IgM$^+$ memory B cells induced in response to *Plasmodium berghei* adopt a germinal centre B cell phenotype during secondary infection

Halina M. Pietrzak[1, 2], Lisa J. Ioannidis[1, 2] , Diana S. Hansen[1, 2*]

[1]The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia.

[2]Department of Medical Biology, University of Melbourne, Parkville, Victoria, 3010, Australia.

Running title: IgM$^+$ memory B cells induced by malaria

* Corresponding author Diana S. Hansen, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia. Phone: +61 3 93452469; Fax: +61 3 93470852; Email: hansen@wehi.edu.au

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Abstract

Emerging evidence started to delineate multiple layers of memory B cells, with distinct effector functions during recall responses. Whereas most studies examining long-lived memory B cell responses have focussed on the IgG^+ memory B cell compartment, IgM^+ memory B cells have only recently started to receive attention. It has been proposed that unlike IgG^+ memory B cells, which differentiate into antibody-secreting plasma cells upon antigen re-encounter, IgM^+ memory B cells might have the additional capacity to establish secondary germinal centre (GC) responses. The precise function of IgM^+ memory B cells in the humoral immune response to malaria has not been fully defined. Using a murine model of severe malaria infection and adoptive transfer strategies we found that IgM^+ memory B cells induced in responses to *P. berghei* ANKA readily proliferate upon re-infection and adopt a GC B cell-like phenotype. The results suggest that that IgM^+ memory B cells might play an important role in populating secondary GCs after re-infection with *Plasmodium,* thereby initiating the induction of B cell clones with enhanced affinity for antigen, at faster rates than naive B cells.

Key Words

Malaria, immunity, memory B cells, germinal centres

Introduction

Malaria is one of the most serious infectious diseases affecting humans, causing 250 million clinical cases annually and leaving 3 billion people at risk of infection globally (World Health Organisation, 2017). Blood stage parasite infection is responsible for all clinical symptoms associated with malaria (Schofield and Grau 2005), which can range from relatively mild and uncomplicated disease to severe complications including cerebral malaria, respiratory distress, hypoglycaemia, renal failure and pulmonary oedema (Miller et al. 2013). Children under five years of age, with low levels of pre-existing immunity, are most vulnerable to these severe manifestations of disease. Individuals living in malaria endemic regions acquire clinical immunity to the disease only after repeated exposure to the parasite over a number of years. This type of immunity is non-sterilising, but can prevent clinical episodes by substantially reducing parasite burden (Marsh and Kinyanjui 2006), commonly resulting in asymptomatic infection of adults. Parasite specific antibody responses are known to play an important role protective immunity to malaria (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991) and engage in various effector functions during blood stage infection including the prevention of erythrocyte invasion and sequestration (Bolad and Berzins, 2000; Stanisic *et al.*, 2009), promotion of complement mediated lysis, and opsonisation for phagocytic parasite clearance (Schmidt *et al.*, 2015).

The development of long-lived, antibody-mediated immunological memory requires the establishment of germinal centre (GC) structures. Following initial interaction with antigen, naive B cells in lymphoid organs either differentiate into short-lived plasma cells, or migrate deeper into the B cell follicles where they establish a GC. Within the GCs, activated B cells undertake somatic hypermutations in their Ig genes, followed by selective survival of B cells producing antibodies with higher affinity. GC establishment and function requires a

specific subset of T cells- named T_{FH} cells (Crotty 2011). Recent studies investigated how these processes are modulated in malaria in order to identify immunological processes which underpin the slow acquisition of immunity. Epidemiological studies revealed that the development of IgG^+ memory B cells specific for *P. falciparum* antigens is delayed in individuals residing in areas of high seasonal malaria transmission, who are vulnerable clinical episodes of disease (Weiss et al. 2010). Consistent with those findings, using a rodent infection model we previously showed that that the same inflammatory cytokines that contribute to the induction of malaria disease symptoms such as IFN-γ, upregulate the expression of the transcription factor T-bet in T_{FH} cells, which prevents their normal differentiation and capacity to provide help for the induction of plasma cell and IgG^+ memory B cells (Ryg-Cornejo et al. 2016).

