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Monitoring B cell alloresponses in rats

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ABSTRACT

Antibody-mediated rejection is a major cause of graft failure in organ transplantation. For this reason, B cell responses are of particular interest to transplantation research. Rats are important model organisms for transplant studies, but B cell alloimmune assays and B cell subset markers are poorly established in rats. We alloimmunized rats by donor blood injection using the high responder rat strain combination Brown Norway (donor) and Lewis (recipient) rats. Using splenocytes from alloimmunized and control rats, we established assays to assess allospecific B cell proliferation and the capacity to generate allospecific B memory cells and alloantibody-secreting cells after antigenic rechallenge *in vitro* using a mixed lymphocyte reaction. Furthermore, we defined a simple gating and sorting strategy for pre- and post-germinal center follicular B cells, as well as non-switched and switched plasmablasts. Our protocols for assessing B cell alloresponses and B cell subsets in rats may help to accelerate research into the role of B cells and manipulation of humoral alloresponses in transplant research.

1. Introduction

Model organisms remain an important pillar of basic and translational research, especially in the context of interventional and transgenic studies in disease models. Rats serve as important model organisms for various human conditions, including allogeneic solid organ and bone marrow transplantation and autoimmune diseases. Autoimmune disease models using rats include the membranous nephropathy model Heymann nephritis (Salant et al., 1989), the rheumatoid arthritis model adjuvant arthritis (van Eden et al., 2001), experimental autoimmune encephamyelitis (Mannie et al., 2009), as well as models of hypertension (Eissler et al., 2011) and autoimmune type diabetes (Mordes et al., 2004).

Compared to mice, rats are significantly larger, which has been advantageous for surgical and microsurgical techniques, including organ transplantation. Furthermore, the availability of rapid generation of transgenic animals using modern techniques may give rats wider appeal for experimental transgenic studies. However, in comparison to experimental techniques using mouse or human material, many markers and experimental protocols are poorly established in rats. Furthermore, optimal reagents for rat experiments are less readily available compared to reagents for mouse or human studies.

In models of allogeneic transplantation, the combination of rat strains determines the strength of alloresponses. The major histocompatibility complex of the rat, the RT1 complex, has been fully genotyped and consists of class I (RT1A), class II (RT1B and RT1D) and class III regions (Gunther and Walter, 2001). In transplant models, combinations of rat strains with full mismatches in MHC haplotypes, such as Brown-Norway (BN) and Lewis (LEW), serve as high-responder combinations, while combinations with partial MHC and non-MHC haplotype mismatches, such as F344 and LEW, serve as low-responders (Shrestha and Haylor, 2014). Low responders have been used to model chronic

Abbreviations: AB, antibody; ABMR, antibody-mediated rejection; Bcl-6, B cell lymphoma 6; Blimp-1, B lymphocyte-induced maturation protein-1; BN, Brown Norway rat; CD, cluster of differentiation; DSA, donor-specific antibodies; GC, germinal center; HLA, human leukocyte antigen; HPRT, hypoxanthine-guanine-phosphoribosyl-transferase; ICOSL, inducible T cell costimulatory ligand; IRF-4, Interferon Regulatory Factor 4; Ig, immunoglobulin; IL-21R, interleukin-21 receptor; LEW, Lewis rat; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PAX-5, paired box protein 5; TCMR, T cell mediated allograft rejection; XBP-1, X-box binding protein 1.

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rejection (Adams et al., 1993; Hancock et al., 1993; Orloff et al., 1999). We have previously used a full MHC-mismatch combination of strains to best model human kidney transplantation (Kuhne et al., 2017; Steines et al., 2020a; Steines et al., 2021). BN rats contain $RT1^n$, while LEW rats contain $RT1^{1\ 6}$. In models of kidney transplantation, this combination elicits acute cellular and humoral rejections (Kuhne et al., 2017).

Although T cell mediated allograft rejection (TCMR) may still occur despite modern immunosuppressive protocols, it can usually be effectively managed using anti-rejection therapies. Antibody-mediated rejection (ABMR), however, is more difficult to treat and represents a major cause of allograft failure (Sellares et al., 2012). B cells have received much attention as precursors of antibody-secreting cells in this context (Crespo et al., 2015; Thaunat et al., 2012). The mechanisms and molecules involved in B cell alloactivation and differentiation are of particular interest to transplant researchers with a view of finding targets for intervention to prevent or treat ABMR (Sicard et al., 2016; Chen et al., 2018). In addition, other functions of B cells beyond antibody generation have been explored (Chesneau et al., 2014; Viklicky et al., 2013; Newell et al., 2015). These include antigen-presentation and costimulation, as well as regulatory functions (Cherukuri et al., 2014). Several B cell targeting therapies have shown efficacy in the treatment of autoimmune diseases (Cherukuri et al., 2014; Falk and Jennette, 2010). Thus, therapies targeting B cells are being explored in organ transplantation (Banham et al., 2018; Clatworthy, 2011; Pilat et al., 2021). Studying B cell responses *in vivo* in translational transplant models may help to investigate novel targets to prevent rejection and prolong allograft survival in the future.

This increased interest in B cells in the context of allogeneic transplantation requires the establishment of adequate experimental protocols. Since B cell subset markers and activation protocols are poorly established in rats, we used an alloimmunization setting to establish these experimental protocols. Here, we report on several important methods for studying alloimmune B cell responses and specific B cell subsets in rat strains widely used in transplantation research and hope to thereby facilitate and accelerate research in this field.

2. Methods

2.1. Allo-immunization protocol

Animal experiments were approved by local authorities (Regierung von Unterfranken) and experiments were performed according to animal protection laws. Male BN served as donors and male LEW as recipients (Charles River Laboratories, Sulzfeld, Germany). Alloimmunization was performed by intraperitoneal ($n = 3$) or intravenous (n = 3) injection of BN donor blood. We used whole blood taken from BN tail veins and injected 0,5 ml of heparinized BN blood iv/ip on d0, d7 and d14 into LEW recipients. Blood was drawn on d21 after first BN blood injection and rats were sacrificed on d28 after the first BN blood injection, at which point serum and spleens were collected for further analysis. Serum was frozen for alloantibody analysis and spleens were used for flow cytometry and mixed lymphocyte reaction (MLR). Untreated Lewis rats were used as controls. Fig. 1 shows a schematic overview of the experiments (Fig. 1).

