



Published in final edited form as:

J Immunol Methods. 2006 December 20; 317(1-2): 175–185.

Quantitation of Rare Memory B Cell Populations by Two Independent and Complementary Approaches

Ian J. Amanna* and Mark K. Slifka*,†

* Vaccine and Gene Therapy Institute Oregon Health & Science University 505 NW 185th Avenue, Beaverton, OR 97006, USA

Abstract

Current methodology for quantitation of memory B cells (MBC) in clinical samples is limited and often does not allow for detection of multiple MBC specificities in a single assay. Here we describe two independent approaches to antigen-specific MBC quantitation. First, a sensitive flow cytometry (FC) assay was developed for simultaneous quantitation of two prototypical B cell antigens, tetanus and diphtheria. Second, an ELISA-based MBC limiting dilution assay (LDA) was developed that provides quantitative analysis of up to 8–12 different MBC frequencies from a single blood sample. Cross-validation studies indicated that MBC numbers measured by FC correlated significantly with the frequencies obtained by LDA ($p=0.0002$). These two functionally distinct approaches will be useful for accurate quantitation of rare MBC populations specific for either simple antigens (e.g. tetanus and diphtheria) or complex antigens (e.g. vaccinia or other viruses), and is amenable for use in a variety of model systems.

Keywords

B cell memory; Flow cytometric analysis; Limiting dilution analysis; Multiple species

1. Introduction

Enumeration of antigen-specific memory B cells (MBC) in humans is technically challenging, due to their relatively low frequencies in peripheral blood (Leyendeckers et al., 1999). Flow cytometry (FC), even with its high-throughput capabilities, can be hampered by nonspecific binding of some antigens, especially repetitive or complex multi-protein antigens such as intact virus particles (Doucett et al., 2005). In addition, few options are available for examining multiple antigen-specific MBC populations from a single experiment, which would be of great utility when analyzing rare clinical samples.

To overcome these limitations, we have developed two distinct MBC quantitation methods that are applicable to a wide variety of model systems. These assays use FC staining to directly detect antigen-specific MBC by virtue of their affinity/avidity for cognate antigen, or MBC numbers are determined through functional analysis based on antibody production following *in vitro* polyclonal stimulation of purified peripheral B cells under limiting dilution assay (LDA) conditions. Eight parameter FC was used to simultaneously detect tetanus toxoid- (TT)

†Correspondence should be addressed to: Mark K. Slifka, Vaccine and Gene Therapy Institute, Oregon Health & Sciences University, 505 NW 185th Avenue, Beaverton, OR 97006, USA, Phone: (503) 418-2753, Fax: (503) 418-2755, E-mail: slifkam@ohsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and diphtheria toxoid- (DT) specific MBC in human peripheral blood. The ELISA-based LDA was also able to detect TT- and DT-specific MBC, with the potential to quantitate 5 to 10 different antigen-specific MBC populations from a single blood sample. One distinct advantage of developing two independent approaches to MBC quantitation (one based on antigen binding and the other based on antigen-specific antibody secretion) is that these assays can be internally cross-validated in terms of both sensitivity and specificity. Moreover, MBC quantitation by the LDA method is highly amenable to any antigen-specific model system that has a pre-existing ELISA protocol. The ability to enumerate a variety of both simple and complex antigen-specific MBC populations simultaneously will provide a better understanding of the roles that MBC play in the induction, maintenance and anamnestic phases of the humoral immune response following infection or vaccination.

2. Methods

2.1. Animal Studies

BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, ME. RM were bred in colonies at the Oregon National Primate Research Center. To induce TT- and DT-specific humoral immune responses, mice were immunized i.p. with 100 μ l of Tripedia[®] (Aventis-Pasteur), followed by two booster immunizations (d21 and d43). Rhesus macaques (RM) were immunized with 500 μ L Tripedia[®], followed by three booster doses administered at one month intervals. Murine splenocytes were obtained after euthanasia and used immediately or after cryopreservation. Peripheral blood was collected from RM into heparinized tubes, peripheral blood mononuclear cells (PBMC) were purified on Histopaque[®]-1007 (Sigma-Aldrich) and cryopreserved. The Oregon Health & Science University (OHSU) Institutional Animal Care and Use Committee approved all animal use protocols.

2.2. Human Subjects

Umbilical cord blood (UCB) samples were obtained from discarded umbilical cords of full-term deliveries, purified on Histopaque[®]-1007 (Sigma-Aldrich) and cryopreserved. Adult volunteers provided written informed consent and completed an extensive medical history questionnaire (including TT and DT vaccination dates) before participation in the study. At the indicated time points, peripheral blood was collected into heparinized tubes, PBMC were purified on Histopaque[®]-1007 (Sigma-Aldrich) and cryopreserved. All human studies were approved by the Institutional Review Board for OHSU.

2.3. Conjugation of MBC detection reagents

Recombinant tetanus toxin C-fragment (Calbiochem) was biotinylated using the EZ-Link[™]Sulfo-NHS-LC-Biotin kit (Pierce), according to the manufacturers protocol and dialyzed against PBS/0.01% NaN₃ to remove excess biotin. Purified diphtheria toxin (Calbiochem) was conjugated to the succinimidyl ester of either Pacific Blue[™] or Alexa Fluor[®] 700 (Molecular Probes). For both conjugates, 250 μ g of protein was incubated with dye at a molar ratio of 3.5:1 moles of dye per mole of protein for 1 hour at room temperature and purified over P-30 fine resin columns (Bio-Rad). HSA (Sigma-Aldrich) was biotinylated or conjugated to Alexa Fluor[®] 700 as described above.

2.4. MBC analysis by FC

Cryopreserved mouse splenocytes were stained with α -CD19 (Caltag, clone 6D5) and α -IgD (Southern Biotech, clone 11–26). Human PBMC were stained with α -CD20 (Caltag, clone HI47) and α -IgD (BD Biosciences, clone IA6-2). RM PBMC were stained with α -CD20 (Caltag, clone HI47) and α -IgD (Southern Biotech, Goat pAb, δ heavy chain specific). To enumerate TT- and DT-specific MBC, cells ($10\text{--}30 \times 10^6$) were stained with 0.3 μ g of rTT.C-

FITC (List Biological Laboratories), 0.1 μg of rTT.C-biotin, 0.16 μg of DT-Pacific BlueTM, and 0.33 μg of DT-Alexa Fluor[®] 700. Control stains were carried out with 0.1 μg of HSA-biotin or 0.16 μg of HSA-Alexa Fluor[®] 700. Cells were stained in 50 μl volumes for 1h at 4[°]C, washed, and incubated with streptavidin-APC (diluted 1:500, Molecular Probes) for 30 min at 4[°]C. Cells were washed again and fixed with 2% formaldehyde in PBS. Events were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.) with live cell gating based on FSC x SSC characteristics.

