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Integrin-mediated interactions between B cells and follicular dendritic cells influence germinal center B cell fitness[1]

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Abstract

Integrin-ligand interactions between germinal center (GC) B cells and antigen-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. Here we show that while integrins αLβ2 and α4β1 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 β2-integrin deficiency and α4-integrin blockade also did not affect the GC response against a particulate antigen. However, the combined integrin deficiency did cause B cells to be outcompeted in splenic GC responses against a soluble protein antigen and in mesenteric lymph node GC responses against gut-derived antigens. Similar findings were made for β2-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 antigen acquisition, proliferation rates or pAKT levels. In summary, our findings provide evidence that αLβ2 and α4β1 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 αvβ3 and adhere to vitronectin and milk fat globule EGF-factor-8 protein. Integrin β3-deficient B cells contributed in a slightly exaggerated manner to GC responses suggesting this integrin has a regulatory function in GC B cells.

Keywords

integrin; adhesion molecules; germinal center; B lymphocyte; follicular dendritic cells; vitronectin; MFGE8

Introduction

Several *in vitro* studies over the last 25 years have highlighted the ability of GC B cells to undergo integrin αLβ2 (LFA1)- and α4β1-mediated adhesive interactions with FDCs (1–5). αLβ2 and α4β1 on the GC B cell bind cell adhesion molecules ICAM1 and VCAM1, respectively, that are upregulated on GC FDCs (5, 6). MADCAM1, a ligand for both of the α4-containing integrins, α4β7 and α4β1, has also been detected on FDCs (4). As well as

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promoting cell-cell adhesion, both β1- and β2- 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 α4β1 and αLβ2 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 where the kinase 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 antigen to receive positive selection signals from T follicular helper (Tfh) cells (16, 17). Much of the antigen 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 antigens from lipid bilayers can be augmented by αLβ2- and α4β1-ligand interactions (19–21). Whether such interactions are important for antigen 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 (ECM) components. Although the GC is relatively devoid of collagens, laminin and fibronectin, studies in human tissue show the GC light zone contains the 70kD glycoprotein vitronectin (VN) (6). VN binds a number of integrins, including αvβ3 (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 αvβ3 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.

Here we report that neutralization of β2- and α4-containing integrin function has varying impacts on GC B cells depending on the type of response being mounted. During the polyclonal response to sheep red blood cells (SRBCs), cells without β2 and α4 integrin function were able to participate efficiently in the GC response, indicating that these integrins are not universally required for antigen capture or GC B cell survival. Importantly, however, during the response of B cells to a soluble protein antigen, β2- and α4-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 αvβ3 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 Laboratories. Integrin Itgb2$^{-/-}$ mice (27) were backcrossed to C57BL/6J for six generations. Hy10 mice with knock-in expression of immunoglobulin 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. Itgb1$^{fl/fl}$mice (30), VCAM1$^{fl/fl}$mice (31) and Itgb3$^{-/-}$ mice (32) were from Jackson 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

BM chimeras were generated by mixing equal amounts of the indicated BM types and injecting 2–5×10^6 BM cells in to lethally irradiated recipients as described (33). Mice were reconstituted for 6–12 weeks before immunization or analysis. SRBC immunizations were i.p. with 2 × 10^8 cells (Colorado Serum Company). 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 where the latter were from Itgb2$^{+/-}$ intercrossed mice. The ratio of Itgb2$^{-/-}$ and Itgb2$^{+/-}$ Hy10 B cells in control GCs varied between experiments, likely 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 intraperitoneally with 50ug duck egg lysozyme–ovalbumin (DEL-OVA) in the Sigma Adjuvant System. Some of the mice were intravenously injected with 200ug integrin α4 antibody (PS/2; Bio X Cell) or MADCAM1 antibody (MECA367; Bio X Cell) at the indicated time. Some of the mice were also injected with 50ug anti-DEC205-OVA together with anti-α4 as indicated. The Eα-GFP construct was kindly provided by Mark Jenkins (Univ. Minnesota) and the protein was produced, chemically conjugated to HEL and purified as described (34). Mice were immunized i.v. with 20ug HEL-Eα-GFP 4hr prior to analysis.

Flow cytometry

Spleen and lymph nodes 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; antibody to T cell and B cell activation antigen (GL7; BD Biosciences); anti-Mouse Eα52-68 peptide bound to I-A^b (Y-Ae,

eBioscience); anti-integrin β1 (MB1.2; Chemicon); anti-integrin β2 (C71/16; BD Biosciences); anti-integrin β3 (2C9.G2; Biolegend); anti-integrin β7 (M293; BD Biosciences); anti-integrin α4 (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 7um in thickness were cut and fixed in cold acetone. Sections were stained with anit-ICAM1 (3E2; BD Biosciences); anti-VCAM1 (429; BD Biosciences); anti-MADCAM1(MECA367; Bio X Cell); anti-IgD (11-26c.2a; Biolegend); anti-Vitronectin (347317; R&D); 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 10ug/ml ICAM1, VCAM1, MADCAM1, Vitronectin or MFGE8. Vitronectin were from Abcam. All the other reagents were from R&D Systems.