2.2. Flow cytometry

We isolated spleen cells as previously described (Steines et al., 2020b). Briefly, spleens were macerated, filtered through 70 μm and 30 μm filters and mononuclear cells separated using ficoll gradient centrifugation. Single cell suspensions were blocked using 10% bovine serum albumin in PBS and stained. Fc-Block (InnovexBiosciences NB30930, Richmond, USA) was used before staining. We used the following antibodies to detect T and B cells and B subsets, see Tables 1 and 2.

2.3. Donor-specific antibodies

DSA were measured by flow crossmatch as previously described (Kuhne et al., 2017; Steines et al., 2021). BN donor splenocytes were incubated with heat-inactivated LEW recipient serum or MLR supernatants for 30 min at 4 °C, washed and stained with the following antibodies, see Table 3. Finally, cells were stained and gated for CD3-FITC (eBioscience/Thermofischer 11–0030) to exclude non-specific binding

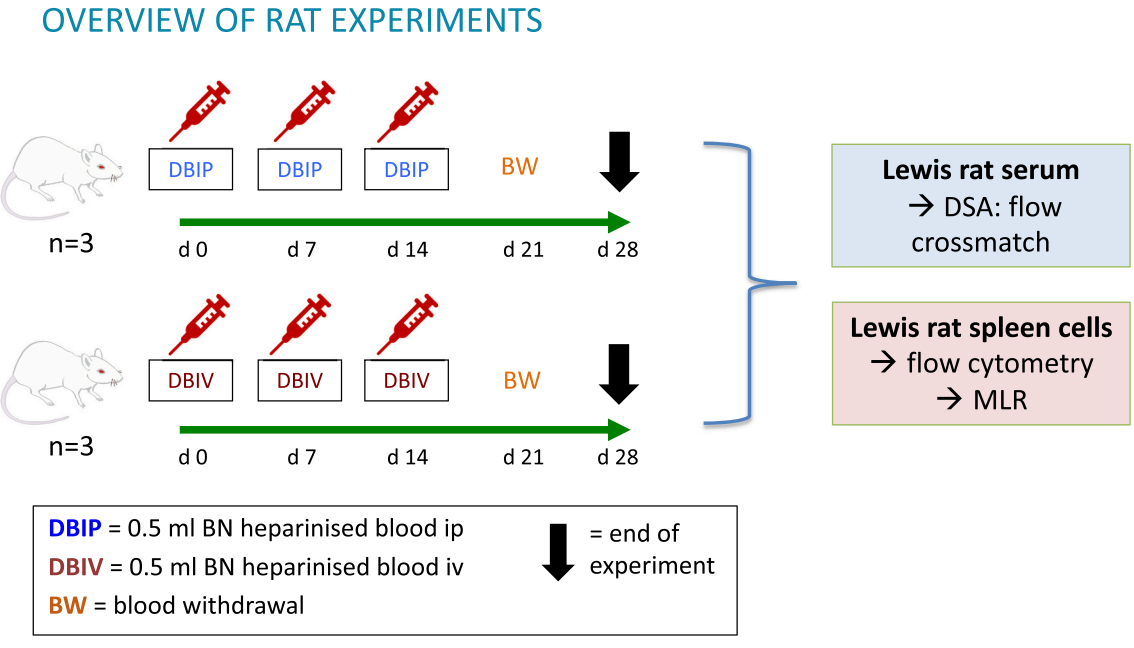

Fig. 1. Schematic overview of alloimmunization protocol. Lewis (LEW) rats were immunized with Brown Norway (BN) rat heparinized whole blood *via* intravenous ($n = 3$) or intraperitoneal (n = 3) injection on days 0, 7 and 14. On day 21 blood was withdrawn for DSA analysis. On day 28, alloimmunized rats were sacrificed, serum was preserved for DSA analysis and spleen cells used for mixed lymphocyte reactions (MLR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
T and B cells (MLR PANEL).

Primary antibody	source
fixable viability dye eFluor780	Invitrogen, 65-0865
anti-rat CD3-PerCPeFluor710	Invitrogen, 46-0030-82
anti-rat CD4-BV510	BD, 740138
anti-rat CD45R(B220)-PeCy7	Invitrogen, HIS24 25-0460
anti-rat IgM-FITC	Biolegend, 408905
anti-rat CD27-biotin	Biorad, MCA4701B
CellTrace Cell Proliferation Kit Yellow	Invitrogen, C34567

Secondary antibody	source
Streptavidin BV421	Biolegend, 405,226

B cell subsets were stained using the following antibody panel:

Table 2
B cell subset PANEL.

Primary antibody	source
fixable viability dye eFluor780	Invitrogen, 65-0865
anti-rat CD3-APC	Invitrogen, 46-0030
anti-rat CD45R(B220)-PeCy7	Invitrogen, HIS24 25–0460
anti-rat IgM-PE	Biolegend, 408,910

Table 3
Antibodies for DSA detection.

Primary antibody	source
anti-rat CD3-FITC	Invitrogen, 46–0030
mouse Anti-rat IgM-PE	eBio,12–0990
chicken Anti-rat IgG-AlexaFluor647	Invitrogen, A21472

Primary antibody	source
anti-rat CD3-PerCP-eFluor710	Invitrogen, 46–0030
mouse Anti-rat IgG1-APC	eBioscience, 17–4812
mouse Anti-rat IgG2a-FITC	Invitrogen, 11–4817
mouse Anti-rat IgG2b-PE	eBioscience, 12–4815
mouse Anti-rat IgG2c-biotin	BD, 553909
Secondary antibody	source
Streptavidin BV421	Biolegend, 405,226

by Fc-receptors.

In addition, complement-mediated cytotoxic activity was measured using a previously described CDC assay (Kuhne et al., 2017). In brief, BN donor splenocytes were incubated with heat-inactivated recipient serum or MLR supernatants for 30 min at 4 °C. After washing, rabbit complement (BAG 7018, Lich, Germany) was added and incubated for 2 h at 24 °C. Cells were then washed and stained for propidium iodide (PI) (Invitrogen, P3566). PI-positive cells were measured by flow cytometry. As background control, medium was added instead of serum. For complete lysis, FixPerm (Thermo Fischer, Waltham, USA) was used. Percent Lysis was calculated using the formula: ("PI $^{+}$cells in sample" – "PI^{+}cells in medium")/("PI^{+}cells in FixPerm" – "PI^{+}cells in Medium") × 100.