2.5. Human MBC limiting dilution analysis

Initial optimization experiments with whole PBMC indicated that antibody responses were inhibited at high cell concentrations and detection of low frequency antigen-specific MBC (<20/10⁶ PBMC) required B cell purification prior to *in vitro* culture. B cells were isolated from total human PBMC through positive selection with human CD20 MicroBeads (Miltenyi Biotec) according to the manufacturers protocol using LS columns (Miltenyi Biotec). Purified CD20⁺ B cells were resuspended in RPMI, 1X Pen/Strep and 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids (Invitrogen), 20 mM HEPES (Cambrex), 50 μM β -ME, and 10% heat-inactivated FBS (HyClone). Cells were cultured in serial 2-fold diluted doses (10 wells per dose), starting with 50,000 to 100,000 purified B cells per well at the highest dose with 5,000 irradiated (1,200 RAD) NIH/3T3 cells or NIH/3T3.huCD40L cells (a generous gift of Jeff Viera, University of Washington, provided by Scott Wong, Oregon Health & Science University), in 96-well flat-bottom plates in a final volume of 200 μl per well. Cells were incubated with *Staphylococcus aureus* Cowan strain (SAC) lysate diluted 1:10,000 (Protein A, Sigma-Aldrich, P7155), pokeweed mitogen (PWM) diluted 1:1,000,000 (generous gift from Dr. Shane Crotty, La Jolla Institute for Allergy & Immunology), lipopolysaccharide (LPS, *E. coli*, 0111:B4) at 10 $\mu\text{g}/\text{ml}$ (List Biological Laboratories), phosphorothioated human CpG-2006 motif at 1 $\mu\text{g}/\text{ml}$ (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3', Integrated DNA Technologies) (Hartmann et al., 2000), 16 ng/ml hIL-2 (Amgen), 10 ng/ml hIL-6, and 17 ng/ml hIL-10 (Peprotech). To determine background absorbance values, supernatants were used from 8 wells incubated with NIH/3T3 feeder cells only. Plates were incubated at 37 $^{\circ}\text{C}$ and 6% CO₂ for 6 days.

IgG-specific MBC frequencies were calculated by assaying LDA supernatants with antigen-specific ELISA as previously described (Slifka and Ahmed, 1996; Hammarlund et al., 2003). Briefly, half-area, 96-well microtiter plates (Corning-Costar, high binding) were coated at 50 $\mu\text{l}/\text{well}$, overnight at 4 $^{\circ}\text{C}$, with optimally diluted antigen. For TT- and DT-specific ELISA, plates were coated with 2 $\mu\text{g}/\text{ml}$ rTT.C or DT (Calbiochem) in PBS. Virus-specific ELISA was performed with optimized inactivated vaccinia lysate (vaccinia virus Western Reserve), measles lysate (rubeola, Biodesign) or mumps lysate (Enders strain, Biodesign). For production of vaccinia lysate, BSC40 cells were grown to 75–80% confluency in T-150 flasks (15–20 x 10⁶ cells per flask) and infected at an MOI of 0.1 in ~1 ml of medium (DMEM, 1X Pen/Strep, 2 mM L-glutamine, and 1 mM sodium pyruvate). After cells had reached ~100% cytopathic effect (CPE, ~48 hours post-infection), cells were harvested by cell scraping and centrifuged at 3000 g for 15 minutes at 4 $^{\circ}\text{C}$. Cells were resuspended in 10 mM Tris-HCl, pH=8.0, and subjected to three rounds of freeze-thaw cycle with vortexing in between. Vaccinia viral lysate was inactivated with a final concentration of 3% H₂O₂ for 2 hours at room temperature. After coating overnight, ELISA plates were stored long-term (up to several months) at -20 $^{\circ}\text{C}$, or used fresh. Plates were thawed, the coating flicked out, and blocked at 150 $\mu\text{l}/\text{well}$ with PBS-T (PBS with 0.05% Tween20, v/v) plus 5% (w/v) non-fat dry milk for 1 hour at room temperature. Block buffer was flicked out and plates were washed once with PBS-T (~150 $\mu\text{l}/\text{well}$), and tamped dry on paper towels. Supernatants from LDA cultures were then replicated onto the ELISA plates, at 15–25 $\mu\text{l}/\text{well}$, and incubated at room temperature for 1 hour. As a precautionary measure against exposure to human blood-borne pathogens, ~10 $\mu\text{l}/\text{well}$ of

10% H₂O₂ (diluted in block buffer) was added to each well and incubated at room temperature for 30 minutes. Plates were then washed 6 times with PBS-T (~150 µl/well) and tamped dry on paper towels. Detection antibody (goat α-human IgG(γ)-HRP, Jackson ImmunoResearch Laboratories) was diluted 1:1000 in block buffer, added at 50 µl/well and incubated for 1 hour at room temperature, followed by washing as above. Substrate (O-phenylenediamine, Sigma-Aldrich) was diluted to 0.4 mg/ml in citrate buffer (0.05 M, pH=5.0) and H₂O₂ was added to a final concentration of 0.01% (v/v) just prior to use. Substrate was added to plates (50 µl/well) and allowed to develop for 20 minutes. Development was stopped with 50 µl/well of 1M HCl, and optical densities were read at 490 nm on a VersaMax plate reader (Molecular Devices). LDA cultures were scored as positive if they demonstrated optical densities 4-fold over the average of the background observed in eight negative control wells (NIH/3T3 cells only).

2.7. Serum ELISA

Antigen-specific ELISA was performed to determine relative serum antibody titers. Full size 96-well ELISA plates were coated with optimal concentrations of antigen and ELISA was carried out essentially as described above for quantitating antibody production in LDA cultures. For quantitating serum antibody titers, heat-inactivated serum (56°C, 30 minutes) was diluted 1:30 in block buffer [PBS-T, 5% (w/v) non-fat dry milk] and then up to seven 3-fold serial dilutions were performed, with the final well left as a blank control to use for background subtraction. Each ELISA plate included an internal standard/positive control sample for normalization between plates and between experiments performed on different days. For detection of antigen-specific antibody responses, mouse α-human IgG(γ)-HRP antibody (BD Pharmingen, diluted 1:1000) was used. After development, antibody end-point titers were calculated. After background subtraction of the average blank controls, antibody titers were determined by logarithmic transformation of the linear portion of the curve (in our hands, optical densities of 0.05 to 1.50) with 0.1 OD units used as the endpoint and conversion performed on final values. Subjects with serum titers of >200 ELISA units were considered seropositive.

