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New Methods To Analyze B Cell Immune Responses to Thymus-Dependent Antigen Sheep Red Blood Cells

Ellen J. McAllister,*[,†,‡] John R. Apgar,[§] Charlotte R. Leung,[§] Robert C. Rickert,[‡] and Julia Jellusova*[,†,‡]

B cells contribute critically to an effective immune response by producing Ag-specific Abs. During the immune response to so-called "thymus-dependent Ags," activated B cells seek T cell help and form germinal centers. In contrast, thymus-independent Ags generally do not induce germinal center formation. In the germinal center, B cells undergo somatic hypermutation, affinity-based clonal expansion, and differentiation to produce plasma cells and memory B cells. Valuable insight into these processes has been gained by using model hapten–carrier complexes or SRBCs. SRBCs induce robust germinal center formation in mice. Therefore, this Ag is commonly used to study germinal center responses. In contrast to haptenated Ags, thus far it has been difficult to measure the titer of Ag-specific Abs or the expansion of Ag-specific B cells after immunization with SRBCs. We have developed new, simple methods to access these parameters, thus providing new tools to study germinal center and Ab responses. ***The Journal of Immunology*****, 2017, 199: 2998–3003.**

B cell immune responses can be classified into two types based on the nature of the Ag and the involvement of additional cells. Thymus-independent Ags are structures that activate B cells in a polyclonal manner or Ags with repetitive motifs that induce B cell activation and Ab secretion without the need for T cell help (1). Thymus-dependent Ags are typically proteins that induce a full immune response only after activated B cells have received additional costimulatory signals from other cell types, such as T cells. After activated B cells receive T cell help, they initiate germinal center (GC) formation (2). In the GC, Ab diversification occurs through somatic hypermutation, affinity-based clonal selection, and expansion (3). Furthermore, through Ig class switch recombination, the H chain of the produced Abs and, thus, their function can change (4). The final output of the GC is the differentiation of GC B cells to plasma cells, which secrete large quantities of Abs, and memory B cells, which mediate sustained protection against previously encountered pathogens. These processes are crucial for an effective immune response, and considerable effort has been put into elucidating how B cell activation, clonal expansion, Ab secretion, and B cell differentiation are regulated. Important tools in this endeavor have included model Ags consisting of hapten-conjugated proteins (e.g., 4-hydroxy-3-nitrophenylacetyl–chicken γ globulin) and SRBCs. Unlike hapten–carrier conjugates, SRBCs do not require the presence of an adjuvant to induce a robust GC response. Although SRBCs are well suited to study GC formation and plasma cell differentiation, it has been difficult to analyze levels of SRBC-specific Abs in serum from immunized mice. Protocols to analyze levels of SRBC-specific Abs by ELISA are available; however, because of the complexity and potential technical difficulties of these protocols, determining the levels of SRBC-specific Abs remains a challenge (5, 6). We have developed a simple, reliable, and time- and cost-effective method to simultaneously assess levels of SRBC-specific Abs of different isotypes in serum from immunized mice. In addition, we present an assay to stain for SRBC-binding B cells. This stain can be included in a multiparameter flow cytometric assay, thus providing the possibility to study the fate and phenotype of Ag-binding B cells after SRBC immunization. In summary, these two new methods provide novel tools to study GC responses.

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Abbreviations used in this article: AU, arbitrary unit; FSC, forward scatter; GC, germinal center; MFI, mean fluorescence intensity; NP-KLH, 4-hydroxy-3-nitrophenylacetyl (31)–keyhole limpet hemocyanin; SSC, side scatter.

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Materials and Methods

Mice

Female and male adult wild-type mice of mixed FVB and C57BL/6 background were used for all experiments. Animals were maintained in the animal facility of the Sanford Burnham Prebys Medical Discovery Institute or the animal facility of the Max Planck Institute of Immunobiology and Epigenetics. Protocols were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with institutional guidelines and regulations.

SRBC immunization

One milliliter of citrated sheep blood (Colorado Serum, Denver, CO or Cedarlane Laboratories, Burlington, ON, Canada) was washed twice with 50 ml of PBS and resuspended 1:10 in PBS (0.4 ml of packed SRBCs and 3.6 ml of PBS). One hundred microliters of the SRBC suspension was injected i.p. into mice. Mice were sacrificed 7 or 9 d after the immunization. Blood was collected for serum on the day of immunization (day 0) and on the day of sacrificed (day 7 or 9).

