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New insights into immune mechanisms of antiperlecan/LG3 antibody production: Importance of T cells and innate B1 cells

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Autoantibodies against perlecan/LG3 (anti-LG3) have been associated with increased risks of delayed graft function, acute rejection, and reduced long-term survival. High titers of anti-LG3 antibodies have been found in de novo renal transplants recipients in the absence of allosensitizing or autoimmune conditions. Here, we seek to understand the pathways controlling anti-LG3 production prior to transplantation. Mice immunized with recombinant LG3 produce concomitantly IgM and IgG anti-LG3 antibodies suggesting a memory response. ELISpot confirmed the presence of LG3-specific memory B cells in nonimmunized mice. Purification of B1 and B2 subtypes identified peritoneal B1 cells as the major source of memory B cells reactive to LG3. Although nonimmunized CD4-deficient mice were found to express LG3-specific memory B cells, depletion of $CD4^+$ T cells in wild type mice during immunization significantly decreased anti-LG3 production. These results demonstrate that B cell memory to LG3 is T cell independent but that production of anti-LG3 antibodies requires T cell help. Further supporting an important role for T cells in controlling anti-LG3 levels, we found that human renal transplant recipients show a significant decrease in anti-LG3 titers upon the initiation of CNI-based immunosuppression. Collectively, these results identify T cell targeting interventions as a means of reducing anti-LG3 levels in renal transplant patients.

KEYWORDS
animal models, autoantibody, B cell biology, basic (laboratory) research/science, cell death: apoptosis, cellular biology, clinical research/practice, immunobiology, kidney transplantation/nephrology

Abbreviations: ANA, antinuclear antibodies; ASC, antibody-secreting cells; AT1R, angiotensin II type 1 receptors; CD4dep, $CD4^+$ T cell-depleted; CHUM, Centre hospitalier de l'Université de Montréal; CIPA, Comité Institutionnel de Protection des Animaux; CNI, calcineurin inhibitor; DAMPs, danger-associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; ESRD, end-stage-renal disease; FBS, fetal bovine serum; GC, germinal center; HLA, human leucocyte antigen; HRP, horseradish peroxidase; IFA, incomplete Freund's adjuvant; IL-2, interleukin-2; MSA, mouse serum albumin; PBS, phosphate-buffered saline; PC, peritoneal cavity; RFP1, red fluorescent protein; SEM, standard error of the mean.

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1 | INTRODUCTION

Allogeneic antibodies targeting human leucocyte antigens (HLA) play a central role in the development of antibody-mediated allograft rejection.[1-6] Mounting evidence suggests that autoreactive antibodies also contribute to rejection and can have an adverse impact on graft outcome in kidney, heart, and lung transplant patients.[7-18] Autoantibodies against angiotensin II type 1 receptors (AT1R), vimentin, collagen V, tubulin, and perlecan/LG3 (anti-LG3) have been associated with accelerated allograft rejection in animal models of kidney, heart, and lung transplantation and in transplant patients.[8,9,15,19,20] In addition, anti-LG3 antibodies also aggravate ischemia-reperfusion injury (IRI) in renal transplant patients and in murine models through complement activation leading to microvascular rarefaction, fibrosis, and long-term renal allograft dysfunction.[21-23]

Classically, the appearance of autoantibodies was thought to follow episodes of acute rejection leading to the release of danger-associated molecular patterns (DAMPs) that in turn favor autoantibody production. However, in previous studies, anti-AT1R and anti-LG3 autoantibodies have been detected prior to transplantation in patients awaiting a first transplant.[8,24,25] These patients had neither allosensitizing conditions nor classic autoimmune diseases.

In the present study, we sought to understand the mechanisms responsible for anti-LG3 production prior to transplantation. We investigated the crosstalk between T cell and B cell responses in controlling anti-LG3 formation.

2 | MATERIAL AND METHODS

2.1 | Reagents

The mouse perlecan fragment LG3 (aa 3514-3707 with N-terminal His_8G tag) or a secreted form of red fluorescent protein (RFP1) (with C-terminal His_8G tag) were cloned into the pTT5™ plasmid.[26,27] For protein production, 293-6E cells were transfected at 1.8×10^6cells/ml with pTT5/cDNA constructs. Cultures were harvested at 5 days posttransfection and purified by IMAC on Fractogel®-Cobalt.[28] For some experiments, N-terminal His8G tag was removed from the LG3 construct.

Purified mouse serum albumin (MSA) protein was obtained from Alpha Diagnostic International (San Antonio, TX). Endotoxin levels were measured by Limulus Amebocyte Lysate test using Endosafe-PTS spectrophotometer (Charles River Laboratories, Wilmington, MA) for LG3 (with or without His8G tag) and RFP1 and by clot method for MSA. The levels of endotoxin were respectively equal to 0.016 EU/mg, 0.021 EU/mg, and 1.2 EU/mg.

2.2 | Mice

Wild type (WT) female C57BL/6 mice between 5-7 weeks of age were obtained from Charles River (St-Constant, QC, Canada). CD4-deficient mice (B6.129S2-$Cd4^{tm1Mak}$) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments mice were approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the CRCHUM. Mice were anesthetized using isoflurane (2%) by inhalation and sacrificed by cardiac puncture. For the recovery of peritoneal cavity cells, mice were sacrificed by the dislocation of the cervical vertebrae to prevent blood contamination of peritoneal cavity.

2.3 | Immunization and depletion of $CD4^+$ T cells

WT C57BL/6 mice were injected subcutaneously with recombinant LG3, MSA, RFP1 protein (50 µg), or phosphate-buffered saline (PBS), either alone or in association with incomplete Freund's adjuvant (IFA), every 2 weeks for a total of 4 immunizations. Mice were bled every 2 weeks until sacrifice, which occurred either 2 or 13 weeks after the last immunization. Depletion of $CD4^+$ T cells was achieved by injecting InvivoPlus antimouse CD4 (GK1.5 clone) (BioXcell, West Lebanon, NH) intraperitoneally (100 µg/mouse) on 2 consecutive days, starting either 2 days before the first immunization or 2 weeks after the last injection. CD4 depletion was maintained by weekly injections until sacrifice. PBS was used as vehicle control. The efficiency of CD4 depletion was confirmed by cytometry following staining of splenocytes with antimouse CD3e-FITC and antimouse CD4-PerCP-Cy5.5 (BD Biosciences, San Jose, CA).

2.4 | ELISA

Anti-LG3 titers were measured by ELISA. Recombinant LG3 without His8G-tag (1 µg/well) was first coated on 96-well Immulon II HB plates (Thermo Electron, Waltham, MA). Sera were diluted (1/100) and 100 µl/well were added. After washing, the LG3-bound mouse Igs were detected using sheep antimouse IgG (Amersham, Baie d'Urfé, QC, Canada), goat antimouse IgG1, IgG2a, IgG2b, IgG3, or goat antimouse IgM (all from Santa Cruz Biotechnology, Santa Cruz, CA) horseradish peroxidase (HRP) conjugate. Reactions were revealed with tetramethylbenzidine substrate (BD Biosciences) and stopped with H_2SO_4 (1 mol/L). Optical densities (OD) were read using a microplate photometer at 450 nm (Multiskan FC; Thermo Fisher Scientific, Nepean, ON, Canada).

Total IgG and antinuclear antibodies (ANA) levels were assessed using Mouse IgG total Ready-SET-Go Kits (Affymetrix eBioscience, Santa Clara, CA) and ANA Mouse Bioassay Kits (US Biological Life Sciences, Marblehead, MA), respectively, according to the manufacturer's instructions.

