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B cell αv Integrins regulate TLR-driven autoimmunity

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Abstract

Systemic Lupus Erythematosus (SLE) is defined by loss of B cell tolerance, resulting in production of autoantibodies against nucleic acids and other cellular antigens. Aberrant activation of Toll-like receptors (TLRs) by self-derived RNA and DNA is strongly associated with SLE in patients and in mouse models, but the mechanism by which TLR signaling to self-ligands is regulated remain poorly understood. Here we show that αv integrin plays a critical role in regulating B cell TLR signaling to self-antigens in mice. We show that deletion of αv from B cells accelerates autoantibody production and autoimmune kidney disease in the Tlr7.1 transgenic mouse model of SLE. Increased autoimmunity was associated with specific expansion of transitional B cells, extrafollicular IgG2c-producing plasma cells and activation of CD4 and CD8 T cells. Our data show that αv-mediated regulation of TLR signaling in B cells is critical for preventing autoimmunity, and indicate that loss of αv promotes escape from tolerance. Thus, we identify a new regulatory pathway in autoimmunity and elucidate upstream signals that adjust B cell activation to prevent development of autoimmunity in a mouse model.

Introduction

A hallmark of systemic lupus erythematosus (SLE) is the production of high levels of class-switched IgG autoantibodies that form pathogenic immune complexes. Appearance of autoantibodies often precedes disease, and loss of B cell tolerance is a critical initiating event for SLE. Although autoantibodies can arise against a wide range of self-antigens,

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Author Contributions

M.A., F.R. and A.L-H. designed and performed experiments. Additional assistance in experimental design, execution and analysis were provided by S.S, E.G. and L.K. Autoantibody array experiments and analysis were performed by Q.Z.L. and M.Y. J.A.H. provided reagents and advised on experimental design and data interpretation. M.A., L.M.S. and A.L-H. designed and conceived the study and wrote the paper.

nucleic acids and associated nuclear antigens, including DNA, RNA, histones and ribonucleoproteins, dominate the autoantigen repertoire. There is increasing evidence from human genetic studies and mouse models of SLE that recognition of self-derived nucleic acids by Toll-like receptors (TLRs) contributes to this loss of tolerance and production of autoantibodies. Polymorphisms and copy number variations in *TLR7*, which recognizes single stranded RNA, and in downstream signaling components such as IRF7 are associated with susceptibility to SLE (1–5). Supporting a causative role for TLR signaling in SLE, overexpression of *TLR7,* either due to the Y-linked autoimmune accelerator (Yaa) translocation (6–8), or through transgenic manipulation (9), causes lupus-like autoimmunity in mice.

TLR7 is expressed by conventional and plasmacytoid dendritic cells (cDC and pDC) and B cells, and all these cell types become activated in SLE and contribute to autoimmunity. TLR7 signaling in DCs triggers production of inflammatory and immune-stimulatory cytokines, most notably type-I IFNs, which are secreted at high levels by pDCs and are associated with severe autoimmunity in human patients and in mouse models (10). In B cells, TLR7 and TLR9 synergize with the B cell receptor (BCR) (11–13) to promote expansion of B cells specific for nucleic acids and associated antigens and production of autoantibodies (14). TLR signaling also promotes antigen presentation in DCs and B cells, driving T cell- mediated autoimmunity (15, 16). Confirming the importance of TLR signaling in SLE, deletion of Tlr7 and Tlr9 significantly attenuates anti-nucleic acid autoantibody production and autoimmune pathology in a mouse model of SLE (17). Conditional knockout studies reveal that TLR signaling in B cells is critical for many aspects of autoimmunity, including autoantibody production, T cell activation and glomerular nephritis. Disruption of DC-intrinsic TLR signaling, in contrast, does not prevent autoantibody production or nephritis, although it is required for development of dermatitis and production of type-I IFN by pDCs (16). Furthermore, the DC activation observed in this mouse model of autoimmunity is dependent on B cell-intrinsic TLR signaling, suggesting that B cell activation is an essential early step in autoimmune disease (16, 18). However, it remains unclear what mechanisms exist to regulate B cell TLR signaling in response to self-antigens, and how loss of these mechanisms in B cells impacts development of autoimmunity.

We have recently identified a regulatory role for the integrin αvβ3 in TLR signaling in B cells (19, 20). We have shown that αvβ3 promotes recruitment of the autophagy component LC3 to TLR-containing endosomes, which in turn promotes endosome fusion with lysosomes, terminating TLR signaling. Deletion of either αv or β3 integrins from B cells delays TLR trafficking, resulting in increased signaling *in vitro* and increased B cell proliferation and antibody production *in vivo* (19). Disruption of this regulatory mechanism specifically affects responses to antigens associated with TLR ligands, causing expansion of plasma and memory B cells, and increased production of high affinity, IgG2a/c class-switched antibodies (20). We have previously reported that αv-knockout mice also develop serum anti-dsDNA autoantibodies as they age, leading us to hypothesize that this regulatory mechanism contributes to B cell tolerance to self-TLR ligands (19). The αv-knockout mice thus provide us with a unique model to alter the strength of B cell TLR signaling and disrupt tolerance. To confirm whether loss of this mechanism can lead to development of

autoimmunity, we have crossed B cell-specific αv integrin knockout mice with the Tlr7.1 tg model of autoimmune disease. Here we show that deletion of αv from B cells promotes production of autoantibodies to a wide range of autoantigens, including RNA and snRNPs, leading to increased antibody and complement deposition in kidneys. This is associated with a major expansion of IgG2c class-switched plasmablasts, increased inflammatory cytokine production by B cells and activation of CD4 and CD8 T cells. Together, these data identify αvβ3 as a physiological regulator of autoreactive B cell activation, and show that alterations in the strength of TLR signaling in B cells can accelerate autoimmunity.

Material and Methods

Mice

αv-CD19 (19) and TLR7.1 tg mice (9) have been described previously. All mice were housed under specific pathogen-free conditions at Benaroya Research Institute. All animal experiments were performed under appropriate licenses and institutional review within local and national guidelines for animal care.

Antibodies and reagents

Anti-mouse antibodies used for flow cytometry include the following: CD95(Jo2), CD138(281–2), CD4(RM4–5), CD38(90), MHC-II(2G9), CD23(B3B4), CD80(B7–1), CD44(IM7), B220(RA3–6B2), CD11b(M1/70), IgM(R6–60.2), IgD(11–26c.2a), CD19(1D3) and Mouse BD Fc block (2.4G2) from BD Biosciences. CD8α(53–6.7), GL7(GL7), CD86 (GL1), CD21(7E9), CD69(H1.2F3), CD25(PC61) and CD73 (Ty/11.8) were from Biolegend. Anti-NFκ-B (D14E12), anti-LSD1 (C69G12) and horseradish peroxidase conjugated anti-rabbit IgG were from Cell Signaling Technology. Alkaline phosphatase conjugated anti-mouse IgG-AP, alkaline phosphatase conjugated anti-mouse IgG2c-AP and anti-mouse IgG(H+L) were from Southern Biotech. PNA-FITC was from Vector laboratories and R848 was from Invivogen.