To analyze Ab production during a secondary immune response, mice were immunized on day 0 and again on day 14, as described. Blood was

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collected on days 0, 7, 14, 21, and 27. To prepare serum, blood was allowed to coagulate for ≥1 h and was spun down for 10 min at 10,000 rpm, and the supernatant was collected and frozen at −20°C until needed.

Immunization with 4-hydroxy-3-nitrophenylacetyl (31)–keyhole limpet hemocyanin

Mice were immunized i.p. with 50 μg of 4-hydroxy-3-nitrophenylacetyl (31)–keyhole limpet hemocyanin (NP-KLH; LGC Biosearch Technologies, Petaluma, CA) in PBS + Imject Alum (Thermo Fisher Scientific, Rockford, IL) on day 0 and were sacrificed on day 7. Blood was collected on days 0 and 7.

Rapamycin injection

Mice were immunized with SRBCs on day 0, as described, and injected with 60 μl of rapamycin solution (1.2 mg/ml rapamycin; LC Laboratories, Woburn, MA) in PBS + 5% polyethylene glycol 400 + 5% Tween-20 per 10 g of body weight on days 1, 2, 3, 4, 5, and 6. Control mice were injected with vehicle only. Mice were sacrificed on day 7, and blood was collected.

Bone marrow reconstitution

μMT mice were sublethally irradiated (5 Gy) and injected i.v. with bone marrow cells obtained from the femurs of $Nik^{+/-}$ and $Nik^{-/-}$ mice. The mice were sacrificed 9 wk later, and the frequency of splenic B cells was analyzed by flow cytometry. Alternatively, chimeric mice were immunized with SRBCs, as described, and sacrificed on day 9. Serum was prepared from blood obtained on days 0 and 9.

Detection of SRBC-specific Abs in serum

SRBCs were prepared as described. Twenty microliters of SRBCs were mixed with mouse serum (1 μl undiluted serum or 1, 2, or 4 μl from a 1:10 serum dilution) and incubated on ice for 20 min. Samples were washed with FACS buffer (PBS + 1% FBS + 0.01% sodium azide) and stained for 20 min on ice with 100 μl of FACS buffer + anti-mouse IgM allophycocyanin or eFluor 450 (1:100; eBioscience, San Diego, CA) + anti-mouse IgG1 PE or anti-mouse IgG FITC (1:100; BD Pharmingen, San Diego, CA) + anti-mouse B220 allophycocyanin–eFluor 780 (1:100; eBioscience). Samples were washed with 1 ml of FACS buffer and resuspended in 100 μl of FACS buffer. Data were collected using a FACSCanto flow cytometer or BD LSR II flow cytometer (both from BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR). The forward scatter (FSC) and side scatter (SSC) parameters were chosen to allow for the discrimination of intact SRBCs and small debris.

Loading SRBCs with Cell Proliferation Dye eFluor 670

SRBCs were prepared as described. Fifty microliters of SRBCs from the final dilution were mixed with 1 ml of 10 μM Cell Proliferation Dye eFluor 670 (eBioscience; hereafter called eFluor 670) in PBS and incubated at room temperature for 10 min. The cells were washed with FACS buffer, resuspended in 1 ml of FACS buffer, and used for staining.

Detection of SRBC-binding B cells

Mice were immunized, as described, and sacrificed 7 d after immunization. Spleens were isolated, homogenized using glass slides, and incubated in 500 μl of ACK (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) on ice to lyse RBCs. Cells were washed and incubated in 100 μl of FACS buffer with primary Abs for 20 min on ice. The following primary Abs were used: 1:100 anti-B220 eFluor 780 or PE-Cyanine 7 (eBioscience) + 1:100 anti-FAS PE-Cy7 or PE (BD Pharmingen) + 1:100 GL7-Biotin or FITC (eBioscience). Cells were washed and resuspended in 100 μl of FACS buffer + 1:100 Streptavidin-PE (eBioscience) and 20 μl of SRBCs loaded with eFluor 670. Cells were stained for 20 min on ice, washed, resuspended in FACS buffer, and measured using a FACSCanto flow cytometer or BD LSR II flow cytometer (both from BD Biosciences). Analysis was done with FlowJo software (TreeStar). Doublet discrimination was not performed during analysis.