Statistics

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

Results

β2 and α4 integrin and integrin-ligand expression in the GC

By flow cytometric analysis, splenic GC B cells had elevated αL and β2 integrin expression compared to follicular B cells (Fig. 1A). Integrin β1 levels were slightly elevated whereas β7 was reduced (Fig. 1A), suggesting GC B cells shift from a mixture of α4β1 and α4β7 heterodimers to predominantly expressing α4β1. 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 αLβ2 in GC B cells. Both cell types adhered strongly to VCAM1 and weakly to MADCAM1 (Fig. 1C).

Intact GC response to complex particulate antigen

To test whether β1 integrin-mediated interaction with FDC-expressed VCAM1 was important during the GC response we generated mixed chimeras by reconstituting irradiated wild-type mice with $Itgb1^{f/f}$ or$^{f/+}$ $Mb1Cre^{+}$ $CD45.2^{+}$ BM and wild-type $CD45.1/2^{+}$ BM. Eight days following SRBC immunization there was an under-representation of $CD45.2^{+}$ GC B cells compared to 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 to their heterozygote controls (Fig. 2B). We confirmed that there was efficient ablation of integrin β1 from Itgb1$^{f/f}$ 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 αLβ2 and α4β1 integrins in GC B cells, we induced GCs in Itgb2$^{-/-}$: wild-type mixed BM chimeras and then treated them with anti-α4 blocking antibody from day 7–14 after SRBC immunization. Analysis of GC B cells at the time of isolation confirmed that their α4 integrins were saturated with the antibody (Fig. 2D). Even under these conditions, where αLβ2, α4β1 and α4b7 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 αLβ2 and α4β1 in response against soluble protein antigen

We considered it likely that the contribution of integrins to the GC response may vary depending on the form of antigen. To test if integrin function is required during the response to a soluble protein antigen, we intercrossed lysozyme-specific Ig heavy chain knockin and light 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 recipients that were then immunized with a conjugate of duck egg lysozyme (DEL) and ovalbumin (OVA; DEL-OVA) in monophosphoryl lipid A (MPL)-based adjuvant (28). In a first experiment, the impact of α4 integrin blockade during the early phase of the response was tested. Treating with antibody at days 1, 3 and 5 followed by analysis at day 7 showed the GC response was unaffected by α4-antibody treatment (Fig. 3B). These data suggested that β2 and α4 integrin function on B cells was not crucial for entry into the GC response against a soluble antigen. 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 α4-neutralizing antibody on days 7, 9, 11 and 13, or only on day 12, and analyzed on day 14. Under these conditions, the β2-deficient GC B cells were underrepresented compared to the WT GC B cells. This effect was more striking with the longer period of treatment (Fig. 3C).

To confirm that the α4-blocking antibody was acting by inhibiting GC B cell interaction with FDC-expressed VCAM1, we generated mice lacking VCAM1 on FDCs by intercrossing VCAM1$^{f/f}$ 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 to littermate control hosts, there was a significant reduction in GC participation at day 14 (Fig. 3E). Additionally blocking MADCAM1 by treatment with a neutralizing antibody had only a mild effect in further reducing the response. Since 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 β2 integrin and VCAM1 deficiency. Analysis of the fraction of GC B cells with a light zone (CXCR4^{lo}CD86^{hi}) phenotype showed comparable frequencies for integrin-function deficient and control cells (Fig. 3F). We attempted to perform experiments to

confirm that the B cell β2-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, 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 antigens was influenced by integrin deficiency. Analysis of Itgb2$^{-/-}$:wild-type mixed BM chimeras 8–12 weeks after reconstitution showed that β2-deficiency alone had no effect on GC participation but when combined with VCAM1-deficiency from FDCs, the β2-deficient GC B cells became under-represented (Fig. 3G). In summary, these data provide *in vivo* evidence that GC B cell participation in the splenic response against a soluble antigen and in the mucosal response against gut-derived antigens is augmented by αLβ2 and α4β1 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 antigen 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 duck egg lysozyme (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 antigen 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 antigen to the B cells irrespective of the efficiency of antigen encounter via the B cell receptor (42). The DEC205-OVA antibody was effective at delivering antigen *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 antigen, there was no measurable rescue of Itgb2$^{-/-}$cell participation in the GC response (Fig. 4B). As an approach to test the efficiency of antigen acquisition, mice were injected with HEL-Eα-GFP at the peak of the GC response and then examined for the amount of GFP capture and Eα-peptide display, the latter being detected using the I-A^{b}-Eα specific antibody Y-Ae (34). When analyzed 4hrs after HEL-Eα-GFP injection, Itgb2$^{-/-}$ and control Hy10 B cells showed equivalent amounts of antigen capture and MHC class II-peptide display (Fig. 4C). BrdU labeling experiments showed the integrin-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 where the cells are fixed with PFA during isolation in an attempt to maintain their signaling molecules in the same status as within the GC (35). Signaling via β2 and β1 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 to cells treated with calf intestinal phosphatase (CIP) that serve as a

background control. However, integrin-deficient GC B cells did not show any alteration in pAKT levels compared to the matched control cells (Fig. 4E).