2.5 | Cell isolation

Cells were isolated from the spleen, bone marrow, or peritoneal cavity as described.[29,30] B1 cells and B2 cells were purified from the peritoneal cavity cells. In brief, total B cells were first purified using Easysep mouse pan-B cell isolation kit (STEMCELL Technologies, Vancouver, BC, Canada). Next, B1 cells ($CD23^-$) and B2 cells ($CD23^+$) were enriched from purified B cells using Easysep release mouse PE positive selection kit (STEMCELL) in combination with antimouse CD23-PE (BD Biosciences). The efficiency of cell enrichment was evaluated by

cytometry using antimouse CD19-PE-Cy7 (BD Biosciences) and antimouse CD23-PE. Purity of B1 cell and B2 cell suspension was respectively about 85%-91% and >98%.

2.6 | Stimulation of memory B cells

Splenocytes, bone marrow cells, and peritoneal cells were cultured in RPMI-1640 medium supplemented with L-glutamine, 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (all from Invitrogen Canada Inc, Burlington, ON, Canada), at 37°C in a humidified atmosphere containing 5% CO_2. Murine cells were seeded at 4×10^6 cells/ml (splenocytes and bone marrow cells) or 2×10^6 cells/ml (peritoneal cavity cells) and memory B cells were stimulated with R-848 (1 μg/ml) (Enzo Life Sciences, Farmingdale, NY) in combination with recombinant murine IL-2 (10 ng/ml) (PeproTech, Dollar-des-Ormeaux, QC, Canada) for 3 days.

2.7 | ELISpot assays

To detect antigen-specific responses, 96-well ELISpot IP filter plates, 0.45 μm, clear (Millipore Canada Ltd., Etobicoke, ON, Canada) were coated with 5 μg of recombinant LG3 without the His8G-tag or MSA diluted in Dulbecco's phosphate buffered saline (D-PBS; Wisent Bioproducts, St-Bruno, QC, Canada). To measure the number of total IgM and IgG-antibody-secreting cells, plates were coated with 1.5 μg of goat antimouse IgG (H + L) F(ab'2) (Sigma-Aldrich, Canada Ltd., Toronto, ON, Canada). Activated cells were added for 18-24 hours at 37°C in a 5% CO_2 humidified incubator. Following washing, bound Ig were detected using goat antimouse IgG (Fc specific)-alkaline phosphatase or goat antimouse IgM (μ specific)-alkaline phosphatase antibodies (both from Sigma-Aldrich). ELISpots were developed using 100 μl/well of 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (MabTech, Inc, Cincinnati, OH) to visualize the spots. The reaction was stopped by tap water. The plates were read by an automated ELISpot reader and data were analyzed by ImmunoSpot Analyzer 5.1 software (Cellular Technology Limited, Cleveland, OH).

2.8 | Flow cytometry analysis

The percentage of viable naïve ($CD62L^{high}$, $CD44^{low}$), effector ($CD62L^{low}$, $CD44^{high}$), and memory ($CD62L^{high}$, $CD44^{high}$) $CD4^+$ T cells was determined by cytometry using antimouse CD3e-APC (Biolegend, San Diego, CA), antimouse CD4-PE-Cy, antimouse CD62L-PE (both from Affymetrix eBioscience), antimouse CD44-BB515 mAbs (BD Biosciences), and Live/dead Fixable aqua dead cell stain kit (Molecular Probes, Eugene, OR) to gate on viable cells. The cells were acquired by cytometry using BD LSRII flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed with FlowJo software 10.0 (Ashland, OR).

2.9 | Human study

We performed a retrospective cohort study among the first 31 participants of the Montreal Renal Transplant Biobank. In brief, starting from July 1, 2008 at the Centre Hospitalier de l'Université de Montréal (CHUM), subjects who received a kidney allograft were approached for participation in the biobank, with a 95% enrollment rate. All patients received calcineurin inhibitor (CNI)-based immunosuppression after transplantation with 2 different types of induction regimens. Recipient sera were sampled immediately before and 1-month posttransplantation and banked at −80°C. Anti-LG3 antibodies were measured with a locally developed ELISA on sera collected at both time points, as previously described.[8]

2.10 | Statistical analysis

The results are expressed as mean ± SEM. All statistical analysis were performed with GraphPad Prism 3.0 software (La Jolla, CA) using the appropriate parametric (paired *t* test, unpaired *t* test, and repeated measures one-way analysis of variance [ANOVA]) and nonparametric tests (Wilcoxon signed-rank test and Mann-Whitney test). P values < .05 were considered statistically significant. Simple linear regression was performed to determine the factors associated with changes in anti-LG3 levels pre- and posttransplantation.

3 | RESULTS

3.1 | Antibody reactivity to LG3 can occur in the absence of inflammation

Inflammatory conditions associated with the production of DAMPs are known to favor the formation of autoantibodies. To assess the importance of inflammation for the production of anti-LG3 autoantibodies, WT mice were immunized with recombinant LG3 or PBS as vehicle control in the presence or absence of IFA. As expected, LG3-immunization in the presence of adjuvant triggered strong antibody reactivity to LG3 as measured by the production of high titers of anti-LG3 IgG and IgM antibodies (Figure 1A,B) in all mice. Immunization with IFA alone did not induce the production of anti-LG3 antibodies, demonstrating that inflammation favors but is not sufficient for triggering the production of anti-LG3 antibodies (Figure 1A,B). Immunization with LG3 in the absence of IFA also favored the production of anti-LG3 antibodies, albeit at lower titers and not in all mice. About 41% of mice immunized with LG3 in the absence of IFA showed significantly increased titers of anti-LG3 IgG and IgM antibodies (Figure 1C,D). Anti-LG3 titers remained significantly increased in immunized mice until 13 weeks after the last injection (Figure 1E).

Mice express 4 IgG subclasses: IgG1, IgG2a, IgG2b, and IgG3. IgG2a, IgG2b, and IgG3 subclasses activate complement whereas IgG1 is not complement fixing.[31] Knowing that rejection-accelerating anti-LG3 antibodies are of complement fixing isotypes both in humans and in mice,[8] we evaluated which subclasses of anti-LG3 IgG are produced in the presence or absence of IFA (Figure 1F). Our results showed that anti-LG3 IgG1, IgG2a, IgG2b, and IgG3 are strongly produced after LG3-immunization with IFA. The 4 IgG subclasses were