2.4. Mixed lymphocyte reaction (MLR)

We used splenocytes from allo-immunized LEW recipients as responder cells and splenocytes from BN rats as stimulator cells. Lewis cells were labeled with CellTrace Cell Proliferation Kit Yellow (Invitrogen, C34567) to assess proliferation. BN splenocytes were irradiated with 45Gy. Stimulator and responder cells were incubated at a 1:2 ratio (400.000 cells/well) in complete medium containing RPMI 1640 + GlutaMAX-I (Gibco, cat. 61870), 10% fetal bovine serum (Sigma-Aldrich, cat. F7524), 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, cat. 15140122), 1% MEM non-essential amino acids (Sigma-Aldrich, cat. M7145), 10 mM HEPES (Gibco, cat. 15630-056), 1 mM sodium pyruvate (Gibco, cat. 11360039), 50 µM ß-mercaptoethanol (Gibco, cat. 31350-010), 2 mM L-glutamine (Gibco, cat. 25030-024) and 1% MEM vitamin solution (Thermo Fisher Scientific, cat. 11120052) for 4 and 8 days at 37 °C and 5% CO_2. Irradiated LEW splenocytes were used for stimulator cells as negative controls (autologous stimulation). At the end of the incubation period, cells were analyzed using flow cytometry using the antibody panels above (d4) and supernatants were frozen for DSA analysis (d8). A schematic overview of MLR set-up is shown in Fig. 3.

2.5. Cell sorting of B cell subpopulations

We used a BD FACSAria IIu cell sorter to isolate B cell subsets from spleens of non-immunized LEW rats after staining with IgM-PE (Invitrogen, 12–0990), CD45R-Pe-Cy7 (Invitrogen, 25–0460) and CD3-APC (Invitrogen, 17–0030) using a sort strategy depicted in Fig. 7a-b. Sorted cells were captured in 100 µl lysis buffer (NucleoSpin RNA Plus XS kit, Macherey-Nagel, Germany, cat. 740,990.50) and frozen at −20 °C for later RNA isolation.

2.6. Real-time PCR

We used RT-qPCR to further characterize sorted B cell subsets. qPCR was performed as previously described (Kuhne et al., 2017). Briefly, total RNA was extracted using NucleoSpin RNA Plus XS (Macherey-Nagel, Germany, cat. 740,990.50). DNAse was used to degrade trace genomic DNA. RNA was reverse transcribed into cDNA using Oligo(dT) primer (Promega, Mannheim, Gemany, cat. C110A), RNasin (Promega, Mannheim, Germany, cat. N251B), and Sensiscript Kit (QIAGEN, cat. 205,213) for 1 h at 37 °C. A ViiA7 detection system was used (Applied Biosystems, Darmstadt, Germany) after applying QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany, cat. 204,145). The sequences of primers are listed in table S1. Copy numbers of target genes were normalized to expression of house-keeping gene hypoxanthine-guanine-phosphoribosyl-transferase (HRRT) and shown as delta CT values. The z-score was calculated from delta CT values for each sample and target gene using the formula $z = (\chi-\mu)/\sigma$, where z is the deviation from the mean, x is sample value, µ is the mean and σ is the standard deviation. Mean z-scores from each sorted B cell subset and sorted T cells are shown in a heat map using a color scale for up- or down-regulation of gene expression.

2.7. Statistical analysis

Data was analyzed using GraphPadPrism (Version 9.0, San Diego, USA) and is shown as mean ± SEM. Statistical analysis was performed by Mann-Whitney test for non-paired analyses, Wilcoxon Test for paired analyses or ANOVA for multiple analyses. *P*-values ≤0.05 were considered statistically significant.

3. Results

3.1. Production of donor-specific antibodies after allo-immunization

We used BN rats (RTa1^{n}) as donors and LEW rats (RTa1^{l}) as recipients for allo-immunization due to the complete MHC-incompatibility of the species. We injected whole blood from BN rats into LEW recipients intraperitoneally ($n = 3$) or intravenously (n = 3). For increased alloantibody production, we allo-immunized LEW rats with a first injection of BN donor blood (d0), followed by 2 booster immunizations after 7 days each (d7 and d14). Fig. 1 shows a schematic overview of allo-immunizations.

We analyzed DSA formation in recipient LEW rat sera on d21 and d28 after the initial allo-immunization. We found that IgM and IgG DSA were significantly elevated at both d21 and d28 compared to control sera from non-immunized LEW rats (Fig. 2a-b). CDC assay demonstrated that

