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SHORT COMMUNICATION

Early B blasts acquire a capacity for Ig class switch recombination that is lost as they become plasmablasts

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Rapid production of neutralizing antibody can be critical for limiting the spread of infection. Such early antibody results when B-cell blasts mature directly to plasmablasts without forming germinal centers. These extrafollicular responses can involve Ig class switch recombination (CSR), producing antibody that can readily disseminate through infected tissues. The present study identifies the differentiation stage where CSR occurs in an extrafollicular response induced by 4-hydroxy-3-nitrophenyl acetyl (NP) conjugated to Ficoll (NP-Ficoll). To do this, we took advantage of the antigen dose dependency of CSR in this response. Thus, while both 30 and 1 μg NP-Ficoll induce plasmablasts, only the higher antigen dose induces CSR. Activation-induce cytidine deaminase (AID) is critical for CSR and in keeping with this a proportion of NP-specific B-cell blasts induced by 30 μg NP-Ficoll express AID. None of the B blasts responding to the non-CSR-inducing 1 μg dose of NP-Ficoll express AID. We confirmed that CSR occurs in B blasts by demonstrating the presence of rearranged heavy-chain transcripts in B blasts in the 30 μg response. CSR in this extrafollicular response is confined to B blasts, because NP-specific plasmablasts, identified by expressing CD138 and Blimp-1, no longer express AID and cannot undergo CSR.

Key words: Antibodies · B cells · cell differentiation · spleen

Supporting Information available online

Introduction

The rapid induction of neutralizing antibody can be a critical factor in limiting the spread of the extracellular infection [1–3]. This is achieved by direct antigen-driven differentiation of B cells into B blasts and then plasmablasts and plasma cells without going through an affinity maturation stage in germinal centers [4–6]. For this reason, the rapid route to antibody production is commonly referred to as the extrafollicular response, and these lead to the production of nonswitched and switched antibodies. While the induction of class switch recombination (CSR) seems to happen at the follicle – T-zone interphase [6], the earliest class-switched B cells have been observed in follicles [7]. The object of the present study is to identify differentiation stages during extrafollicular antibody responses when CSR occurs, and whether these relate to germinal center (GC) or plasmablast differentiation. To do this, we have studied B cells responding in vivo to the thymus-independent type 2 (TI-2) antigen 4-hydroxy-3-nitrophenyl acetyl conjugated to Ficoll (NP-Ficoll). The characterization of cells at the single-cell level early in the response has been facilitated by the use of F1 hybrids of QM and C57BL/6 mice (QMxB6 mice). These hybrids have one copy of a targeted

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insertion of a rearranged NP-specific Ig heavy chain [8]. The 5% of B cells that have a productive lambda light chain rearrangement in these mice are specific for NP [9].

NP-Ficoll induces an impressive extrafollicular antibody response in QMxB6 mice. In addition, at high antigen doses, it can induce GCs [10]. These GCs, however, are abortive, for the GC B cells enter apoptosis at the stage when their continued survival depends on antigen-specific selection by T cells. In this study, we use a combination of flow cytometry with single-cell sorting and real-time RT-PCR to identify responding B blasts from plasmablasts and GC-founding cells. Differentiation of B blasts toward plasmablasts or GC B cells is tightly regulated by a network of transcription factors. We used the expression of three of these to distinguish B blasts, plasmablasts, and GC-founding cells. The main transcription factor associated with confirmed differentiation toward the plasmablast phenotype is Blimp-1, encoded by the gene Prdm1 [11]. Blimp-1 in combination with surface expression of CD138 is used to identify plasmablasts. The master regulator of GC B-cell commitment is Bcl6. Bcl6 and Blimp-1 mutually repress each other [12–14]. The coexpression of activation-induced cytidine deaminase (AID) with Bcl6 is used to identify GC-founding cells. AID is essential for CSR and is required for Ig V-region hypermutation in GCs [15]. It is well documented that CSR occurs in GCs [16]. The question addressed below is whether AID is expressed in B blasts or plasmablasts or both of these cell types as they develop along the extrafollicular pathway, and whether this expression is associated with CSR.