Flow cytometry and cell sorting and stimulations

Spleen cells were harvested in PBS/ 0.5% BSA/ 2 mM EDTA and depleted of red blood cells (ACK lysis buffer, GIBCO). Single cell suspensions blocked with Fc Block (BD Biosciences) were stained with a combination of fluorochrome tagged antibodies for surface markers and Live/dead cell marker (Thermo Fisher) at 4°C for 30 min. For sorting of spleen marginal zone (MZ), follicular (FO) and transitional (T1) B cells, RBC lysed single cell suspensions were labeled with anti-B220, anti-CD23 and anti-CD21 and anti-CD24 antibodies then sorted with FACs Aria-II (BD Bioscience). For studies on T cell activation and proliferation, FACs sorted B cells or total B cells after enrichment (Stem cell technologies) were plated on to 96 well plated in complete RPMI-1640 (10% fetal bovine serum 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin, and 50μM 2-β-mercaptoethanol). Magnetically sorted naïve CD4 T cells (Miltenyi Biotec) were added to the B cells with or without 2.5 μg/ml anti-CD3(145–2C11; BD Biosciences). Cells were cultured at the ratio of 1:4 B cells to T cells. CD4 T cell activation was measured at 48 hrs by flow cytometry using activation specific markers. For measurement of proliferation, cells

were pulsed with 1μCi/ well tritiated thymidine ([^{3}H]-TdR) for 18 hrs prior to harvest; incorporation was determined by liquid scintillation spectrometry.

Cytokine array

For analysis of cytokines produced by B cell populations, FACs sorted MZ, FO or T1 B cells were plated in 96 well plates at the density of 100,000 cells per well and stimulated with TLR7 ligand R848 (5μg/ml). Supernatants were harvested 48 hrs later and cytokine production measured by Legendplex bead-based multi-analyte flow assay kit from Biolegend, according to manufacturer's protocol. Cytokine production was analyzed by FACs Calibur and quantified using Legendplex software (Biolegend).

Western blot

For analysis of NFκB activation, FACs sorted MZ, FO and T1 B cell subsets were stimulated for indicated times with TLR7 ligand R848 (5μg/ml). Nuclear extracts were prepared by lysing cells in hypotonic nuclear extraction buffer (1M Hepes, PH7.5, 5M NaCl, 0.5M EDTA PH8, 50% glycerol, 10% Igepal, 10% TritonX100) for 10 min followed by centrifugation at 1500g for 5 min at 4°C to pellet the nuclei and nuclei were resuspended in RIPA buffer. Lysates were centrifuged for 10 min at 4°C at 14000g and supernatant was collected as nuclear fraction. Proteins were quantified by BCA assay (Pierce), separated by electrophoresis using NuPage-Bis-Tris gels (Invitrogen) and blotted onto PVDF membranes. Non-specific binding was blocked with 5% BSA in TBS-Tween (0.1%) followed by incubation with primary antibodies overnight at 4°C. Horseradish peroxidase conjugated secondary antibodies were added for 1 hr at room temperature and developed using ECL reagents (Millipore).

ELISA

For detection of anti-RNA antibodies, Immunol 2HB microtiter plates were first treated with poly-L-Lysine and then coated with 10μg/ml yeast RNA (Sigma Aldrich) diluted in PBS. Plates were blocked with 2% BSA, 2% fetal calf serum, 0.1% Tween-20, 0.02% sodium azide in PBS and sera was added in serial dilution and incubated overnight at 4°C. Plates were washed and bound antibodies detected by adding alkaline phosphatase-conjugated goat anti-mouse IgG or IgG2c (Southern Biotech) diluted in blocking buffer for 60 min at 37°C. Secondary antibodies were detected by using disodium p-nitrophenyl phosphate substrate (Sigma Aldrich) and absorbance (OD) read at 405 nm. For detection of total Ig in culture, supernatants plates were coated with 10 μg/ml goat anti-mouse IgG (heavy and light chain specific) (Southern biotech) in PBS at 4°C overnight. Plates were blocked as above and incubated with various dilutions of cell culture supernatants in PBS. After incubation with alkaline phosphatase conjugated IgG or IgG2c antibodies (Southern Biotech), color was developed as above with disodium p-nitrophenyl phosphate substrate.

Autoantigen array

Autoantigen microarrays were performed at University of Texas Southwestern Medical Center, Microarray core facility, Dallas, Texas (https://microarray.swmed.edu/products/product/autoantigen-microarray-panel-i/).

ANA

For ANA assays diluted serum (1/40) was added to fixed Hep-2 ANA slides (MBL-Bion) and FITC conjugated goat anti-mouse IgG was used as detection antibody. Fluorescent images were obtained using Nikon Ti (Eclipse) inverted microscope at 40X magnification with constant exposure.

Kidney immunofluorescence staining

Mouse kidneys were embedded in OCT compound and snap frozen over dry ice. 6μM sections were cut on cryostat and mounted on super-frost plus slide and fixed with ice cold acetone for 5 min. After rehydration with PBS, slides were blocked with PBS/ 5%BSA and stained with IgG-FITC (Invitrogen Alexa 488 IgG CAT A11001) or C3-FITC (MP Biomedicals CAT 855500). Images were acquired using Nikon Ti (Eclipse) inverted microscope with Ultraview Spinning Disc (CSU-X1) confocal scanner (Perkin Elmer).

Results

Deletion of αv from B cells increases splenomegaly in Tlr7.1 tg mice

To determine whether B cell αv integrins affected autoimmunity driven by TLR signaling, we used an established model in which the *Tlr7* gene is overexpressed from a BAC transgene (Tlr7.1 tg mice (9)), resulting autoimmunity associated with expansion of autoreactive B cells (9, 21). αv-CD19 mice (19) were crossed with Tlr7.1 tg mice to generate αv-CD19.Tlr7 mice; littermates hemizygous for the αv-flox allele, but with the same CD19-Cre and Tlr7.1 tg alleles served as controls (referred to as Tlr7.tg controls). Tlr7.tg mice develop exacerbated immune dysregulation with age, often requiring euthanasia from 3 months of age (9, 22). αv-CD19.Tlr7 mice showed increased incidence of sudden death or development of severe autoimmune defects such as anemia that required euthanasia compared with littermate controls. This was most pronounced in female mice, resulting in approximately 40% mortality by 10 weeks of age (Fig 1). αv-CD19.Tlr7 mice also had significantly larger spleens than both Tlr7.tg littermates and non-transgenic controls and as with mortality, the effects of αv deletion on splenomegaly was most prominent in females (Fig 1). Non-transgenic αv-CD19 mice do not have splenomegaly, indicating that this increase in spleen size was due to a synergistic effect between αv-deletion and Tlr7 overexpression. Furthermore, αv-CD19.Tlr7 developed splenomegaly earlier than littermate controls. Almost all of the αv-CD19.Tlr7 mice analyzed had enlarged spleens by 6–8 weeks of age, whereas only 30% of Tlr7.tg control mice had significantly enlarged spleens at this age, and had not yet developed the severe splenomegaly and widespread autoimmune inflammation reported for this strain at 10–12 weeks (9, 21, 23). These data therefore supported our hypothesis that deletion of αv from B cells increased or accelerated autoimmunity in TLR7.tg mice.