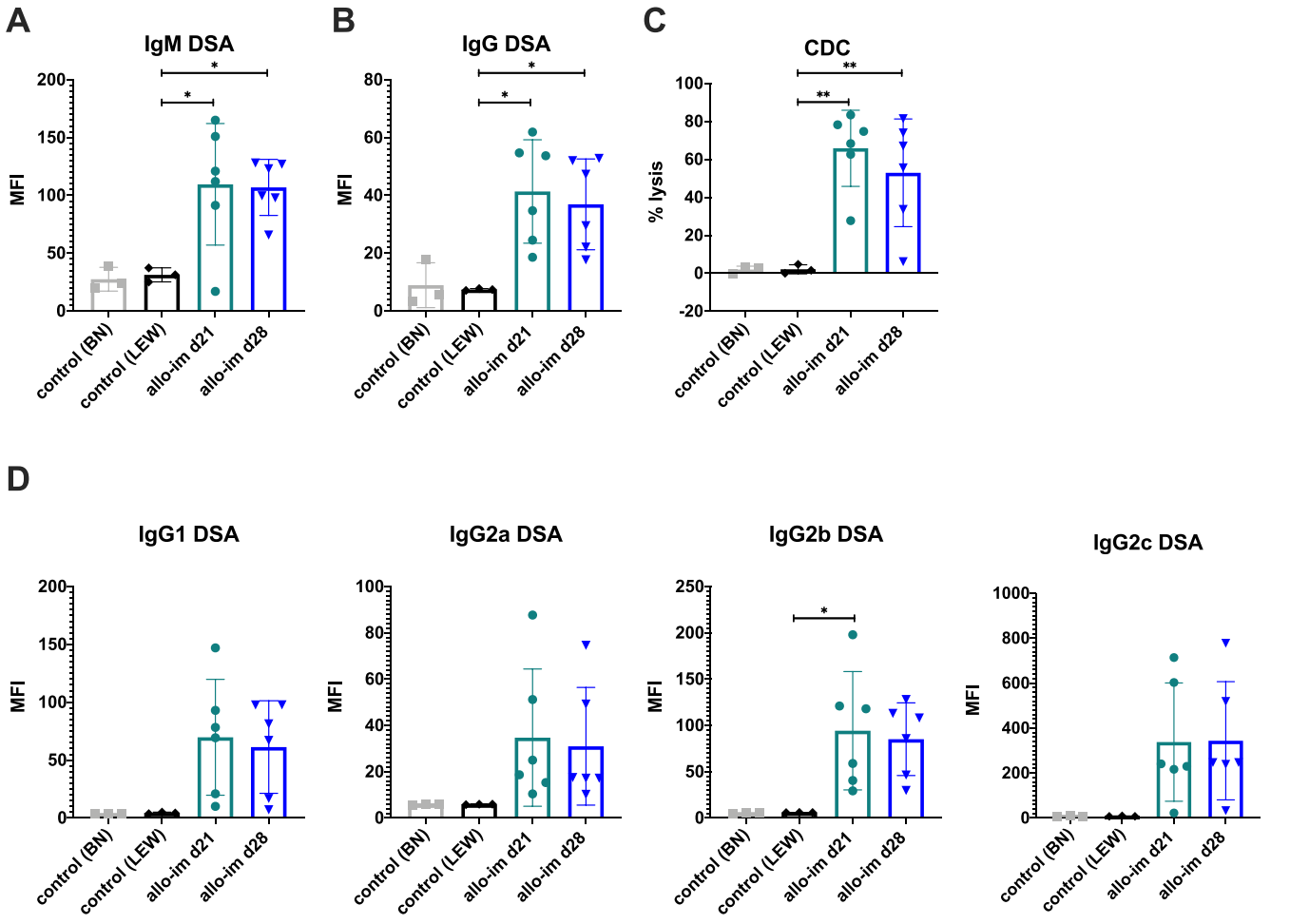

Fig. 2. Effect of alloimmunization on DSA formation. DSA were measured in serum at d21 and d28 after first alloimmunization by flow crossmatch. (A) shows IgM DSA and (B) IgG DSA as mean fluorescence intensity (MFI). (C) shows complement-dependent cytotoxicity (CDC) of serum samples as percent lysis of donor BN cells. (D) shows flow crossmatch results of DSA according to IgG subclass. Sera from untreated BN and LEW rats were used as controls. Data is shown as individual data points and mean ± SEM; statistical significance between groups is shown as $*p \leq 0.05$, $**p < 0.01$ and $***p < 0.005$.

DSA were complement-activating at these time points (Fig. 2c). Analysis of DSA IgG subclasses showed that DSA of all IgG subclasses trended higher in response to immunization compared to controls, but only DSA of IgG2b were significantly elevated compared to controls (Fig. 2d). This may be explained by mixed responses of rats to immunization in DSA IgG subclasses IgG1, IgG2a and IgG2c. We have shown the responses of each individual (fig. S1a); it appears that total IgG DSA is best reflected by IgG subclasses IgG1 and IgG2c, although one rat (A3) did not follow this pattern (fig. S1a). Overall, the distribution of IgG subclass DSA responses in MFI was similar between rats (IgG2c > IgG2b > IgG1 > IgG2a). The route of allo-immunization administration did not result in significantly different DSA responses in terms of DSA MFI, although there was a trend to higher MFI after iv administration compared to ip administration (fig. S1b).

3.2. Detection of allo-specific T and B cell proliferation

Using a MLR set-up as shown in Fig. 3, we were able to detect potent recall responses of allo-specific T and B cells. T cells from allo-immunized LEW rats proliferated substantially upon antigenic restimulation with BN stimulator cells, but not when autologous LEW cells were used as stimulators (Fig. 4a). T cells from non-immunized Lew rats also did not proliferate in this MLR setting, demonstrating the specificity of the assay for recall responses of allo-immunized cells (Fig. 4a). Allo-specific B cell proliferation was also detected by MLR assay and was also specific to BN antigenic rechallenge in allo-immunized LEW rats, but not in non-immunized LEW controls (Fig. 4b). Overall, the proliferative response of T cells was stronger than that of B cells in this setting.

3.3. Generation of allo-specific B memory cells

Next, we quantified the amount of allo-specific B memory cells generated during antigenic restimulation with BN stimulator cells in the MLR experiment. To this end, we analyzed $CD3^{-}CD45R^{+}CD27^{+}$ memory B cells and found a significant increase in the number of memory B cells upon antigen stimulation in an MLR using BN stimulator cells compared to LEW stimulator cells (Fig. 5).

3.4. Differentiation of DSA-secreting cells in vitro

We analyzed the production of DSA by cells from allo-immunized rats after re-stimulation with allo-antigen by assessing DSA content in

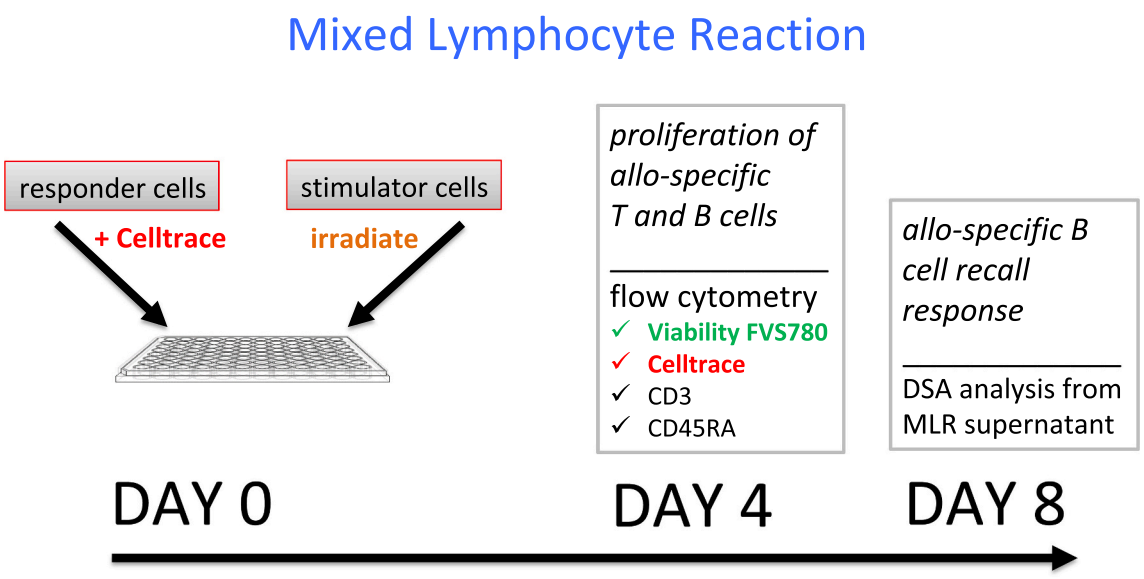

Fig. 3. Schematic overview of mixed lymphocyte reaction (MLR). Splenocytes from alloimmunized LEW rats (responder cells) were isolated at d28 after initial alloimmunization and labeled with a proliferation dye (Celltrace), while donor BN rat splenocytes were isolated and irradiated. Responder and stimulator cells were then incubated together. B cell ($CD45R^{+}CD3^{-}$) and T cell ($CD3^{+}$) proliferation after stimulation with alloantigen (irradiated BN splenocytes) or autologous (irradiated LEW splenocytes) was measured on d4 of MLR. On d8 of MLR, culture supernatants were removed for DSA analysis.