Results and discussion

CSR occurs in B blasts rather than plasmablasts responding to NP-Ficoll

To identify where and when CSR occurs in the response to NP-Ficoll, it is necessary to summarize the pattern of migration and differentiation of NP-specific B cells in responses to NP-Ficoll. As in WT mice [17] NP-specific cells from QMxB6 mice move from the marginal zone and follicles to the T zone within 8 h of immunization with 30 μg NP-Ficoll (Fig. 1A, top two panels). The responding cells enter cell cycle by 24 h after immunization (data not shown). By 48 h postimmunization proliferation has increased the number of NP-specific cells (Fig. 1B, top) and B blasts are spread throughout the white pulp (Fig. 1A, lower left). The white arrowheads in this photomicrograph identify the first $CD138^+$ plasmablasts, which have appeared where the red pulp abuts to the T zone. This is reflected by an increase in NP^+CD138^+ cells seen by flow cytometry (Fig. 1B, bottom). By 72 h large accumulations of $CD138^+$ plasmablasts fill the red pulp, although there are still many B blasts in the white pulp (Fig. 1A, center bottom panel). Clusters of $Bcl6^+$ NP-specific GC-founding B blasts first appear in the center of follicles at 72 h, whereas Bcl6 expression is not seen prior to immunization or in nonresponding naïve B cells (Fig. 1A, bottom right). Quantitative immunohistology shows that by 72 h a significant proportion of the plasmablasts have switched to IgG3 (Fig. 1D, left panel).

To determine whether CSR induced by 30 μg NP-Ficoll takes place in the B blasts, or plasmablasts, or both of these cell types, responding B cells were sorted by flow cytometry (Fig. 1C). NP-specific B cells were identified as $B220^+$ cells that bind NP-conjugated to phycoerythrin (PE). At 24 h after immunization, NP-PE binding by NP-specific cells is markedly reduced, probably due to competition for BCR ligation with the immunogen and/or BCR internalization. To detect NP-specific cells at this time point, mice were immunized with NP-FITC-Ficoll and FITC-positive cells were sorted (cells shown in red in Fig. 1B inset). The expression of CD138 (blue gate) was used to distinguish plasmablasts from B blasts (red gate, which includes GC founding cells).

Bulk sorts of NP-specific cells at 24 h intervals post-immunization were analyzed to identify the appearance of heavy-chain transcripts containing Iμ apposed to IgG3 heavy-chain genes (Fig. 1D, center panel). These rearranged heavy-chain transcripts can only be expressed in cells that have completed CSR to IgG3 [18], and are a good indicator of the frequency of class-switched cells, as the Iμ exon is constitutively active [19]. There is a significant accumulation of the Iμ-Cγ3 transcripts in the B-blast population sorted at 48 h. Both the plasmablast and the B-blast populations have higher levels of these transcripts at 72 h (Fig. 1D, center).

These observations indicate that CSR does occur in B blasts in this response but not if this process continues in plasmablasts. To probe this, the same bulk sorts were tested for the expression of *Aicda* transcripts. This shows that some cells in the B-blast fractions at 48 and 72 h express AID, whereas the plasmablast fraction does not (Fig. 1D, right panel). At this time, UNG mRNA has also been induced (Supporting Information Fig. 1), but blasts still express Pax5 mRNA, the main transcription factor associated with B-cell phenotype, and do not express XBP1 mRNA, which drives Ig secretion in plasma cells (Supporting Information Fig. 1) These results show that by the time cells responding to NP-Ficoll express CD138 they have lost the capacity to initiate further CSR. At 48 h after immunization, B blasts are found in all compartments of the white pulp. Class-switched B blasts, as opposed to GC blasts, have also been identified in follicles and T zones at the early stages of T-dependent responses [7, 20]. The precise features of the microenvironments within the white pulp where CSR occurs in B blasts remain to be determined.