2.8. Statistical Analysis

MBC precursor frequencies were calculated by the semi-logarithmic plot of the percent of negative cultures versus the cell dose per culture, as previously described (Miller et al., 1977) and frequencies were calculated as the reciprocal of the cell dilution at which 37% of the cultures were negative for antigen-specific IgG production. Cell doses which yielded 0% negative wells were excluded, since this typically resides outside of the linear range of the curve and artificially reduced the MBC precursor frequency. Comparisons of MBC frequencies determined by FC or LDA were modeled through linear regression, with associated p-values, using the least squares method in Excel (Microsoft Corporation). By analyzing only the LDA with at least 4 positive wells (out of 10 wells containing 100,000 purified B cells per well at the highest dose) the estimated limit of detection for antigen-specific MBC by LDA is ~5/10⁶ B cells, or ~1/10⁶ PBMC. Note: sensitivity of MBC quantitation by LDA or FC may be increased further by simply increasing the number of B cells that are assayed.

3. Results

3.1. MBC quantitation by FC using multiple fluorochrome-antigen conjugates

To develop a method for quantitation of TT- and DT-specific MBC, we prepared fluorochrome-conjugated TT and DT reagents that were initially optimized using naive BALB/c mice or mice vaccinated with Tripedia® (Diphtheria and Tetanus Toxoids and Acellular Pertussis Vaccine, adsorbed). Optimal amounts of each staining reagent were determined by comparing antigen-specific MBC frequencies within the CD19⁺IgD⁻ (i.e. immunoglobulin class-switched) B cell population. Unfortunately, when using only a single color fluorochrome reagent (e.g. TT-FITC

or TT-APC) to quantitate antigen-specific MBC, we observed substantial non-specific staining of B cells from the naïve/unvaccinated control mice, resulting in a poor signal-to-noise ratio (Fig. 1). Based on these preliminary studies, it was clear that the specificity of MBC quantitation by FC required improvement in order to be suitable for detecting rare MBC populations. To achieve this result, we tested a dual staining approach similar to one previously described in other murine studies involving B cell receptor transgenic B cells (Townsend et al., 2001). In this approach, MBC were detected on the basis of dual labeling with a specific antigen coupled to two different fluorochromes. Antigen-specific MBC were defined as the cells that had sufficient affinity/avidity to simultaneously bind both forms of the optimized target antigen. To test this dual-staining FC approach to MBC quantitation in a non-transgenic model system, we gated on class-switched B cells ($CD19^+IgD^-$) to improve the signal-to-noise ratio and determined specific antigen binding of both TT reagents (Fig. 1 & 2b). TT-immune and naïve mice were analyzed and B cells staining positive for both fluorochrome reagents (as defined by the oval MBC region gate) were counted as antigen-specific MBC. Interestingly, two TT double-positive populations were observed when staining mouse splenocytes, with one population showing a fluorescent staining intensity that was approximately 10-fold lower than the other in the TT-APC channel (TT^{lo} vs TT^{hi}). A similar population was not observed for either RM or human PBMC (Fig. 3). Further investigation revealed that the TT^{lo} population was $CD11b^+$, whereas the TT^{hi} population was $CD11b^-$ (data not shown). This TT^{lo} population may represent myeloid cells binding serum Ig through surface Fc receptors, as reported previously (Bell and Gray, 2003), and was therefore excluded from MBC analysis.

A similar analysis was performed for human PBMC, using CD20 instead of CD19 to identify B cells, with an adult volunteer at ~5 months after booster vaccination serving as the immune subject. Due to the inherent difficulties in finding adult human subjects who are naïve to TT or other common vaccine antigens, we utilized umbilical cord blood (UCB) samples obtained on the day of birth as a source of mature naïve B cell controls. For both mice and humans, these naïve controls demonstrated very low non-specific staining ($=1$ TT-binding B cell per 10^6 B cells). To confirm and extend these results with a different clinically relevant antigen, we stained MBC with DT reagents coupled to either Pacific Blue™ (DT-PB) or Alexa Fluor® 700 (DT-Alexa700) in the same samples in which TT-specific staining was performed. These fluorochrome labels were chosen for DT because they are distinct from the ones used to measure TT-specific MBC and thereby allow for simultaneous quantitation of two different MBC populations within a single FC experiment. Similar to TT (Fig. 2b), the DT reagents provided highly specific MBC staining (Fig. 2c), demonstrating the utility of the dual staining approach for simultaneously measuring MBC specific for two different vaccine antigens. As an added specificity control, both TT and DT double-positive MBC populations were gated and examined for fluorescent staining of the reciprocal MBC population (Fig. 2d). None of the TT-positive MBC bound DT antigens and none of the DT-positive MBC bound TT antigens. The same result was observed in mice (data not shown) and shows that the MBC populations measured in these studies are not due to B cells binding antigen in a non-specific manner.

3.2. Determination of MBC frequencies with internal negative controls

The dual staining approach described here provides sensitive detection of MBC, but this method of quantitation can become complicated if appropriate naïve/negative controls cannot be obtained for comparison and validation of antigen staining specificity. In genetically identical strains of inbred mice, this is rarely an issue because naïve mice are available and provide appropriate controls for comparison with vaccinated animals. In contrast, humans [as well as non-human primates (NHP)] are genetically diverse and age/gender matched naïve controls are often not available, especially if one studies humoral immune responses to common pathogens or vaccine antigens. In our preliminary clinical studies, cells purified from UCB were used as negative controls (Fig. 2), but this may underestimate the degree of non-specific

staining that exists in individual adults who have a diverse and genetically dissimilar MBC compartment. To address this potential caveat, we developed internal negative antigen staining controls by labeling human serum albumin (HSA) with the same fluorochromes used for the TT and DT antigens. To test this approach, cryopreserved mouse splenocytes, RM PBMC, or human PBMC were divided into two fractions: one stained with paired antigen reagents (TT-FITC + TT-APC, DT-PB + DT-Alexa700), and the other stained with mismatched antigen reagents (TT-FITC + HSA-APC, DT-PB + HSA-Alexa700) (Fig. 3). This improved, second generation staining approach provided quantitative and simultaneous enumeration of MBC for both TT (Fig. 3) and DT (data not shown) in murine, NHP, and human clinical samples, without requiring additional age/sex matched genetically identical naive controls for comparison.

