

Novel approach for identifying memory B cells during immune responses

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Summary

Measurement of antibody production is a critical feature in vaccine design. However, the production and persistence of memory B (B^{mem}) cells may be more informative for long-term humoral protection against pathogens. In addition to the frequency of available B^{mem} cells, the heterogeneity of B^{mem} cells may influence protective responses. To determine the level of heterogeneity, production and persistence of B^{mem} cells with time after encountering pathogens, we utilized the AmpliGrid platform developed by Advalytix AG in concert with 7-color polychromatic flow cytometry to identify B cell subpopulations during ongoing immune responses to a model protein antigen. Flow cytometric analysis and cell sorting was performed on a 3-laser FACSria system. Antigen-binding B cells participating in germinal center (GC) and post-GC responses were sorted directly onto AmpliGrid slides. Subsequent single cell analysis using on-chip 1 μ l RT-PCR and sequencing reactions revealed that responding B cells express V_H gene segments known to contribute in responses to the hapten NP, providing proof of principle for using the AmpliGrid platform to determine V_H gene utilization. After the initial response, we find evidence of memory B cells persisting following antigen encounter. This preliminary work suggests that this experimental approach will be beneficial in the evaluation of experimental vaccines.

Methods:

Two novel methods were used in the current work:

- 1) Adaptation of cell sorting by FACS with BL-3 containment, and the sorting of single cells onto AmpliGrid slides.
- 2) Use of a new technology platform for molecular and genetic analysis of single cells, the AmpliGrid system.



Figure 2. Biohazard sorting and analysis of cells from immunized mice using FACSria located inside a biological safety cabinet Biohood Baker II (top panel). System is equipped with an aerosol management system (BD Biosciences) and self-designed remote-control unit (bottom panel). Remote-controlled sorting approach was developed for sorting cells infected with airborne pathogens.