Integrin αvβ3 expression and function in GC B cells

By genome wide gene expression analysis, mouse GC and follicular B cells, were found to have a 2–3 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 β3 and αv integrin chains are upregulated on GC versus follicular B cells (Fig. 5A). The secreted proteins VN and MFGE8 are ligands for αvβ3 (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 while follicular B cells showed only weak adhesion to VN (Fig. 5C). Adhesion of GC B cells to both VN and MFGE8 was β3 dependent (Fig. 5C). To test whether αvβ3 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 to their representation in the follicular compartment, Itgb3$^{-/-}$ cells were slightly over-represented 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 antigen was affected. Itgb3$^{-/-}$ Hy10 B cells and control Hy10 B cell and OTII T cells were transferred to syngeneic recipients and the mice were analyzed 14 days after immunization with DEL-OVA in MPL-based adjuvant. Under these immunization conditions there was no impact of β3-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 αLβ2 and α4β1 integrins play an essential role in GC responses. Their role was thought to include provision of necessary trophic signals and augmentation of antigen capture. Our findings above contrast with the simplest version of this model by showing that B cells lacking αLβ2- and α4β1-function continue to be able to mount GC responses against a particulate antigen. However, these integrins did participate in the GC B cell response against a soluble protein antigen and undefined gut-derived antigens. During the protein antigen response, we were not able to observe defects in antigen 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 αvβ3 and we show that VN is a conserved component of the GC light zone. β3-integrin deficiency had a mild augmenting effect on the GC response to particulate and commensal antigens. Taken together, our findings suggest a highly contextual involvement of integrin-ligand interactions in the GC response, with their contributions likely depending heavily on the type of antigen 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 α4β1-VCAM1 dependent manner (1), several *in vitro* experiments showed α4β1- and αLβ2-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 αLβ2 and α4β1 are not essential for GC B cell survival. An explanation for this discrepancy likely lies in the greater availability of trophic factors *in vivo* than are provided in the *in vitro* cultures. In addition, while integrin-ligand interactions are likely 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 receptor-2 (S1PR2) responding to a repulsive gradient of sphingosine-1-phosphate (S1P) promotes GC B cell clustering over the FDC network (50). When GC B cells lack S1PR2, 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 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.

α4β1 and αLβ2 integrin-mediated adhesion can greatly augment the ability of follicular B cells *in vitro* to acquire antigen 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 antigen (19, 51, 52). Given these convincing data we were surprised that our studies did not readily reveal defects in antigen acquisition by integrin-deficient GC B cells. We suspect that there are a number of explanations for this apparent discrepancy. First, the forms of antigen tested in the lipid bilayer studies and in our *in vivo* studies are not identical. For example, it is likely that the antigens 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 antigen capture (54). Second, the properties of the FDC membrane may facilitate B cell capture of certain types of antigen as aggregates or in vesicular (‘iccosome’) 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 naïve lymphocytes and dendritic cells do not require integrin-mediated adhesion for motility in lymphoid tissues. Therefore, the integrin-mediated spreading and contraction functions revealed i*n 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 DCs 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 αvβ3 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 αvβ3, MFGE8, is a well-defined marker of the GC FDC network and it plays a role in promoting apoptotic cell uptake by tingible 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 an 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 αvβ3 ligand, VN may act to help limit membrane damage by inhibiting terminal cytolytic complement pathway activity (61). αvβ3 can also bind the RGD-containing proteins fibronectin, fibrinogen, von Willebrand's factor, thrombospondin and osteopontin (22). GCs contain detectable fibronectin (unpubl. obs.) but whether any of the additional αvβ3 ligands are present is not yet defined. Although the αvβ3 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 αvβ3 on GC B cells may have a role in sensing MFGE8 during antigen uptake. Recent studies in dendritic cells have shown that MFGE8 directs internalized apoptotic material to lysosomes and when it is absent, apoptotic antigen is less rapidly destroyed and more efficiently presented (62, 63). Perhaps by targeting phosphatidylserine-bearing antigens internalized via the BCR for rapid lysosomal degradation, MFGE8-αvβ3 antagonizes antigen presentation to Tfh cells. The slightly augmented GC participation of β3-deficient B cells is consistent with such a negative regulatory role. It will be valuable in future studies to determine whether β3-integrin deficiency in B cells predisposes to production of anti-apoptotic cell autoantibodies. There is also evidence that, in contrast to most integrins, αvβ3 can transmit pro-apoptotic signals depending on the extent of ligand engagement (64–68). Thus, the slight overgrowth of β3-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 here, GC B cells have been reported to upregulate the α6 chain (69). This integrin chain can pair with β1 and β4 and its induction may account for the elevated β1 but slight reduction in α4 staining observed by flow cytometry (this study and (69)). GC B cells express little or no β4 chain ((69) and unpubl. obs.). α6β1 is a laminin binding integrin (70). In the studies so far reported, laminin is sparse within the GC but can be abundant at the GC perimeter ((71) and unpubl. data), suggesting that if α6β1 is functional in GC B cells it may have a distinct role to the other integrins where 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 α4β1 being redundant with other β1-containing integrins in GC B cells, mice lacking *Itgb1* from all hematopoietic cells mounted normal GC responses against a haptenated-protein antigen (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 β1-containing integrins in other hematopoietic cell types.