Emerging evidence started to delineate multiple layers of memory B cells, with distinct effector functions during recall responses (Dogan et al. 2009). Whereas most studies examining long-lived memory B cell responses to infection and vaccination have focussed on the IgG^+ memory B cell compartment, IgM^+ memory B cells have only recently started to receive attention. Evidence from murine studies has shown that this compartment remains stable (Good-Jacobson and Tarlinton 2012) and recognises antigen with low affinity (Pape et al. 2011) compared to class-switched memory B cells. Unlike IgG^+ memory B cells, which rapidly differentiate into antibody-secreting plasma cells upon antigen re-encounter, recent studies in humans and murine models suggest that IgM^+ memory B cells have the additional capacity to establish secondary GC responses, undertake further rounds of somatic hypermutation and affinity selection to remodel their existing B cell receptor specificity, as well as class switch recombination (Taylor et al. 2012; Seifert et al. 2015). As such, it has been proposed that IgM^+ memory B cells may assist in the maintenance of long-term

immunity by replenishing the IgG^+ memory B cell pool when reactivated by their specific antigen.

Recent studies revealed that IgM antibody responses specific for a range of *P. falciparum* surface antigens are associated with protection from symptomatic malaria (Arama et al. 2015; Boyle et al. 2019), suggesting a potential role for IgM^+ memory B cells in the acquisition of clinical immunity to the infection. Only a few studies have identified IgM^+ memory B cells in malaria-exposed individuals and in malaria mouse infection models (Wendel et al. 2017; Krishnamurty et al. 2016; Stephens et al. 2009), with those induced by murine malaria found to rapidly differentiate into plasma cells upon antigenic re-stimulation (Krishnamurty et al. 2016). Memory B cells isolated from malaria-exposed individuals have been shown to undertake Ig somatic hypermutations (Triller et al. 2017) and accumulate further mutations after acute infection (Wendel et al. 2017) raising the intriguing possibility that IgM^+ memory B cells might re-enter GC reactions during a secondary infection. To address that question, here we have used a murine model of severe malaria to investigate the differentiation capacity of IgM^+ memory B cells during re-infection. Our results revealed that this compartment adopts a GC B cell-like phenotype upon parasite-specific antigenic re-stimulation.

Materials and methods

Mice and infections

Eight to twelve-week-old C57BL/6 mice were used for all experiments in this study. Mice were infected with 5.0 x 10^4 *P. berghei* ANKA pRBCs via intravenous (i.v.) tail vein injection. In most experiments, mice were treated with a dose chloroquine (10 mg/kg) and

pyrimethamine (10 mg/kg) intraperitoneally (i.p.) at the onset of severe malaria symptoms on day 5 post-infection (p.i.), followed by treatment with drinking water containing chloroquine and pyrimethamine (10 mg/kg) for 7 days. Parasitemia was assessed from Giemsa-stained smears of tail blood. All experiments conducted with approval from the Walter & Eliza Hall Institute Animal Ethics Committee.