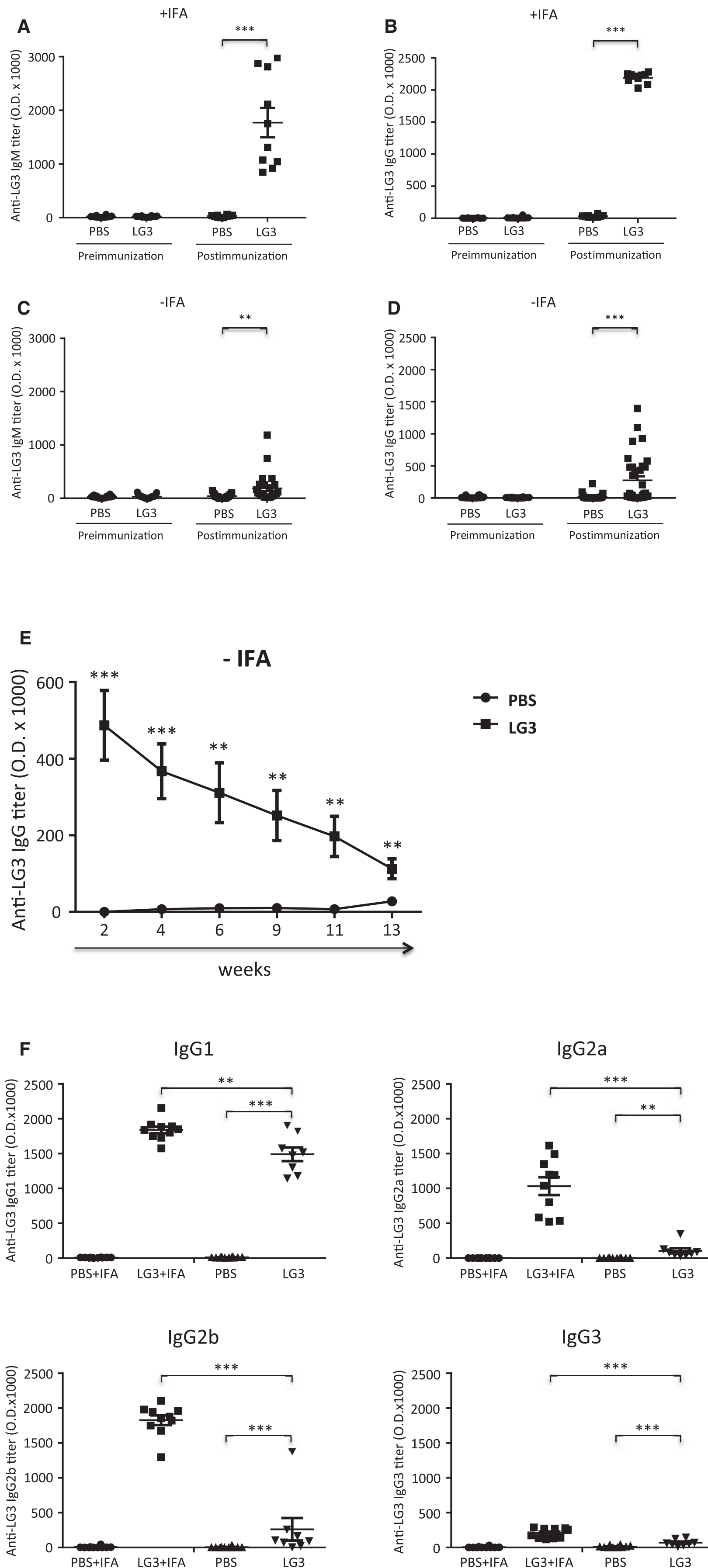


FIGURE 1 Effect of LG3 immunization ±IFA on antibody reactivity to LG3. WT C57BL/6 mice were immunized with LG3 (50 μg/sc every 2 weeks for a total of 4 injections) or control PBS in the presence (A,B,F) or absence of IFA (C-F). Anti-LG3 IgM (A,C) and IgG (B,D) titers were evaluated in the serum of mice preimmunization and postsacrifice by ELISA. After the last injection, the levels of anti-LG3 IgG were evaluated in the serum of mice every 2-3 weeks by ELISA (E). Anti-LG3 IgG1, IgG2a, IgG2b, and IgG3 titers were evaluated in the serum of mice postsacrifice by ELISA (F). Results shown are the mean ± SEM of at least N = 10 (A-D,F) or N = 6 (E). ****P* < .001; ***P* < .01 (unpaired *t* test [A,E,F]; Mann-Whitney test [B-D])

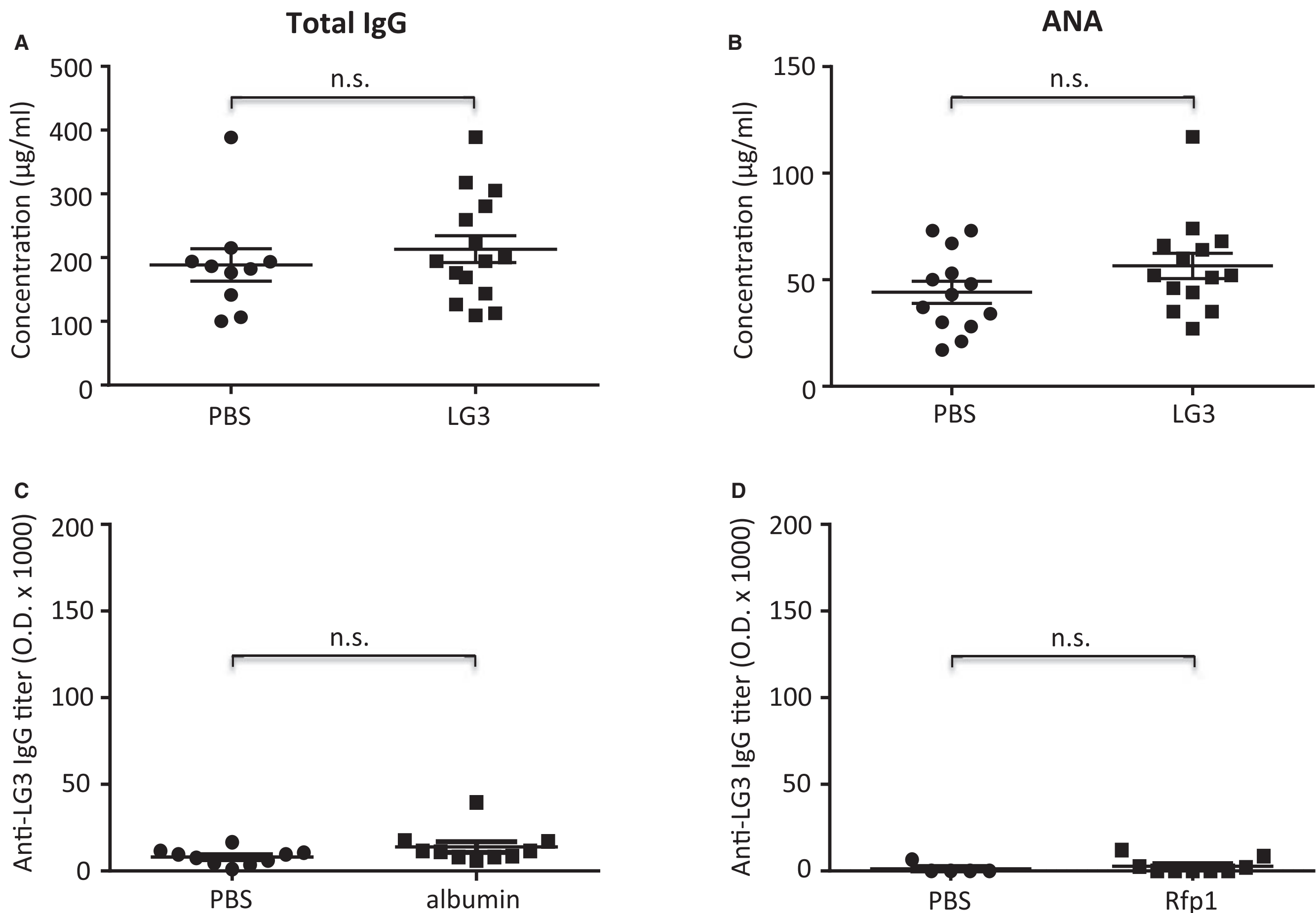


FIGURE 2 Evaluation of the specificity of LG3-immunization on antibody reactivity to LG3. WT C57BL/6 mice were immunized with LG3 (A,B), mouse serum albumin (C), RFP1 (D) (50 μg/sc every 2 weeks for a total of 4 injections), or control PBS (A-D). The levels of total IgG (A), antinuclear antibodies (ANA) (B), and anti-LG3 IgG (C,D) were evaluated in the serum of mice postsacrifice by ELISA. Results shown are the mean ± SEM of at least N = 10 (A-C) or N = 5 (D). ns, not significant (unpaired *t* test)

also significantly increased in mice immunized with LG3 alone but with dramatically lower levels for IgG2a, IgG2b, and IgG3 subclasses (IgG1: 1.22-fold lower; IgG2a: 10-fold lower; IgG2b: 7-fold lower; IgG3: 4.2-fold lower). These results suggest that inflammation is not a prerequisite for anti-LG3 production. However, when inflammation is present, it favors the production of complement-fixing anti-LG3 isotypes.

Keeping in mind that various autoantibodies have been described prior to transplantation, we tested whether immunization with LG3 fosters a broad autoimmune response. Immunization with LG3 did not modulate total IgG levels (213 ± 20 μg/ml [LG3] vs 189 ± 28 μg/ml [PBS]) (Figure 2A) nor ANA concentration (57 ± 15 μg/ml [LG3] vs 44 ± 12 μg/ml [PBS]) (Figure 2B). This indicates that anti-LG3 production is not the consequence of a generalized B cell hyperactivity. To assess the specificity of the anti-LG3 response, we evaluated whether immunization with proteins other than LG3 can lead to anti-LG3 production. WT mice were immunized with mouse serum albumin (MSA), an endogenous protein (Figure 2C), or red fluorescent protein (RFP1) (Figure 2D), the latter being produced through similar cloning and purification methods as LG3. Our results showed that neither MSA nor RFP1-immunization induced anti-LG3 production demonstrating the specificity of the anti-LG3 response.