Expansion of extrafollicular plasma cells in αv-CD19.Tlr7 mice

To understand the role of αv-deficient B cells in early development of autoimmunity, and to avoid possible confounding factors due to extensive immune dysregulation, early mortality and sex, we focused our analysis of B cells on young (6–8 week) female αv-CD19.Tlr7

mice. Tlr7.tg mice are reported to have a reduction in total spleen B cells, accompanied by an increase in the frequency of transitional cells and reduction in marginal zone (MZ) B cells (9, 21). We therefore first analyzed spleen B cells in αv-CD19.Tlr7 and Tlr7.tg control mice, using CD21/ CD23 and CD24 staining to identify transitional, MZ and follicular (Fo) B cell subsets. We did not observe any statistically significant changes in total B cell numbers in 7–8 week-old Tlr7.tg mice compared with either non-tg controls or αv-CD19.Tlr7 mice (Fig 1C). $CD21^{high}$ $CD23^{low}$ MZ B cell numbers were also not statistically significantly different between TLR7.tg strains and controls, although the relative proportions of MZ B cells were much more variable in αv-CD19.Tlr7 mice than in Tlr7.tg or non-tg controls, suggesting that they may be in the process of developing the reduction of MZ B cells previously reported for Tlr7.tg mice. Germinal center (GC) B cells are also reported to be expanded in Tlr7.tg mice, but they were not present at high numbers in these young mice and their frequencies were similar between Tlr7.tg controls and αv-CD19.Tlr7 mice (Supp Fig 1). We did observe significantly higher proportions of $CD21^{mid\text{-}low}$ $CD23^{low}$ B cells in αv-CD19.Tlr7 mice compared with Tlr.tg and non-transgenic controls (Fig 1D–E). This population includes immature and transitional B cells, which have been reported to expand in Tlr7.tg mice. We therefore used additional antibody panels to investigate changes in these and other B cell populations further. The percentage of immature B cells (based on expression of CD93) was not affected by αv-deletion (Supp Fig 1). The relative proportions of Transitional 1 (T1: $B220^{+}$ $CD24^{high}$ $CD21^{low}$) or T2 ($B220^{+}$ $CD24^{high}$ $CD21^{mid}$) B cells also did not change significantly between Tlr7.tg and αv-CD19.Tlr7 mice except for a small increase in IgM^{high} $CD23^{mid}$ T2 cells (Fig 1 F–G and Supp Fig 1). Instead, the increase in $CD23^{low}$ cells seems to be driven by the emergence of a population of $B220^{+}$ $CD24^{-}$ $CD21^{mid}$ cells. These cells are uniformly IgM^{low} and do not express high levels of CD11b or CD11c, which are seen on activated ‘age-associated B cells’ associated with mouse autoimmunity models (24) (Supp Fig 1). Based on these data we concluded that many of the previously reported effects of Tlr7 overexpression on B cell populations are yet to develop in the young mice used for these experiments.

In contrast, we observed clear effects of Tlr7 overexpression and αv deletion on spleen plasma cells. The frequency of CD138+ plasma cells was increased in Tlr7.tg mice compared with non-tg controls, and this was even further enhanced in αv-CD19.Tlr7 mice (Fig 1 H–J). Furthermore, the proportion of plasma cells that had undergone class-switching to IgG2c was markedly increased in αv-CD19.Tlr7 mice compared with Tlr7.tg controls (Fig 1H–J). We also analyzed B cell phenotypes in older αv-CD19.Tlr7 mice and Tlr7.tg controls that had survived to 10–13 weeks of age. At this age, we observed similar increases in Immature/ Transitional, CD24- and IgG2c+ plasma cells in both αv-CD19.Tlr7 mice and Tlr7.tg controls (Supp Fig 1). These data are consistent with the earlier expansion of autoreactive B cells in the αv-CD19.Tlr7 mice and loss of the αv-CD19.Tlr7 mice with severe autoimmunity. Hence, taken together, these data indicated that deletion of αv did not directly affect the proportion of immature cells in the spleen, but instead influenced their activation and/or differentiation into class-switched antibody-producing cells during initiation of autoimmunity.

αv deletion has differential outcomes on activation of B cell subsets

Based on our previous studies we hypothesized that deletion of αv caused increased B cell activation by increasing TLR7 signaling(19, 20). To test this, we measured activation of the transcription factor NF-κB upon TLR stimulation in purified B cell subsets from αv-CD19.Tlr7 and Tlr7.tg control mice. Treatment with the TLR7 ligand R848 induced stronger activation (nuclear localization) of NF-κB p65 in T1 cells from αv-CD19.Tlr7 mice compared with controls (Fig 2A), confirming that loss of αv increases TLR signaling in these B cells, similar to our previous observations in MZ and GC B cells (19, 20). R848 also triggered NF-κB nuclear localization in Tlr7.tg Fo B cells, but this was not significantly increased by αv deletion. However, Fo cells from αv-CD19.Tlr7 mice showed high levels of NF-κB nuclear localization in the absence of exogenous TLR7 ligands, which was not seen in Tlr7.tg control Fo B cells (Fig 2A). We attribute this to basal activation of autoreactive Fo B cells by endogenous ligands encountered during purification or from cellular debris in culture, and these data therefore support our model that deletion of αv increases TLR7 signaling. MZ B cells normally express high levels of TLR7 and respond robustly to TLR stimulation. Consistent with this, MZ B cells from both αv-CD19.Tlr7 and control Tlr.tg mice showed very high levels of TLR signaling even in the absence of exogenous ligands, with no clear difference between the genotypes (Fig 2A). Thus, while we can see the effect of loss of αv in increasing TLR signaling in transitional and Fo B cells, this is harder to see in the MZ B cells due to massive increase in basal TLR7 signaling.

To understand how αv-mediated changes in TLR signaling affect B cell responses, we first measured cytokine production after Tlr7 stimulation *in vitro*. Fo and MZ B cells from αv-CD19.Tlr7 both showed significant increases in cytokine production after stimulation when compared with Tlr7.tg control or non.tg controls (IL-10 and IL-6 production by Fo B cells, and IFN-β production by MZ B cells) (Fig 2B). T1 B cells produced little or no detectable TNF-α, IL-6 or IFN-β after stimulation, but did secrete low levels of IL-10, which was increased in Tlr7.tg T1 cells, and further increased in αv-CD19.Tlr7 T1 cells (Fig 2B). Tlr7.tg transitional B cells have been shown to respond to TLR7 ligands by differentiating into IgG2c-producing cells (21). In agreement with this previous report, transitional Tlr7.tg B cells differentiated into IgG2c-producing cells after stimulation with R848 (Fig 2C), as seen by IgG2c production in supernatants. Under the same conditions, cultures of MZ and Fo B cells also differentiated into IgG2c-producing cells, although at considerably lower levels than in T1 cultures. αv-deletion greatly increased IgG2c production in T1 cells, with cultures from αv-CD19.Tlr7 mice producing 2–3-fold higher levels of IgG2c antibody than the equivalent cells from Tlr7.tg controls (Fig 2C). Together these data demonstrate that αv normally regulates TLR7 signaling in transitional, MZ and Fo B cells, and deletion of αv- leads to increased Tlr7 responses in Tlr7.tg B cells. This has distinct functional consequences in B cell subsets, such that loss of αv leads to increased cytokine production by MZ and Fo cells while it increases differentiation of transitional cells to IgG2c-producing cells.

