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

Jennifer L. Marshall, Yang Zhang, Lalit Pallan, Mei-Chi Hsu, Mahmood Khan, Adam C. Cunningham, Ian C. M. MacLennan, Kai-Michael Toellner

MRC Centre for Immune Regulation, the School of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom

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Correspondence to:
Kai-Michael Toellner
MRC Centre for Immune Regulation,
Room 432, IBR,
University of Birmingham,
Birmingham B15 2TT,
United Kingdom
Tel.: +44 121 41 58687
FAX: +44 121 41 43599
Email: K.M.Toellner@bham.ac.uk

Abbreviations: QM: Quasi Monoclonal, CSR: Class switch recombination, NP: 4-hydroxy-3-nitrophenyl acetyl, TI-2: thymus-independent type 2, QMxB6 mice: F1 hybrids of quasimonoclonal and C57BL/6 mice

Abstract

Rapid production of neutralizing antibody can be critical for limiting the spread of infection. Such early antibody results when B blasts mature directly to plasmablasts without forming germinal centres. 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. To do this we took advantage of the antigen dose dependency of CSR in this response. Thus, while both 30 μg and 1 μg NP-Ficoll induced plasmablasts, only the higher antigen dose induces CSR. Activation-induced cytidine deaminase (AID) is critical for CSR and in keeping with this a proportion of NP-specific B 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, for NP-specific plasmablasts, identified by expressing CD138 and Blimp1, no longer express AID and cannot undergo CSR.

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 centres [4-6]. For this reason the rapid route to antibody production is commonly referred to as extrafollicular responses, and these lead to the production of non-switched and switched antibodies. While the induction of 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 GC or plasmablast differentiation. To do this we have studied B cells responding *in vivo* to the thymus-independent type 2 (TI-2) antigen – 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 quasimonoclonal (QM) and C57BL/6 mice (QMxB6 mice). These hybrids have one copy of a targeted insertion of a rearranged NP-specific Ig heavy chain [8]. The 5 percent of B cells that have a productive lambda light chain rearrangement in these mice are specific for NP [9].

NP-Ficoll induces an impressive extra-follicular antibody response in QMxB6 mice. In addition, at high antigen doses it can induce (GC) [10]. These, 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 towards 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 towards the plasmablast phenotype is

Blimp1, encoded by the gene Prdm1 [11]. Blimp1 in combination with surface expression of CD138 is used to identify plasmablasts. The master regulator of GC B cell commitment is Bcl6. Bcl6 and Blimp1 mutually repress each other [12-14]. The co-expression of AID with Bcl6 is used to identify GC founding cells. As mentioned above AID is essential for CSR and in GC is required for Ig V-region hypermutation [15]. It is well documented that CSR occurs in GC [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 wild type mice [17] NP-specific cells from QMxB6 mice move from the marginal zone and follicles to the T zone within 8h of immunization with 30 μg NP-Ficoll (Fig 1A top two panels). The responding cells enter cell cycle by 24h after immunization (not shown). By 48h post immunization 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 72h large accumulations of $CD138^+$ plasmablasts fill the red pulp, although there are still many B blasts in the white pulp (Fig 1A centre bottom panel). Clusters of $Bcl6^+$ NP-specific GC-founding B blasts first appear in the centre of follicles at 72h, while Bcl6 expression is not seen prior to immunization or in non-responding naive B cells (Fig 1A bottom right). Quantitative

immunohistology shows that by 72h 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 24h 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 24h intervals post immunization were analyzed to identify the appearance of heavy chain transcripts containing Iμ apposed to IgG3 heavy chain genes (Fig 1D centre 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-γ3 transcripts in the B blast population sorted at 48h. Both the plasmablast and B blast populations have higher levels of these transcripts at 72h (Fig 1D centre).

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 48h and 72h express AID, while the plasmablast fraction does not (Fig 1D right panel). At this time UNG mRNA has also been induced (suppl. 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 (suppl. 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 48h 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 remains to be determined.