3.2 | Memory B cells specific for LG3 are present in nonimmunized WT mice

We observed that most mice immunized with LG3 showed concomitant (10/17) increases in anti-LG3 IgM and IgG antibodies, suggesting a memory response (Figure 3A). To assess the memory response to LG3, we used ELISpot assays to evaluate the presence of memory B cells specific to LG3 in the spleen, bone marrow, and peritoneal cavity (PC) of nonimmunized WT mice (Figure 3B). MSA was used as negative control. We did not observe significant MSA-specific antibody-secreting cells (ASC) in the spleen, bone marrow, and PC of WT mice. In contrast, LG3-specific IgM-secreting ASC (IgM-ASC) were detected in all compartments (spleen: 120 ± 18; bone marrow: 42 ± 2; PC: 108 ± 24 per 2×10^4 cells) (Figure 3C). LG3-specific IgG-ASC were also identified in all three compartments (spleen: 54 ± 9; bone marrow: 38 ± 6; PC: 313 ± 85 per 5×10^5 cells), but with a six- to eight-fold higher magnitude in the PC (Figure 3D). Collectively, these data demonstrate the presence of memory B cells specific to LG3 in nonimmunized WT mice and identify peritoneal cavity B cells as a major source of memory B cells reactive to LG3.

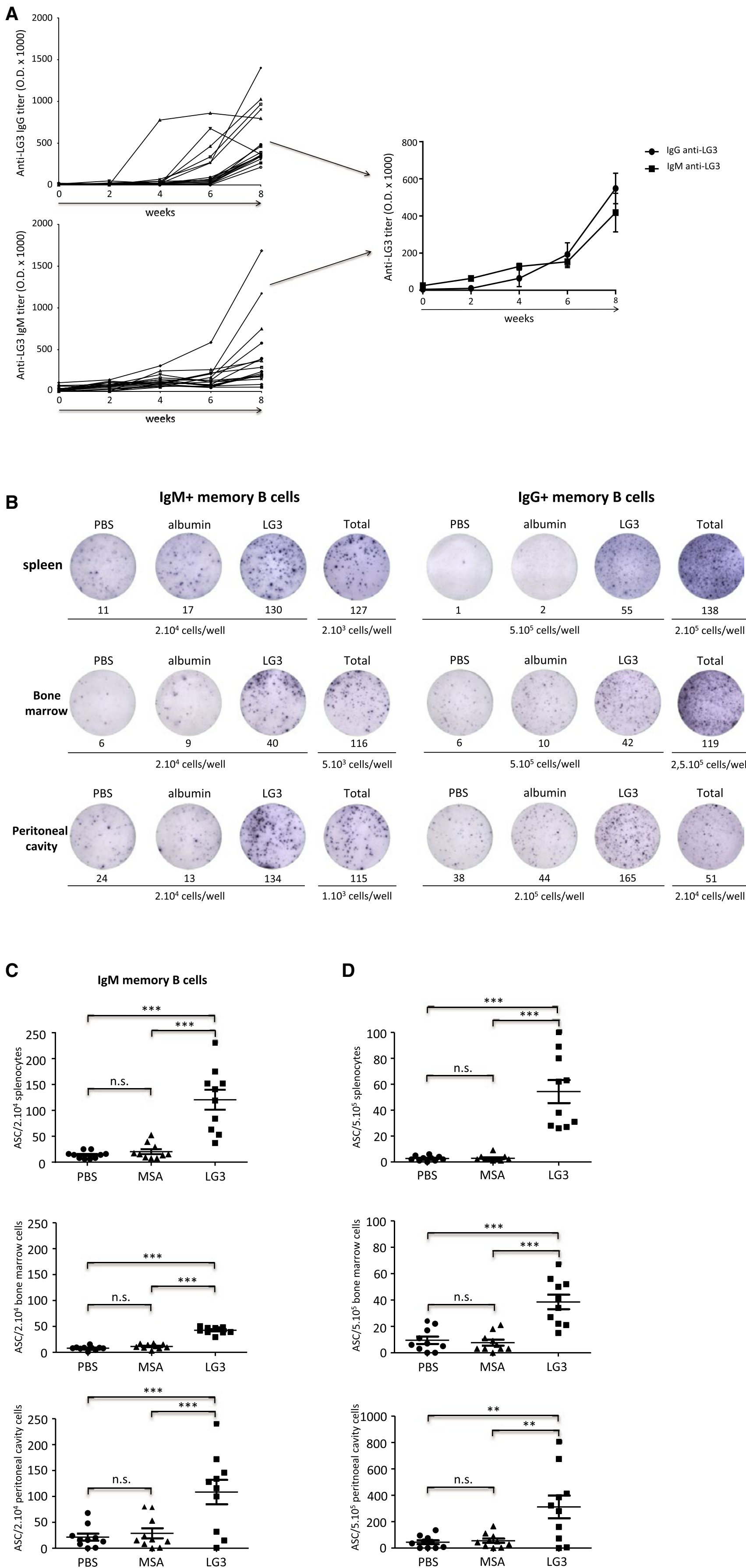


FIGURE 3 Evaluation of memory B cells specific to LG3. (A) WT C57BL/6 mice were immunized with LG3 (50 μg/sc every 2 weeks for a total of 4 injections). Circulating levels of anti-LG3 IgG and IgM were evaluated in the serum of mice every 2 weeks by ELISA. Results shown are individual data for each mouse (left panel; each mouse is represented by the same symbol in the 2 graphs) or the mean ± SEM of N = 17 (right panel). (B-D) C57BL/6 WT mice, 5-7 weeks, unimmunized with LG3 were sacrificed. Splenocytes, bone marrow cells, and peritoneal cavity cells were isolated and incubated with R848+IL-2 to stimulate memory B cells. After 72 hours, cells were subsequently cultured for another 24 hours in ELISpot 96-well to detect IgG or IgM antibody-secreting cells (ASC) specific to LG3, mouse serum albumin (MSA), or control PBS. (B) Image shows results of representative ELISpot assay. (C,D) The graphs depict the frequency of LG3-, MSA-, and PBS-specific IgM- or IgG-ASC per 2×10^4 or 5×10^5 cells, in the spleen, the bone marrow, and the peritoneal cavity. Results shown are the mean ± SEM of 10 independent experiences. $^{**}P < .01$; $^{***}P < .001$; ns, not significant (1-way ANOVA with Holm-Sidak's multiple comparison test) [Color figure can be viewed at wileyonlinelibrary.com]

The peritoneal cavity contains 2 major B cell subclasses: conventional B2 cells and B1 cells. To determine which subclass of memory B cells is reacting with LG3 in the PC of WT nonimmunized mice, we performed an ELISpot assay using B1 or B2 cells isolated from the PC (Figure 4). In these mice, B cells accounted for about 14%-20% of total cells in the PC, of which 58%-67% are B1 cells and 33%-42% are B2 cells (Figure 4A). We detected the presence of ASC in B1 and B2 cell populations. However, the quantity of total IgM-ASC was about seven-fold lower in B2 cells in comparison with B1 cells and total IgG-ASC were not detected in the B2 cell population (Figure 4B) indicating that B1 cells are the main B cell subclass with IgG memory in the PC. We identified the presence of LG3-specific memory B cells in the B1 cell population (LG3-specific IgM-ASC: 2.7% ± 0.2% of total IgM-ASC; LG3-specific IgG-ASC: 17.3% ± 1.2% of total IgG-ASC [Figure 4C]). In contrast, we did not observe significant LG3-specific ASC in B2 cell population (Figure 4D). The quantity of IgM and IgG-ASC reactive to LG3 was respectively about 10- and 22-fold lower in B2 cell populations compared to B1 cells (Figure 4E,F). These data demonstrate that B1 cells are the main subset of B cells in the PC with memory specific to LG3.