Flow cytometry

Splenocytes suspensions were incubated with anti-CD16/CD32 antibody. Cells were washed twice prior to incubation with fluorescent antibodies for 30 minutes at 4°C. To detect of IgM^+ memory B cells, splenocytes were stained with: APC anti-CD21 (7G6, Miltenyi Biotec), FITC anti-IgM (Il/41, BD Biosciences), PE anti-IgD (11-26c.2a, BD Biosciences), PerCP Cy5.5 anti-CD19 (1D3, BD Biosciences), BV650 anti-Fas/CD95 (Jo2, BD Biosciences), PE/Cy7 anti-CD38 (90, BioLegend) and BV421 anti-CD138 (281-2, BioLegend). To detect GC B cells splenocytes were stained with the following antibodies: PerCP Cy5.5 anti-CD19 (1D3, BD Biosciences), PE anti-GL7 (GL7, BD Biosciences), FITC anti-CD38 (90, BioLegend), BV785 anti-CD45.1 (A20, BioLegend) and PE/Cy7 anti-CD45.2 (281-2, BioLegend). To detect plasma cells, splenocytes were stained with the following antibodies: PerCP Cy5.5 anti-CD19 (1D3, BD Biosciences), FITC anti-IgM (Il/41, BD Biosciences), PE anti-IgD (11-26c.2a, BD Biosciences), BV421 anti-CD138 (281-2, BioLegend), BV785 anti-CD45.1 (A20, BioLegend) and PE/Cy7 anti-CD45.2 (281-2, BioLegend). For detection of proliferating cells or intranuclear transcription factors, cells were fixed and permeabilised with 200µl of eBioscience permeabilisation buffer for 1hr at 22°C, then washed and stained with AF594 anti-Ki67 (SOIA15), or with APC anti- Bcl-6 (GC B cells, BD Biosciences) or

APC anti-Blimp-1 (Plasma cells, BD Biosciences) for a another hour. After staining, cells were washed twice, and analysed either by flow cytometry on a BD LSRFortessa™ X-20 cell analyser (BD Biosciences), or sorted on BD FACSAria™ III/W FACS cell sorters (BD Biosciences). Data was analysed using FlowJo v12 software (Treestar Inc.).

Adoptive transfer

Ly5.1$^+$ C57BL/6 mice were infected with 5.0 x 10^4 *P. berghei* ANKA parasitized red blood cells (pRBCs) and drug cured at day 5 p.i.. On day 21 p.i., CD19$^+$CD21$^-$CD138$^-$Fas$^-$ CD38$^+$IgD$^-$IgM$^+$ cells were isolated by sorting on a BD FACS Aria III system (BD Biosciences) with BD Diva software. Isolated IgM$^+$ memory B cells were adoptively transferred (1.0 x 10^6/mouse) into Ly5.2$^+$ C57BL/6 recipients, which were infected with 5.0 x10^4 *P. berghei* ANKA pRBCs the following day. Spleen cells from recipient animals were collected on day 3 p.i. for assessment of immune responses.

Statistical analysis

Statistical analysis was performed in Prism version 8 (GraphPad Software Inc.). The Student's t test was used for analysis of data. Information on replicates/error/significance are indicated in the figure legends.

Results

IgM$^+$ memory B cells can be detected after infection with Plasmodium berghei

We have previously shown that *P. berghei* ANKA infection results in the induction of IgG^+ memory B cells (Ryg-Cornejo et al. 2016; Ly et al. 2019). To determine if this infection also induced IgM^+ memory B cells, C57BL/6 mice were infected with 5.0 x 10^4 pRBCs. As *P. berghei* ANKA infection is lethal in C57BL/6 mice, infected animals were cured with anti-malarial drugs after onset of symptoms at day 5 p.i. to allow the assessment of immune responses at later time-points as described (Ryg-Cornejo et al. 2016). After full resolution of parasitemia on day 24 p.i., splenocytes were extracted from malaria-exposed mice, stained with a panel of fluorescent antibodies and analysed by flow cytometry using the gating strategy delineated in Figure 1A. Memory B cells were identified among live lymphocytes by high expression of CD19, CD38 and IgM, and low expression of IgD, CD138, the marginal zone B cell marker CD21 and the GC B cell marker Fas (Figure 1A). A population of $CD19^+CD38^+IgM^+$ memory B cells was identified at day 24p.i. The absolute number of IgM^+ memory B cells was 3 times higher in *P. berghei* infected mice compared to naïve controls (Figure 1B). Thus *P. berghei* ANKA infection induces IgM^+ memory B cells that can be detected in the spleen two weeks after parasitemia has been controlled.