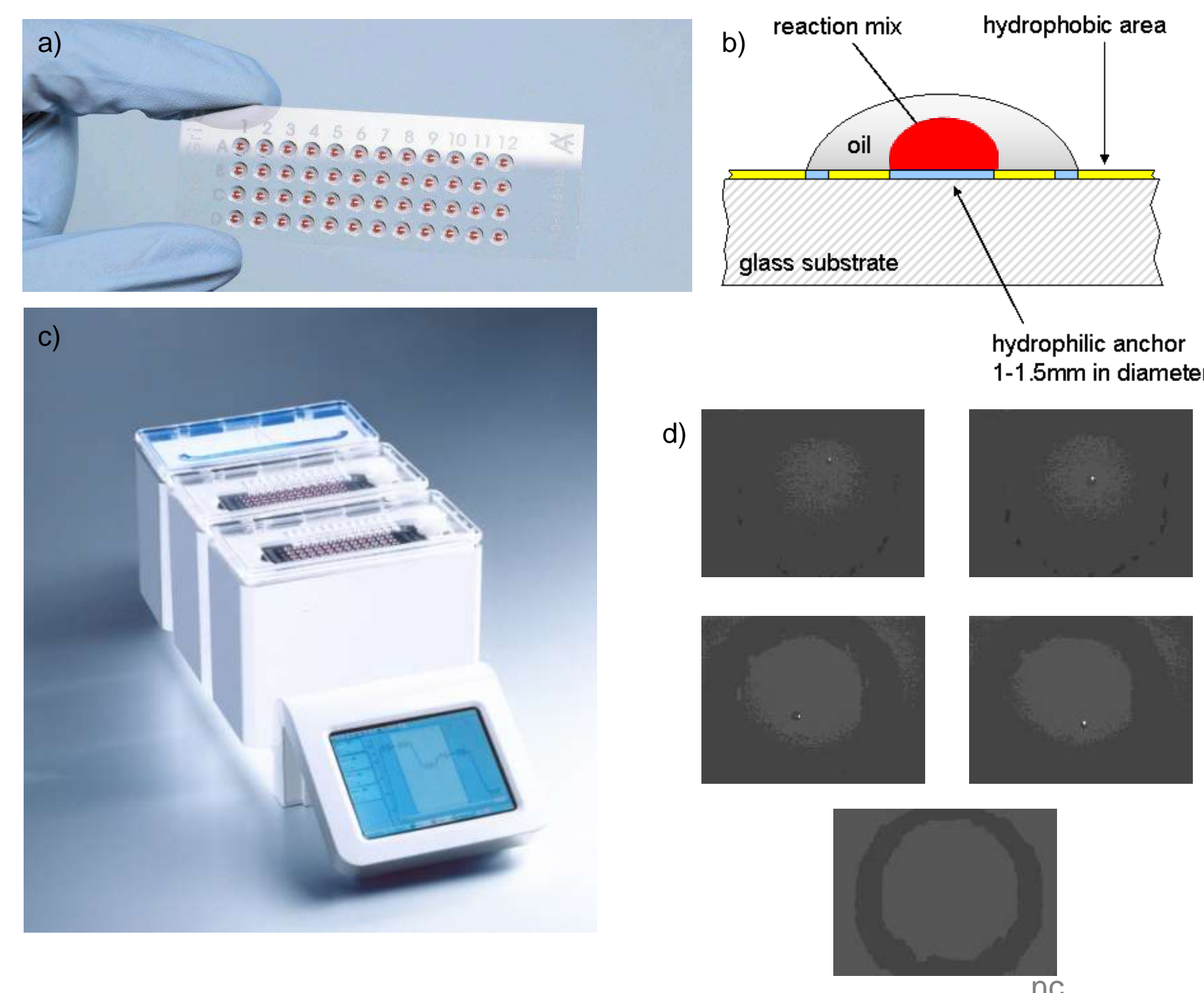


Figure 3. AmpliGrid and AmpliSpeed platform to assess IgH gene heterogeneity in antigen-specific B cells. Single GC and post-GC B cells were sorted directly onto AmpliGrid slides (a, b), and lyophilized. 1 μ l RT-PCR reactions were performed in one-step using pan V_H and C_{μ} (or $C_{\gamma 1}$) primers, and the AmpliSpeed cyclor (c). With AmpliGrid, cells can be visualized by microscopy (d); nc = no cell.