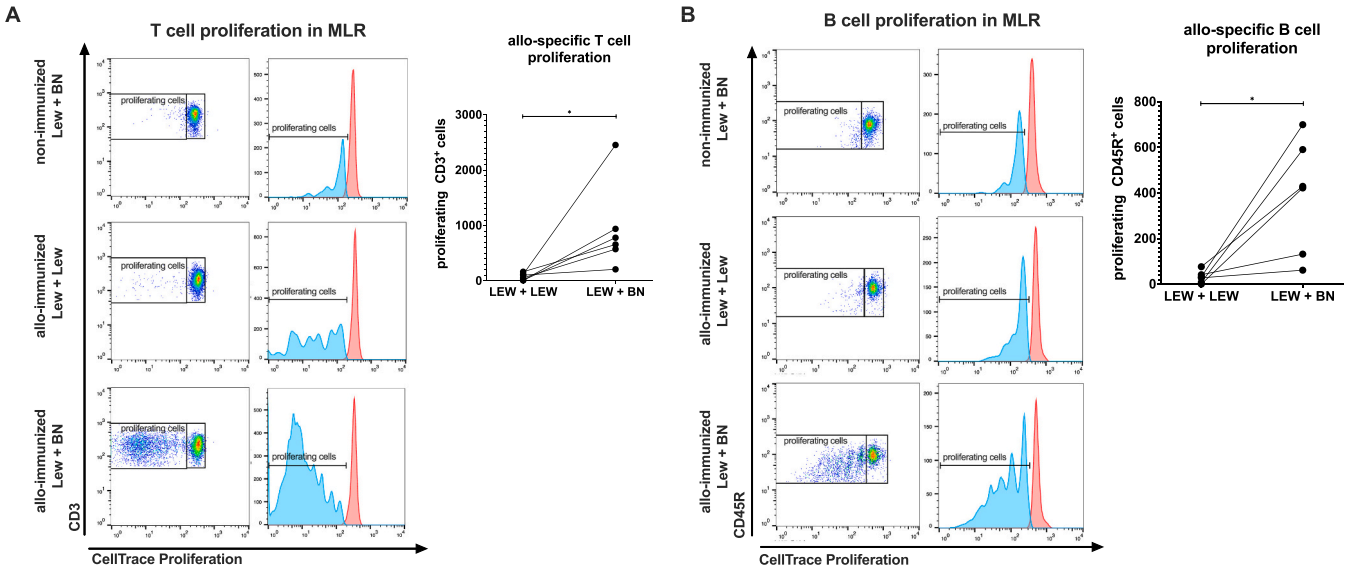

Fig. 4. Allo-specific proliferation of T and B cells after allogeneic or autologous antigen stimulation by MLR. Splenocytes were isolated from alloimmunized and non-immunized (control) LEW rats and restimulated *in vitro* by irradiated BN (allogeneic Ag) or LEW (autologous Ag, control) splenocytes in an MLR. Dilution of Celltrace proliferation dye shows proliferating T (A) and B (B) cells. Representative dot plots and histograms are shown, where $Celltrace^{low}$ events show proliferating cells (blue trace) and $Celltrace^{high}$ non-proliferating cells (red trace). Graphs are showing summary of data of proliferating T or B cells after MLR stimulation with LEW (control) or BN stimulator cells from each allo-immunized rat as connected data points; statistical significance between groups is shown as $^{*}p \leq 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

supernatants from MLR. We found that cells from allo-immunized rats produced IgM and IgG DSA *in vitro* only when re-stimulated by allo-antigen, but not after stimulation with autologous antigen (Fig. 6a-b). Cells from non-immunized LEW rats did not produce DSA upon stimulation with allo-antigen (Fig. 6a-b). Complement-dependent cytotoxicity could be detected in supernatants from allo-antigen restimulated cells from immunized recipients, but this was not statistically significantly higher than in controls (Fig. 6c). Similarly, IgG subclasses of DSA produced after allo-antigenic *in vitro* restimulation could also be determined, but detected levels were not significantly higher than in controls, except in IgG2c (Fig. 6d).

3.5. Phenotyping of B cell subsets

Since rat B cell subsets are poorly characterized and sort experiments may offer valuable information on B cell subsets' gene and protein expression, we sorted B cell subsets for further analysis. To this end, we established a simple FACS staining protocol based on CD45R, CD3 and IgM expression (Fig. 7a) and defined four distinct B cell subpopulations consistently identified in rat spleen (Fig. 7a, Table 4).