Statistical analysis

Data points were tested for normal distribution using the Shapiro–Wilk normality test, and statistical significance was determined using the *t* test

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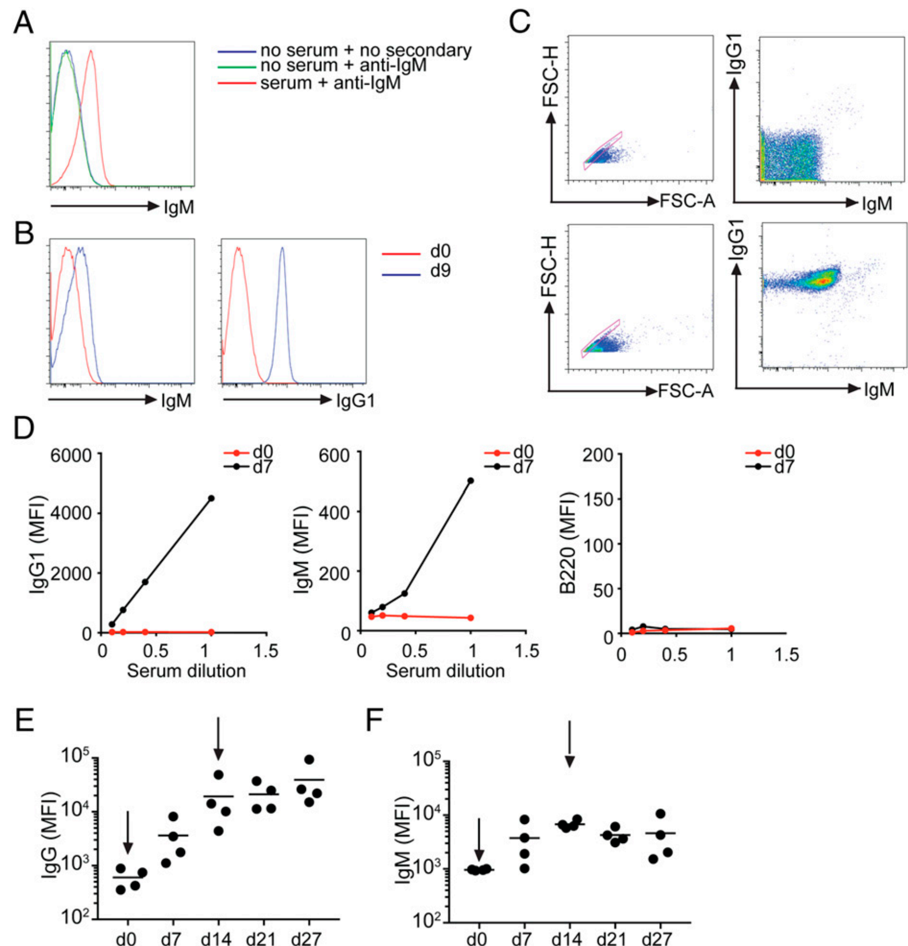

FIGURE 1. Detection of SRBC-specific Abs in serum. (**A**) MFI of SRBCs labeled with serum from SRBC-immunized mice, stained with anti-mouse IgM (red), SRBCs only stained with the secondary Ab (green), and unstained SRBCs (blue). (**B**) SRBCs labeled with serum obtained before immunization (red) or 9 d after SRBC immunization (blue) and stained with anti-mouse IgM (left panel) and anti-mouse IgG1 (right panel). (**C**) SRBCs labeled with serum obtained before immunization (upper panels) or 7 d after SRBC immunization (bottom panels) and stained simultaneously with anti-mouse IgM and anti-mouse IgG1. (**D**) SRBCs labeled with 0.1, 0.2, 0.4, and 1 μl of serum obtained from mice before and 7 d after immunization. The cells were stained with anti-mouse-IgG1 (left panel), anti-mouse IgM (middle panel), and an irrelevant Ab (anti-mouse B220) in a second step. The graphs show the obtained MFIs plotted against the respective dilution. (**E** and **F**) Mice were immunized on day 0 with SRBCs and boosted on day 14. Blood was collected on the indicated days. Shown are MFIs of SRBCs labeled with 0.4 μl of serum and stained with anti-IgM (E) and anti-IgG (F). Each circle represents an individual mouse. Arrows indicate immunization with SRBCs.

or the ANOVA test, as indicated in the figure legends. GraphPad Prism (GraphPad) was used for statistical analysis. Significant differences are marked with an asterisk as follows: ***$p \leq 0.001$, ****$p \leq 0.0001$.