B cell activation, diversification and generating memory

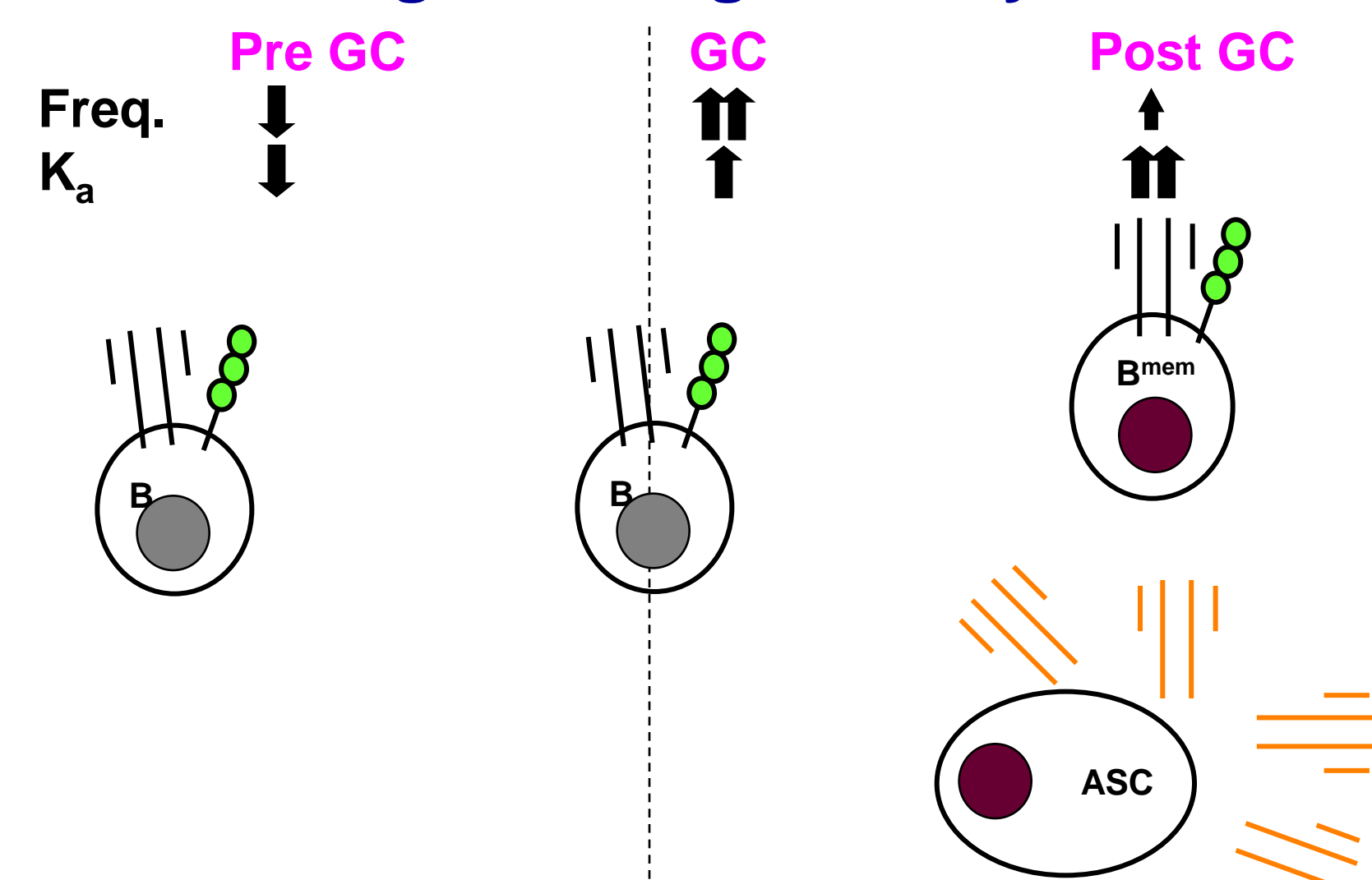


Figure 1. Major checkpoints towards developing B cell memory. The goal of the current work is to identify GC and memory B cells, and to define these cells based on IgH gene sequencing.

Introduction

-A typical measure of vaccine value is production of high-titer, antigen-specific serum antibody by plasma cells.

-While the lifespan of plasma cells is debated, it is generally acknowledged that memory B cells are long-lived, and provide a precursor population for continued production of plasma cells (1-4). Therefore, for long-term protective antibody, memory B cells may provide a more accurate measure of vaccine effectiveness.

-Memory B cells can be identified by multi-parameter flow cytometry. The number of memory B-cells is very low in the spleen (frequency 1-10 per 1,000,000 of splenocytes). The known phenotype of murine B-cells is expression of CD38 and B220, class-switched antibody, ability to bind the lectin peanut agglutinin (PNA), and presence of somatically mutated variable (V) gene segments (5,6), but it little is known about subsets and additional antigens expressed on memory B-cells.

Results:

Humoral responses in immunized and naive mice

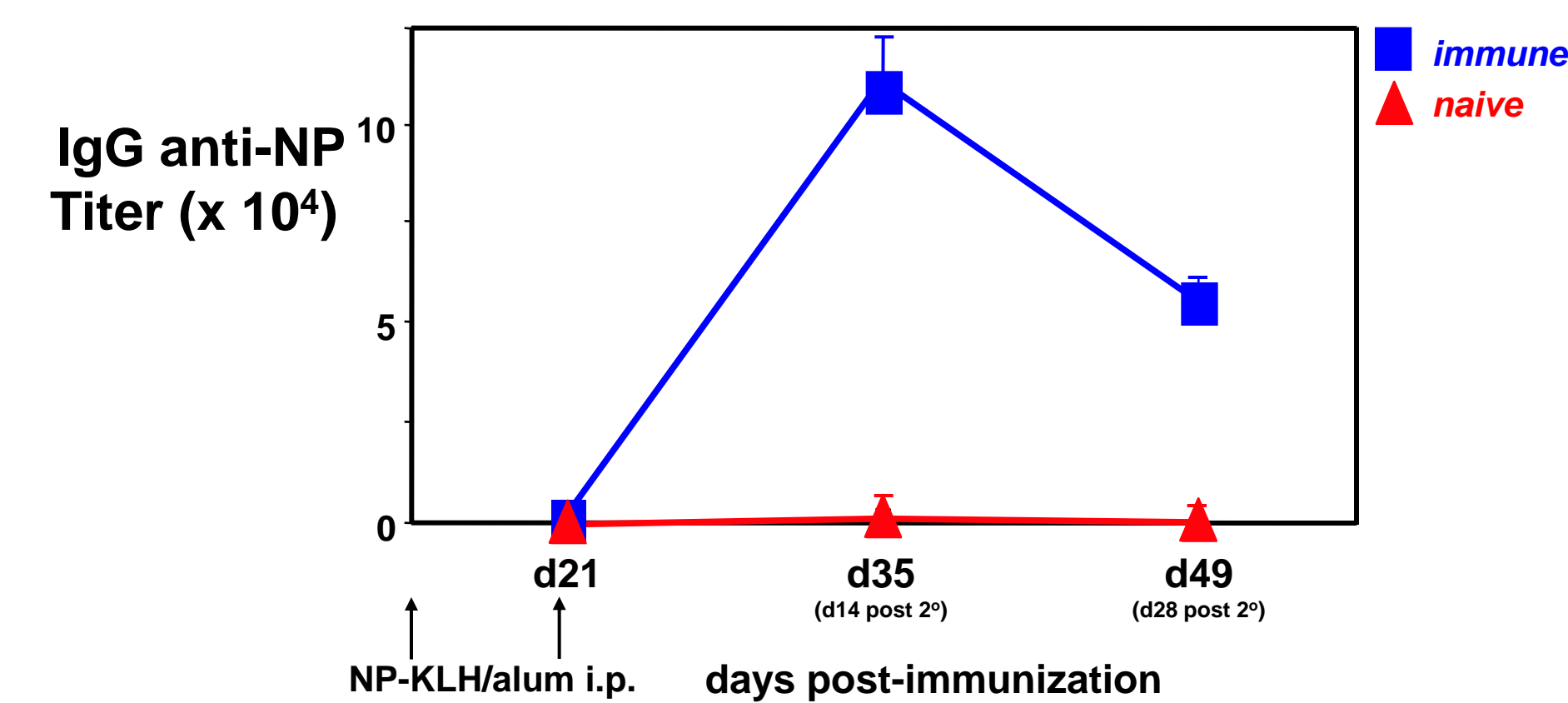


Figure 4. Model system to assess generation of antigen-experienced cells. Immunized (n=5) and naive (n=5) wild-type mice were analyzed by ELISA for production of antigen-specific antibody. Shown are serum titers of the IgG anti-NP response.