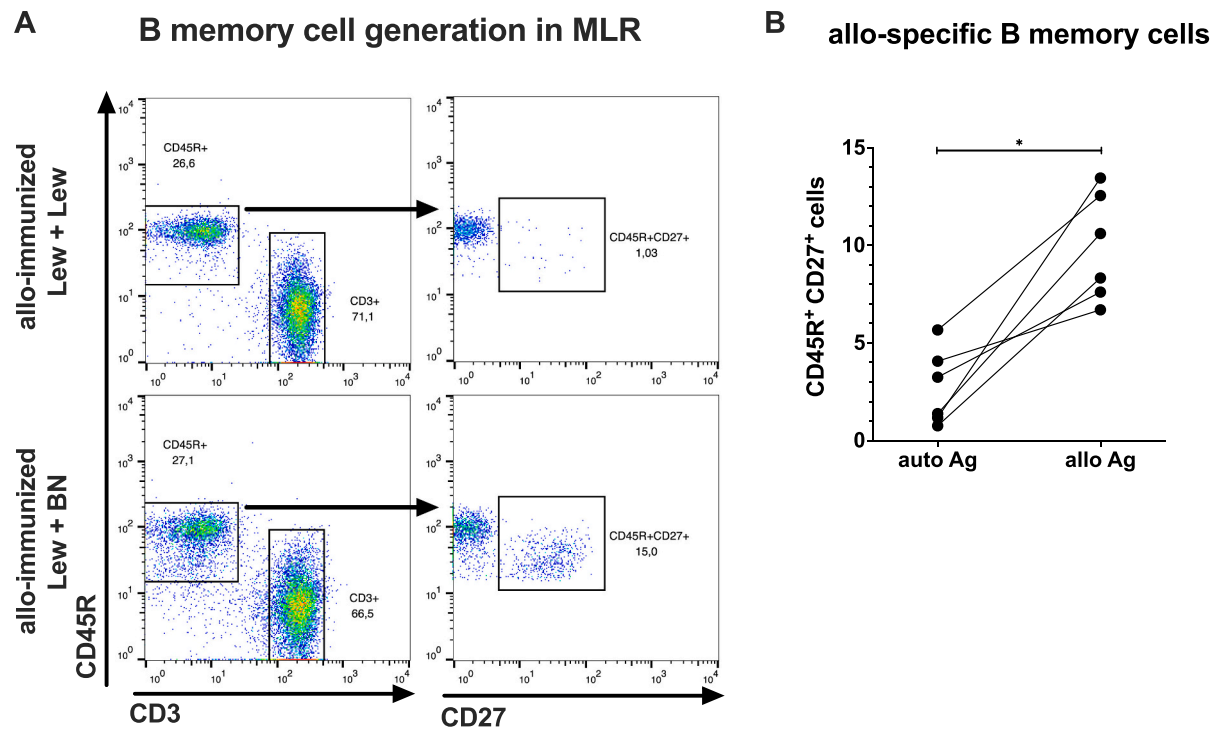

Fig. 5. Generation of allospecific B memory cells. Representative flow cytometric data (A) and summary data (B) of B memory cell ($CD45R^+CD3^-CD27^+$) generation after allogeneic or autologous antigen stimulation by MLR are shown. Splenocytes were isolated from alloimmunized rats and restimulated by irradiated BN (allo Ag) or LEW (auto Ag) splenocytes in an MLR. Data is shown as individual data points; statistical significance between groups is shown as $*p \leq 0.05$.

We then sorted the four populations using the protocol shown in Fig. 7b. Cells were sorted directly in PBS buffer and then subjected to RNA isolation followed by qRT PCR for specific target genes listed in Fig. 7c. We then performed qPCR of target genes isolated mRNA from sorted B cell populations. We were interested in the expression of genes related to differentiation stages of splenic B cells. Relative gene expression on the sorted B cell populations are displayed using a heatmap (Fig. 7c).

We added $CD3^+$ sorted T cells as a negative control and determined the expression of CD28, which was absent in all sorted B cell subsets but highly expressed in $CD3^+$ T cells. PAX-5 is a pan-B cell lineage transcription factor, which was highly expressed in B cell subsets from sort gates 2, 3 and 4, but was absent from cells in sort gate 1 and T cells (Fig. 7c).

The high expression of IgD, CXCR5, as well as Bcl-6 and IL-21 receptor in $CD3^-$ $CD45R^+$ IgM^{int} cells (sort gate 2 in Fig. 7b)(Fig. 7c), suggests that these cells represent naïve and germinal center B cells. These cells co-express IgM but are IgG negative. Furthermore, they express the important co-stimulatory molecules CD40 and ICOSL (Fig. 7c), which are critical in T cell dependent B cell activation.

$CD3^-$ $CD45R^+$ IgM^{hi} cells (sort gate 3) also express CXCR5, placing them in the B cell follicle (Fig. 7c). However, these cells no longer express IgD meaning they are antigen-experienced B cells. Since Bcl-6 and IL-21 receptor expression are low, these cells are post-germinal center, with moderate residual expression of CD40 and ICOSL (Fig. 7c). Upregulated IRF-4 expression may signify the initiation of plasmablast differentiation, while the plasmablast factors XBP-1 and Blimp-1 are not yet expressed (Fig. 7c).

$CD3^-$ $CD45R^{lo}$ IgM^{hi} cells (sort gate 4) do not express the follicular homing receptor CXCR5 and they do not express IgD (Fig. 7c). Although surface staining of these cells shows high IgM expression, IgM mRNA expression is residual, but no IgG expression is observed (Fig. 7c). This cell population also expresses IRF-4, a factor of plasmablast differentiation, and in addition, has upregulated Blimp-1 (Fig. 7c), another factor of the plasmablast/plasma cell program.

Finally, $CD3^-$ $CD45R^{+/lo}$ IgM^- cells (sort gate 1), lack IgD and IgM expression, but do express IgG (Fig. 7c), suggesting these cells are Ig class-switched cells. The plasmablast/plasma cell differentiation factors XBP-1 and Blimp-1 are upregulated, while the B cell lineage factor PAX-5 is down-regulated. Overall, these cells are likely to correspond to IgG^+ class switched plasmablasts.

Table 5 summarizes the splenic B cell differentiation stage likely corresponding to the four B cell subsets analyzed. Having further characterized these B cell subsets, the surface staining pattern of CD45R and IgM may be used for immunophenotyping and sorting of rat splenic B cell subsets.

4. Discussion

Since ABMR is a major cause of allograft failure, studies of B cells and mechanisms of B cell alloactivation in the context of transplantation are pertinent to defining effective immunosuppressive strategies to prevent and treat ABMR. Rats are important model organisms for transplantation studies, but cell subset markers and immunological protocols focusing on B cells are poorly established in rats. Using an alloimmunization experiment of MHC-mismatched rat strains, we established important experimental protocols to investigate the alloactivation of B cells, including the expansion of allospecific B memory cells, the capacity to differentiate into DSA-secreting plasmablasts, the Ig isotype, IgG subclass and complement-activating properties of generated DSA. Furthermore, we characterized specific B cell subsets using gene expression analysis and provided a sorting strategy for further analysis of these subsets.