Single-cell analysis confirms that a proportion of extrafollicular B blasts express AID, while plasmablasts do not

To determine the proportion of B blasts that express AID transcripts and whether these have transcriptional regulation similar to emerging extrafollicular plasmablasts or GC founding cells, multiplex real-time RT-PCR was carried out on single cells. Figure 2A–D show representative individual experiments in which single NP-specific cells were sorted into the wells of

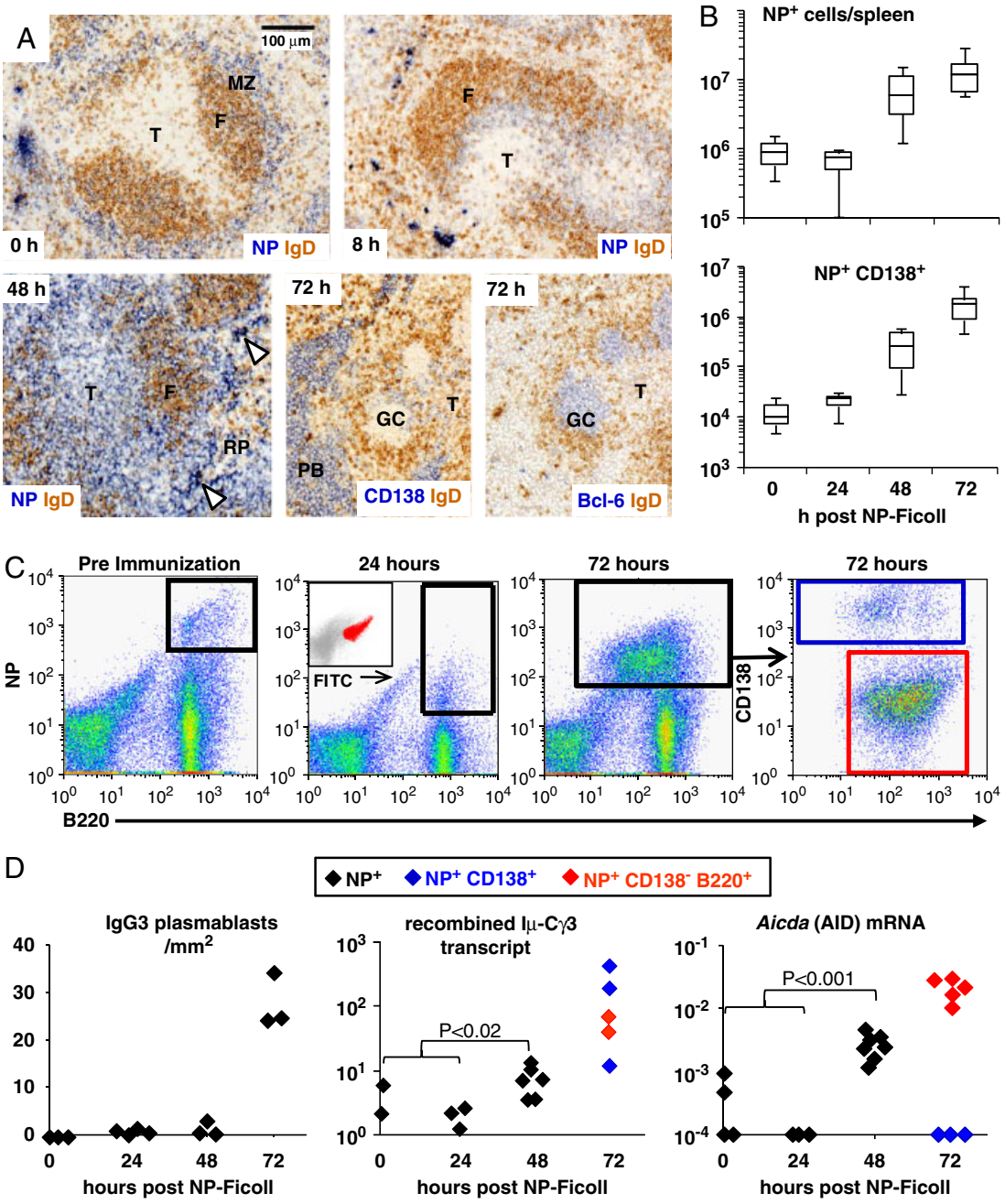

Figure 1. The emergence of plasmablasts, GC-founding cells, and Ig class switching. QMxB6 mice were immunized i.p. with 30 μg NP-Ficoll. (A) The location and differentiation of NP-specific B cells in the spleen are shown by immunohistology. Before immunization (top left, 0 h), NP-binding B cells (blue) are located mainly in the marginal zone (MZ) and follicles (F). Follicles are identified by the presence of IgD^+ B cells (brown). By 8 h (top right), the NP-specific B cells are located in the outer T zone (T). At 48 h (bottom left) NP-binding cells fill the white pulp; arrowheads indicate darker-staining plasmablasts at the red pulp (RP)-T zone junction. At 72 h $CD138^+$ red pulp plasmablasts (PB) stained for CD138 (blue) crowd the red pulp (bottom center) and clusters of $Bcl6^+$ germinal center-founding cells fill the follicle centers (bottom right). (B) Total numbers of NP-binding cells and $CD138^+$ NP-binding plasmablasts and plasma cells, determined by flow cytometry. Boxes represent median and lower and upper quartile, whiskers represent minimum and maximum values. (C) Flow cytometric sorting of NP-binding B cells responding to NP-Ficoll. In all, 5% of C57B6xQM mouse B cells are NP-specific preimmunization (left); 24 h after immunization B cells bind NP-PE less efficiently (middle left), and are detected via NP-FITC-Ficoll staining used as immunogen (red, inset); 72 h after immunization NP-binding B cells are divided into two fractions (right): $CD138^-$ $B220^+$ and $CD138^+$. (A–C) In total, 4–12 animals were used per time point in three independent experiments. (D) Increase in the number of IgG3 expressing cells, assessed by immunohistochemistry (left). The relative amounts of recombined Iμ-Cγ3 heavy-chain transcripts (center) or *Aicda* mRNA (right) assessed by real-time RT-PCR in NP-binding cells sorted using the gates shown in (B). The colors of the diamonds correspond to the colors of the gates in (C). Each diamond represents data from sorted cells of one mouse from three independent experiments. Statistics indicate Mann–Whitney *U*-test results.