Strategy for isolating antigen-experienced B cells

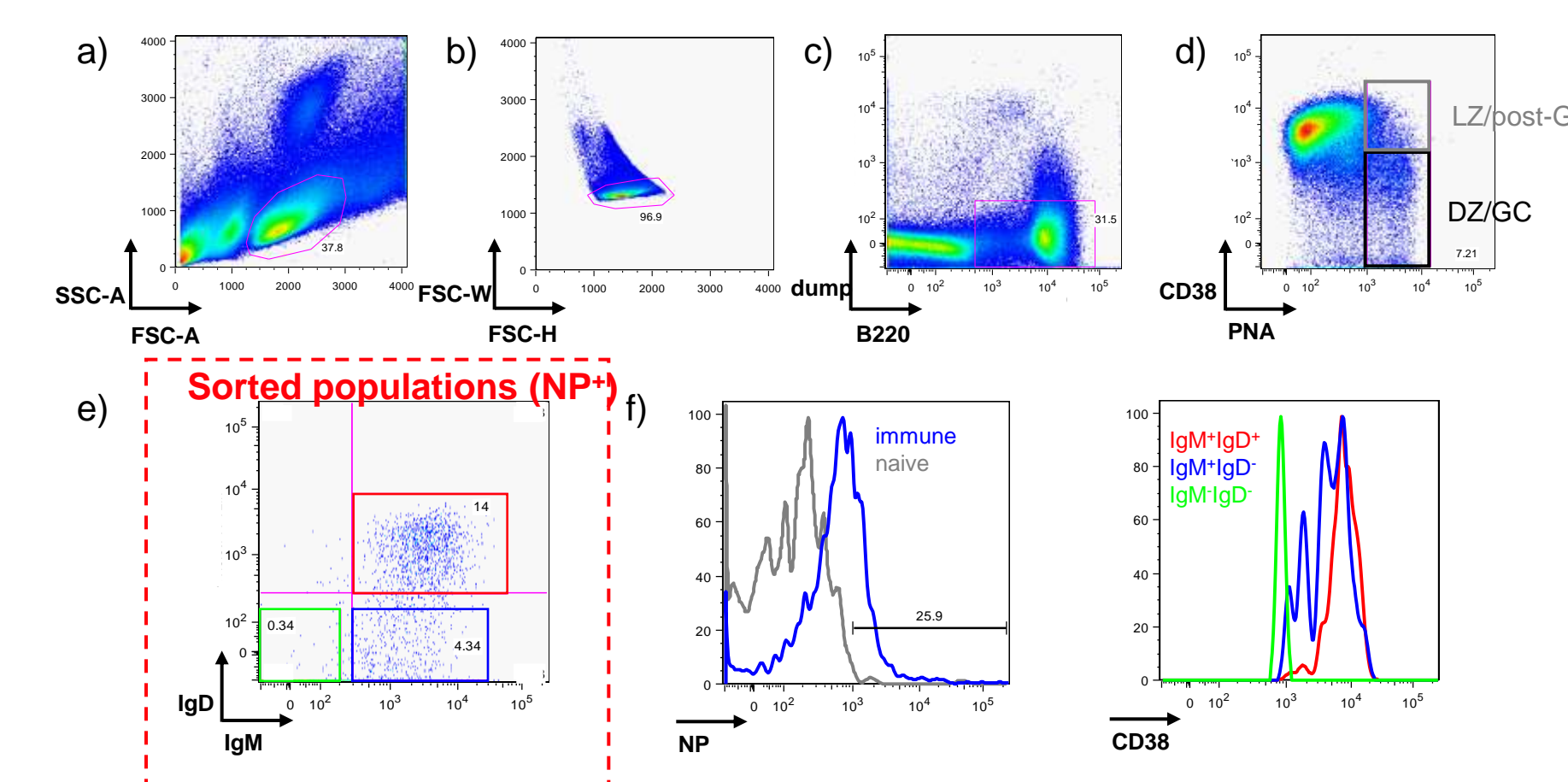


Figure 5. Identifying and sorting GC subpopulations. 7 color, 9-parameter FACS was used to identify B cell subpopulations following immunization (d14 example shown). Population gating scheme is from a-f. In addition to live-gating using FSCW x FSCW, viability dye was added to exclude dead cells.