We used an alloimmunization experiment based on injection of

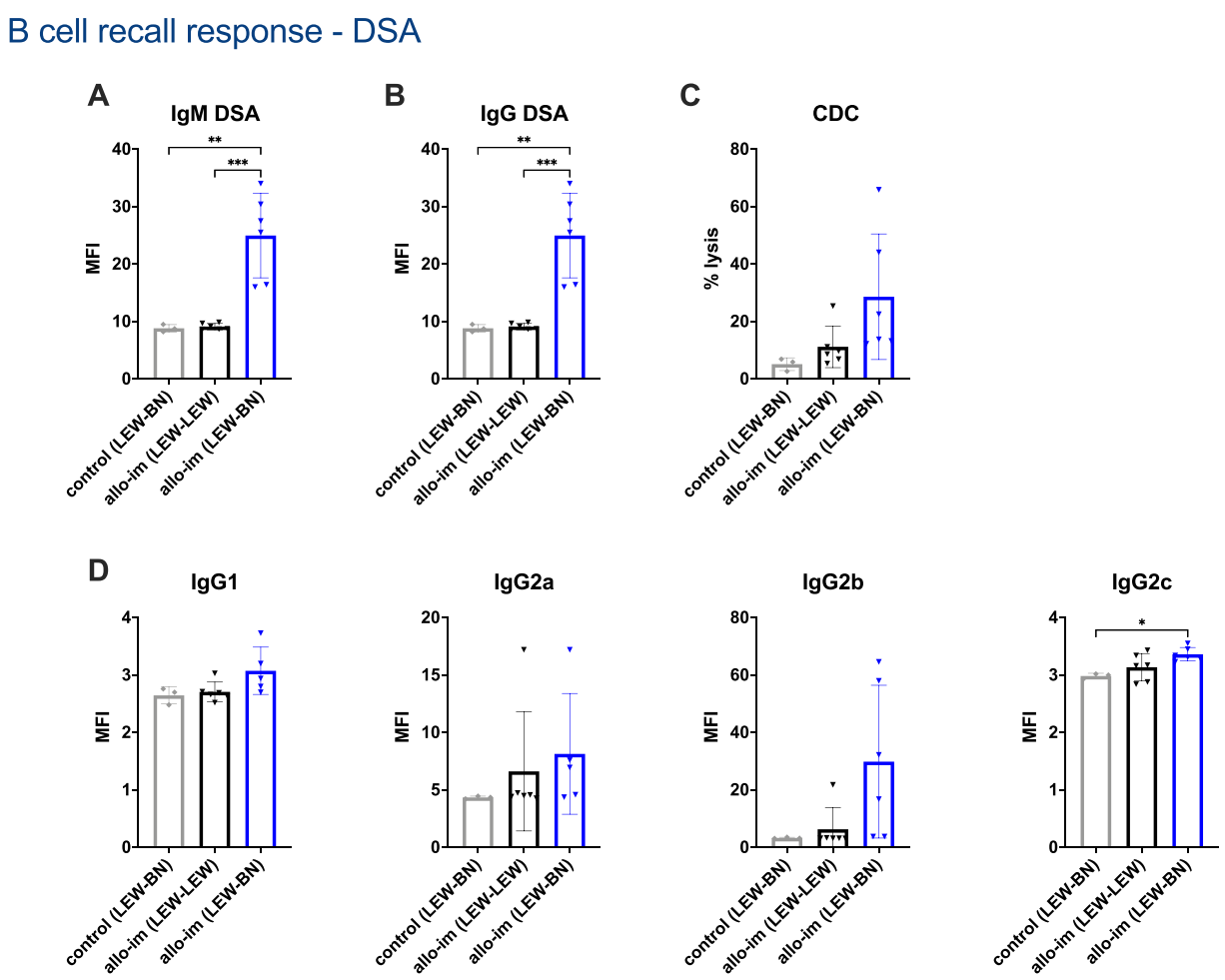

Fig. 6. Generation of alloantibody-secreting cells. Splenocytes were isolated from non-immunized (control) and alloimmunized LEW rats and restimulated by irradiated BN (allo Ag) or LEW (auto Ag) splenocytes in an MLR. DSA were measured from MLR supernatants by flow crossmatch for IgM (A), IgG (B), CDC (C) and IgG subclasses (D). Data is shown as individual data points and mean ± SEM; statistical significance between groups is shown as *p ≤ 0.05, **p < 0.01 and ***p < 0.005.

donor BN leukocytes into LEW recipient rats with multiple booster injections to generate allospecific lymphocytes for *ex vivo* analysis. Alloimmunization was confirmed in immunized rats by serological analysis of DSA. The methods for characterization of DSA by Ig isotype, IgG subclass and complement-dependent cytotoxicity assay have previously been described by our group (Kuhne et al., 2017; Steines et al., 2021).

Measuring B cell frequency in peripheral blood or organs does not provide information about allospecificity of B cells. The MLR is a functional assay, which has been used for many years to assess allospecific T cell responses; however, B cell responses have not been routinely investigated in this setting. We show that allospecific B cell proliferation can be effectively measured using an MLR in allosensitized rats. This may provide important information on B cell recall responses when assessing novel interventions in translational rat transplant models.

Similarly, our *ex vivo* MLR setting may be used to determine the capacity of allospecific B cells to differentiate into effector and memory cells. We showed that cells from alloimmunized rats can produce DSA *in vitro,* but require antigenic restimulation. This suggests *in vitro* differentiation of antigen-activated B cells into DSA-producing plasmablasts. We have shown that the DSA produced by these cells *in vitro* can be characterized using flow crossmatch and CDC assays regarding important functional features, such as Ig isotype, IgG subclass and complement-activating capacity. These characteristics of DSA have recently been linked to clinical outcome in kidney transplantation (Lefaucheur et al., 2016; Loupy et al., 2013). Using these assays, interventions may be tested which modulate or interfere with the differentiation of antibody-secreting effector cells and affect functional properties of DSA. In addition, we showed that *ex vivo* MLR experiments can provide a measure of B memory cell expansion upon alloantigenic restimulation. The impact of allospecific B memory cells on allograft outcomes and their susceptibility to various treatments has not yet been well defined; assays which address this can help to further our understanding of the role of allospecific B memory cells in transplantation.