3.3 | $CD4^+$ T cells are central to the production of anti-LG3 antibodies

To evaluate the impact of LG3 on $CD4^+$ T cell function, we studied the effect of LG3-immunization on the naïve, effector, and memory $CD4^+$ T cell populations. WT mice were immunized with LG3 or PBS, as previously described, and the expression of CD44 and CD62L on $CD4^+$ T cells from splenocytes was analyzed by cytometry (Figure 5A). Our results showed that LG3-immunization decreased the percentage of naïve $CD4^+$ T cells (73.2% ± 0.8% [PBS] vs 66.3% ± 1.2% [LG3]) and increased the percentage of effector $CD4^+$ T cells (16.7% ± 0.6% [PBS] vs 20.2% ± 0.9% [LG3]) in the spleen. No significant difference was observed between the 2 groups for the percentage of memory T cells (4.2% ± 0.1% [PBS] vs 4.4% ± 0.2% [LG3]) (Figure 5B). We then evaluated the importance of $CD4^+$ T cells in the production of anti-LG3 antibodies by immunizing WT and $CD4^+$ T cell-depleted mice with LG3. Our results showed that $CD4^+$ T cell-depleted mice failed to develop anti-LG3 IgM (Figure 5C) and IgG (Figure 5D). Taken together, these results show that LG3 modulates $CD4^+$ T cell phenotype and that $CD4^+$ T cells are necessary for anti-LG3 production.

3.4 | T cell help is not required for B cell memory to LG3

To evaluate the importance of CD4+ T cells in B cell memory to LG3, we assessed the presence of memory B cells in nonimmunized $CD4^{-/-}$ mice by ELISpot (Figure 6A). IgM-ASC specific to LG3 were present in the spleen (100 ± 14 per 2×10^4 cells), bone marrow (34 ± 11 per 2×10^4 cells), and PC (99 ± 26 per 2×10^4 cells) of $CD4^{-/-}$ mice (Figure 6B). We also detected the presence of LG3-specific IgG-ASC in all compartments (spleen: 33 ± 4; bone marrow: 11 ± 3; PC: 151 ± 69 per 5×10^5 cells) (Figure 6C). These results indicate that $CD4^+$ T cells play a central role for anti-LG3 production but are not essential for the generation of memory B cells specific to LG3. We also evaluated the percentage of LG3-specific memory B cells over total memory B cells in the spleen, bone marrow, and peritoneal cavity. Our results showed reduced numbers of memory B cells specific to LG3 in the spleen (LG3-specific IgM-ASC: 1.9-fold lower; LG3-specific IgG-ASC: 1.6-fold lower) and bone marrow (LG3-specific IgM-ASC: 1.6-fold lower; LG3-specific IgG-ASC: 6.7-fold lower) of $CD4^{-/-}$ mice in comparison with WT mice (Figure 6D,E). In contrast, there was no significant difference between WT and $CD4^{-/-}$ mice for the number of LG3-specific memory B cells in the PC. These results suggest that $CD4^+$ T cells contribute to B cell memory to LG3 in the spleen and bone marrow but memory B cells specific to LG3 are present in the PC independently of T cell help.

3.5 | Immunosuppressive regimens targeting T cell function in renal transplant patients reduce anti-LG3 levels

Having shown that, although B cell memory to LG3 is present in nonimmunized mice, the production of anti-LG3 antibodies is T cell dependent, we sought to evaluate the importance of $CD4^+$ T cells for maintaining anti-LG3 production once it is established. WT mice were immunized with LG3 as previously described. Two weeks after the last injection, mice were depleted of $CD4^+$ T cells or not (Figure 7A) and anti-LG3 IgG titers were measured every week in both groups until sacrifice (Figure 7B,C). The results obtained showed that during CD4 depletion anti-LG3 titers decreased faster in CD4dep mice compared to WT mice. Indeed, anti-LG3 IgG titers significantly decreased by 35% between weeks 8 and 13 of CD4 depletion whereas anti-LG3 IgG titers remained elevated and stable in WT mice. Collectively, these results show that both initiation and maintenance of anti-LG3 production are $CD4^+$ T cell dependent.

To study the impact of immunosuppression on anti-LG3 production in humans, we evaluated whether levels of anti-LG3 antibodies decrease after transplantation in association with the initiation of immunosuppression. We tested circulating anti-LG3 levels immediately prior to or 1 month posttransplantation, in 31 de novo renal transplant recipients receiving CNI-based immunosuppression in combination with mycophenolate mofetil and corticosteroids. Patient characteristics are found in Table 1. Anti-LG3 levels were significantly reduced at 1 month posttransplantation compared to pretransplant levels (Figure 7D). No variable included in Table 1 was associated with the change in anti-LG3 values posttransplant. Collectively, these results identify T cell help as a target of intervention for dampening humoral immunity against LG3.

4 | DISCUSSION

In the present work, we show that exposure to the perlecan fragment LG3, even in the absence of adjuvant, leads to the production