Recall responses of antigen-experienced B cells

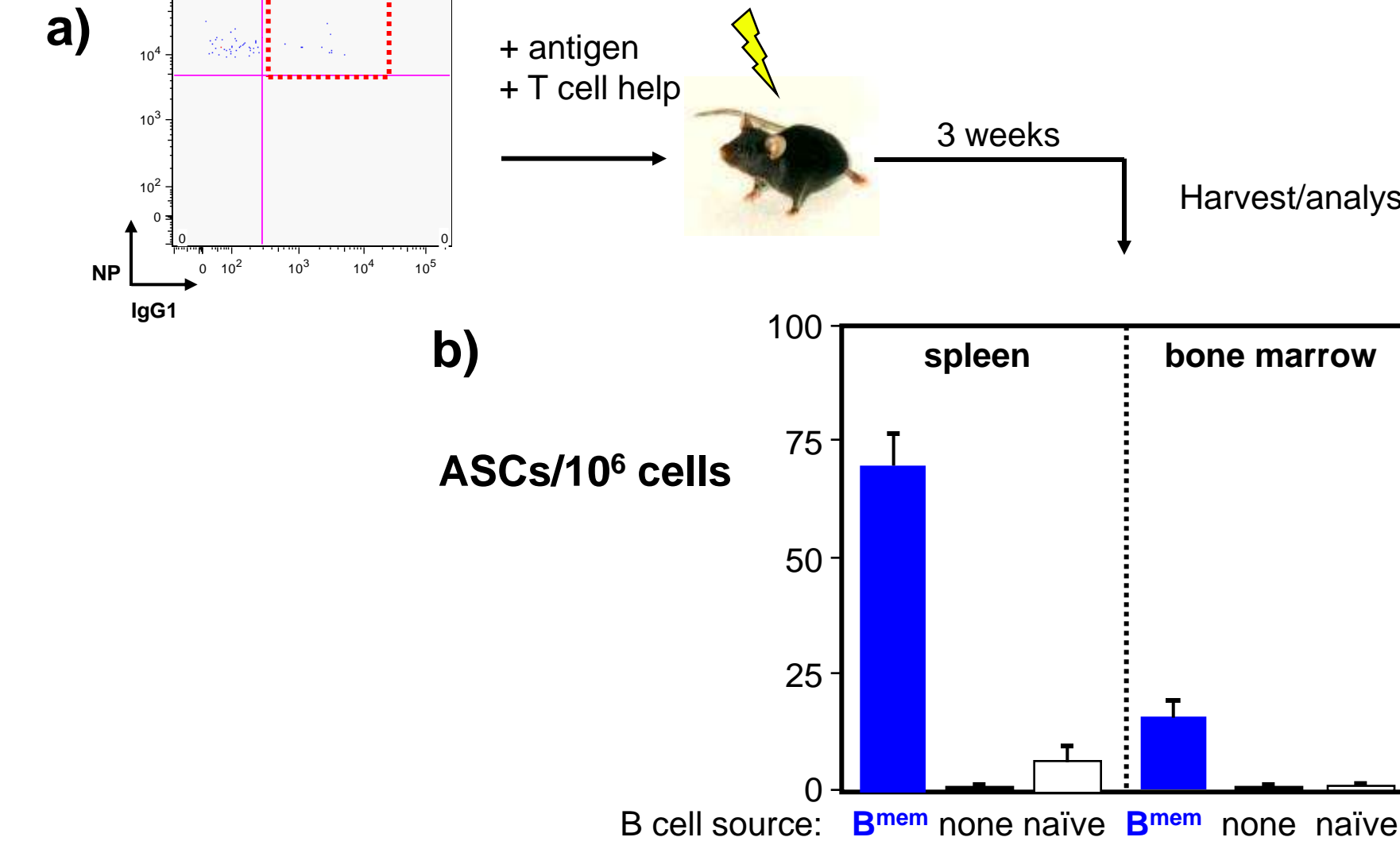


Figure 6. Functional analysis of antigen-experienced (B^{mem}) cells. NP+IgG1⁺ were isolated (2 months after immunization) by a similar strategy to that shown in figure 5, and adoptively transferred with helper T cells and antigen into sublethally-irradiated wild-type mice (a). Recipient mice were analyzed 3 weeks after transfer for NP-specific IgG1 responses; antibody-secreting cell data are shown in (b); 1-3 mice/group; control mice received no B cells (black), or naive B cells (white). No NP+IgG1⁺ B cells were detected in naive mice.