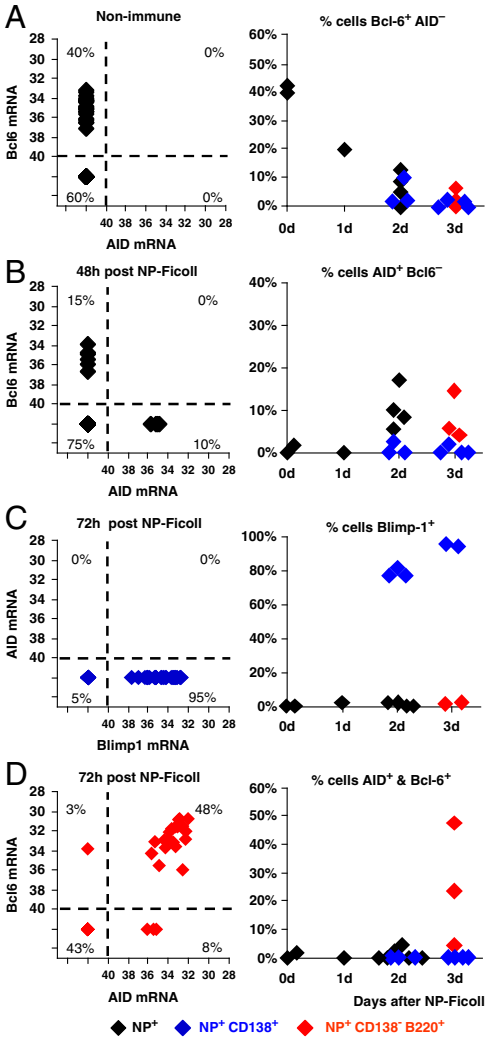

Figure 2. Transcriptional differentiation of B blasts, plasmablasts, and GC-founding cells. Single cells from the spleens of QMxB6 mice immunized with 30 μg NP-Ficoll were FACS-sorted into 384-well plates using the gates shown in Fig. 1C. The mRNA levels for AID and Bcl6, or AID and Blimp-1 in each cell were determined by real-time RT-PCR. (A) Left: Bcl6 and AID mRNA expression in nonimmune NP-binding B cells. Each diamond corresponds to one cell. Data are from one representative animal. Right: the percentage of cells that are $Bcl6^+$ AID^- in individual mice (each diamond corresponds to one mouse, derived from three independent experiments. Color of diamond indicates population as in Fig. 1C and D. (B) Left: Bcl6 and AID mRNA expression at 48 h in NP-binding B cells. Right: the percentage of cells from different sorted subsets that are AID^+ $Bcl6^-$. (C) Left: AID and Blimp-1 mRNA expression at 72 h in individual NP-binding $CD138^+$ cells. Right: percentage of cells in each subset expressing Blimp-1 and no AID mRNA. (D) Left: expression of Bcl6 and AID mRNA in NP-binding $B220^+$ $CD138^-$ cells. Right: The percentage of cells from the sorted cell subsets that are AID^+ and $Bcl6^+$. Numbers on the horizontal and vertical axes in the single-cell PCR plots (left graphs) represent the PCR cycle when signal above threshold was reached (C_t), which corresponds to a log2 scale of mRNA quantity per cell. Percentages indicate the proportion of cells in each quadrant.

384-well plates on the left hand side. They were then assessed for the expression of mRNA for AID with Bcl6, or AID with Blimp-1. To exclude empty wells, each well was assessed for the constitutively expressed β2-microglobulin – as described in the *Materials and methods*. Graphs on the right-hand side show a summary of the single-cell RT-PCR results from all the individual experiments.

Neither AID nor Blimp-1 was expressed in NP-PE-binding $B220^+$ cells before immunization (Fig. 2B and C, right side). By contrast, some 40% of these cells express Bcl6 message (Fig. 2A). Despite the presence of Bcl6 mRNA, naïve B cells express little or no Bcl6 protein at levels detectable by immunoenzymatic staining (Fig. 1A and [21]). Bcl6 mRNA is progressively lost following immunization (Fig. 2A), and is only re-expressed in germinal center-founding cells 72 h after immunization (Fig. 2D). The appearance of cells coexpressing Bcl6 and AID mRNA at high levels (median 10× higher) coincides with the appearance of clusters of NP-specific cells in follicle centers that express Bcl6 protein, as shown by immunohistology (Fig. 1A, bottom right).

Figure 2B confirms that AID is induced in B blasts. AID mRNA is first found in B blasts 48 h post-NP-Ficoll. At this stage, when no $AID^+/Bcl6^+$ coexpressing GC-founding cells are present (Fig. 2D, right), AID mRNA is expressed in the absence of Bcl6 mRNA in around 10% of B blasts. Cells with this phenotype are still present at 72 h (Fig. 2B, right). In striking contrast, $CD138^+$ NP-binding cells do not express AID. This even applies to the small number of early plasmablasts that are present at 48 h after immunization. At this stage, a median of 80% of the $CD138^+$ cells express Blimp-1 mRNA, whereas >95% of these cells express Blimp-1 at 72 h. The presence of a significant minority of $CD138^+$ cells that do not express Blimp-1 raises the question whether this regulator of plasmablast differentiation is the sole repressor of AID. Out of 683 cells studied in total, only 3 showed coexpression of Bcl6 and Blimp-1.