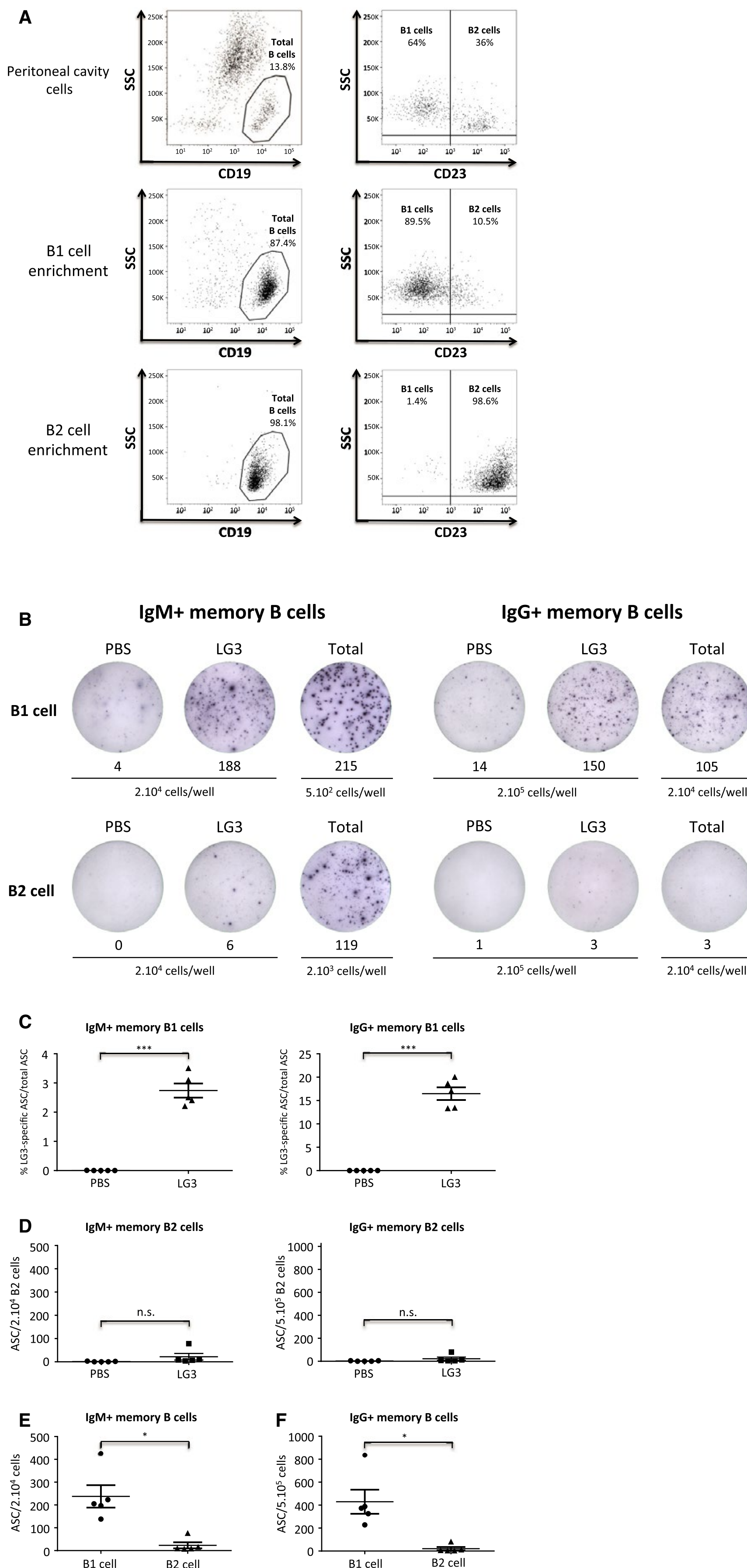


FIGURE 4 Determination of the role of B1 and B2 cells in the LG3-specific memory. C57BL/6 WT mice, 5-7 weeks, unimmunized with LG3 were sacrificed. Peritoneal cavity cells from 12 mice were pooled together. B cells have been purified using Pan-B cell enrichment kit. Enrichment in B1 cells and B2 cells have been processed using anti-CD23 PE in combination with PE-positive selection kit. B1 cells and B2 cells were cultured at 37°C, in the presence of R848+IL-2 to stimulate memory B cells. After 72 hours, cells were cultured for another 24 hours in ELISpot 96-well to detect total ASC and IgG or IgM-ASC specific to LG3 or control PBS. (A) The expression of CD23 receptor was measured by cytometry on the surface of CD19+ B cells to evaluate efficiency of B1 ($CD23^{-}$) and B2 cell ($CD23^{+}$) enrichment. (B) Results of representative ELISpot assay. (C,D) The graphs depict the percentage of LG3-specific IgM-ASC per total IgM ASC or the percentage LG3-specific IgG-ASC per total IgG ASC. (E,F) The graphs compare the frequency of LG3-specific IgM-ASC (E) and the frequency of LG3-specific IgG-ASC (F) between B1 and B2 cell populations. The number of spots in PBS condition has been subtracted from the total number of LG3-specific spots. Results shown are the mean ± SEM of 5 independent experiences. *P < .05; ***P < .001 ns, not significant (Wilcoxon signed-rank test) [Color figure can be viewed at wileyonlinelibrary.com]

of anti-LG3 antibodies. This relative ease in inducing immune response to LG3 is likely because of the presence of memory B cells, even prior to immunization, in the spleen, bone marrow but most strikingly in the peritoneal cavity of normal mice. We also found that B1 subsets within the peritoneal cavity are the main source of LG3-specific memory B cells. Mouse B1 cells are the predominant constituents of peritoneal B cells and are also found in the pleural cavity, the spleen, and the bone marrow.[32-35] In contrast to B2 cells, B1 cell development occurs primarily during fetal and perinatal life. B1 cells are effectors of the innate immune system and the main producers of polyreactive antibodies that bind to both microbial antigens and self-antigens, including neo-self epitopes expressed by apoptotic cells such as annexin IV and phosphorylcholine.[36-38] It has been suggested that natural autoantibodies produced by B1 cells favor the clearance of senescent and apoptotic cells and therefore have protective effector functions.[34,39] We previously showed that LG3 is produced by apoptotic endothelial cells downstream of caspase-3 activation and released through the production of exosome-like apoptotic membrane vesicles.[40-42] The presence of a predominant B1 cell memory-response to LG3 in naïve mice suggests that memory to LG3 is likely a normal and innate response to components of membrane vesicles released by apoptotic cells. Interactions between

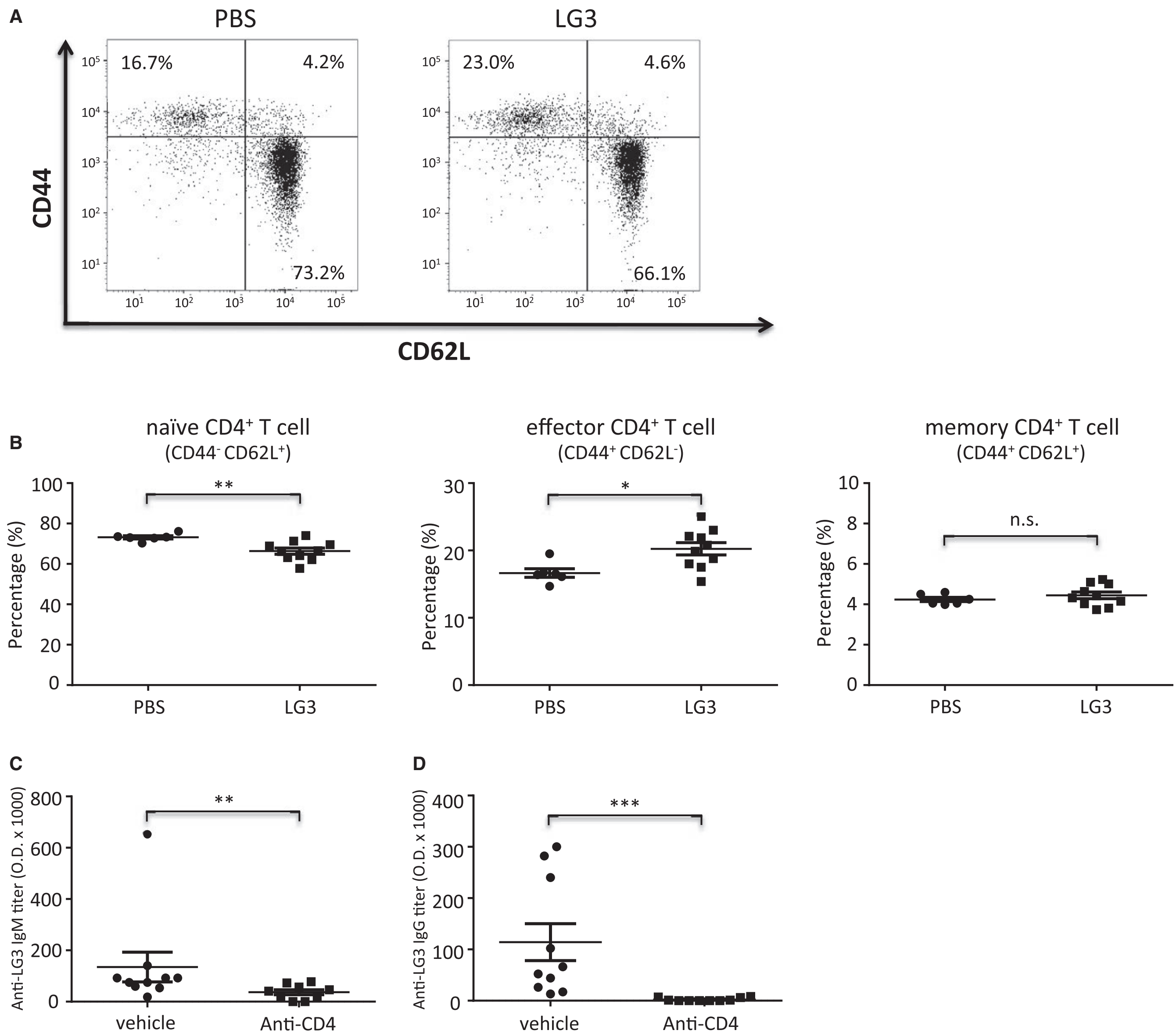


FIGURE 5 Evaluation of the role of CD4+ T cells for the induction of antibody reactivity to LG3. (A,B) C57BL/6 mice were immunized with LG3 (50 μg/sc every 2 weeks for a total of 4 injections) or control PBS. The expression of CD44 and CD62-L receptors were measured by cytometry on the surface of CD4+ T cells from splenocytes of mice immunized with LG3 or control PBS (A) to evaluate the proportion of naïve (CD44− CD62L+), effectors (CD44+ CD62L−), and memory (CD44+ CD62L+) CD4+ T cells (B). (C,D) C57BL/6 mice depleted from CD4+ T cells (anti-CD4) or not (vehicle) were immunized with LG3 (50 μg/sc every 2 weeks for a total of 4 injections). Anti-LG3 IgM (C) and IgG (D) titers were assessed in the serum of mice postsacrifice by ELISA. Results shown are the mean ± SEM of at least N = 10. *$P < .05$; **$P < .01$; ***$P < .001$; ns, not significant (unpaired *t* test [B]; Mann-Whitney test [C,D])