Single cell analysis confirms that a proportion of extrafollicular B blasts and GC founding 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. The left hand set of graphs in Fig 2 show representative individual experiments in which single NP-specific cells were sorted into the wells of 384-well plates. They were then assessed for expression of mRNA for AID with Bcl6, or AID with Blimp1. To exclude empty wells, each well was assessed for the constitutively-expressed β2microglobulin - as outlined in the methods. The right hand set of graphs shows 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 2 B, C right side). By contrast some 40% of these cells express Bcl-6 message (Fig 2 A). Despite presence of Bcl-6 mRNA, naïve B cells express little or no Bcl-6 protein at levels detectable by immunoenzymatic staining (Fig.1A and [21]). Bcl-6 mRNA is progressively lost following immunization (Fig. 2A), and is only re-expressed in germinal centre founding

cells 72h after immunization (Fig. 2D). The appearance of cells coexpressing Bcl-6 and AID mRNA at high levels (median 10x higher) coincides with the appearance of clusters of NP-specific cells in follicle centres that express Bcl6 protein, as shown by immunohistology (Fig 1A bottom right).

Fig 2B confirms that AID is induced in B blasts. AID mRNA is first found in B blasts 48h post NP-Ficoll. At this stage, when no $AID^+/Bcl\text{-}6^+$ coexpressing GC founding cells are present (Fig 2 D 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 72h (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 48h after immunization. At this stage a median of 80% of the $CD138^+$ cells express Blimp-1 mRNA while >95% of these cells express Blimp-1 at 72h. 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 co-expression of Bcl6 and Blimp1.

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 GC than are needed to induce the production of extra-follicular 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 and suppl. 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 48h after immunizing with 1 μg 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.

Concluding remarks

Although CSR is associated with B cell proliferation and differentiation in germinal centres [22, 23], CSR is also induced during extrafollicular plasmablast differentiation. During the initial response to T dependent antigens [6], and in responses to TI-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 centre independent class switching may represent an ancient pathway of AID induction that developed before proper germinal centres evolved, as is seen in lower vertebrates that develop plasma cells and undergo CSR in the absence of germinal centres [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, while germinal centre 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].

Material 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 μg or 1 μg NP40-Ficoll or, when 24h time points were taken, 30 μg NP-fluorescein-Ficoll (Biosearch Technologies, Novato, CA).

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 CD3, 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), 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-phycoerythrin (Biosearch Technologies). Antigen-specific B cells 24h after immunization with NP-FITC-

Ficoll were identified by their FITC uptake and NP-phycoerythrin plus B220-APC staining. Cell sorting was carried out on a MoFlo cell sorter (DakoCytomation). Sorted populations were checked for purity and frozen at -80ºC 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 labelled probe CTGTCTATCCCTTGGTCCCTGGCTGC (Eurogentec, Southampton, UK) in multiplex with β-actin specific primers as described [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).

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 to 32 cells per well served as positive and one row without cells as negative controls. After sorting plates were stored frozen at -80ºC. 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). Blimp1 (CAAGAATGCCAACAGGAAGTATTTT 80nM, CCATCAATGAAGTGGTGGAACTC 100 nM, FAM-TCTCTGGAATAGATCCGCCA-MGB, Applied Biosystems), Aicda (GTCCGGCTAACCAGACAACTTC 60 nM, GCTTTCAAAATCCCAACATACGA 100 nM, TET-TGCATCTCGCAAGTCATCGACTTCGT-BHQ1, Eurogentec). Bcl6 was detected

with primers (CAGACGCACAGTGACAAACCA, 60 nM, ACTGCGCTCCACAAATGTTACA 300 nM) and probe FAM-CAGCCACAAGACTGTCCACACGGGT-BHQ1 (Eurogentec) in multiplex with Aicda, or CalFluor560-CAGCCACAAGACTGTCCACACGGGT-BHQ1 (BioSearch Technologies) in multiplex with Blimp1. Reactions were run for 40 cycles in a 7900 Real-Time PCR System (Applied Biosystems). Data from wells that were positive for β2microglobulin mRNA were plotted as cycle number at which target gene was higher than threshold (Ct) 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 Ct and log2 of the amount of template mRNA (suppl. Fig. 3).