Results

Detection of SRBC-specific Abs in serum

To induce plasma cell generation and production of SRBC-specific Abs, mice were immunized with SRBCs, and blood was collected on days 0 and 7 of the immunization. To measure serum levels of SRBC-specific Abs, we decided to test whether SRBCs can be used for a flow cytometry–based assay. The goal of this assay was to let Abs bind to SRBCs and then detect the bound Abs with fluorescent-labeled secondary Abs specific for murine IgM or IgG1. Intact SRBCs are large enough to be detected by the flow cytometer. Our aim was to test whether the mean fluorescence intensity (MFI) of the bound Abs can be used to reliably assess the concentration of SRBC-specific Abs in the serum sample. First, we incubated intact SRBCs with serum obtained from a mouse at day 7 of immunization and used anti-mouse IgM as a secondary Ab. The immune response to SRBCs peaks 7–10 d after immunization; thus, serum collected at day 7 was expected to contain high levels of SRBC-specific Abs. Indeed, in comparison with SRBCs that have not been stained, we observed a strong MFI shift in the sample stained with serum and anti-mouse IgM (Fig. 1A). To rule out the possibility that the secondary Ab binds in a nonspecific manner to SRBCs, we also incubated SRBCs with the secondary Ab alone. The MFI of this sample was comparable to the unstained sample (Fig. 1A). To rule out that murine Igs bind to SRBCs in a nonspecific manner, we collected serum from mice before immunization and 9 d after immunization and measured IgM or IgG1 levels (Fig. 1B). Samples labeled with serum collected 9 d after immunization showed a strong shift when stained for anti-IgM or anti-IgG1 compared with samples labeled with serum collected from nonimmunized mice (Fig. 1B). To test whether the staining for IgM and IgG1 can be performed simultaneously, we incubated serum-labeled SRBCs with a mixture of anti-mouse IgM and anti-mouse IgG1. Similar to previous experiments, negligible binding of the secondary Abs was detected in samples labeled with preimmune sera, whereas the MFIs shifted strongly for both isotypes in samples labeled with day-7 serum (Fig. 1C). To determine whether this method is suited to reliably analyze the relative concentration of SRBC-specific Abs, we in-

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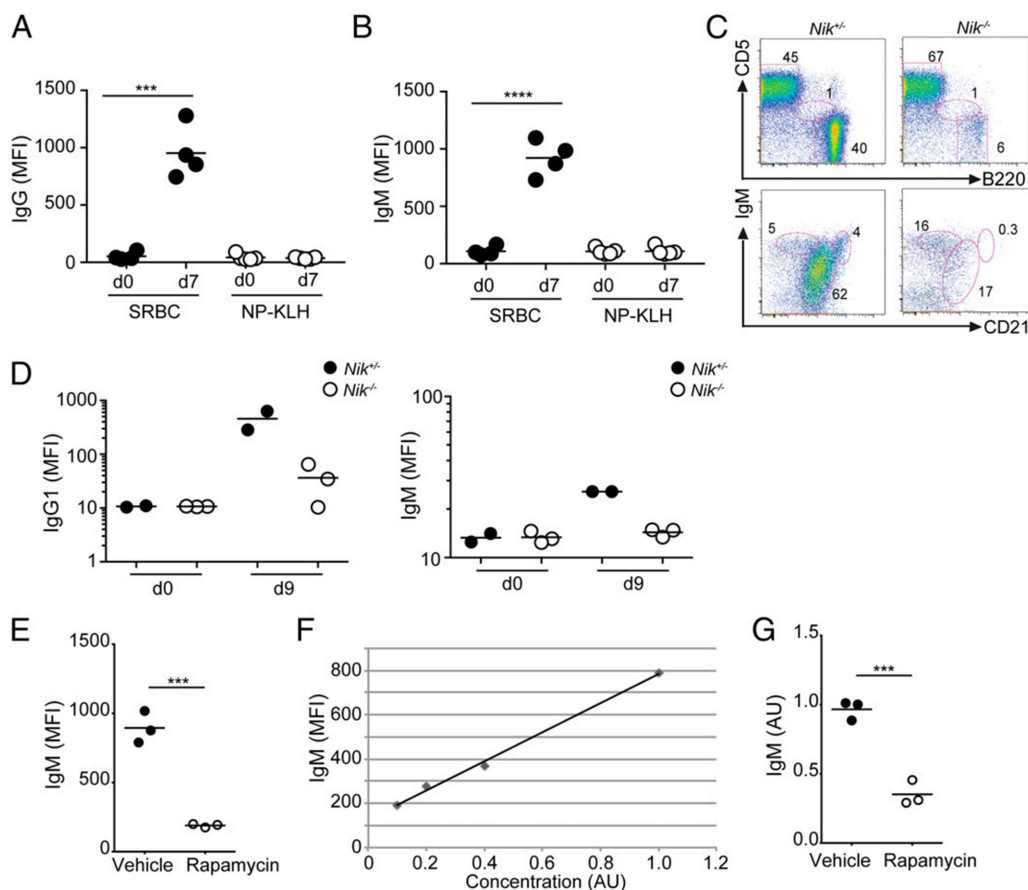