3.3. Optimization of an ELISA-based MBC LDA

The dual staining FC methodology provides a highly sensitive and specific technique for measuring MBC that bind simple protein antigens, but multi-protein complexes such as intact virus particles are prone to substantial levels of non-specific binding when measured by FC (Doucett et al., 2005). To enumerate complex virus-specific MBC populations, we developed an ELISA-based LDA. The underlying theory of LDA predicts that any specific responder cell (whose biological activity can be measured) becomes limiting as the cells are diluted in graded doses (Miller et al., 1977). Based on a Poisson distribution, the fraction of negative cultures can be plotted as a function of the number of cells per culture, and the responder cell frequency is calculated as the reciprocal of the cell dilution at which 37% of the cultures are negative (Miller et al., 1977). MBC bear membrane-bound immunoglobulin (Ig, allowing them to be identified by FC, Fig. 1, 2 and 3), however, to be identified by an ELISA these cells must be stimulated to proliferate and differentiate into antibody-secreting cells. To accomplish this task, we tested several stimulation conditions for the LDA. For example, we compared NIH/3T3 feeder cells to NIH/3T3 cells expressing human CD40L (to provide B cell help via CD40-CD40L interactions) in the presence or absence of B cell mitogens and exogenous cytokines (Fig. 4a). Optimal polyclonal stimulation consisted of previously identified B cell mitogens (Crotty et al., 2003) including SAC lysate, PWM, LPS, a 24-mer DNA oligonucleotide containing an optimized human CpG motif (CpG-2006) (Hartmann et al., 2000), and additional cytokines, IL-2, IL-6, and IL-10. Other cytokines (IL-4, IL-12, IL-18) were tested in various combinations, but provided no further increase in triggering Ig production under LDA conditions (data not shown). Graded doses of whole PBMC were incubated with different combinations of these cytokines and cell supernatants were tested for antigen-specific IgG or total IgG by ELISA. When we compared NIH/3T3 feeder cells with stably transfected NIH/3T3 cells expressing human CD40L (NIH/3T3.huCD40L), in each case the *in vitro* culture of B cells in the presence of CD40L resulted in reduced antibody production (Fig. 4a and data not shown). This might be expected since other studies have found that stimulation by CD40L induces B cells to follow a differentiation pathway towards MBC and not towards antibody-secreting plasma cells (Arpin et al., 1995). An abbreviated summary of four representative stimulation conditions is shown in Fig. 4a. In all subsequent experiments, LDA were performed with the optimal stimulation conditions that used the polyclonal stimulation cocktail with unmodified NIH/3T3 feeder cells. To further expand the utility of the ELISA-based LDA approach, we used half-area 96-well plates for the detection ELISA because these plates require only 15–25 μ l of supernatant for detection of antigen-specific antibody. Using this methodology, the 200 μ l cell culture supernatant from each well of a single polyclonally-stimulated LDA culture can be replica-plated onto 8–10 different ELISA plates, each coated with a different antigen. For example, a single blood sample provided MBC quantitation to multiple specificities including vaccinia, mumps, measles, TT, DT, as well as the total IgG precursor frequency (Fig. 4b). In this experiment, the adult subject had been immunized against each of the antigens (confirmed by antigen-specific serum ELISA) except for vaccinia, and as expected, the MBC frequency in this vaccinia-naive individual was below detection (<5 MBC/

10^6 B cells). In subsequent studies, 0/6 seronegative vaccinia-naïve subjects and 17/17 seropositive vaccinia-immune subjects demonstrated vaccinia-specific MBC responses by this assay, indicating 100% specificity and 100% sensitivity for this antigen (Fig. 4c). Vaccinia-immune subjects ranged from 21 days to 43 years post-primary or secondary immunization. The specificity of this LDA was also tested using naïve B cells isolated from UCB and in each case, no IgG⁺ MBC were detected against any of the described antigens ($n = 3$ UCB donors; data not shown). Thus, highly specific and sensitive quantitation of MBC frequencies can be determined for several simple and complex antigens in a single experiment.

3.4. Correlation between MBC frequencies measured by LDA or FC

To gauge the overall sensitivity of the LDA, the frequencies of TT-specific MBC determined by LDA were compared to the frequencies determined through direct binding of specific fluorochrome-labeled antigen by FC (Fig. 4d). There was a strong correlation between the two methods ($R^2=0.92$, $p=0.0002$). A perfect correlation would result in a 1:1 ratio between the LDA and FC methods and we observed an approximate 0.7:1 ratio. This indicates that the FC approach is a slightly more sensitive method of MBC quantitation, but the LDA conditions developed here are highly comparable in terms of overall sensitivity and have the added feature of being useful for analyzing MBC responses to multiple complex antigens in a single assay. Similar analysis was performed for DT-specific MBC, and again a significant correlation between MBC frequencies determined by the LDA and FC methods was observed ($p=0.00002$; data not shown).

4. Discussion

Enumeration of antigen-specific MBC is critical for in-depth analysis of humoral immune responses following vaccination or infection, but remains challenging due to their relatively low frequencies. In this study, we describe the development of two independent and cross-validated methods for the quantitation of antigen-specific MBC in human clinical samples. The FC approach provides the simultaneous detection of TT- and DT-specific MBC by direct antigen-binding, while the ELISA-based LDA quantitates MBC on the basis of their functional ability to proliferate and differentiate into antibody secreting cells and allows for enumeration of up to 10 antigen-specific MBC populations from a single experiment.

FC offers the ability to directly examine antigen-specific MBC, and has been commonly used to investigate MBC responses in mice (Hayakawa et al., 1987; McHeyzer-Williams et al., 1993). However, frequencies for antigen-specific MBC populations in these animal models can be relatively high, comprising up to ~1% of total splenocytes in certain instances (McHeyzer-Williams et al., 1993). This is in contrast to human clinical samples in which MBC frequencies may be as low as ~0.003% of total B cells for specific populations (Leyendeckers et al., 1999). With such low frequencies, even a limited number of false-positive events can profoundly hinder analysis (Townsend et al., 2001). To increase the specificity of direct MBC staining by FC, a “dump channel” is often used to gate out non-specific, antigen-binding cells (McHeyzer-Williams and McHeyzer-Williams, 2004). In our studies, we avoided extensive negative gating by employing a dual staining approach, similar to that described in a transgenic B cell receptor mouse model (Townsend et al., 2001) and found that it dramatically improved specificity without reducing the sensitivity of MBC quantitation (Fig. 1). This approach was further optimized by the addition of stringent antigen-mismatch staining controls to confirm antigen specificity (Fig. 3). This antigen-mismatch approach, wherein samples are divided into two fractions and stained with dual antigen-specific reagents (e.g. TT-FITC + TT-APC), or a single antigen-specific reagent in addition to an irrelevant “mismatch” fluorescently labeled protein reagent (e.g. TT-FITC + HSA-APC) not only provides an important specificity control but also facilitates optimal placement of the region gate used for MBC quantitation. This

stringent internal specificity control is particularly important for humans and NHP, in which genetically matched, naive/unvaccinated individuals are often not available for comparison.