In summary, our findings provide evidence that integrins αLβ2 and α4β1 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 antigen, 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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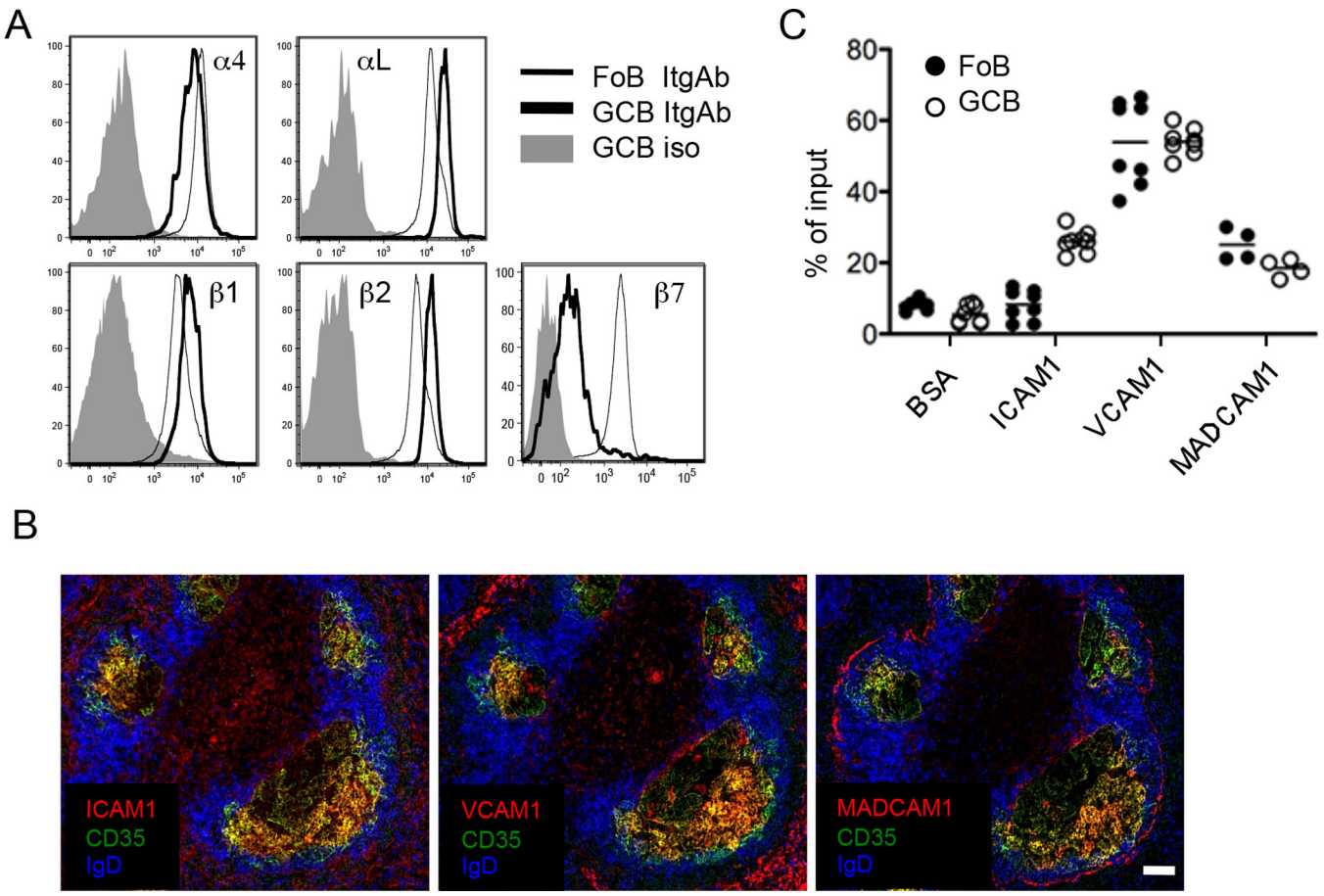

FIGURE 1. α4β1, α4β7 and αLβ2 integrin and ligand expression in the GC

(**A**) C57BL/6 mice were immunized with SRBCs and at day 8 expression of integrins on $B220^{+}IgD^{+}Fas^{-}$ follicular B cells (FoB) and $B220^{+}IgD^{-}Fas^{+}GL7^{+}$ GC B cells (GCB) were analyzed by flow cytometry with chain-specific antibodies (ItgAb). (**B**) Serial sections of an SRBC immunized spleen stained with the indicated integrin ligand antibodies (red), CD35 (green) and IgD (blue). Scale bar, 100um. Data in A and B are representative of more than 3 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 % of input cells that remained adherent and are representative of at least two experiments.