αv deficient B cells drive increased T cell activation

T cell activation by B cells is increasingly appreciated as an important contributor to autoimmune disease (16). We therefore determined whether the increased B cell TLR

responses in αv-CD19.Tlr7 mice may affect T cells. Consistent with previous reports of T cell activation in Tlr7.tg mice, 7–8 week old Tlr7.tg control mice had reduced numbers of spleen T cells and a significant increase in the proportion of activated $CD44^{+}$ $CD62L^{low}$ T cells. The frequency of spleen T cells was similar in Tlr7.tg and αv-CD19.Tlr7 mice, but activation of both CD4 and CD8 subsets was significantly increased in αv-CD19.Tlr7 mice (Fig 3A,B). To determine whether αv integrins affect direct T cell activation by B cells, B cells from αv-CD19.Tlr7 mice or Tlr7.tg controls were co-cultured with naïve CD4 T cells in the presence of anti-CD3 antibodies, and T cell activation and proliferation measured. T cells cultured with αv-knockout B cells had higher expression of the T cell activation markers CD69 and CD25 (Fig 3C,D) and proliferated significantly more than T cells cultured with Tlr7.tg control B cells (Fig 3E). T1, Fo and MZ B cell subsets from αv-CD19.Tlr7 mice all shared this capacity for increased T cell activation, although this was most evident in MZ B cells (Fig 3F). Hence B cell-intrinsic effects of αv deletion promote increased activation of T cells *in vivo,* and our *in vitro* experiments support a direct role for αv-knockout B cells in T cell activation.

αv-deletion increases autoantibody response

To determine whether αv deficient B cells affected development of autoimmunity we examined production of autoantibodies. Autoimmunity in Tlr7.tg mice is associated with high levels of autoantibodies to RNA and associated antigens (9). Serum titers of anti-ssRNA IgG antibodies were increased in αv-CD19.Tlr7 mice compared with littermate Tlr7.tg controls (Fig 4A). Titers of IgG2c anti-ssRNA were also increased (Fig 4A), consistent with the expansion of IgG2cclass-switched plasma cells in αv-CD19.Tlr7 mice. However, IgM anti-ssRNA antibodies, which were elevated in Tlr7.tg controls compared to non-transgenic controls, were not further affected by αv-deletion (Fig 4A). Autoantibodies against the RNA-associated protein snRNP were also increased in αv-CD19.Tlr7 mice compared with Tlr7.tg controls, and in this case IgM, IgG and IgG2c isotypes were all increased (Fig 4B). Autoantibodies were also assessed using a fluorescent ANA assay. Both Tlr7.tg and αv-CD19.Tlr7 mice had ANA positive serum, but exhibited different patterns of staining. While Tlr7.tg controls had nuclear or mixed nuclear/ cytoplasmic staining patterns, most αv-CD19.Tlr7 mice showed staining that was concentrated around the nucleus or in the cytoplasm, indicating that deletion of αv may alter the range of autoantibody targets (Fig 4C). To test this and better understand the effects of αv on autoantibody repertoire, we used an autoantigen array to profile serum antibodies from αv-CD19.Tlr7 mice and controls. αv deletion led to increases in all autoantibodies present in Tlr7.tg mice, with increases in both IgM and IgG isotypes (Fig 4D), indicating that αv-deletion increased all TLR7-driven autoantibody responses.

αv deletion increases kidney antibody deposition and glomerulonephritis

To determine whether the increased serum autoantibodies in the αv-CD19.Tlr7 mice could accelerate autoimmune organ damage, we analyzed antibody and complement deposition in kidneys of the mice at 7–8 weeks. αv-CD19.Tlr7 mice showed higher levels of IgG staining in glomeruli than the Tlr7.tg mice (Fig 5A). αv-CD19.Tlr7 mice also showed prominent glomerular C3 deposition, at much higher levels than in Tlr7.tg control mice (Fig 5B), consistent with increase in IgG2c class-switched autoantibodies in these mice. Since our

analysis was focused on younger mice we did not expect to see severe glomerulonephritis in these mice. However, histological analysis of kidney sections indicated increased glomerular size in αv-CD19.Tlr7 mice compared with controls, supporting acceleration of autoimmune pathology in these mice (Fig 5C). We therefore conclude that deletion of αv integrins from B cells significantly accelerated autoimmunity as measured by splenomegaly, autoantibody production and antibody deposition in organs and this results in increased pathology and mortality.

Discussion

Although TLR7 signaling has been shown to be critical for development of lupus-like autoimmunity in mice (6, 9, 16, 17), little is known about how TLR signaling is normally regulated and how loss of regulation in individual cell types contributes to autoimmune disease. Our results identify αv integrin as a cell-intrinsic regulator of pathogenic TLR signaling, and show that loss of this regulatory pathway in B cells exacerbates TLR7-driven autoimmunity, complementing previous studies that have shown that TLR signaling in B cells is required for autoimmunity. Dysregulated TLR signaling has differential outcomes on B cell subsets, promoting plasma cell differentiation, IgG2c class-switching, cytokine production and T cell activation. Together, this results in increased autoantibody production and tissue damage in the Tlr7.tg model. These data highlight the central role of B cell TLR signaling in autoimmunity and show that increasing TLR signaling in B cells is enough to accelerate autoantibody production and class switching, kidney damage and activation of other immune cells.

Based on our previous work, we propose that deletion of αv from B cells increases autoimmunity through cell-intrinsic dysregulation of B cell TLR signaling (19, 20). Our data support this model, showing that isolated αv-knockout Tlr7.tg B cells respond more strongly to TLR stimulation in culture. This was particularly clear in transitional B cells, where αv-deletion resulted in increased TLR7-driven NF-κB activation, similar to our previous observations in MZ and GC B cells (19, 20), and was also apparent in Fo B cells where αv-knockouts showed higher constitutive NF-κB activation. The high constitutive NF-κB activation in Tlr7.tg MZ B cells confounded our ability to show effects of αv on acute TLR signaling in this subset. However, the increase in T cell activation and cytokine secretion in αv-knockout MZ and Fo B cells confirmed that TLR signaling in these cell subsets is increased when αv is deleted. Further supporting our model, the effects of αv-deletion on B cell TLR responses in culture matched the phenotypes of αv-CD19.Tlr7 mice, including increased IgG2c class switching and T cell activation. Notably, our data also reveal that dysregulation of TLR signaling had distinct effects on different B cell subsets.

The transitional B cell stage represents an early site of encounter of autoreactive B cells with self-antigens in the periphery, and acts as a checkpoint for deletion of strongly autoreactive cells (25, 26). T1 B cells have been shown to be strongly affected by TLR7 overexpression, which leads to expansion of T1 B cells, and drives their differentiation into autoreactive extrafollicular IgG2c-producing plasma cells in the red pulp (21). Supporting a regulatory role for αv in TLR signaling, αv-deletion increased production of IgG2c by transitional cells *in vitro* and resulted in expansion of spleen IgG2c-producing plasma cells *in vivo*.