The dual staining FC approach works well for simple protein antigens such as TT and DT, but staining B cells with highly repetitive and complex multi-protein antigens has previously proven difficult to perform due to substantial non-specific binding (Doucett et al., 2005). To expand the range of antigen-specific MBC populations that can be measured in clinical samples, and to cross-validate the FC approach that we used to measure MBC frequencies specific to single protein antigens, we developed an optimized and highly sensitive ELISA-based LDA for determining MBC numbers. Purified CD20⁺ B cells were stimulated polyclonally *in vitro* under limiting dilution conditions and supernatants from the limiting dilution cultures were tested by ELISA for reactivity against several antigens. One advantage of this LDA over other previously described methods is that the MBC frequencies can be quantitated on the basis of the total B cell population rather than as a proportion of B cell subpopulations capable of antibody production following polyclonal activation (Crotty et al., 2004). Another advantage of this LDA over other LDA methods that use antigen-specific stimulation for MBC quantitation (Slifka and Ahmed, 1996) is that polyclonal stimulation results in antibody production by all potential MBC specificities (instead of antibody responses elicited against only a single antigen) and therefore cell culture supernatants can be used to measure MBC responses specific for virtually any antigen that works well for ELISA quantitation of serum antibody responses. The LDA is a functional assay designed to measure MBC capable of dividing and differentiating into antibody secreting cells and therefore provides different biological information about MBC than that obtained by the FC approach, which only measures antigen-specific binding and not function. On the other hand, the FC approach facilitates clear phenotypic characterization of the antigen-specific MBC populations since multiple internal or cell surface markers can be directly visualized on the antigen-binding cells.

In Fig. 4b, approximately 1/23 CD20⁺ B cells (containing a mixture of naive and antigen-experienced B cells) were identified by this optimized LDA. This was typical of 13 clinical samples tested (average=1/40, range=1/15 to 1/84). However, CD20⁺IgD⁻ B cells comprised just 15.7±5.9% (range = 8.4% to 24.6%) of peripheral B cells in subjects tested ($n = 13$, data not shown) and we reasoned that the low cloning efficiency observed in these experiments was due to naive B cells (~84% of peripheral B cells) being largely incapable of differentiation into IgG-secreting cells under stimulation conditions employed here. The bulk of MBC reside in the class-switched, IgD⁻ B cell population (Hayakawa et al., 1987) and to determine if naive (IgD⁺) B cells responded differently to polyclonal stimulation than Ig-class switched (IgD⁻), we magnetically sorted PBMC into IgD⁺ and IgD⁻ fractions for a subgroup of individuals ($n=4$) and performed the LDA. For the IgD⁺ B cells, total Ig-producing precursor frequencies averaged 1/21 (range=1/7 to 1/33), while IgG-producing precursor frequencies averaged only 1/844 (range=1/154 to 1/2030). In contrast, an average of 1/3.7 (range=1/2.5 to 1/5.8) IgD⁻ B cells produced antibody after *in vitro* polyclonal stimulation under limiting dilution conditions (data not shown). This may still represent an underestimate of MBC activation under LDA conditions since a proportion of the gated CD20⁺IgD⁻ B cells may be circulating marginal zone B cells displaying an IgD^{lo} (as well as IgM⁺CD27⁺) phenotype (Weller et al., 2004) and not necessarily represent antigen-induced MBC. Using these LDA conditions to enumerate TT-specific MBC from clinical samples, we found a significant correlation with the frequencies determined by FC ($p = 0.0002$, Fig. 4). As might be expected when relying on a functional readout, the LDA was somewhat less sensitive than FC, detecting ~70% of the response observed through direct FC staining. However, in contrast to the FC approach, which was limited to analysis of two simple protein antigens, the LDA described here was able to quantitate antigen-specific MBC frequencies for up to 10 different antigens, including complex viral antigens such as vaccinia, measles, and mumps (Fig. 4 and data not shown). This method

will be useful for determining MBC frequencies for any antigen that can currently be studied by ELISA, greatly expanding the utility of this approach for measuring functional MBC responses to multiple antigens.

The mechanisms which maintain MBC, as well as the respective roles of MBC and plasma cells in sustaining long-term antibody responses in humans, remain controversial (Slifka, 2004; Manz et al., 2005; McHeyzer-Williams and McHeyzer-Williams, 2005; Amanna et al., 2006) making the quantitative analysis of antigen-specific MBC a timely and relevant topic. The assays presented here allow for the enumeration of MBC with different antigen-specificities and will aid in addressing these fundamental questions of immunological memory.

Acknowledgements

We thank the volunteers for their time and their participation in this research study; Dr. S. Crotty for the provision of reagents; Dr. S. Shiigi for his FC technical advice; and M. Lewis and E. Hammarlund for human blood collection and processing. This work was supported by National Institutes of Health contract N01 AI40029, PO1 AG023664, RR00334, and RR00163.

References

- Amanna IJ, Slifka MK, Crotty S. Immunity and immunological memory following smallpox vaccination. *Immunological reviews*. 2006 In press
- Arpin C, Dechanet J, Van Kooten C, Merville P, Grouard G, Briere F, Banchereau J, Liu YJ. Generation of memory B cells and plasma cells in vitro. *Science* 1995;268:720–2. [PubMed: 7537388]
- Bell J, Gray D. Antigen-capturing cells can masquerade as memory B cells. *J Exp Med* 2003;197:1233–44. [PubMed: 12756262]
- Crotty S, Aubert RD, Glidewell J, Ahmed R. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. *J Immunol Methods* 2004;286:111–22. [PubMed: 15087226]
- Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol* 2003;171:4969–73. [PubMed: 14607890]
- Doucett VP, Gerhard W, Owler K, Curry D, Brown L, Baumgarth N. Enumeration and characterization of virus-specific B cells by multicolor flow cytometry. *J Immunol Methods* 2005;303:40–52. [PubMed: 16045923]
- Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, Hanifin JM, Slifka MK. Duration of antiviral immunity after smallpox vaccination. *Nat Med* 2003;9:1131–7. [PubMed: 12925846]
- Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, Rasmussen WL, Waldschmidt M, Sajuthi D, Purcell RH, Davis HL, Krieg AM. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J Immunol* 2000;164:1617–24. [PubMed: 10640783]
- Hayakawa K, Ishii R, Yamasaki K, Kishimoto T, Hardy RR. Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigen-binding cells. *Proc Natl Acad Sci U S A* 1987;84:1379–83. [PubMed: 3493491]
- Leyendeckers H, Odendahl M, Lohndorf A, Irsch J, Spangfort M, Miltenyi S, Hunzelmann N, Assenmacher M, Radbruch A, Schmitz J. Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG. *Eur J Immunol* 1999;29:1406–17. [PubMed: 10229109]
- Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. *Annu Rev Immunol* 2005;23:367–86. [PubMed: 15771575]
- McHeyzer-Williams LJ, McHeyzer-Williams MG. Analysis of antigen-specific B-cell memory directly ex vivo. *Methods Mol Biol* 2004;271:173–88. [PubMed: 15146121]
- McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. *Annu Rev Immunol* 2005;23:487–513. [PubMed: 15771579]
- McHeyzer-Williams MG, McLean MJ, Lalor PA, Nossal GJ. Antigen-driven B cell differentiation in vivo. *J Exp Med* 1993;178:295–307. [PubMed: 8315385]