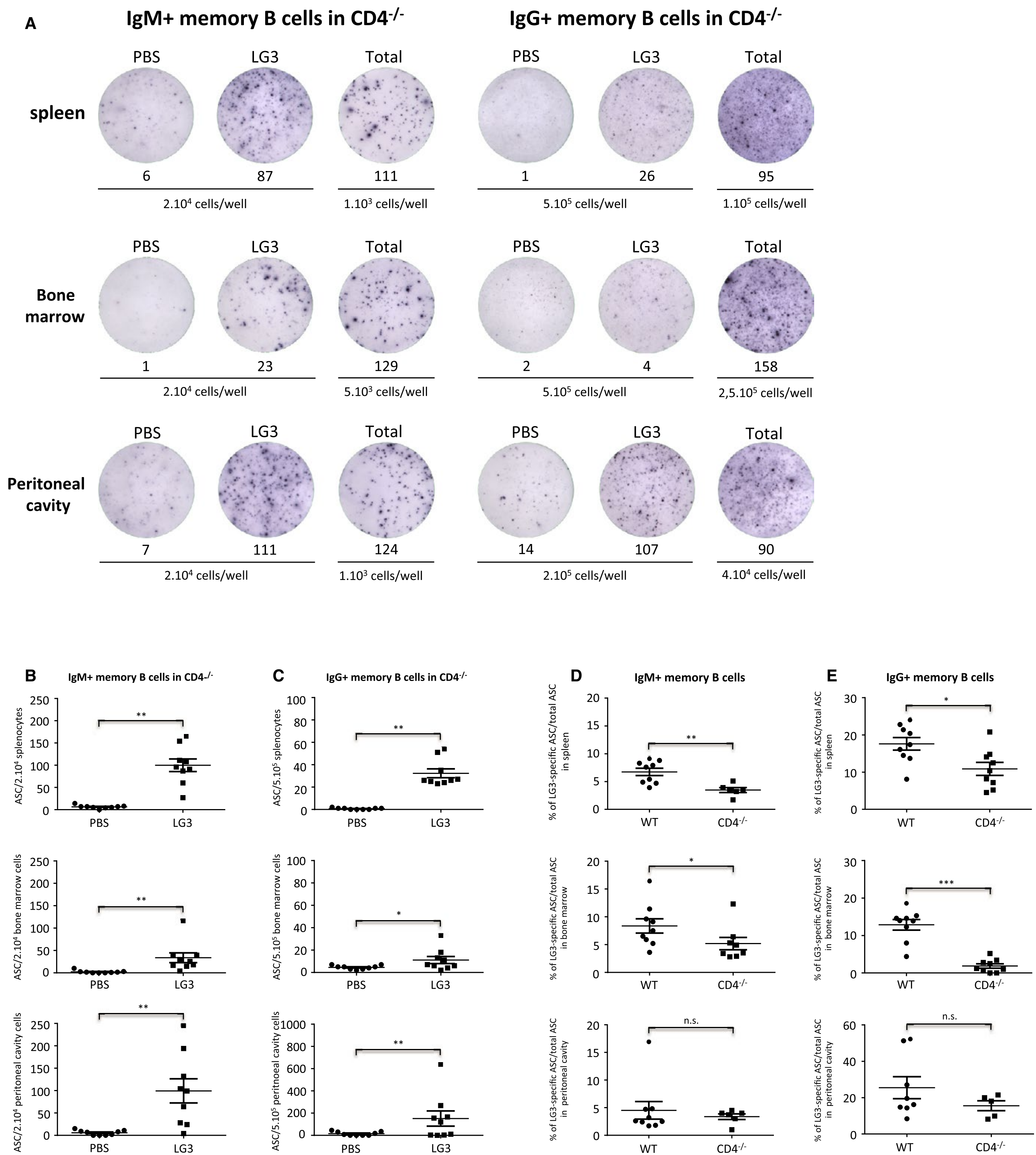
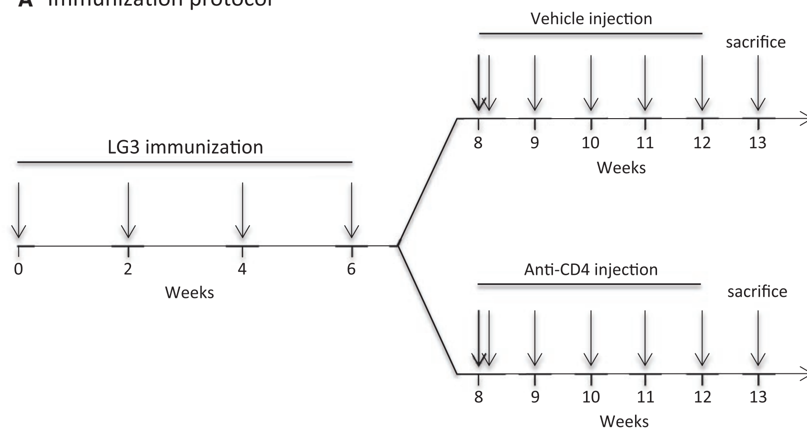


FIGURE 6 Evaluation of the role of $CD4^+$ T cells in the generation of humoral memory specific to LG3. C57BL/6 $CD4^{-/-}$ (A-E) or WT (D,E) mice, 5-7 weeks, unimmunized with LG3 were sacrificed. Splenocytes, bone marrow cells, and peritoneal cavity cells were isolated and cultured with R848+IL-2 to stimulate memory B cells. After 72 hours, cells were cultured for another 24 hours in ELISpot 96-well to detect IgM- or IgG-ASC specific to LG3 or control PBS. (A) Image shows results of representative ELISpot assay. (B,C) The graphs depict the frequency of LG3- or PBS-specific IgM- and IgG-ASC per 2×10^4 or 5×10^5 cells, in the spleen, the bone marrow, and the peritoneal cavity of $CD4^{-/-}$ mice. (D,E) The graphs depict the percentage of LG3-specific IgM-ASC per total IgM-ASC (D) or the percentage of LG3-specfic IgG-ASC per total-IgG ASC (E), in the spleen, the bone marrow, and the peritoneal cavity of $CD4^{-/-}$ or WT mice. Results shown are the mean ± SEM of at least 9 (B,C) or 5 (D,E) independent experiences. **P* < .05; ***P* < .01; ****P* < .001; ns, not significant (Wilcoxon signed-rank test [B,C]; Mann-Whitney test [D,E]) [Color figure can be viewed at wileyonlinelibrary.com]

anti-LG3 antibodies and LG3 present on apoptotic membrane vesicles could potentially help the organism clear remnants of apoptotic cells.

Several studies show an association between polyreactive natural antibodies, kidney allograft injury, and reduced long-term graft outcome in human.[10,43,44] Memory B cells producing natural antibodies with reactivity to multiple HLA alleles, DNA, and self-antigenic structure were found in the blood of patients with kidney graft rejection.[45] As we reported, a high frequency of memory B1 cells specific to LG3 is found in the peritoneal cavity of naïve mice. We do not exclude the possibility that antibodies with reactivity to LG3 also react to a broad range of other antigens. Further studies are needed to evaluate the potential role of polyreactivity in the response to LG3.