Curiously, αv deletion did not appear to affect numbers of transitional cells in Tlr7.tg mice, although we have previously shown that transitional B cell numbers are increased in αv-CD19 mice (19) indicating that αv does regulate expansion of this B cell population in the absence of pathogenic TLR signaling. However, we did observe a previously unreported $B220^{+}$ $CD24^{-}$ $CD21^{mid}$ population that develops in Tlr7.tg mice and is greatly expanded in αv-CD19.Tlr7 mice. This population appears to be distinct from the age associated B cell populations seen in mouse autoimmunity models (24, 27, 28). The concurrent expansion of these cells and IgG2c-producing cells in young αv-CD19.Tlr7 mice, and our observation that transitional cells produce more IgG2c in culture than other B cell subsets lead us to speculate that many of the IgG2c-producing cells we observe in αv-CD19.Tlr7 mice develop directly from an immature or transitional population as extrafollicular plasma cells (21). However, additional studies will be needed to confirm this. Recent studies of SLE patients have identified a new immature/transitional population of effector B cells that are poised to differentiate into autoantibody-producing plasma cells(29, 30), and develop through an extrafollicular pathway in response to TLR7 signaling. Thus, our data are in agreement with other studies of mouse models and SLE in highlighting the important role of TLR regulation at the immature stage of B cell development, and suggest that activation of autoreactive immature cells and conversion to extrafollicular plasma cells is an important early step in autoimmunity.

Our data also point to important effects of αv-mediated regulation of TLR signaling on functions of other B cell subsets, including cytokine production and T cell activation, which were more pronounced in MZ and Fo B cells than transitional cells. T cell activation is an important feature of lupus-like autoimmunity and in mouse models, requires B cell TLR signaling (16). Our data show that dysregulated TLR signaling in B cells can further increase T cell activation. Whether this is due to increased cytokine production by B cells, or changes in processing or presentation of antigens remains to be determined.

Regulation of TLR signaling by αvβ3 requires components of the autophagy pathway. Specifically, αvβ3 promotes recruitment of LC3 to TLR-containing endosomes, which is required for lysosomal fusion. Deletion of LC3b or a component of the LC3 conjugation machinery, *Atg5*, cause increased B cell responses to TLR ligands, reproducing the phenotype of αv-knockout cells (19). We would therefore expect that disruption of LC3 recruitment would also increase autoimmunity in the Tlr7.tg model. However, studies from the Huber group report that deletion of *Atg5* from B cells in the Tlr7.tg model has the opposite effect to αv-deletion, reducing autoantibody production and autoimmune pathology (31). These seemingly contradictory findings reflect the involvement of LC3 in many distinct intracellular processes. Atg5 and LC3 are essential for ‘classical’ autophagy (also referred to as macro-autophagy), a process by which cells target, partition and digest intracellular protein complexes, organelles or pathogens, via formation of a characteristic double membrane autophagosome (32). Classical autophagy is essential for plasma cell homeostasis, and mice lacking *Atg5* and other components of the LC3-lipidation pathway have fewer plasma cells and impaired antibody production (33, 34). Effects on plasma cells therefore explain the reduction in autoantibodies in *Atg5*-knockout mice. In contrast, αvβ3-mediated activation of Atg5 and recruitment of LC3 to Tlr endosomes occur via a non-canonical autophagy pathway, independent of macro-autophagy. This pathway, which

requires Rubicon and reactive oxygen species (ROS) (19, 20), appears similar to the LC3-associated phagocytosis (LAP) pathway described by Green, Sanjuan and Martinez (35–37). Disruption of LAP in mice results in SLE-like inflammatory responses (38), suggesting that non-canonical autophagy may also play an immune-regulatory role for non-canonical autophagy in myeloid cells, although it remains to be seen whether LAP involves αv and if it regulates TLR signaling in myeloid cells. These different and potentially opposing roles for core autophagy components in immune cells may explain the complex and poorly understood role of autophagy in SLE and other autoimmune diseases (32, 39–41).

We have previously shown that αvβ3 directs the intracellular trafficking of TLRs and their ligands to lysosomes, where they are degraded and signaling is terminated (19). Deletion of αv or β3 delays TLR trafficking, resulting in increased and prolonged signaling (19). Although previous studies have focused on the role of TLR trafficking in delivering TLRs to endosomes (42–45), regulating ligand engagement (46–52) or determining signaling through NF-κB and IRFs (53–57), our studies identified an additional and critical role for the rate of trafficking in regulating TLR signaling. Our finding that αv-CD19 mice develop spontaneous autoantibodies (19) and accelerate TLR7-driven autoimmunity suggest that this αv-mediated regulatory pathway is particularly important in preventing autoimmunity to self-derived TLR ligands such as nuclear antigens. αv integrins have long been known to be involved in clearance of apoptotic cells and other cellular debris which serve as a rich source of autoantigens and nucleic acid TLR ligands. We propose that αvβ3 functions as a co-receptor for these self-derived TLR ligands, and regulates TLR-ligand signaling to prevent autoimmunity. Supporting this model, deletion of MFG-E8 also promotes autoimmunity in mice (58), and directs the intracellular trafficking of apoptotic material in DCs (59). The recent findings that another integrin, αMβ2 also acts as a co-receptor for TLR ligands (60), and regulates TLR signaling in the context of autoimmunity (61, 62), suggest that this mechanism of TLR regulation may not be limited to αvβ3 integrins. Together, these data point to the importance of regulating the strength of TLR signaling in B cells, for preventing pathogenic B cell activation by self-antigens. Moreover, these data highlight the underlying role of molecules such as integrins and autophagy proteins that regulate intracellular trafficking events in adjusting immune signaling and maintaining tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