- Miller RG, Teh HS, Harley E, Phillips RA. Quantitative studies of the activation of cytotoxic lymphocyte precursor cells. *Immunol Rev* 1977;35:38–58. [PubMed: 330390]
- Slifka MK. Immunological memory to viral infection. *Curr Opin Immunol* 2004;16:443–50. [PubMed: 15245737]
- Slifka MK, Ahmed R. Limiting dilution analysis of virus-specific memory B cells by an ELISPOT assay. *J Immunol Methods* 1996;199:37–46. [PubMed: 8960096]
- Townsend SE, Goodnow CC, Cornall RJ. Single epitope multiple staining to detect ultralow frequency B cells. *J Immunol Methods* 2001;249:137–46. [PubMed: 11226471]
- Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, Plebani A, Kumararatne DS, Bonnet D, Tournilhac O, Tchernia G, Steiniger B, Staudt LM, Casanova JL, Reynaud CA, Weill JC. Human blood IgM “memory” B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 2004;104:3647–54. [PubMed: 15191950]

Abbreviations

DT	diphtheria toxoid
FC	flow cytometry
HSA	human serum albumin
Ig	immunoglobulin
LDA	limiting dilution assay
LPS	lipopolysaccharide
MBC	memory B cell
NHP	non-human primate
PBMC	peripheral blood mononuclear cells
PWM	pokeweed mitogen
RM	rhesus macaque
SAC	<i>Staphylococcus aureus</i> Cowan strain
TT	tetanus toxoid
UCB	umbilical cord blood

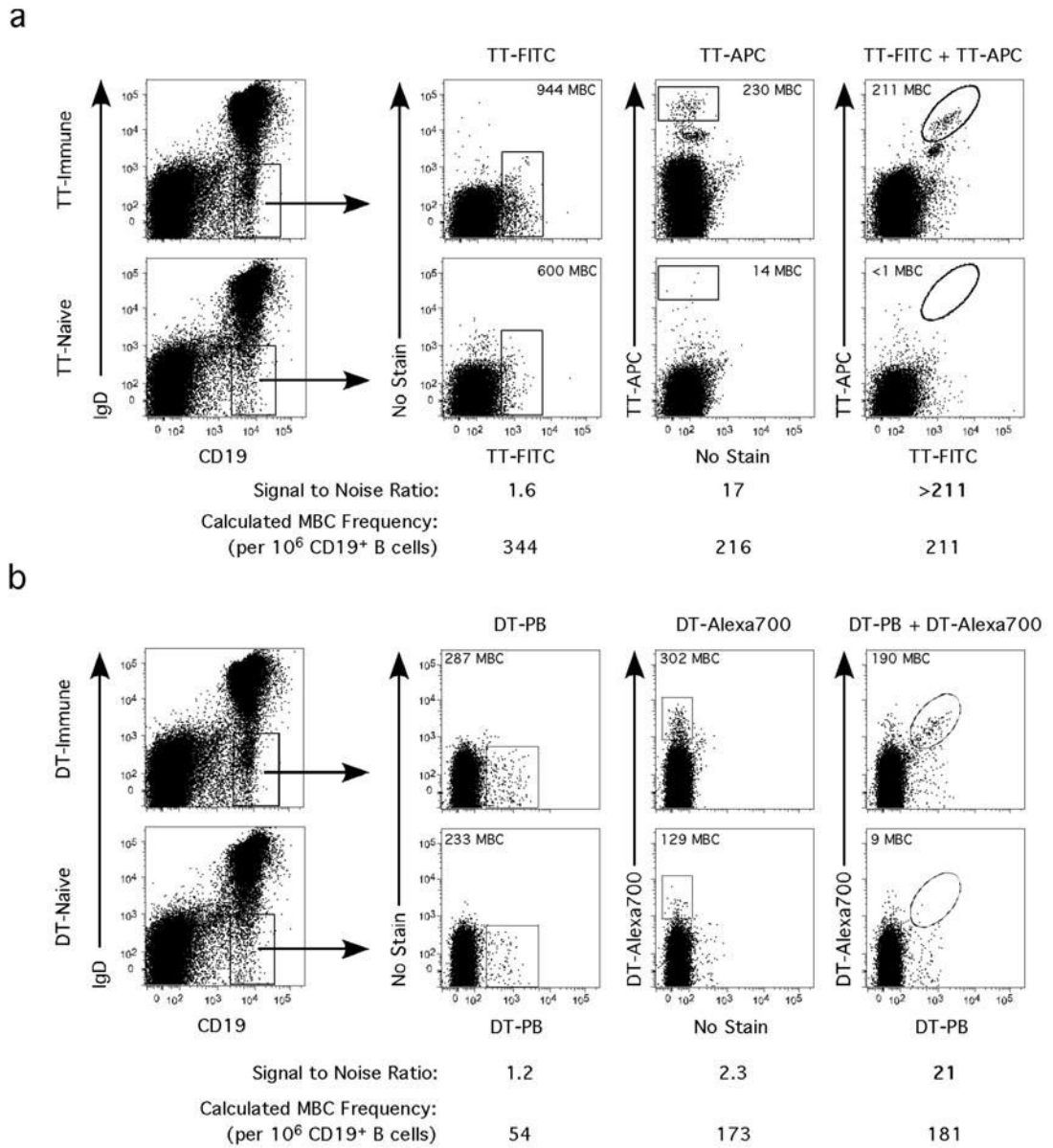


Figure 1. Dual FC staining for MBC increases specificity while maintaining sensitivity
 Splenocytes from DTaP-immune and DTaP-naïve mice were divided into three fractions and stained with a single antigen-specific fluorescent reagent, or dual fluorescent reagents. (a) Live cell gates were established and class-switched B cells were defined as IgD⁻CD19⁺ for both immune and naïve mice. For cells stained only with TT-FITC or TT-APC, a FITC positive gate or APC positive gate was defined. A TT-specific MBC gate was also defined for dual stained cells (TT-FITC + TT-APC). The numbers in each dot plot diagram refer to the frequency per 1x10⁶ CD19⁺ cells. Frequencies listed below the dot plots are immune frequencies with the naïve frequencies subtracted. Signal-to-noise ratios refer to the immune frequencies divided by the naïve frequencies (b) Mice were analyzed as described above using DT-specific fluorescent reagents. Mice were Tripedia®-immune (3 doses) with splenocytes collected ~45 days after the last immunization.