FIGURE 2. Analysis of SRBC-specific Ab titers in different experimental set-ups. (**A** and **B**) Mice were immunized with SRBCs or NP-KLH, and blood was collected on days 0 and 7. The plots shows the summarized MFIs from samples labeled with 0.4 μl of serum obtained before immunization or 7 d after immunization and simultaneously stained with anti-mouse IgG (A) and anti-mouse IgM (B). Each circle represents an individual mouse. (**C**) μMT mice were sublethally irradiated and reconstituted with bone marrow from *Nik*$^{+/-}$ or *Nik*$^{-/-}$ mice. Frequency of B cells in the spleen 9 wk after reconstitution (upper panel) and surface expression of the maturation markers IgM and CD21 on B cells (lower panel). (**D**) μMT-*Nik*$^{+/-}$ and μMT-*Nik*$^{-/-}$ chimeric mice were immunized with SRBCs, and serum was collected 9 d later. SRBC-specific IgG1 and IgM was measured in the serum. Each circle represents an individual mouse. (**E**–**G**) Mice were immunized with SRBCs on day 0 and injected with rapamycin or vehicle on days 1–6. Blood was collected on day 7. (E) MFI of SRBCs labeled with 1 μl of serum. (F) MFIs obtained by labeling SRBCs with 0.1, 0.2, 0.4, and 1 μl of a randomly chosen reference serum were plotted against the concentration (AU). Linear regression was determined and used to calculate concentrations of SRBC-specific IgM in serial dilutions of the serum obtained from the experimental mice. For each serum sample, the concentrations obtained from the different dilutions were multiplied by the dilution factor, and the mean value was calculated and is presented in the graph. Each circle represents the mean value for a serum sample obtained from an individual mouse. ***$p \leq 0.001$, ****$p \leq 0.0001$, *t* test.

cubated SRBCs with serial dilutions of the serum samples. The MFIs for IgM and IgG1 were low in samples labeled with preimmune serum and did not increase with higher serum concentrations, suggesting that the concentration of SRBC-specific Abs in preimmune serum is below detection levels (Fig. 1D). In contrast, the MFIs for IgM and IgG1 increased in a linear fashion with increasing serum concentrations if serum samples from day 7 after immunization were used (Fig. 1D). Thus, within the tested limits, MFI can be used as a relative readout of Ab concentration. As a control, an irrelevant Ab (anti-mouse B220) was included in the staining. Binding of the irrelevant Ab was low independent of serum concentration and the day on which the serum was collected, suggesting that serum from immunized mice does not allow for nonspecific binding of secondary Abs (Fig. 1D). To assess the kinetics of the production of SRBC-specific Abs over the course of the immune response, we measured IgG and IgM levels in serum obtained from mice at days 0, 7, and 14 after the immunization, challenged the mice with a second SRBC immunization on day 14, and measured IgG and IgM levels 7 and 13 d later (days 21 and 27 of the immunization, respectively). As expected, titers of SRBC-specific Abs increased after the immunization, and the increase in IgG was more prominent than the increase in IgM (Fig. 1E, 1F). To rule out the possibility that murine Abs in immunized mice bind to SRBCs in an unspecific manner, we immunized mice with SRBCs and a separate group of mice with NP-KLH. Serum was collected on days 0 and 7 of the immunization. On day 7, both groups of mice are expected to produce high levels of Abs against the respective Ag. As expected, significant levels of SRBC-specific Abs were detected only in the sera of mice immunized with SRBCs. Mice immunized with NP-KLH did not produce significant levels of SRBC-specific Abs, suggesting that murine Igs do not bind SRBCs in a nonspecific manner (Fig. 2A, 2B). To test whether this method is suitable to detect defects in Ab production, we immunized μMT-*Nik*$^{+/-}$ and μMT-*Nik*$^{-/-}$ chimeric mice. In these mice, all B cells originate from hematopoietic stem cells from *Nik*$^{+/-}$ and *Nik*$^{-/-}$ (7) mice, respectively. NIK is a central kinase in the alternative NF-κB pathway and is important for B cell development and survival. μMT mice reconstituted with *Nik*$^{-/-}$ bone marrow develop very few B cells, and the majority of them show a transitional B cell phenotype (Fig. 2C). We immunized these mice with SRBCs and measured SRBC-specific Abs from serum obtained 9 d after immunization. As expected, although levels of SRBC-specific IgM and IgG1 rose in μMT-*Nik*$^{+/-}$ mice after immunization, μMT-*Nik*$^{-/-}$ chimeric mice were not able to mount a strong Ab response (Fig. 2D). To further demonstrate that our method can detect changes in SRBC-specific Ab levels in a biologically relevant setting, we injected SRBC-immunized mice with the mTORC1 inhibitor rapamycin. mTORC1 is a central regulator of B cell metabolism and protein synthesis, and blocking mTORC1 activity in immunized mice prevents clonal expansion of B cells and leads to decreased plasma cell generation (8, 9). Consistently, 7 d after immunization with SRBCs, mice treated with vehicle only showed significantly higher levels of SRBC-specific IgM compared with mice treated with rapamycin (Fig. 2E). To better assess the differences in Ab titers between the two experimental groups, we calculated the concentration of SRBC-specific Abs using a reference serum. To this end, we randomly chose a serum from the group of mice treated with vehicle only, generated serial dilutions from this serum, and measured the MFI of SRBC-bound IgM. The obtained values were plotted against arbitrary units (AU) assigned to the different concentrations, and linear regression was calculated (Fig. 2F). Using the obtained standard curve, the concentration of SRBC-specific IgM in serum from the ex-