Deletion of αv integrins from B cells accelerates TLR-driven autoimmunity

αv-knockout B cells have increased pathogenic TLR signaling

αv regulates cytokine production and differentiation of IgG2c plasma cells

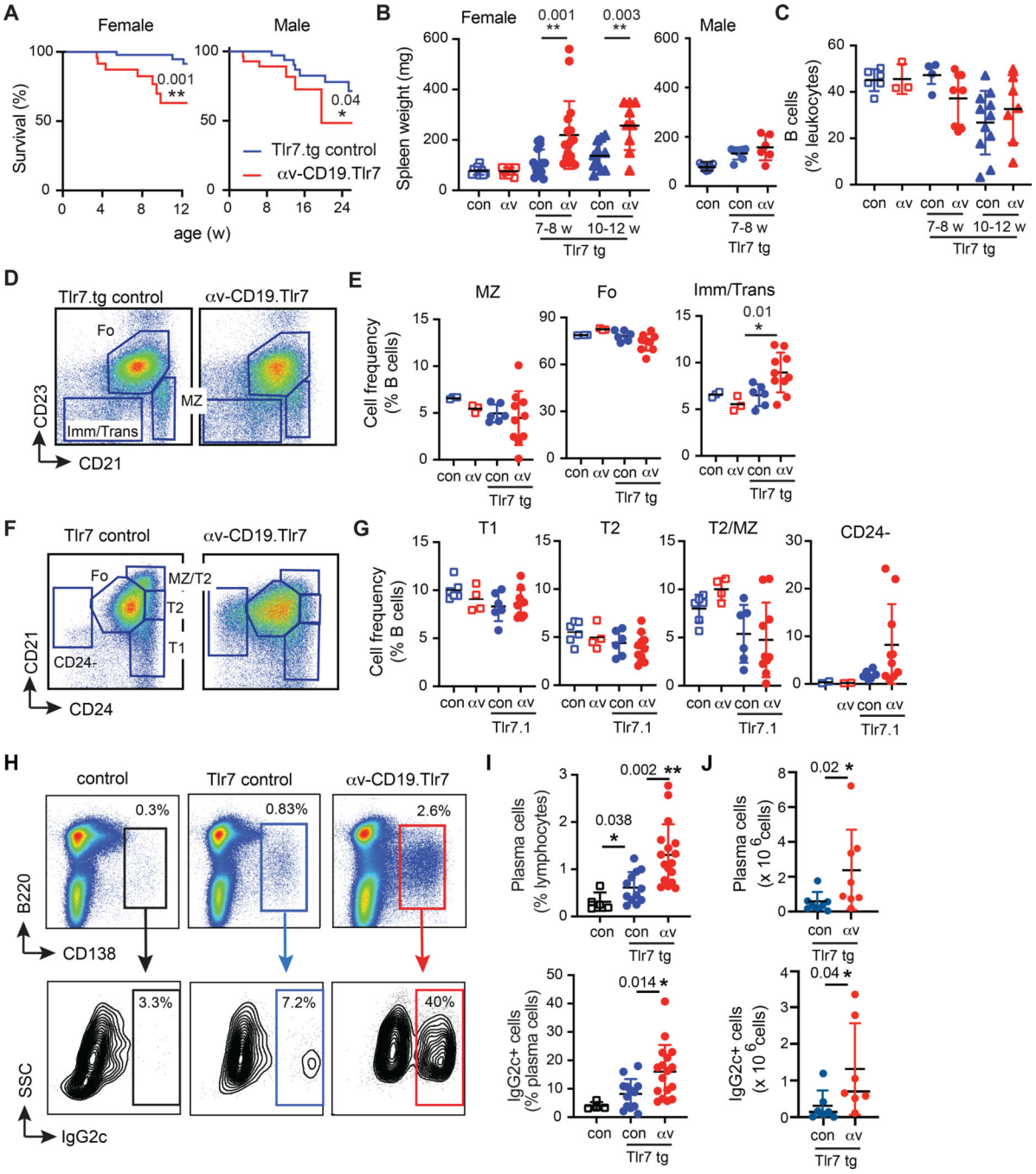

Figure 1: αv deletion promotes expansion of plasma cells:

(A) Survival of female and male Tlr7.tg control and αv-CD19.Tlr7 mice. *n*≥17 mice/ group. **(B)** Spleen weight from control mice (con), αv-CD19 (αv), control Tlr7.tg (con-Tlr7.tg) and αv-CD19.Tlr7 mice (αv Tlr7.tg). Groups analyzed are 7–8 week old male (n ≥ 6/group) and female (n ≥ 10/group), and 10–12 week old females (n ≥7 mice /group). **(C)** Spleen B cell frequency in female mice at 7–8 and 10–12 weeks of age (n ≥ 4 mice/ group). **(D-G)** Splenocytes were gated on $CD19^{+}$ cells and the frequencies of Immature/ Transitional (Imm/ Trans), transitional 1 and 2 (T1, T2), Marginal Zone (MZ), Follicular (Fo), and CD24-

negative B cells determined by flow cytometry as shown. Analysis of non-tg control and αv-CD19 mice are included for comparison. n=3–6/ group of non-transgenic and 6–10/group of transgenic mice. **(H-J)** Spleen plasma cells were identified based on CD138 staining and analyzed for intracellular IgG2c, and quantified by percentage of parent population, n=5 for non-transgenic mice and $n \geq 13$ / group for transgenic mice **(I)** or total number of cells/ spleen (n=8 mice/group) **(J)**. Data are presented as data points from individual mice. p-values <0.05 for comparisons between control Tlr7.tg and αv-CD19.Tlr7 mice are shown, and indicated by * ($p<0.05$) or ** ($p<0.01$). p-values calculated using log-rank test (survival curves, **A**) or Mann-Whitney test.

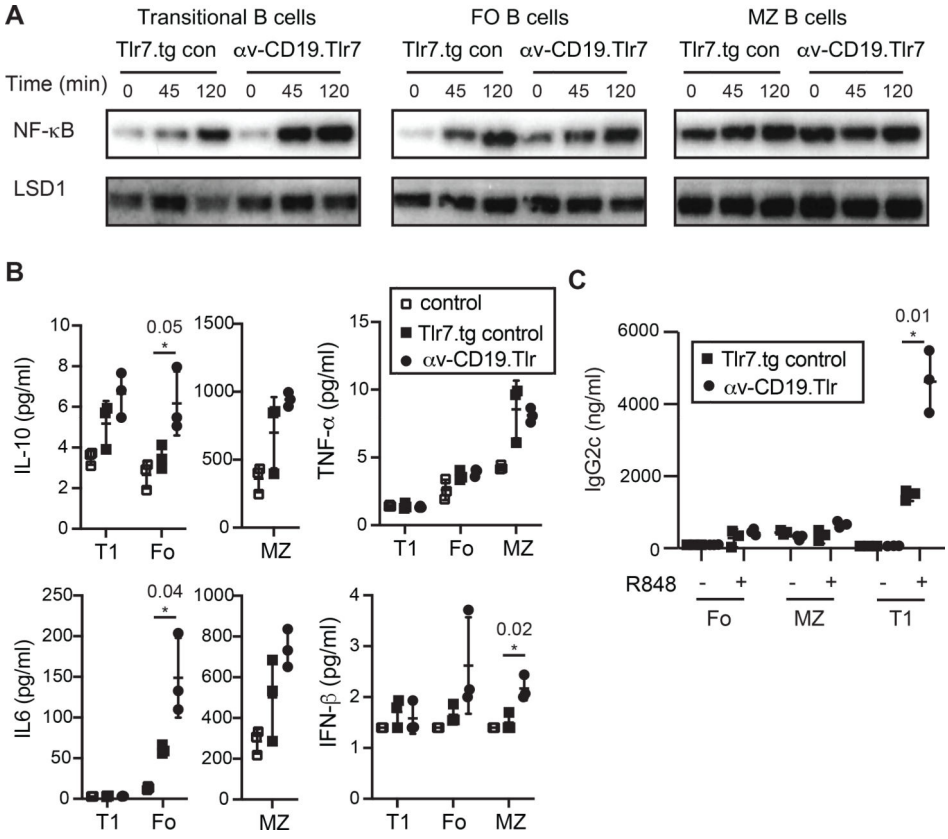

Figure 2: Increased TLR signaling in αv-deficient Tlr7.1 transgenic B cells:

(A) Sorted transitional (T1), marginal zone and follicular B cells from Tlr7.tg control and αv-CD19.Tlr7 mice were cultured with 5μg/ml TLR7 ligand R848 for 0–120 min. Nuclear NF-κB (p65) localization was measured by western blot and LSD1 is shown as nuclear loading control. **(B,C)** Cytokine **(B)** and IgG2c **(C)** levels in supernatants of cultured B cell sub-populations after stimulation with R848 for 24 hrs (cytokines, **B**) or 4 days (IgG2c, **C**). Data shown are mean±SD of replicate cultures from one experiment. *p*-values <0.05 (Student's t-test) for comparisons between control Tlr7.tg and αv-CD19.Tlr7 mice are shown. Similar results were seen in at least 3 independent experiments.