Recent work by our group and others showed an association between the presence of autoantibodies reactive with components of apoptotic cells prior to kidney transplantation and reduced allograft function and survival.[8,10,46] In transplant patients, these autoantibodies are almost exclusively IgG with complement fixing and activating properties (IgG1 and IgG3 in humans). In our study in mice, we show that immunization with Freund's adjuvant, which induces a pronounced inflammatory response, influences the anti-LG3 IgG isotype produced after LG3-immunization. Indeed, the presence of adjuvant leads to the production of high titers of anti-LG3 IgG2a, IgG2b, and IgG3 subclasses known to bind C1q and activate complement via the classical pathway in mice. By contrast, immunization with LG3 alone induces the production of anti-LG3 IgG1. In mice, IgG1 do not bind complement contrary to IgG1 in human. This finding provides a new clue to the mechanisms potentially at play prior to transplantation and implicated in the formation of complement-fixing anti-LG3 IgG isotypes. End-stage-renal disease (ESRD) and dialysis are 2 proinflammatory conditions.[47-49] Also, common complications in ESRD patients, such as sepsis and cardiovascular events, can further enhance the inflammatory response in these



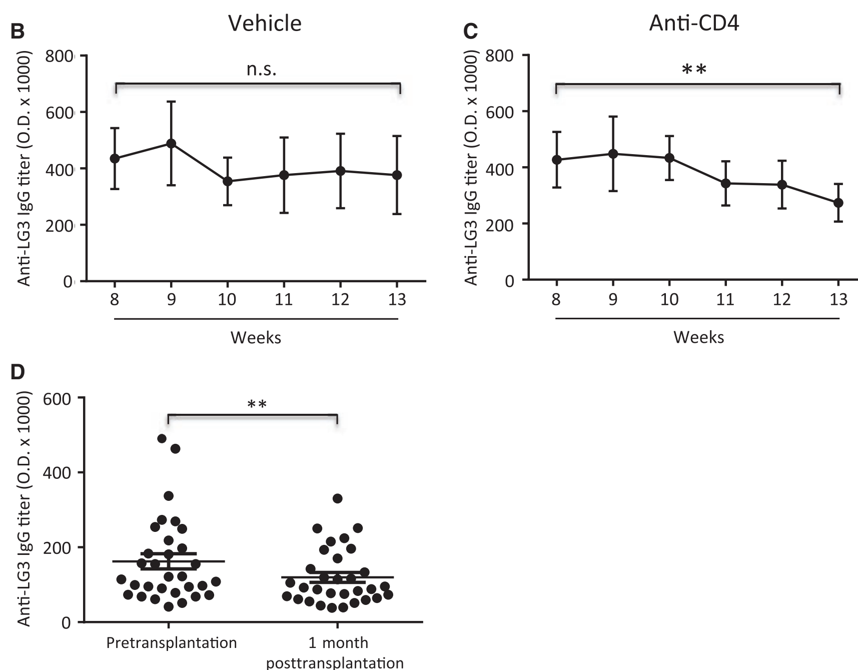


FIGURE 7 Impact of T cell targeting on antibody reactivity to LG3. (A-C) C57BL/6 WT mice were immunized with LG3 (50 μg/sc every 2 weeks for a total of 4 injections). As described in the schematic representation of the experimental timeline (A), at the end of the immunization process, mice with high titers of anti-LG3 IgG were separated into 2 groups and were either depleted from $CD4^+$ T cells (C) or not (B). Anti-LG3 IgG titers were assessed in the serum of mice every week following the first injection of vehicle PBS (B) or anti-CD4 (C) by ELISA. Results shown are the mean ± SEM of 6 independent experiences. $^{**}P < .01$ (one-way ANOVA with Dunnett's multiple comparison test). (D) Pre- and posttransplant levels of anti-LG3 IgG in 31 renal transplant patients. Results shown are the mean ± SEM. $^{**}P < .01$ (paired *t* test)

TABLE 1 Characteristics of renal transplant patients

Variables	Results
Mean age in years (*SD*)	48.0 (13.3)
Race, n (%)	
Caucasian	27 (87)
African American	1 (3)
Other	3 (10)
Male gender, n (%)	19 (61.3)
Cause of chronic kidney diseases, n (%)	
Glomerular diseases	10 (32.3)
Hypersensitive/vascular	4 (12.9)
Diabetic nephropathy	1 (3.2)
Polycystic kidney disease;	7 (22.6)
Urological	5 (16.1)
Autoimmune diseases	1 (3.2)
Unknown	3 (9.7)
Diabetes, n (%)	4 (12.9)
Obesity (BMI > 30 kg/m^2), n (%)	7 (22.6)
Previous pregnancy, n (%)	6 (19.4)
Previous transplantation, n (%)	2 (6.5)
Pretransplant transfusion, n (%)	10 (32.3)
Donor source, n (%)	
Living	9 (29.0)
Deceased, neurological death	20 (64.5)
Deceased after cardiocirculatory arrest	2 (6.5)
Pretransplant PRA (>0%), n (%)	5 (16.1)
Induction immunosuppressive therapy, n (%)	
Antithymocyte globulin	12 (38.7)
Basiliximab	19 (61.2)
Delayed graft function, n (%)	8 (25.8)
Rejection (1st month posttransplant), n (%)	5 (16.1)

BMI, body mass index; PRA, panel reactive antibody; SD, standard deviation.

patients. Our results raise the possibility that proinflammatory conditions prevalent in patients with ESRD could foster immunoglobulin class switching for anti-LG3 antibodies toward complement-fixing isotypes of greater negative impact at the time of transplantation.

It was previously assumed that memory B cells are exclusively formed from conventional B2 cells interacting with CD4-helper T cells leading to germinal center (GC) formation within lymphoid organs. However, recent evidence showed that B1 cells also generate B cell memory, independently of CD4$^+$ T cell help, and GC support.[50-53] This process results in B1 memory with faster but lower-affinity primary response than B2 memory. Consistent with these studies, we here report the presence of memory B cells specific to the autoantigen LG3 even in the absence of CD4$^+$ T cells and we identify B1 cells as the main subset with memory specific to LG3. Our results also show that among B1 cells, peritoneal cells are the ones with the least dependence on T cell help for the generation of LG3-specific memory B cells. This suggests a more pronounced innate-like phenotype in peritoneal B1 cells.

Cyclosporin and tacrolimus have been the mainstay of immunosuppression in solid organ transplantation for many decades. Both types of CNI are potent inhibitors of T cell activation.[54] In addition, CNI are known to dampen humoral immunity by inhibiting the differentiation of T follicular helper from naïve CD4$^+$ T cells and by suppressing naïve B cells.[55] In the present study, our aim was to assess how anti-LG3 antibodies behaved in unselected patients after transplantation with the onset of immunosuppression. This study was not designed or powered to detect the impact of changes in anti-LG3 antibodies on transplantation outcomes, and we observed no association between the change in anti-LG3 values posttransplant and patient outcomes or any other clinical variable. However, we show that initiation of CNI-based immunosuppression in renal transplant patients is associated with decreasing anti-LG3 IgG. This observation suggests that CNI can dampen humoral immunity specific to LG3 although we cannot exclude that other factors such as mycophenolate mofetil and corticosteroids also played a role in our observations. Our results point to the possibility that immunosuppressive regimens targeting T cell function could be used prior to transplantation in patients with high anti-LG3 levels as a means of decreasing anti-LG3 levels and potentially preventing the increased risk of delayed graft function, acute rejection, and reduced long-term survival associated with high anti-LG3 levels.[8,21]

In summary, our study provides novel insights into pathways responsible for the production of anti-LG3 autoantibodies. We show that humoral memory specific to perlecan/LG3 is present within the normal repertoire of WT mice and depends in large part on peritoneal B1 cells. Production of anti-LG3 antibodies is not present in naïve normal mice and develops after immunization through CD4$^+$ T cell-dependent pathways. CNI-based immunosuppression is identified as a means of reducing anti-LG3 levels in renal transplant patients, which also concurs to an important role for T cells in controlling anti-LG3 production.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by *American Journal of Transplantation*.

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