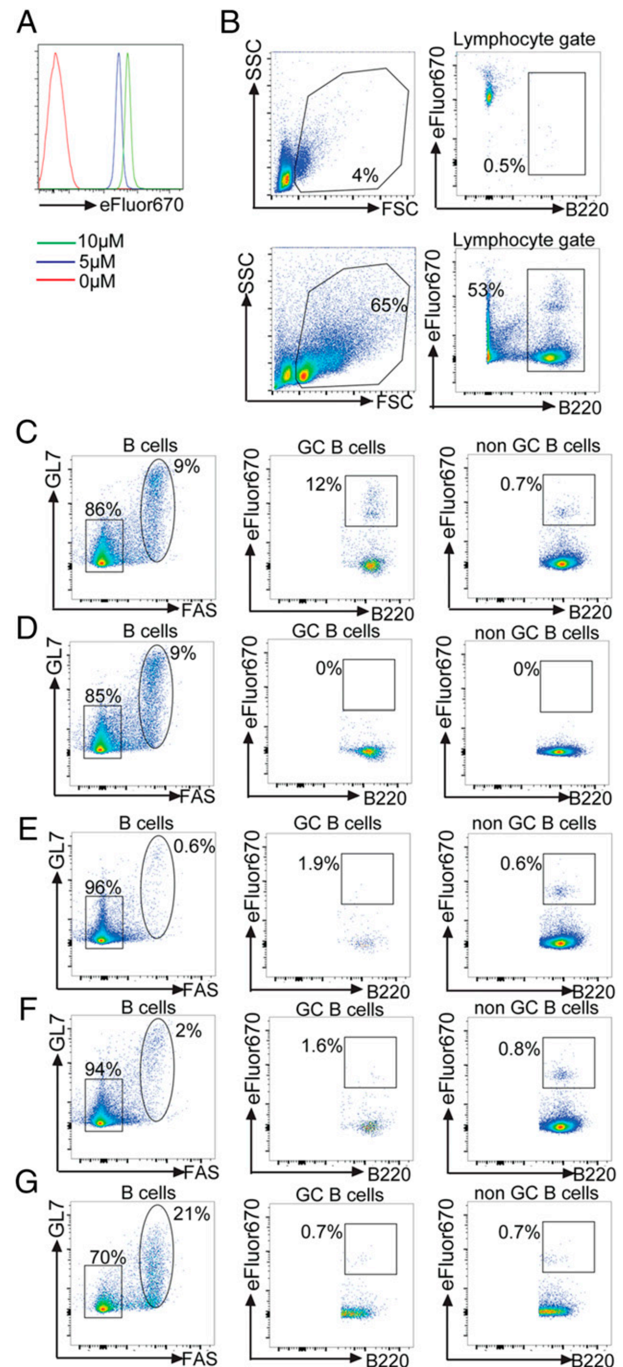

FIGURE 3. Detection of SRBC-specific B cells. (**A**) SRBCs were loaded with the indicated concentration of eFluor 670, and fluorescence was measured. (**B**) FSC/SSC profile of SRBCs loaded with eFluor 670 and their fluorescence intensity plotted against the signal detected in the PE-Cy7 channel, which was used in subsequent samples to measure B220 fluorescence (upper panel). FSC/SSC profile of splenic cells stained with SRBCs and B220 PE-Cy7 (lower panel). (**C**) Flow cytometric analysis of splenic B cells from an SRBC-immunized mouse. eFluor 670–loaded SRBCs were included in the stain. (**D**) Flow cytometric analysis of splenic B cells. eFluor 670–loaded SRBCs were not included in the stain. (**E**) Flow cytometric analysis of splenic B cells from a mouse that has not been immunized with SRBCs. eFluor 670–loaded SRBCs were included in the stain. (**F**) Flow cytometric analysis of splenic B cells from a mouse that has been immunized with NP-KLH. eFluor 670–loaded SRBCs were included in the stain. (**G**) Flow cytometric analysis of B cells from Peyer's patches from a mouse that has not been immunized. eFluor 670–loaded SRBCs were included in the stain.

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perimental mice was calculated (Fig. 2G). In our experiment, the mean concentration of SRBC-specific IgM in mice injected with vehicle only was 2.8 times higher than in mice treated with rapamycin (0.97 versus 0.35 AU), suggesting that rapamycin treatment does lead to an impaired humoral immune response. In summary, we show that, by using our method, differences in the production of SRBC-specific Abs in immunized mice can be measured and quantified.

Detection of SRBC-specific B cells

Since we demonstrated that binding of SRBC-specific Abs can be measured by flow cytometry on SRBCs, we decided to test whether binding of SRBCs to the surface-bound BCR can also be detected. For this assay, it was first necessary to label the SRBCs with a fluorescent dye. We chose eFluor 670, which stains any cellular proteins containing primary amines. We found that the dye strongly labels SRBCs in a concentration-dependent manner (Fig. 3A, 3B). Furthermore, we verified that SRBCs differ in their FSC/SSC properties from lymphocytes, making it possible to use FSC/SSC as one of the parameters to distinguish between free SRBCs and SRBCs bound by SRBC-specific B cells (Fig. 3B). Next, we tested whether the labeled SRBCs can be used to detect SRBC-specific B cells in a mixed population of splenic B cells. To this end, we immunized mice with