The antigen dose dependency of AID induction and switching induced by NP-Ficoll

We have previously reported that higher doses of NP-Ficoll are required to induce the NP-specific B cells of QM mice to produce GCs than are needed to induce the production of extrafollicular plasmablasts [10]. Serendipitously, on reviewing this earlier study, we identified an effect of antigen dose on CSR. While 1 μg of NP-Ficoll is sufficient to induce a significant number of NP-specific B blasts to mature into plasmablasts (Fig. 3A, B and Supporting Information Fig. 2), it does not induce CSR (Fig. 3D). The expression of the proliferation marker Ki-67 distinguishes the plasmablasts induced by 1 μg NP-Ficoll from the background plasma cells seen in QMxB6 mice, which typically are not proliferating (Fig. 3A and C). NP-binding plasmablasts and B blasts were sorted from mice 48 h after immunizing with 1 or 30 μg of NP-Ficoll and the expression of AID message was again assessed by single-cell real-time RT-PCR (Fig. 3E). AID was expressed by a proportion of the B blasts from mice immunized with 30 μg of NP-Ficoll, but was not expressed in any cells from mice immunized with 1 μg of NP-Ficoll, confirming that early expression of AID is related to CSR in extrafollicular B differentiation.

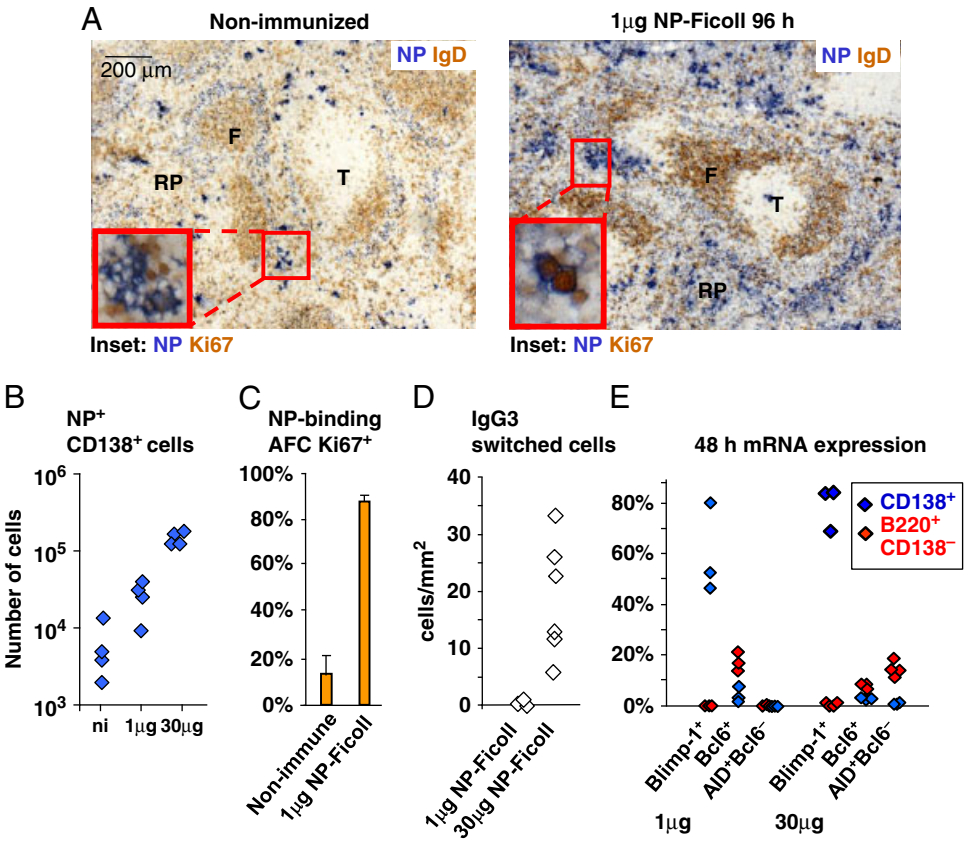

Figure 3. The antigen dose dependency of AID-induction and switching induced by NP-Ficoll. QMxB6 mice were immunized i.p. with either 30 or 1 μg of NP-Ficoll. (A) The induction of NP-specific plasmablasts (blue) in the red pulp of mice by 1 μg NP-Ficoll either before immunization (left), or 96 h after immunization (right) is shown. Red boxes show magnifications of the same clusters of plasma cells or plasmablasts in serial sections stained for the proliferation-associated marker – Ki-76 (brown) and NP-binding (blue). (B) Flow cytometric quantification of numbers of $CD138^+$ NP-binding cells in the spleen of mice immunized 72 h after immunization with 1 or 30 μg NP-Ficoll. (C) The proportion of NP-specific plasmablasts that are $Ki\text{-}67^+$ in spleen sections 96 h after immunization with 1 μg NP-Ficoll, as shown in (A). Data are mean+SD of $n = 4$ mice. (D) Assessment of the numbers of plasmablasts switched to IgG3 in sections of spleen taken 96 h after immunizing with 1 or 30 μg. (E) The proportion of NP-binding cells from the spleens of QMxB6 mice 48 h after immunization with 1 or 30 μg NP-Ficoll that express mRNA for Blimp-1, or Bcl6, or AID without Bcl6 assessed by single-cell real-time RT-PCR. (A, B, D, and E) Data are representative of two independent experiments.

Concluding remarks