High-efficiency 1 μ l RT-PCR

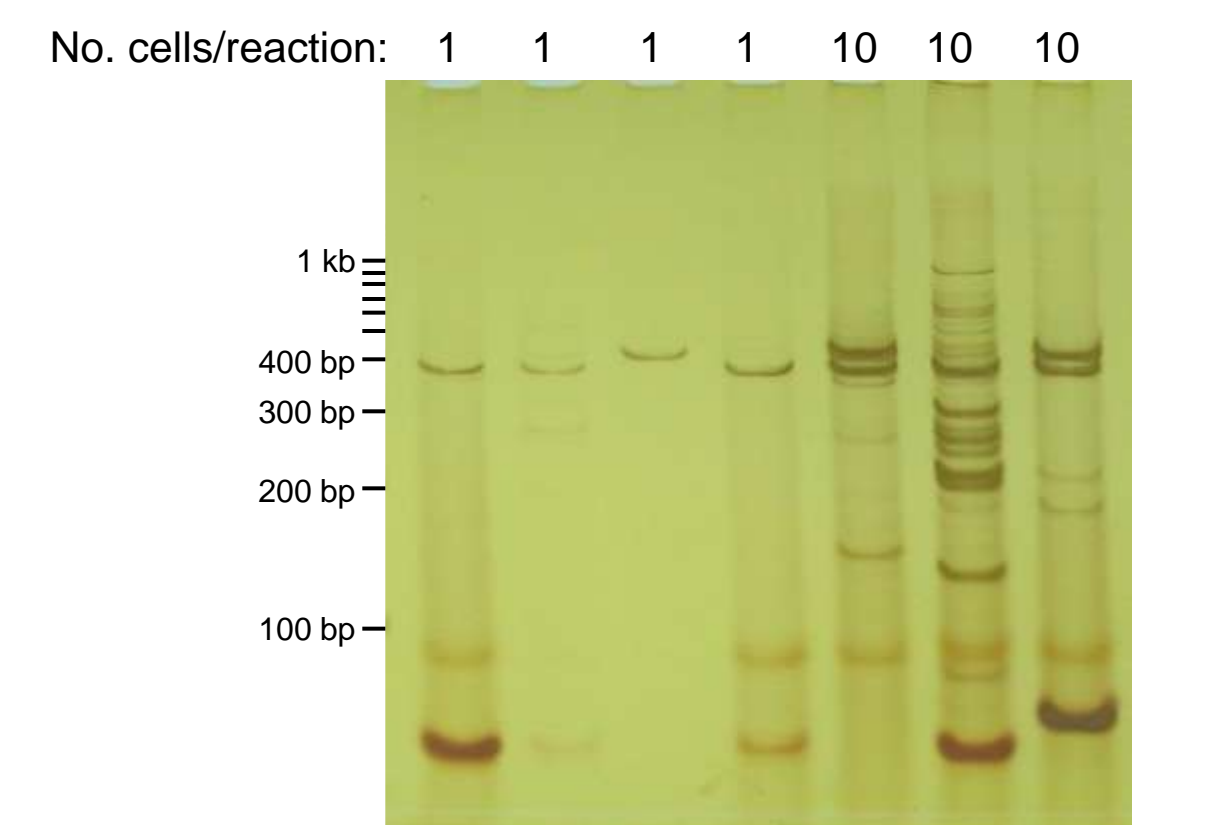


Figure 7. Sensitivity and efficiency of IgH RT-PCR using AmpliGrid. Following single- (left lanes) or ten-cell (right lanes) sorting onto AmpliGrid slides, VDJ- C_{μ} cDNA was analyzed by polyacrylamide gel electrophoresis. Shown is a representative silver-stained gel. Alternatively, bands were excised from an EtBr-stained gel and sequenced (see figure 8). Products for all 6 reactions in this experiment are shown, demonstrating relative high-efficiency. Total B cell RNA in picogram range can also be used (data not shown).

Representative sequence from single GC B cell

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Ms VH186.2 GAAGCCTGGGGCTTCAGTGAAGCTGTCTGCAAGGCTTGGCTACACCTTACCAGCTA 293
Spl  $\mu$ 739 -----C-----A-T-----A-----G-A-----GT-----C
Ms VH186.2 CTGGATGCACCTGGGTGAAGCAGAGGCTGGAGCGGGCTTGGATGGATTGAAGGATTGA 353
Spl  $\mu$ 739 -----A-----AAA-C-----CA-----T
Ms VH186.2 TCCTAATGATGGTGTACTAAGTACAACGAGAAGTCAAGAGCAAGGCCACTGACTGT 413
Spl  $\mu$ 739 -----GGAAG-----A-A-----C-----T-GA-----G-----C
Ms VH186.2 AGACAACCCTCCAGCAGCAGCTACATGCAGCTCAGCAGCCTGACCTCTGAGGACTCTGC 473
Spl  $\mu$ 739 -----T-----A-----A-----
Ms VH186.2 GGTCTATTATTGTGCAAGA 533
Spl  $\mu$ 739 -----C-TC-----
    
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Figure 8. Comparison of nucleotide sequences of an expressed V_H gene from an NP-specific GC B cell to $V_H186.2$. Regions of identity are indicated by "-"; numbers to the right of sequence indicate nucleotide position downstream of transcription start site (5). Sequence shown is from d21 GC B cell, and shares 92% identity to $V_H186.2$, a well-characterized gene segment used in responses to hapten NP.

Conclusions:

- 1) We report the use of multi-parameter 7-color flow cytometry to identify different subsets of GC and post-GC B cells.
- 2) Coupled with single cell IgH gene RT-PCR and sequencing on the AmpliGrid slide-based platform, V_H genes known to be important for NP responses were identified, demonstrating the validity of AmpliGrid in single cell analysis.
- 3) A similar approach is being employed to dissect immune responses to clinically relevant antigens.

Literature cited:

- 1) Slifka et al. 1998. Immunity 8(3): 363.
- 2) Manz et al. 1997. Nature 388(6638): 133.
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