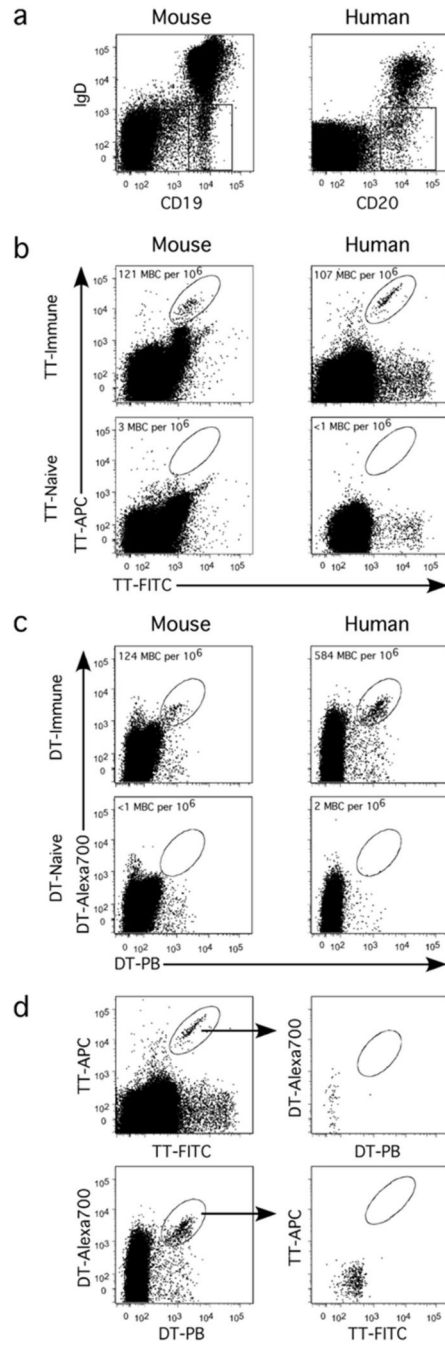


Figure 2. Enumeration of TT- and DT-specific MBC by FC

Mouse splenocytes or human PBMC were analyzed for the presence of TT- or DT-binding MBC FC. (a) Live cell gates were established and Ig class-switched B cells were defined as IgD⁻CD19⁺ (mice) or IgD⁻CD20⁺ (human subjects). (b) TT binding within class-switched B cells was identified using TT-APC and TT-FITC conjugated reagents. TT-specific MBC frequencies were calculated from the region gates as shown and indicate the number of TT binding B cells per 10⁶ CD19⁺ (mouse) or CD20⁺ (human) B cells. (c) DT-specific MBC were measured as described for TT, except that DT-Alexa Fluor® 700 and DT-Pacific Blue™ conjugated DT reagents were used. (d) Human TT-specific and DT-specific MBC populations were gated and analyzed for staining of fluorochromes specific for the reciprocal MBC

populations as an added test of antigen specificity. Immune mice were vaccinated with Tripedia® (3 doses) with splenocytes prepared 1.5 months after the last immunization (representative of 3 mice in 3 experiments). An adult volunteer at ~5 months post-tetanus/diphtheria booster immunization is shown (representative of 13 subjects from 7 experiments), and naïve human cells were collected from UCB (representative of 3 subjects from 3 experiments).

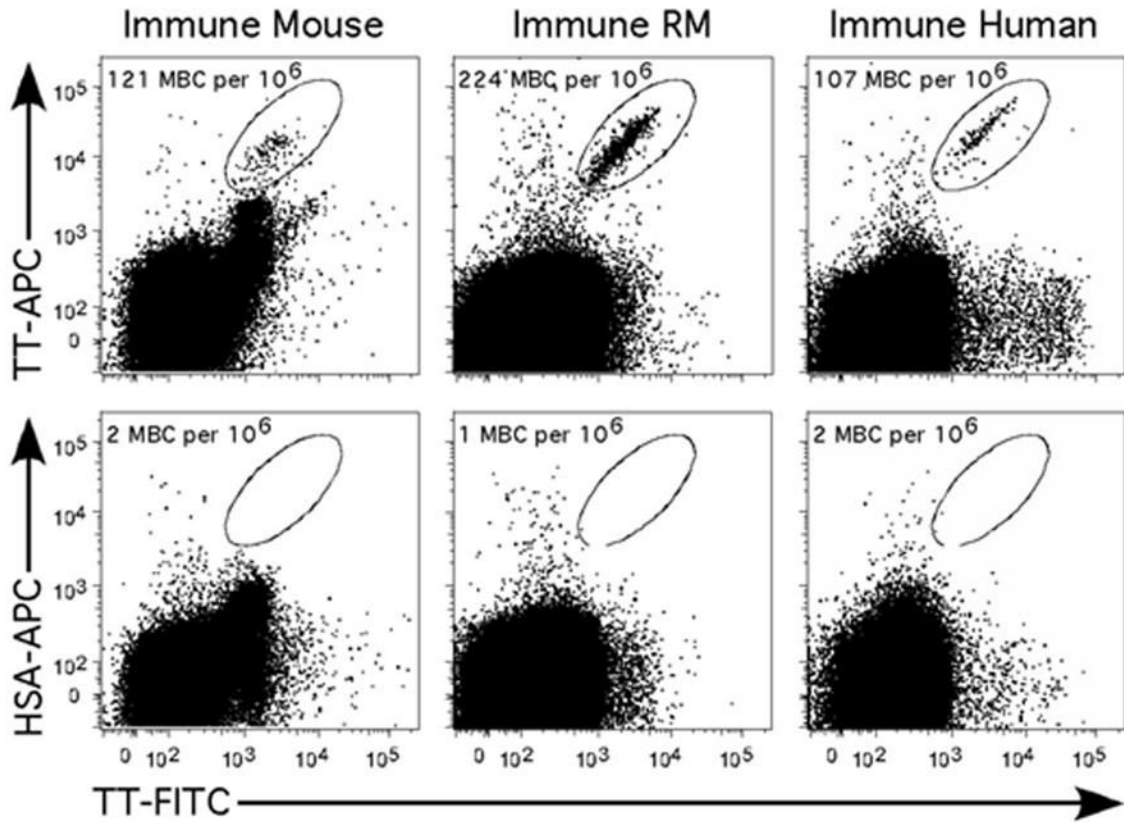


Figure 3. Quantitation of TT-specific MBC in three mammalian species with internal specificity controls