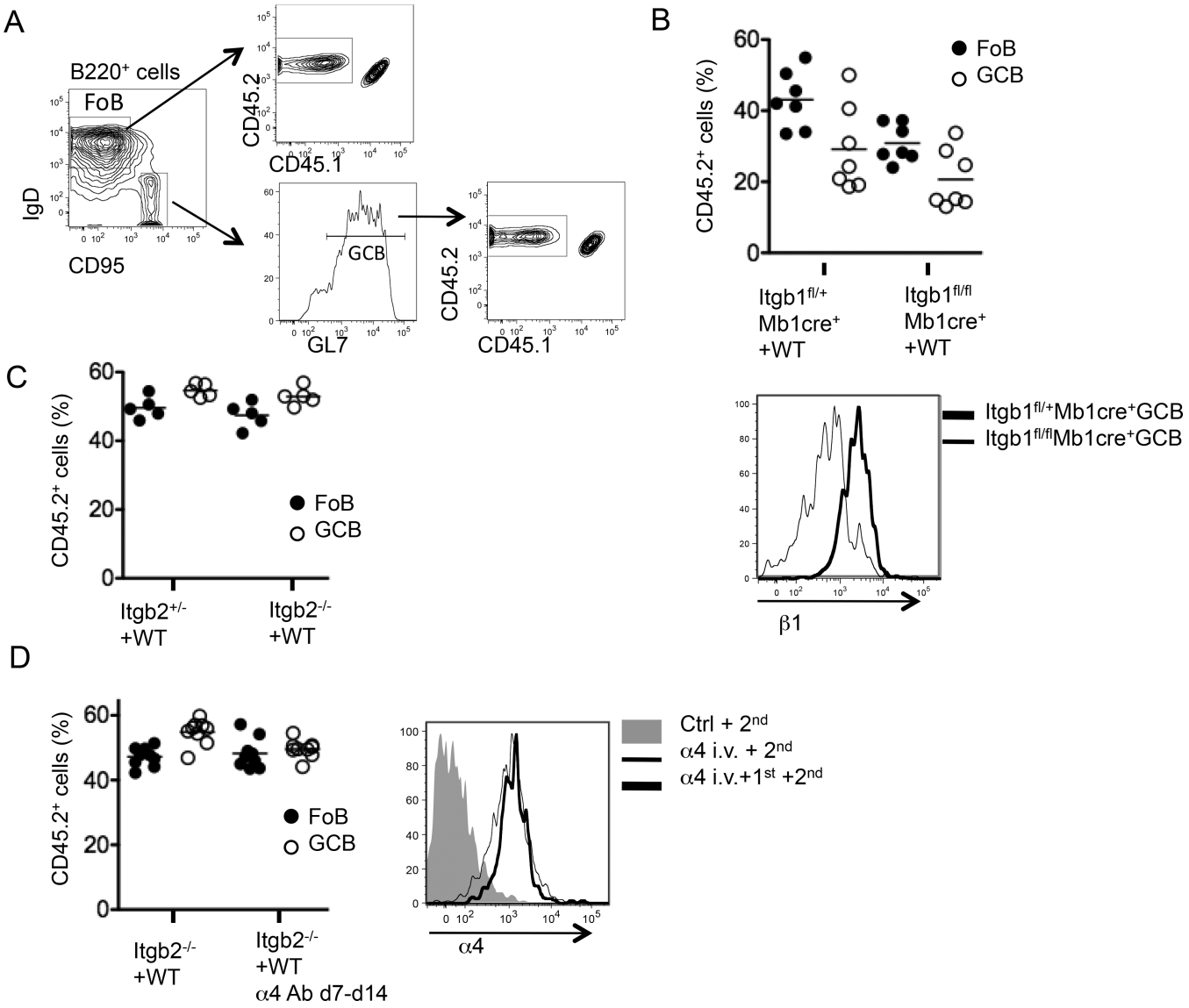

FIGURE 2. β1 and β2 integrin-deficient B cells mount intact GC response to SRBCs

(**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$^{fl/+}$Mb1cre$^+$ or Itgb1$^{fl/fl}$Mb1cre$^+$ (CD45.2$^+$) BM chimeras, immunized i.p. with SRBCs 8 days before analysis. Contribution of CD45.2$^+$ cells to follicular and GC B cells was plotted. Each dot represents one mouse. Bottom panel, integrin β1 staining of GC B cells from Itgb1$^{fl/+}$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 days before analysis. Each dot represents one mouse. Data in (B, 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 α4 antibody every other day from day 7 after immunization and analyzed at day 14. Contribution of CD45.2$^+$ cells to follicular and GC B cells was plotted. Right panel, GC B

cells from α4 antibody injected mice were stained with anti-rat secondary antibody alone (α4 i.v. + 2nd), α4 antibody plus anti-rat secondary antibody (α4 i.v.+1st +2nd) or an isotype control antibody and anti-rat secondary (Ctrl + 2nd). Each dot represents one mouse. Data are representative of three independent experiments.