SRBCs and sacrificed them 7 d later. At this time, GCs have formed (Fig. 3C, 3D), containing a population of cells expressing BCRs that can bind epitopes on SRBCs. We used anti-FAS, GL7, and anti-B220 Abs to discriminate between GC B cells and non-GC B cells and included eFluor 670–labeled SRBCs to stain for SRBC-specific B cells (Fig. 3C). As expected, we found a higher percentage of cells that have bound SRBCs in the population of GC B cells in comparison with non-GC B cells (Fig. 3C). B cells positive for eFluor 670 were not detected in samples that have not been incubated with eFluor 670–labeled SRBCs (Fig. 3D). As a control, we used splenocytes from unimmunized mice and stained the cells with the same Abs and eFluor 670–labeled SRBCs. As expected, few B cells bound to SRBCs (Fig. 3E). To exclude the possibility that GC B cells bound eFluor 670–labeled SRBCs in an unspecific manner, we determined the frequency of SRBC-binding B cells in the population of splenic GC B cells from mice immunized with NP-KLH. In addition, we also analyzed SRBC binding by GC B cells found in Peyer's patches, where GCs form spontaneously in response to gut microbiota (Figs. 3F, 3G, 4A). As expected, GC B cells formed after NP-KLH immunization or in response to a diverse set of Ags originating from intestinal microbiota contained few SRBC-binding cells (Figs. 3F, 3G, 4A). In conclusion, eFluor 670–labeled SRBCs can be used to detect SRBC-specific B cells, and significantly more SRBC-binding cells are present in GC than in non-GC B cells (Fig. 4A). After a productive GC response, memory B cells are generated to enable a faster immune response upon a secondary challenge. Memory B cells in mice are a heterogeneous group of cells, with a subset expressing the markers CD80 and CD73 (10). We sought to determine whether SRBC-binding cells in mice immunized with SRBCs also contain B cells of a memory B cell phenotype, to this end, we immunized mice with SRBCs on day 0, followed by a secondary immunization on day 14 to boost the humoral immune response, and sacrificed the mice on day 27. SRBC-binding cells present in the spleen on day 27 consisted of ~40% of B cells negative for the GC marker GL7 and positive for the memory B cell marker CD80 (Fig. 4B). Additionally, we found these cells to be positive for CD73, suggesting that eFluor 670–labeled SRBCs can be used to label Ag-specific memory B cells (Fig. 4B). In summary, we show that eFluor 670–labeled SRBCs can be used to detect Ag-specific B cells after a SRBC immunization, providing a new tool to analyze the humoral immune response to this Ag.