Mouse splenocytes, RM PBMC, and human PBMC were analyzed for the presence of TT-specific MBC. Live cell gates were determined and class-switched B cells were defined as IgD^-CD19^+ (mice) or IgD^-CD20^+ (RM and humans). Frequencies shown indicate the number of TT-binding B cells per 10^6 total B cells ($CD19^+$ or $CD20^+$ lymphocytes) within the designated region gates. To determine the optimal TT-specific MBC gate, cells from the same sample in each species were divided into two fractions, and stained with either TT reagents (TT FITC + TT-APC) or labeled with antigen-mismatched reagents (TT-FITC + HSA-APC). Splenocytes from Tripedia®-immune mice were analyzed at 1.5 months after the last of three immunizations (representative of 3 mice from 3 experiments). RM were Tripedia®-immune with PBMC collected ~3 years after the last of four Tripedia® immunizations (representative of 6 RM tested in 3 experiments). An adult volunteer at ~6 months post-tetanus/diphtheria booster immunization is shown (representative of 13 subjects from 7 experiments).

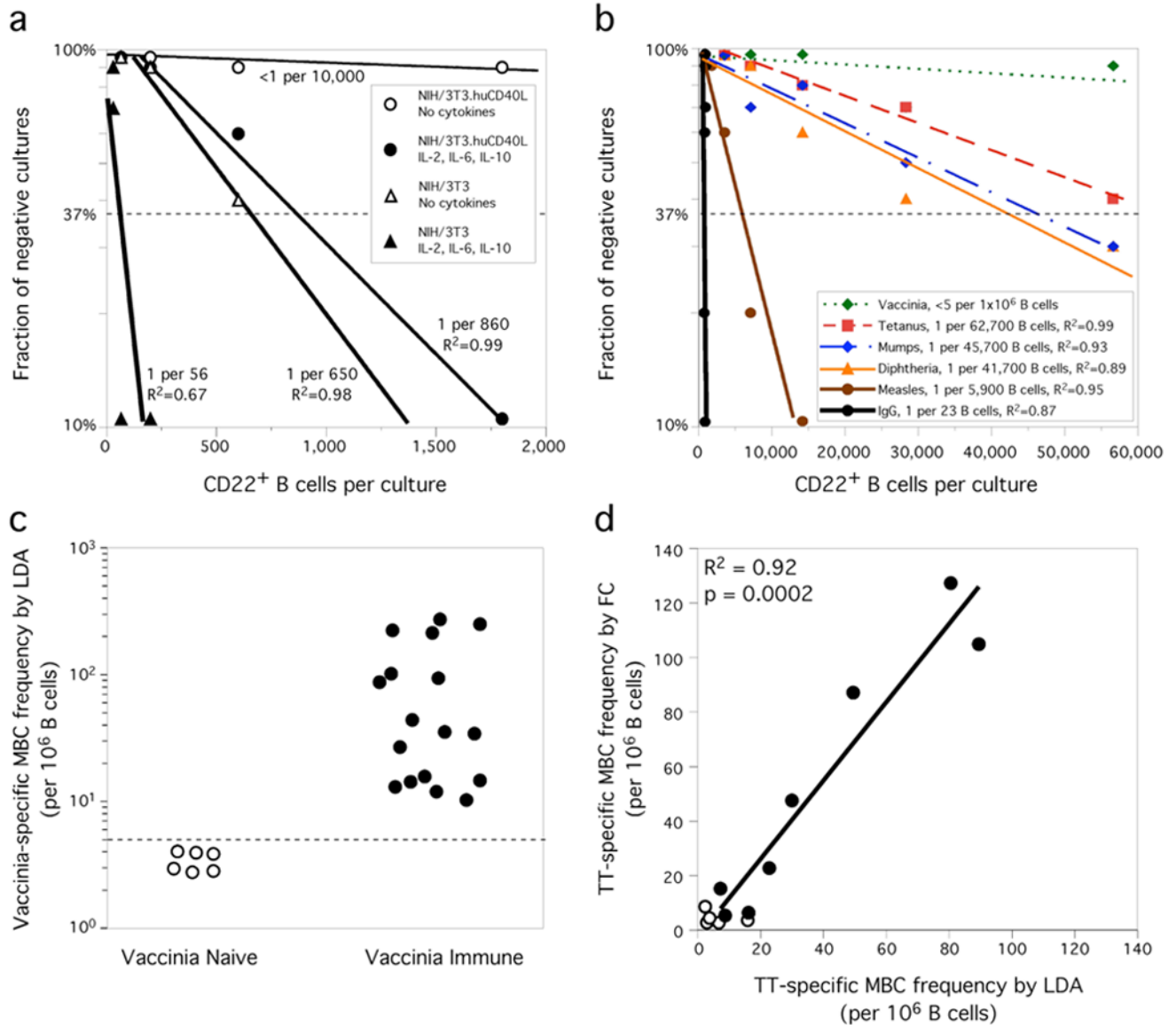


Figure 4. Optimization and validation of a human multi-antigen MBC LDA

(a) LDA was performed with human PBMC, using different feeder cells with or without an optimal cytokine cocktail. IgG-specific ELISA performed on LDA supernatants identified cultures containing IgG-producing B cells. The percent of negative cultures was plotted versus the number of CD22⁺ B cells per culture. Regression analysis was performed as described (Miller et al., 1977), and MBC frequencies (per CD22⁺ B cell) were calculated from the 37% intercept. (b) Using optimal culture conditions of NIH/3T3 feeder cells and IL-2, IL-6, IL-10 (as well as SAC, PWM, LPS and CpG-2006), purified peripheral B cells were analyzed by LDA. MBC were detected by testing LDA supernatants (15–25 μ L/per test) on antigen-specific ELISA plates (vaccinia, measles, mumps, TT, DT) or by an anti-IgG ELISA for quantitating total IgG-secreting cultures. The subject shown was seropositive for each antigen except vaccinia. (c) Vaccinia-specific MBC frequencies were determined by LDA for both vaccinia naïve ($n = 6$) and vaccinia immune ($n = 17$) subjects to demonstrate the specificity and sensitivity of the assay. (d) TT-specific MBC frequencies were compared by FC and LDA. PBMC from adult human donors ($n = 13$) were analyzed for TT-specific MBC as described in

Fig. 3. Purified B cells from the same PBMC samples were analyzed for TT-specific MBC by LDA and compared to the frequencies derived from FC. Least-squares linear regression was used to compare the data sets, and calculate the associated p-value. Open symbols represent data points that were at or below detection limits by either FC or LDA and were not included for correlation analysis.