IgM^+ memory B cells adopt a germinal-centre B cell phenotype upon malaria re-infection

Having established that an IgM^+ memory B cell compartment is detectable after infection with *P. berghei*, we next sought to understand the functional capacity of this cell subset upon re-infection. To that end, congenically marked $Ly5.1^+$ C57BL/6 mice were infected with *P. berghei*, then drug cured at day 5 p.i. as described above. On day 21 p.i., IgM^+ memory B cells were isolated by cell sorting following the gating strategy shown in Figure 1A, and adoptively transferred into $Ly5.2^+$ congenically marked recipient mice (Figure 2A). Flow cytometry analysis indicated that the sorted population was ~93% pure (Supplementary

Figure 1). Recipient mice were then infected with *P. berghei* ANKA and on day 3 p.i. the phenotype of transferred $CD19^+$ donor and recipient cells was examined by flow cytometry (Figure 2B). Rapid proliferation upon re-stimulation is a key feature of antigen-experienced memory cells (Tangye et al. 2003; Good et al. 2009). To determine if adoptively transferred IgM^+ memory B cells from malaria exposed donors rapidly proliferated in response to secondary infection like conventional memory B cells do, the expression of Ki67 was assessed within $Ly5.1^+$ and $Ly5.2^+$ $CD19^+$ cells. Consistent with a memory-like phenotype, $Ly5.1^+$ donor cells readily proliferated only three days after re-infection with *P. berghei* ANKA (Figure 2C) while $Ly5.2^+$ cells did not.

Blimp-1 and Bcl-6 are transcription factors required for the differentiation of B cells into plasma cells and GC B cells, respectively (Shapiro-Shelef et al. 2003; Crotty et al. 2010). Thus, the expression of these transcription factors was examined among $Ly5.1^+$ B cells from malaria-exposed donors and $Ly5.2^+$ recipient controls in order to determine the differentiating capacity of IgM^+ memory B cells upon antigenic re-stimulation. No Blimp-1 upregulation could be detected among donor or recipient B cells on day 3 p.i. (not shown). In contrast, Bcl-6 expression could be readily detected among $Ly5.1^+$ B cells from malaria-exposed donors and was significantly higher to that of recipient controls (Figure 2D), suggesting that adoptively transferred IgM^+ MBCs might adopt a GC B cell-like phenotype during re-stimulation. To investigate this further, frequencies of plasma cells and GC B cells were examined on day 3p.i. in $Ly5.1^+$ donor and $Ly5.2^+$ recipient B cells. Analysis of $Ly5.1^+$ donor cells in recipient mice that were left uninfected, was included as controls. No plasma cells ($CD138^+CD19^+$) could be detected emerging from the $Ly5.1^+$ donor compartment or $Ly5.2^+$ recipients upon malaria infection or from $Ly5.1^+$ cells adoptively transferred into uninfected controls (Figure 2E). Instead, nearly 20% of $Ly5.1^+$ cells from exposed mice downregulated expression of CD38 and upregulated the GC B cell specific marker GL7 when

challenged with *P. berghei* but not when recipient mice were left uninfected (Figure 2E) . Together, these results indicate that IgM^+ memory B cells induced in response to malaria adopt a GC B cell-like phenotype in response to secondary infection.

Discussion

Recent evidence support the concept that IgM^+ memory B cells have the ability to remodel their B cell receptor specificity by accessing secondary GC reactions after encounter with cognate antigen during re-infection. This response has been postulated to be beneficial for responding to antigenic variants of the original pathogen; a desirable feature to efficiently recognise polymorphic malarial antigens. The present study provided evidence for the functional capacity of IgM^+ memory B cells generated during malaria infection to acquire a GC B cell-like phenotype upon a secondary challenge.