Although CSR is associated with B-cell proliferation and differentiation in germinal centers [22, 23], CSR is also induced during extrafollicular plasmablast differentiation. During the initial response to T-dependent antigens [6], and in responses to thymus-independent type 2 antigens, it is the only pathway of productive B-cell differentiation and CSR [17]. We show here that AID induced by NP-Ficoll is expressed at lower levels than in GC blasts and is not coexpressed with Bcl6. Germinal center independent class switching may represent an ancient pathway of AID induction that developed before proper germinal centers evolved, as is seen in lower vertebrates that develop plasma cells and undergo CSR in the absence of germinal centers [24]. CSR occurring before affinity maturation happens may seem counterproductive, as a switch from IgM to IgG leads to a loss of avidity of the resulting antibody. On the other hand, infections often induce efficient extrafollicular plasmablast differentiation, whereas germinal center development is delayed. The gain of additional effector function from switched immunoglobulin may be critical in providing early protective immunity from life-threatening infections [25].

Materials and methods

Animals and immunizations

QM mice [8] (backcrossed to C57BL/6J for >10 generations) were bred under specific pathogen-free conditions in the Biomedical Services Unit, University of Birmingham. QMxB6 mice were generated by crossing mice homozygous for the NP-specific (VH17.2.25-DSP2.3-JH4) Ig heavy-chain segment of QM mice and κ light chain deficient with C57BL/6 mice to generate mice with one copy of the NP-specific QM IgH and κ light-chain genes. Animal experiments were licensed by the British Home

Office according to the Animals Scientific Procedures Act 1986 and approved by the University of Birmingham Biomedical Ethical Review Subcommittee.

Immunizations

Mice were immunized i.p. with either 30 or 1 μg NP40-Ficoll or, when 24 h time points were taken, 30 μg NP-fluorescein-Ficoll (Biosearch Technologies, Novato, CA, USA).

Antigen-specific B-cell staining and isolation

Location of antigen-specific idiotype-positive B cells in frozen spleen sections was detected by immunohistology as described previously [6]. Briefly, acetone-fixed frozen spleen sections (6 μm) were stained using rat anti-mouse IgM, IgD, or CD138 (BD Biosciences, Oxford, UK), rat anti-mouse IgG3 (Serotec, Oxford, UK), sheep anti-mouse IgD (The Binding site, Birmingham, UK), rabbit anti-mouse Bcl6 1/30 (Santa Cruz Biotechnology, CA, USA), or NP conjugated to rabbit Ig. Secondary antibodies conjugated to biotin or horseradish peroxidase were applied. The biotinylated secondary antibodies were detected using biotin-conjugated StreptABComplex-alkaline phosphatase complex (Dako, Ely, UK).