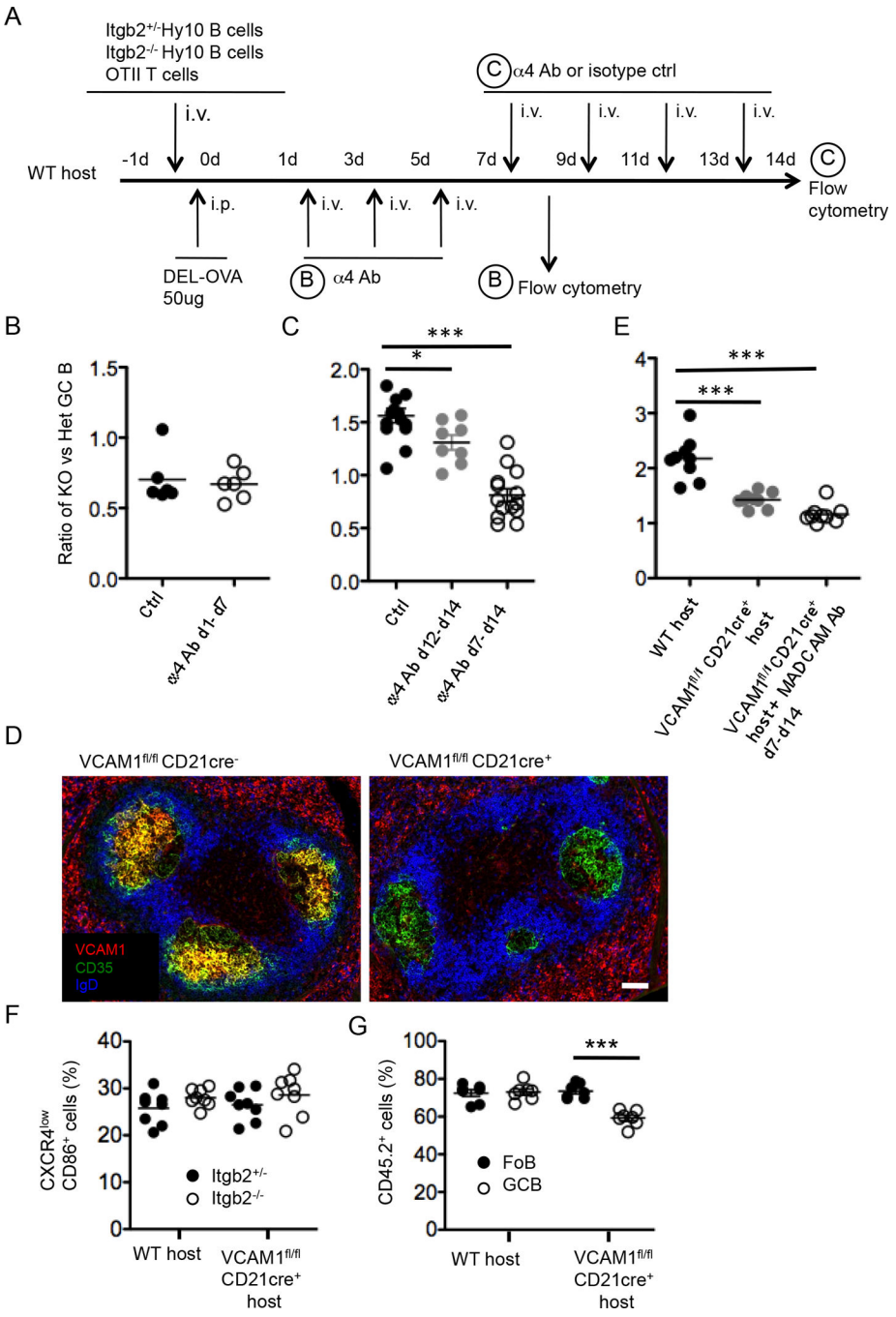

FIGURE 3. Role of integrins αLβ2 and α4β1 in the GC response against soluble antigen

(**A**) Schematic diagram of experimental procedures used for panels B and C. Wild-type mice were adoptively transferred with congenically marked lysozyme specific integrin Itgb2$^{+/-}$ and Itgb2$^{-/-}$ Hy10 B cells plus ovalbumin specific OT-II T cells, immunized with DEL-OVA and treated with antibodies as indicated. (**B**) Mice were treated with saline (Ctrl) or α4 blocking antibody 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 α4 blocking antibody from day 7 or day 12 for every other day and analyzed at day 14. Data are representative of at least three experiments. The ratio of Itgb2$^{-/-}$ and Itgb2$^{+/-}$ Hy10 B cells in control GCs varied between experiments, 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 which were transferred with Hy10 B cells plus OT-II T cells and DEL-OVA immunized 14 days before. Scale bar, 100um. (**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 antibody from day 7 for every other day. Analysis was on day 14. Data are representative of at least three independent experiments. (**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.