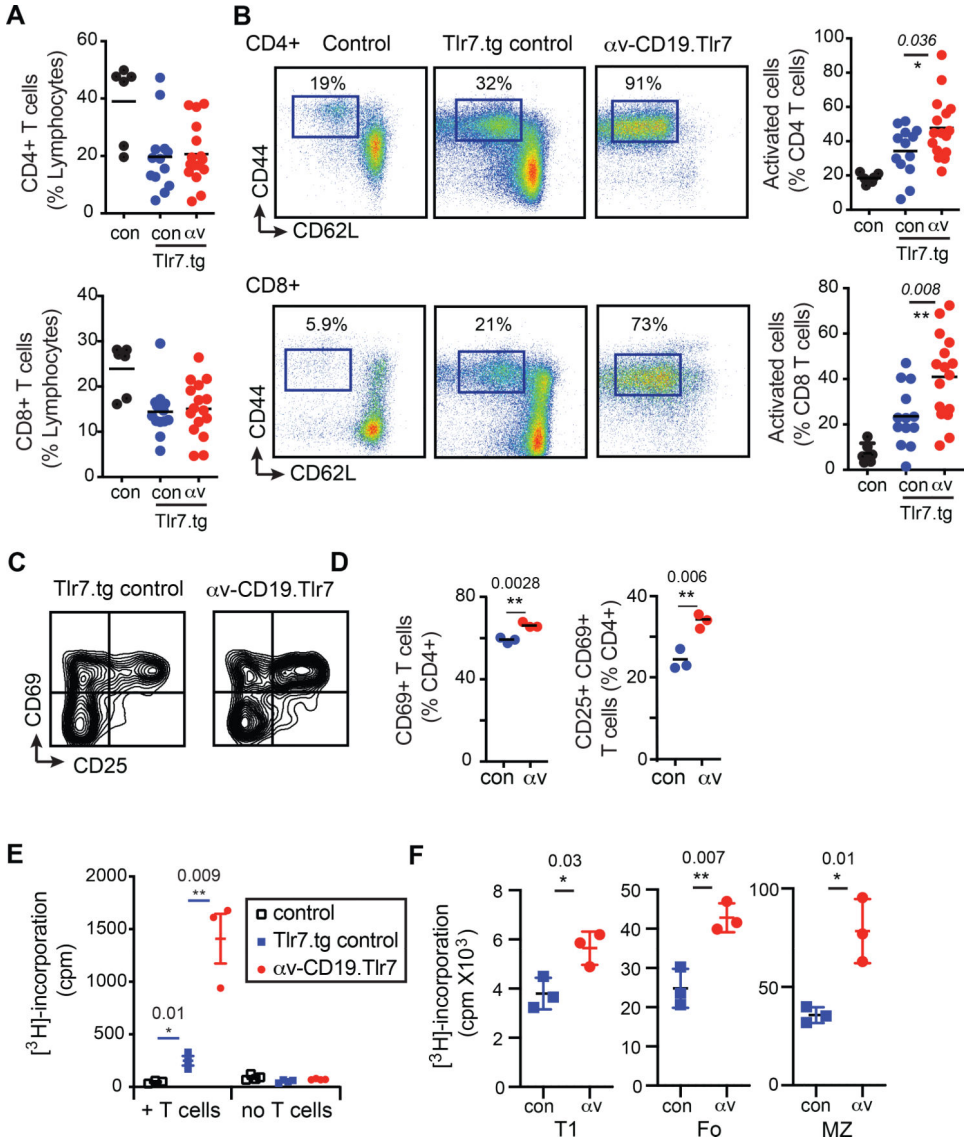

Figure 3: Increased T cell activation in αv-CD19.Tlr7.1 transgenic mice:

(A,B) Spleens from 7–8 week old wild-type control mice (con), Tlr7.tg controls (con-Tlr7.tg) and αv-CD19.Tlr7 mice (αv-Tlr7.tg) were analyzed for $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells as a frequency of total splenocytes by FACS **(A)**. CD4 and CD8 were further analyzed for expression of CD44 and CD62L cells as shown **(B)**. Each data point represents a single mouse. *p*-values <0.05 (Mann-Whitney test) for comparisons between control Tlr7.1 tg and αv-CD19.Tlr7.1 tg mice are shown, and indicated as by * ($p<0.05$) or ** ($p<0.01$) (n=6 non-transgenic, $n\geq13$/group transgenic mice). **(C-E)** B cells from TLR7.tg controls and αv-CD19.Tlr7 mice were cultured with $CD4^+$ T cells from control mice in the presence of anti-CD3. T cell activation (based on CD25 and CD69) **(C,D)** and cell proliferation measured by $[^3H]$-thymidine incorporation **(E)** were measured after 3 days**. (F)** Sorted transitional, marginal zone and follicular B cells from control TLR7.1 transgenics (con-Tlr7.1) and αv-CD19.Tlr7.1 transgenic mice (αv-Tlr7.1) were cultured with control $CD4^+$ T cells in the presence of anti-CD3, and cell proliferation measured after 48 hr. All data shown

are mean±SD of replicate cultures from one experiment. Similar results were seen in at least 3 independent experiments. *p*-values <0.05 (Student's t-test) for comparisons between control Tlr7.1 tg and αv-CD19.Tlr7.1 tg mice are shown, and indicated as by * ($p<0.05$) or ** ($p<0.01$).