Immunostaining of single-cell splenocyte suspensions following immunization with NP-Ficoll used B220-FITC (eBioscience), CD138-APC, or CD138-biotin, followed by streptavidin-PerCpCy5.5 or streptavidin-APC (BD Biosciences), and NP-PE (Biosearch Technologies). Antigen-specific B cells 24 h after immunization with NP-FITC-Ficoll were identified by their FITC uptake and NP-PE plus B220-APC staining. Cell sorting was carried out on a MoFlo cell sorter (DakoCytomation). Sorted populations were checked for purity and frozen at −80°C immediately after sorting.

Real-time RT-PCR

RNA was extracted from frozen cell pellets. Iμ-Cγ3 transcript was detected by real-time RT-PCR with primers TCTGGACCTCTCCGAAACCA and ACCGAGGATCCAGATGTGTCA together with the FAM-BHQ-labeled probe CTGTCTATCCCTTGGTCCCTGGCTGC (Eurogentec, Southampton, UK) in multiplex with β-actin-specific primers as described previously [25]. AID, Pax5, UNG, and XBP1 were detected using TaqMan gene expression assays Mm00507774_m1, Mm00435501_m1, Mm00449156_m1, and Mm00457359_m1 (Applied Biosystems, Foster City, CA, USA).

Single-cell RT-PCR

Single cells were sorted into wells, containing 1 μL nuclease-free water, of 384-well PCR plates using an automatic cell cloning unit of a MoFlo cell sorter. Serial dilutions of 0–32 cells per well served as positive and one row without cells as negative controls. After sorting, plates were stored and frozen at −80°C. Triplex real-time RT-PCR used primers in limiting concentrations with QuantiTect Multiplex RT-PCR buffer (Qiagen, Crawley, UK) in a final volume of 6 μL. The following primers and probes were used: β2-microglobulin (CTGCAGAGTTAAGCATGCCAGTAT 100 nM, ATCACATGTCTCGATCCCAGTAGA 100 nM, NED-CGAGCCCAAGACC-MGB, Applied Biosystems). Blimp-1 (CAAGAATGCCAACAGGAAGTATTTT 80 nM, CCATCAATGAAGTGGTGGAACTC 100 nM, FAM-TCTCTGGAATAGATCCGCCA-MGB, Applied Biosystems), Aicda (GTCCGGCTAACCAGACAACTTC 60 nM, GCTTTCAAAATCCCAACATACGA 100 nM, TET-TGCATCTCGCAAGTCATCGACTT CGT-BHQ1, Eurogentec). Bcl6 was detected with primers (CAGA CGCACAGTGACAAACCA, 60 nM, ACTGCGCTCCACAAATGTTACA 300 nM) and probe FAM-CAGCCACAAGACTGTCCACACGGGT-BHQ1 (Eurogentec) in multiplex with Aicda, or CalFluor560-CAGCCACAAGACTGTCCACACGGGT-BHQ1 (BioSearch Technologies) in multiplex with Blimp-1. Reactions were run for 40 cycles in a 7900 Real-Time PCR System (Applied Biosystems). Data from wells that were positive for β2-microglobulin mRNA were plotted as cycle number at which target gene was higher than threshold (C_t) in reverse order, which corresponds to mRNA quantity per cell on a log2 scale. Preliminary experiments showed that this setup produced semi-quantitative results with good negative correlation between C_t and log2 of the amount of template mRNA (Supporting Information Fig. 3).

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **AID:** activation-induced cytidine deaminase · **CSR:** class switch recombination · **NP:** 4-hydroxy-3-nitrophenyl acetyl · **PE:** phycoerythrin · **QM:** quasimonoclonal · **QMxB6 mice:** F1 hybrids of QM and C57BL/6 mice

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