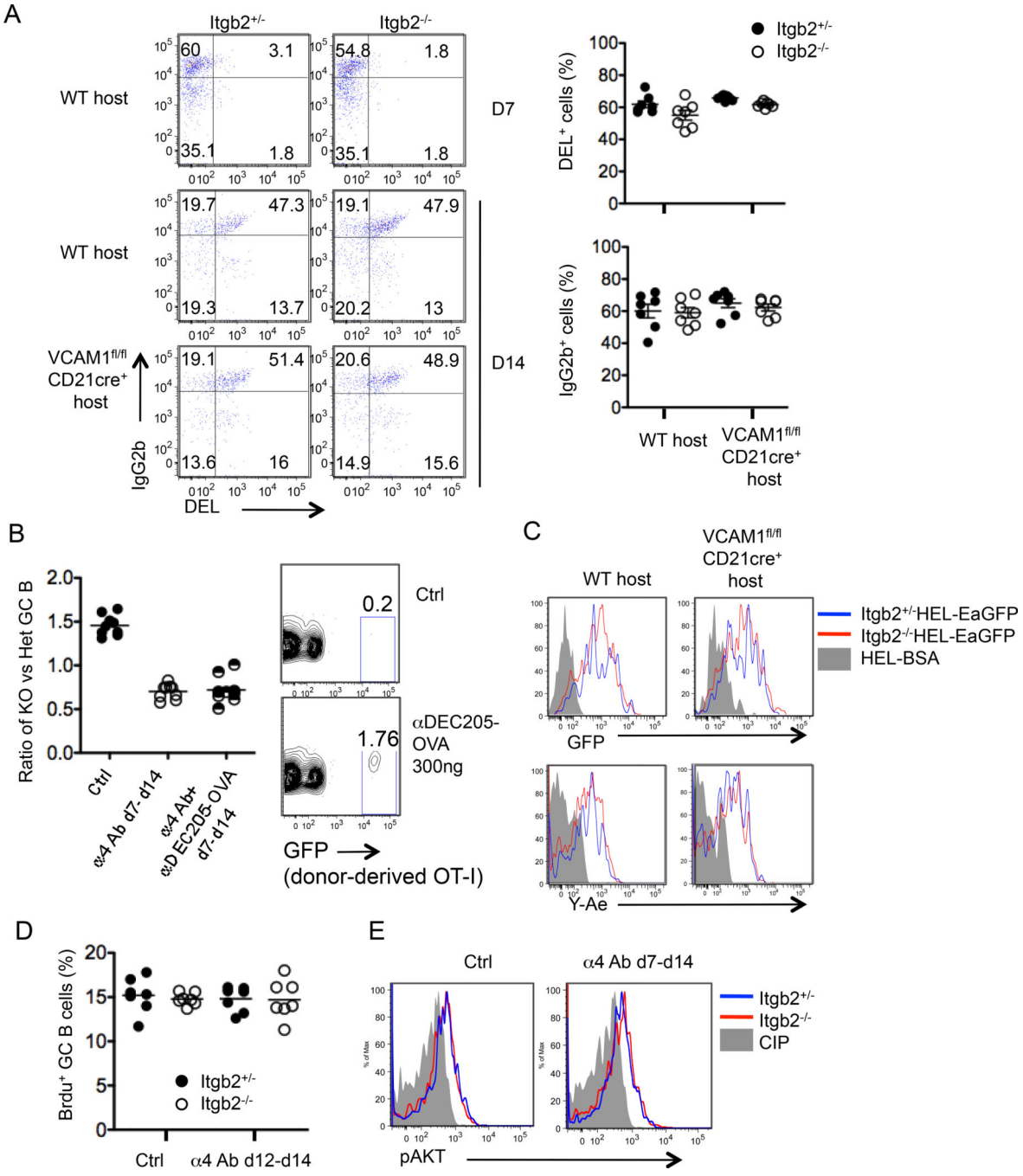

FIGURE 4. Integrin-deficient GC B cell antigen acquisition, affinity maturation and turnover

(**A**) Integrin $\beta2^{+/-}$ and $\beta2^{-/-}$ Hy10 B cells plus OT-II T cells were transferred into wild-type or $VCAM^{fl/fl}CD21cre^{+}$ 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 percent of cells in each quadrant. Graphs on right show percent of DEL-binding or $IgG2b^{+}$ cells at 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 α4 antibody or α4 antibody plus anti-DEC205-OVA (50ug) from day 7 for every other day and analyzed at day 14. Each dot represents one mouse. Data are representative of two independent experiments. Right plots show frequency of OVA-specific OT-1 CD8 T cells in spleens of recipient mice 3 days after injection of saline (Ctrl) or anti-DEC205 OVA (300ng). (**C**) Mice of the type in A were injected with 20ug HEL-Eα-GFP or HEL-BSA on day 14. Four hours later, they were analyzed for GC B cell GFP level and Eα peptide display using Y-Ae antibody. Data are representative of two independent experiments. (**D**) Mice of the type in B were injected with 2mg Brdu on day 14 and analyzed 30 minutes 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.