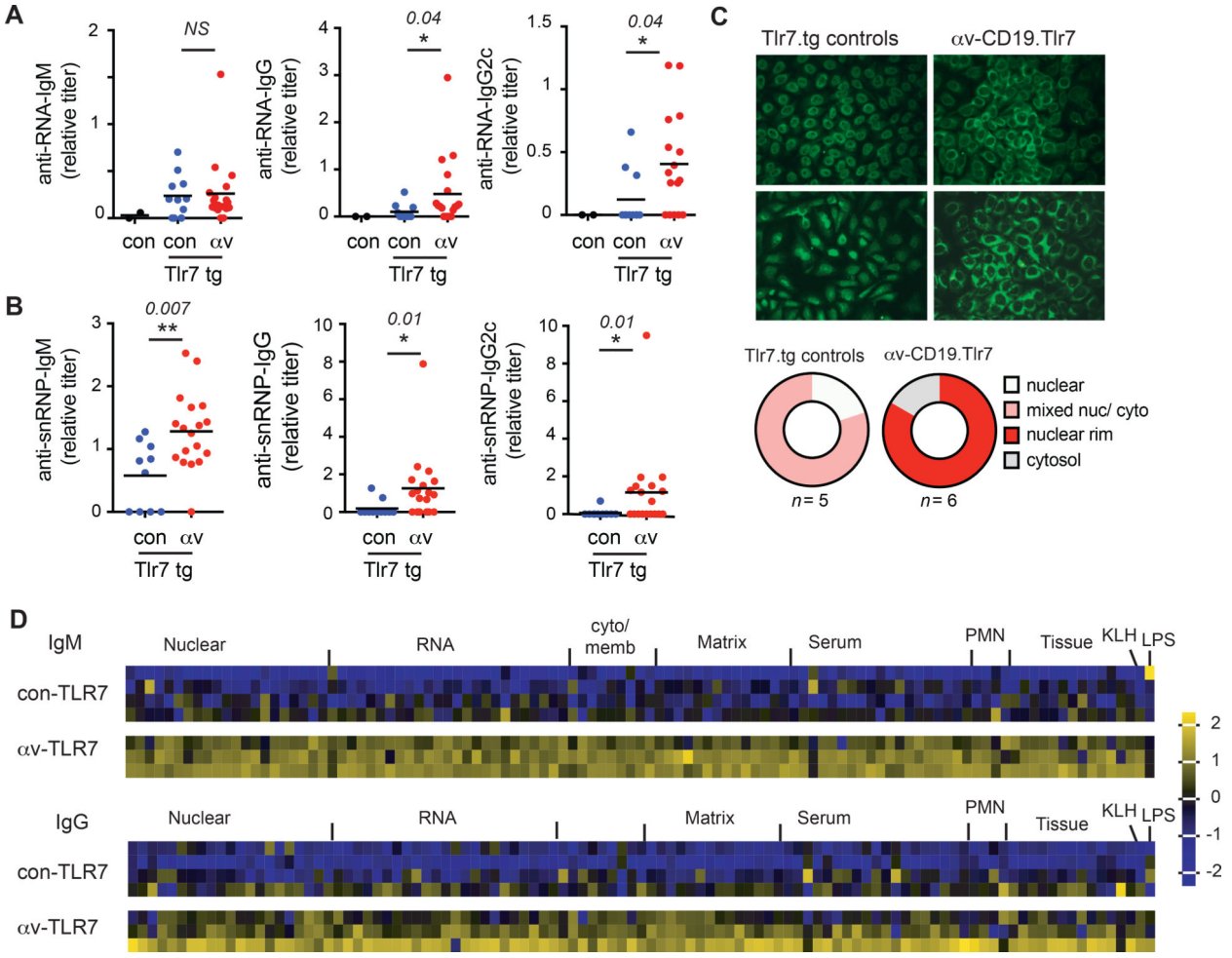

Figure 4: αv deletion from B cells increases autoantibody production:

(A) (A-B) Serum titers of anti-RNA and anti-snRNP antibodies from 7–8 week old wild-type control, control Tlr7.1 tg and αv-CD19.Tlr7.1 tg mice (*n*≥11/group) **(C)** HEp-2 cell staining patterns of serum antibodies from 7–8 week old control Tlr7.1 tg and αv-CD19.Tlr7.1 tg mice. Two representative images are shown per genotype. Pie charts show summary of patterns for 5–6 mice/ genotype. **(D)** Serum IgM and IgG autoantibodies from 7–8 week old control Tlr7.1 tg (n=4) and αv-CD19.Tlr7.1 tg (n=3) mice measured using an autoantibody array chip containing 88 specific autoantigens. Data are shown as a heat map of Z-scores. Autoantigen class is shown, along with control antibodies (anti-KLH and anti-LPS).

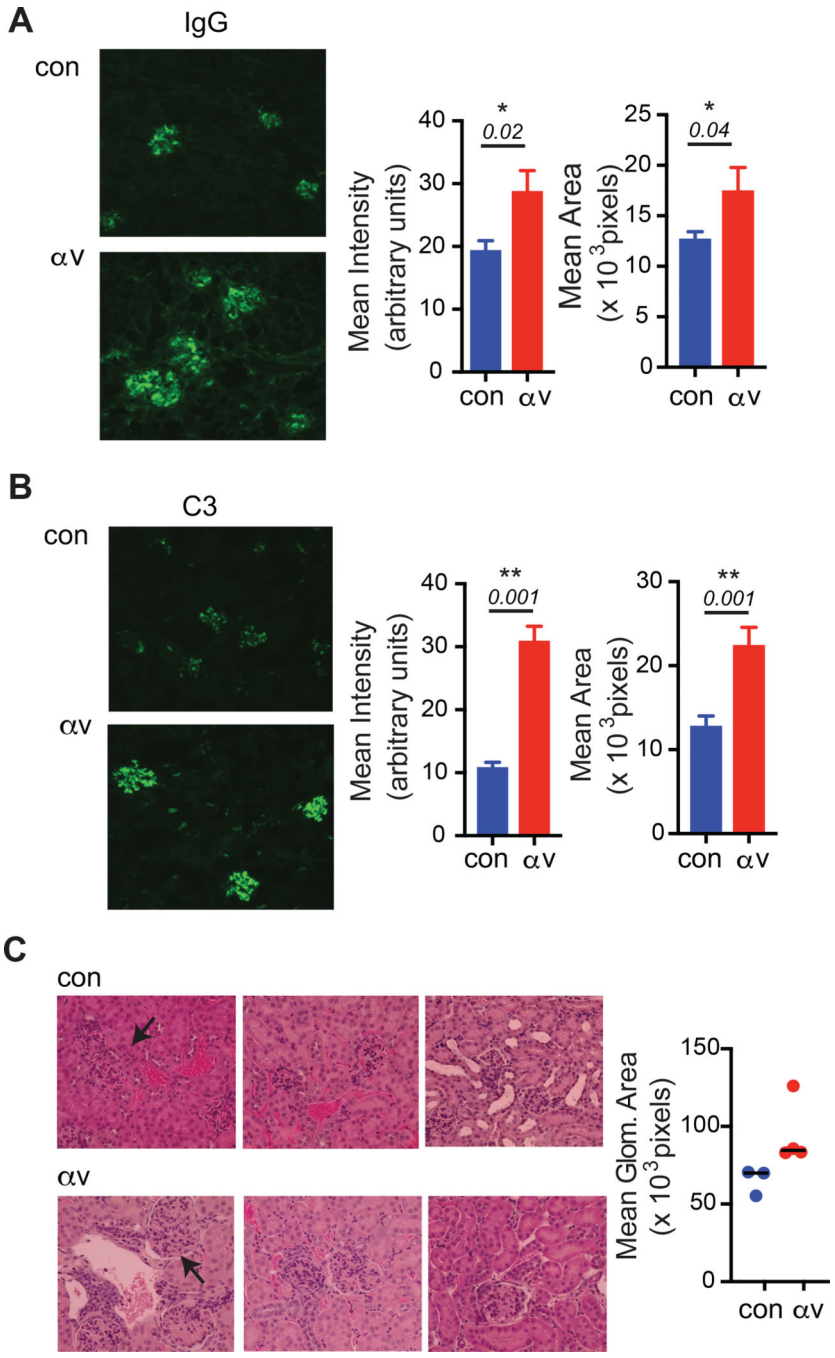

Figure 5: αv-deletion increases kidney antibody deposition and glomerulonephritis:

(A-B) Glomerular immune complex deposits determined by immunofluorescence staining for IgG **(A)** and complement C3 **(B)**. Left panels show representative images from 7–8 week old control Tlr7.1 tg and αv-CD19.Tlr7.1 tg mice. Right panels show intensity and area of staining. **(C)** Histological analysis of hematoxylin and Eosin stained representative sections. Graph represents quantification of glomerular size. Data are mean±SD for at least *n*=3 mice/

group. *p*-values <0.05 (Mann-Whitney test) for comparisons between control Tlr7.1 tg and αv-CD19.Tlr7.1 tg mice are shown, and indicated as by * ($p<0.05$) or ** ($p<0.01$).