Discussion

In this article, we present two new methods to analyze B cell responses to SRBCs. SRBCs are a commonly used Ag that are frequently utilized to induce GC formation. Measuring the Ab response to SRBCs has also been used to assess immunotoxicity of chemical compounds (5, 6). Thus, methods facilitating the analysis of SRBC-induced humoral immune responses can be used in different experimental set-ups. In this article, we describe a simple and robust method to measure SRBC-specific Abs. We show that serum from SRBC-immunized mice can stain SRBCs in a concentration-dependent manner and that serum from nonimmunized mice or from mice immunized with a different Ag does not significantly stain SRBCs. We have used this method to test whether we can detect defects in Ab secretion in NIK-deficient mice, as well as in two previous studies in mice with a B cell–specific survivin and Gsk3 deficiency (11, 12). These molecules play essential roles in B cell development and proliferation (11–14); thus, these mice were expected to show defects in Ab production. Indeed, our method revealed impaired Ab production in all three of these mouse strains, demonstrating that this method is sensitive enough to detect defects in Ab production in genetically modified mice. Furthermore, we have shown that this method can be used to assess the inhibitory activity of chemical compounds, such as rapamycin, on Ab secretion. To date, analyzing the production of SRBC-specific Abs after SRBC immunization has been problematic because of weak attachment of SRBCs to ELISA plates, the complex procedure of

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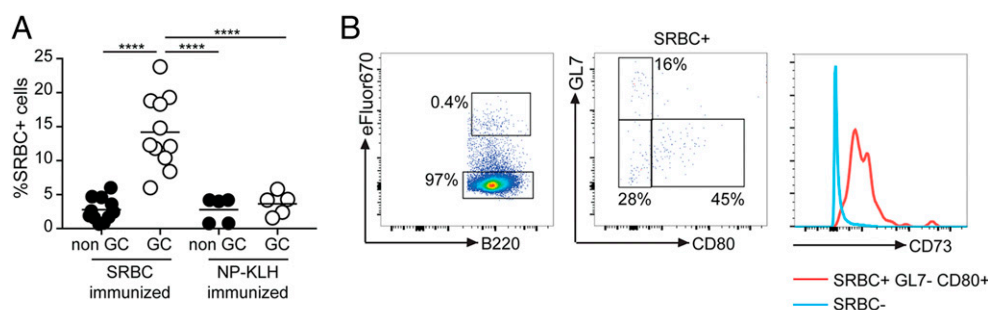

FIGURE 4. Binding of SRBCs to GC and memory B cells. (**A**) Summary of the percentage of SRBC-binding non-GC and GC B cells in mice immunized with SRBCs or NP-KLH and sacrificed 7 d later. Each circle represents an individual mouse. (**B**) Mice were immunized with SRBCs on day 0, boosted on day 14, and sacrificed on day 27. The plot shows SRBC binding of all B cells (left panel), expression of GL7 and CD80 by SRBC-binding B cells (middle panel), and CD73 expression by $GL7^{-}CD80^{+}SRBC^{+}$ cells (right panel). The plots are representative for five mice. ****$p \leq 0.0001$, ANOVA test.

preparing SRBCs for coating, and reactivity of mouse serum with sheep hemoglobin (5, 6). In comparison with the described ELISA protocols, our method has the advantage of being less time consuming and providing the opportunity to simultaneously measure SRBC-specific Abs of different isotypes. In addition, only 1.6 μl of serum is sufficient to produce four serial serum dilutions and to reliably assess SRBC-specific levels. Thus, the small sample size needed for the assay makes the experiments less stressful for the animals. Furthermore, we have developed an assay to stain for SRBC-binding GC B cells by flow cytometry. In the first step, SRBCs are labeled with a fluorescent dye. Subsequently, these cells are used to label B cells in a mixed splenic population. The ability to label SRBCs with a fluorescent dye could also be used in additional experimental set-ups. For example, these cells could be injected into mice, and the initial interaction of the Ag with different immune cells could be monitored by intravital imaging. We have used the stained cells to identify SRBC-binding B cells and have shown that the population of GC B cells contains, as expected, more SRBC-binding B cells in comparison with non-GC B cells. The ability to identify Ag-specific B cells allows us to specifically analyze the phenotype of these cells, to track their fate, and to exclude a polyclonal Ag–independent GC B cell expansion in any genetically modified mouse model of choice.

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Disclosures

The authors have no financial conflicts of interest.

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