Differentiation into antibody-secreting plasma cells is a widely accepted re-stimulatory role of isotype-switched memory B cells (Tangye and Tarlinton, 2009) and a few studies have reported strong plasma cell responses also mediated by IgM^+ memory B cells in response to viral infection (Zabel *et al.*, 2014). In contrast, other work in parasitic and bacterial infections demonstrated the capacity of IgM^+ memory B cells to re-enter GCs during recall responses (Yates *et al.*, 2013; Kenderes *et al.*, 2018). Thus , the available information supports the idea that different pathogens might direct IgM^+ memory B cells towards various differentiation fates (McHeyzer-Williams *et al.*, 2018). In malaria, previous studies using the mild *Plasmodium chabaudi* murine infection model, detected antigen specific IgM^+ memory B cells that appear to differentiate into plasma cells after re-stimulation (Krishnamurty et al. 2016). Unlike *P. chabaudi*, the *P. berghei* ANKA infection used in the current study results in the induction of a highly-inflammatory response to

infection, responsible for the development of severe disease symptoms (Hansen, 2012). In that context, IgM^+ memory B cells induced by *P. berghei* ANKA infection failed to differentiate into antibody secreting plasma cells upon re-encounter with antigen and adopted a GC B cell-like phenotype, raising the possibility that inflammatory responses and the cytokine environment induced during infection might also play a role in the differentiation fate of IgM^+ memory B cells upon re-encounter with cognate antigen.

Our findings are consistent with a model in which the IgM^+ memory B cell compartment plays an important role in populating secondary GCs after re-infection with *Plasmodium.* Similar to other infection settings (Pape *et al.*, 2011), malaria-specific IgM^+ memory B cells might mediate the rapid re-seeding of secondary GCs for earlier recall responses, thereby playing a critical role in the immune response by rapidly initiating the induction of B cell clones with enhanced affinity for antigen, at faster rates than naive B cells (Good et al. 2009). Further work will be required to confirm whether IgM^+ memory B cell-derived GC B cells localise in splenic GCs during re-exposure to malaria and if this process is required for the gradual acquisition of protective immunity. Understanding the role of IgM^+ B cells in mediating immunological memory responses to *Plasmodium* parasites and the impact that infection has on the functional capacity of this compartment may be highly-informative for the development of novel therapeutics and vaccines to target malaria.

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Conflict of Interest

None.

Ethical standards

All experiments conducted with approval from the Walter & Eliza Hall Institute Animal Ethics Committee.

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Figure Legends

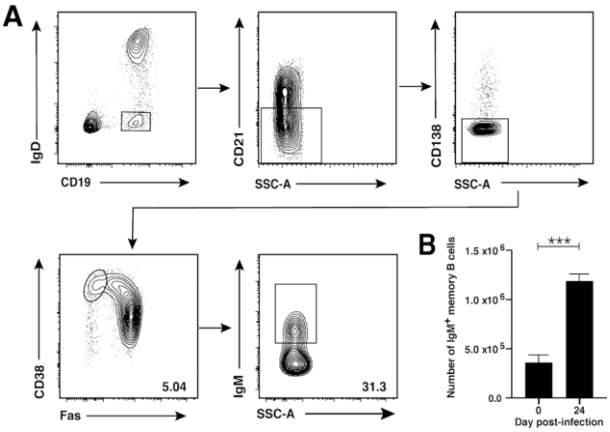

Figure 1: *P. berghei ANKA* infection induces IgM^+ memory B cells. C57BL/6 mice were infected with *P. berghei ANKA* and treated with anti-malarial drugs from day 5 p.i. **(A)** Gating strategy used to identify IgM^+ memory B cells by flow cytometry, depicting gated IgD^-CD19^+ B cells that were negative for CD21 and CD138 to exclude marginal zone B cells and plasma cells, respectively. Memory B cells were then identified by high expression of CD38 and low levels of Fas, to exclude GC B cells. IgM^+ memory B cells were identified among this compartment. **(B)** Absolute number of IgM^+ memory B cells in *P. berghei* ANKA infected mice on day 24 p.i. and naive controls. Error bars represent the mean of 8-10 mice ± SEM, ***p<0.005.