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Legends to figures:

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 are shown by immunohistology. Before immunization (top left, 0h) NP-binding B cells (blue) are located mainly in marginal zone (MZ) and follicles (F). Follicles are identified by the presence of IgD^+ B cells (brown). By 8h (top right) the NP-specific B cells are located in the outer T zone (T). At 48h (bottom left) NP-binding cells fill the white pulp and arrow heads indicate small numbers of darker-staining plasmablasts at the red pulp (RP)-T zone junction. At 72h $CD138^+$ red pulp plasmablasts (PB) stained for CD138 (blue) crowd the red pulp (bottom centre) and clusters of $Bcl6^+$ germinal centre founding cells fill the follicle centres (bottom right). B) Total numbers of NP-binding cells and the numbers of $CD138^+$NP-binding plasmablasts and plasma cells, determined by flow cytometry. C) Flow cytometric sorting of NP-binding B cells responding to NP-Ficoll. Some 5% of C57B6xQM mouse B cells are NP-specific pre immunization (left); 24h after immunization B cells bind NP-phycoerythrin less efficiently (middle left), and are detected via NP-FITC-Ficoll staining used as immunogen (red, inset); 72h after immunization NP-binding B cells are divided into two fractions (right): $CD138^-$ $B220^+$ and $CD138^+$. D) Increase in the number of IgG3 expressing cells, assessed by immunohistochemistry (left). The relative amounts of recombined Iμ-Cγ3 heavy chain transcripts (centre) or *Aicda* mRNA (right) assessed by real-time RT-PCR in NP-binding cells sorted using the gates shown in B. Each diamond represents the data from the sorted cells from one mouse. The colours or the

diamonds correspond to the colours of the gates in (C). Statistics indicate Mann Whitney U test results.

Figure 2: Transcriptional differentiation of B blasts, plasmablasts, and GC founding cells

Single NP-binding cells from the spleens of QMxB6 mice immunized with 30 μg NP-Ficoll were FACS sorted into the wells of 384-well plates using the gates shown in Figure 1C. The mRNA levels for AID and Bcl6, or AID and Blimp1 in each cell were determined by real time RT-PCR. Left hand column of figures shows the level of indicated mRNAs in individual cells in a single experiment (each diamond corresponds to one cell). Numbers on the horizontal and vertical axes represent the PCR cycle when signal above threshold was reached (C_t), corresponding to a log2 scale of mRNA quantity per cell. Percentages indicate the proportion of cells in each quadrant. The right hand column of figures shows the percentages of cells expressing the indicated mRNAs over time in the different FACS-sorted cell populations, with each diamond representing one experiment.

Figure 3: *The antigen-dose-dependency of AID-induction and switching induced by NP-Ficoll*

QMxB6 mice were immunized i.p. with either 30 μg 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. Left is before immunization, right taken 96h after immunization. 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) NP-binding (blue). B) Flow cytometric quantification of numbers of $CD138^+$ NP-binding cells in the spleen of mice immunized 72h after immunization with 1 μg or 30 μg NP-Ficoll. C) The proportion of NP-specific plasmablasts that are $Ki\text{-}67^+$ before seen on spleen sections 96h after immunization with 1 μg NP-Ficoll (± SD). D) Assessment of the numbers of plasmablasts switched to IgG3 in sections of spleen taken 96h after immunizing with 1μg or 30 μg. E) The proportion of NP-binding cells from

the spleens of QMxB6 mice 48h after immunization with1 μg or 30 μg NP-Ficoll that express mRNA for Blimp1, or Bcl6, or AID without Bcl6 assessed by single cell real time RT-PCR.