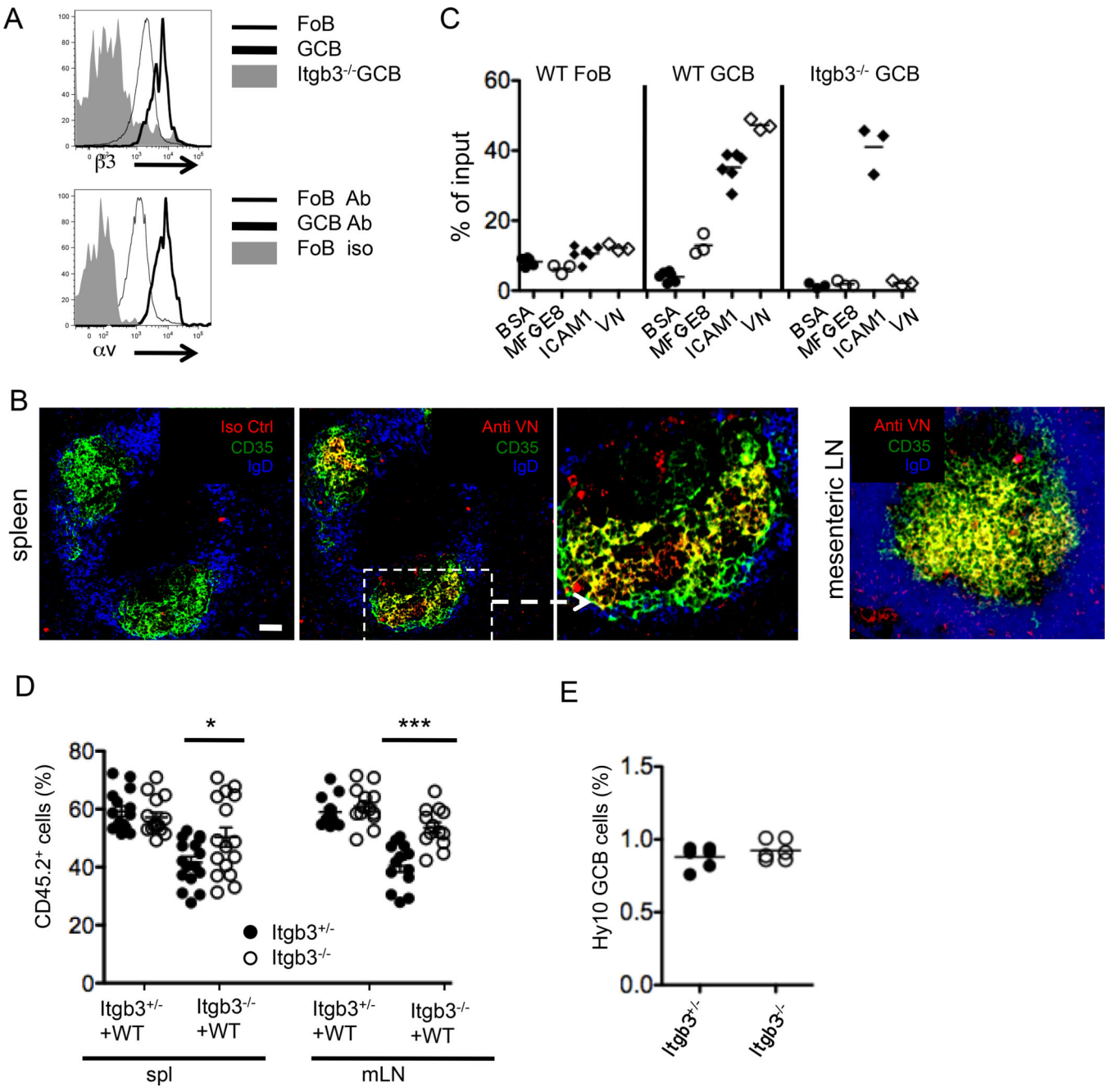

FIGURE 5. Integrin αvβ3 expression and function in GC B cells

(**A**) C57BL/6 mice were immunized with SRBCs and after 8 days examined for expression of integrin αvβ3 on follicular and GC B cells by flow cytometry. Itgb3$^{-/-}$mice or isotype matched antibodies were used as staining controls. Data are representative of two independent experiments. (**B**) Spleen and mesenteric sections from an SRBC immunized mouse were stained with anti-vitronectin (VN) or isotype-matched control. Scale bar, 100um. Data are representative of tissue from more than 3 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 weeks and immunized i.p. with SRBCs 10–12 days before analysis. Data were pooled from three independent experiments and are representative of several additional experiments that involved more than 20 mice. (**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 at day 14. Data are representative of two independent experiments.