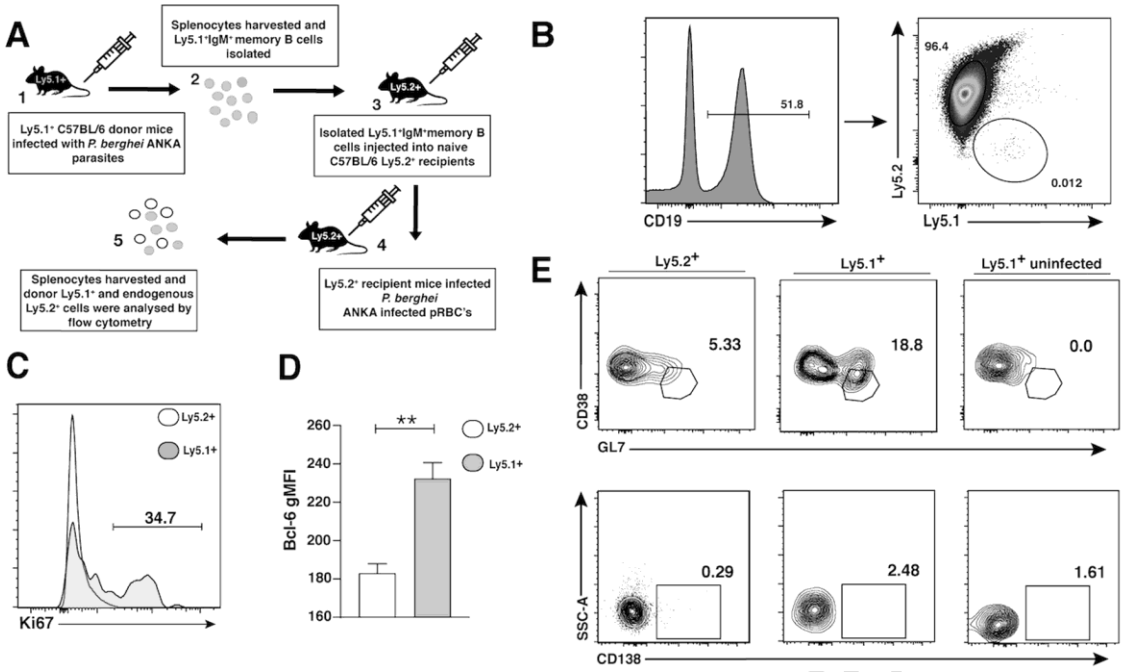

Figure 2: IgM$^+$ memory B cells adopt a GC B cell phenotype during a secondary *P. berghei ANKA* infection. **(A)** Schematic illustrating the adoptive transfer workflow used to assess the functional capacity of IgM$^+$ memory B cells. IgM$^+$ memory B cells isolated from infected C57BL/6 Ly5.1$^+$ mice were adoptively transferred into Ly5.2$^+$ recipient mice. Recipient mice were then challenged with *P. berghei* ANKA, followed by analysis of both the Ly5.1$^+$ donor and Ly5.2$^+$ endogenous compartments 3 days p.i. by flow cytometry. **(B)** Congenically labelled cells were identified among gated CD19$^+$ B cells. **(C)** Percentage of proliferating cells was calculated by assessing Ki67 expression among gated CD19$^+$ Ly5.1$^+$ and Ly5.2$^+$ cells. (**D**) Geometric mean fluorescence intensity (gMFI) for Bcl-6 expression in CD19$^+$Ly5.1$^+$ or Ly5.2$^+$ cells. **(E)** Percentage of CD38^{low}GL7$^+$ GC B cell-phenotype cells (top panels) and CD138$^+$ plasma cells (bottom panels) was calculated among CD19$^+$ Ly5.2$^+$ cells from infected mice and CD19$^+$Ly51$^+$ cells from infected mice and uninfected controls. Data are representative of 2 experiments. Graphs depict means of 4 mice ± SEM, **p<0.01. Representative histograms and dot plots are shown.