B cells may be defined by their differentiation stage. In mice and humans, cell surface markers differentially expressed during various B cell differentiation stages are well defined and may be used to distinguish specific B cell subsets by flow cytometry. In rats, B cell subset classification based on cell surface marker expression is not very well established, although Kroese et al. provided some previous work on rat B cell markers (Kroese et al., 1990). We have established a simple gating strategy for flow cytometric analysis of B cell subsets based on the expression of CD45R (B220) and IgM, thereby defining four different B cell subsets. Based on gene expression of the sorted subpopulations, we have determined the most likely differentiation stage of each subset, including IgG class switched plasmablasts ($CD3^-$ $CD45R^{+/lo}$ IgM^-), pre-GC and GC follicular B cells ($CD3^-$ $CD45R^+$ IgM^{int}), post-GC follicular B cells ($CD3^-$ $CD45R^+$ IgM^{hi}) and non-switched early plasmablasts ($CD3^-$ $CD45R^{lo}$ IgM^{hi}). Since the balance of B cells at different differentiation stages has recently been correlated with clinical outcome in kidney

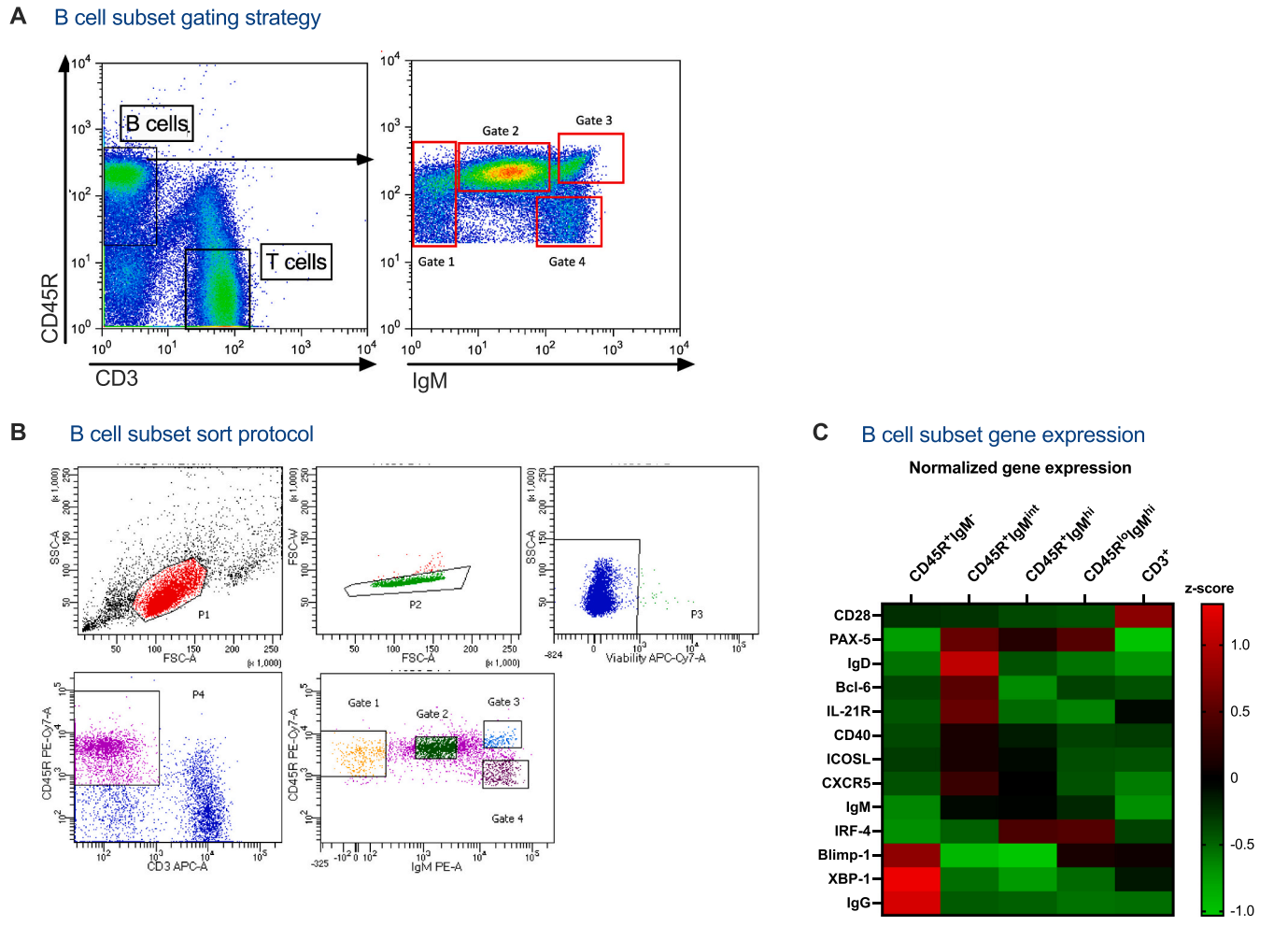

Fig. 7. Gene expression analysis of sorted B cell subsets. (A) Gating strategy for B cell subsets based on CD45R and IgM expression. (B) Sort protocol for cell sorting of B cell subsets based on CD45R and IgM expression. (C) Gene expression of selected genes within sorted B cell populations. Sorted B cell subsets and isolated T cells from 6 LEW rats were analyzed for expression of relevant genes by qPCR. Target gene mRNA expression was normalized to house-keeper HPRT expression. The z-score was calculated from delta CT values per sample using the mean of all samples. Average z-scores from each sorted population are shown using the indicated color scale for increased or decreased gene expression compared to the other populations.

Table 4
Sorted B cell subpopulations and surface marker expression.

Sorted B cell subpopulation	Surface marker expression
Gate 1	$CD3^-$ $CD45R^{+/lo}$ IgM^-
Gate 2	$CD3^-$ $CD45R^+$ IgM^{int}
Gate 3	$CD3^-$ $CD45R^+$ IgM^{hi}
Gate 4	$CD3^-$ $CD45R^{lo}$ IgM^{hi}

Table 5
Sorted B cell subpopulations and surface marker expression.

Sorted B cell population	Surface marker expression	B cell subset
Gate 1	$CD3^-$ $CD45R^{+/lo}$ IgM^-	IgG class switched plasmablasts
Gate 2	$CD3^-$ $CD45R^+$ IgM^{int}	pre-GC and GC follicular B cells
Gate 3	$CD3^-$ $CD45R^+$ IgM^{hi}	post-GC follicular B cells
Gate 4	$CD3^-$ $CD45R^{lo}$ IgM^{hi}	non-switched early plasmablasts

transplant patients (Cherukuri et al., 2017), measurement and further functional or molecular analysis of B cell subsets may be of interest in translational transplant models.

In summary, we provide several protocols for the investigation of B cell alloresponses in rats and hope these may accelerate research in the field.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

Authorships

Steines, Louisa designed experiments, performed data analysis and interpretation and wrote the manuscript. Scharf, Mona performed animal treatments. Hoffmann, Petra contributed to cell sorting experiments and reviewed the manuscript. Schuster, Antonia contributed to data interpretation and reviewed the manuscript. Banas, Bernhard contributed to data interpretation and reviewed the manuscript. Bergler, Tobias contributed to data interpretation and reviewed the manuscript. All authors approved the manuscript before submission.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2021.113212.

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