (CD45.2⁺) cells plus wild-type (CD45.1⁺) cells, reconstituted for at least 6 wk and immunized i.p. with SRBCs 10–12 d before analysis. Data were pooled from three independent experiments and are representative of several additional experiments that involved >20 mice (**p* < 0.05, ****p* < 0.001). **(E)** Wild-type mice were adoptively transferred with lysozyme-specific integrin *Itgb3*^{+/-} or *Itgb3*^{-/-} Hy10 B cells plus OT-II T cells, immunized with DEL-OVA, and analyzed on day 14. Data are representative of two independent experiments.



importance of FDCs in promoting GC B cell viability *in vivo* is supported by the finding that diphtheria toxin-mediated ablation of FDCs leads to rapid death of GC B cells (36). The inability of IKK2-deficient FDCs to support GC responses might reflect both a lack of adhesion molecule expression and reduced production of trophic factors or cues that promote GC B cell clustering.

$\alpha_4\beta_1$ and $\alpha_1\beta_2$ integrin-mediated adhesion can greatly augment the ability of follicular B cells *in vitro* to acquire Ag by promoting spreading and contraction of the cell membrane over lipid bilayers in a manner that facilitates greater contact between the BCR and membrane-associated Ag (19, 51, 52). Given these convincing data, we were surprised that our studies did not readily reveal defects in Ag acquisition by integrin-deficient GC B cells. We suspect that there are a number of explanations for this apparent discrepancy. First, the forms of Ag tested in the lipid bilayer studies and in our *in vivo* studies are not identical. For example, it is likely that the Ags injected *in vivo* become associated with activated fragments of complement, leading to recruitment of complement receptors 1 and 2 on the B cell. These are strong costimulatory receptors (53) that can also augment Ag capture (54). Second, the properties of the FDC membrane may facilitate B cell capture of certain types of Ag as aggregates or in vesicular (“icosome”) structures (55), and this capture may not be enhanced by integrin-mediated membrane spreading and contraction. Third, GC B cells are highly motile *in vivo* and they exhibit a large, probing morphology (28, 56, 57). Findings by us (58) and others (59) have shown that naive lymphocytes and dendritic cells do not require integrin-mediated adhesion for motility in lymphoid tissues. Therefore, the integrin-mediated spreading and contraction functions revealed *in vitro* may be redundant with other cell biological mechanisms acting *in vivo* to promote large areas of membrane contact as GC B cells migrate over and squeeze between the tightly interconnected network of FDC processes. That

said, integrins can augment T cell motility over dendritic cells in the T zone (60), and it remains possible that less efficient movement of integrin-deficient GC B cells over FDCs contributes to their reduced fitness. In future studies, it will be important to visualize the migration dynamics of integrin-deficient GC B cells within the GC.

Our finding that integrin $\alpha_v\beta_3$ is upregulated on the surface of GC B cells and is functional *in vitro* in mediating adhesion highlights a further layer of complexity in dissecting the integrin–ligand contribution to GC responses. One ligand for $\alpha_v\beta_3$, MFGE8, is a well-defined marker of the GC FDC network, and it plays a role in promoting apoptotic cell uptake by tingibile body macrophages (25). We show that a second ligand, VN, is present in the light zone of mouse GCs, consistent with findings for human GCs (46–49). VN is a RGD-containing secreted protein that has a variety of binding partners, including complement C5b-9, plasminogen activator inhibitor-1, and heparin sulfate proteoglycans (61). Whether VN is produced locally in the GC light zone by FDCs or arrives from circulation, where it is abundant, is not yet clear. Beyond a possible role in the GC as an $\alpha_v\beta_3$ ligand, VN may act to help limit membrane damage by inhibiting terminal cytolytic complement pathway activity (61). $\alpha_v\beta_3$ can also bind the RGD-containing proteins fibronectin, fibrinogen, von Willebrand’s factor, thrombospondin, and osteopontin (22). GCs contain detectable fibronectin (J.G. Cyster, unpublished observations), but whether any of the additional $\alpha_v\beta_3$ ligands are present is not yet defined. Although the $\alpha_v\beta_3$ ligands are secreted rather than membrane proteins, it is possible that they function in an overlapping manner with other integrins by promoting motility and spreading of GC B cells. In this regard, it is notable that, based on *in vitro* studies with fibroblasts and epithelial cells, VN is also known as “cell-spreading factor” (22). It is also interesting to consider whether $\alpha_v\beta_3$ on GC B cells may have a role in sensing MFGE8 during Ag

uptake. Recent studies in dendritic cells have shown that MFGE8 directs internalized apoptotic material to lysosomes, and, when it is absent, apoptotic Ag is less rapidly destroyed and more efficiently presented (62, 63). Perhaps by targeting phosphatidylserine-bearing Ags internalized via the BCR for rapid lysosomal degradation, MFGE8- $\alpha_4\beta_3$ antagonizes Ag presentation to T follicular helper cells. The slightly augmented GC participation of β_3 -deficient B cells is consistent with such a negative regulatory role. It will be valuable in future studies to determine whether β_3 integrin deficiency in B cells predisposes to production of anti-apoptotic cell autoantibodies. There is also evidence that, in contrast to most integrins, $\alpha_4\beta_3$ can transmit proapoptotic signals depending on the extent of ligand engagement (64–68). Thus, the slight overgrowth of β_3 -deficient GC B cells observed in our studies might be a consequence of losing an integrin-mediated negative regulatory signal, a possibility that merits future investigation.

As well as the integrins studied in this work, GC B cells have been reported to upregulate the α_6 chain (69). This integrin chain can pair with β_1 and β_4 , and its induction may account for the elevated β_1 but slight reduction in α_4 staining observed by flow cytometry (this study) (69). GC B cells express little or no β_4 chain (69) (J.G. Cyster, unpublished observations). $\alpha_6\beta_1$ is a laminin-binding integrin (70). In the studies reported to date, laminin is sparse within the GC but can be abundant at the GC perimeter (71) (our unpublished data), suggesting that if $\alpha_6\beta_1$ is functional in GC B cells, it may have a distinct role regarding the other integrins in which the ligands are most abundant in association with the FDC network. However, there are multiple forms of laminin (70), and it remains possible that some forms are present in the GC light zone, possibly adding further redundancy to the integrin–ligand interactions in this region. Arguing against $\alpha_4\beta_1$ being redundant with other β_1 -containing integrins in GC B cells, mice lacking *Itgb1* from all hematopoietic cells mounted normal GC responses against a haptenated protein Ag (72). These mice did mount a diminished IgM plasma cell response, but the authors commented that the IgM defect could not be rescued by transfers of wild-type B cells, suggesting that it was due to a role of β_1 -containing integrins in other hematopoietic cell types.

In summary, our findings provide evidence that integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$ play overlapping and context-dependent roles in supporting interactions with FDCs that can augment the fitness of responding GC B cells. The mechanism by which they enhance GC B cell fitness is not yet clear. Our currently favored model is that they contribute small and difficult-to-measure influences on a range of processes, including access to trophic factors, certain forms of Ag, and differentiation factors. That their contribution may often be redundant with contributions by other ligand–receptor systems, possibly including additional integrins, is consistent with the view that the strong evolutionary pressure for mounting GC responses established a highly robust biological system (16).

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Disclosures

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