Atypical and classical memory B cells produce *Plasmodium falciparum* neutralizing antibodies

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Antibodies can protect from *Plasmodium falciparum* (*Pf*) infection and clinical malaria disease. However, in the absence of constant reexposure, serum immunoglobulin (Ig) levels rapidly decline and full protection from clinical symptoms is lost, suggesting that B cell memory is functionally impaired. We show at the single cell level that natural *Pf* infection induces the development of classical memory B cells (CM) and atypical memory B cells (AtM) that produce broadly neutralizing antibodies against blood stage *Pf* parasites. CM and AtM contribute to anti-AtM serum IgG production, but only AtM show signs of active antibody secretion. AtM and CM were also different in their IgG gene repertoire, suggesting that they develop from different precursors. The findings provide direct evidence that natural *Pf* infection leads to the development of protective memory B cell antibody responses and suggest that constant immune activation rather than impaired memory function leads to the accumulation of AtM in malaria. Understanding the memory B cell response to natural *Pf* infection may be key to the development of a malaria vaccine that induces long-lived protection.

Malaria caused by *Plasmodium falciparum* (*Pf*) infection is a leading cause of morbidity and mortality primarily in children in sub-Saharan Africa (Murray et al., 2012). Protective immunity to control clinical disease caused by parasite growth in red blood cells (RBCs) takes years to develop and requires repeated infections (Garnham, 1949). Human serum IgG antibodies can protect from blood stage parasitemia and malaria symptoms as demonstrated by historic passive transfer studies (Cohen et al., 1961). However, in the absence of constant reexposure, serum Ig levels rapidly decline and protection from malaria wanes, suggesting that protective humoral memory is not efficiently formed or is functionally impaired (Struik and Riley, 2004; Dorfman et al., 2005; Langhorne et al., 2008). Indeed, repeated *Pf* infection is associated with the accumulation of circulating atypical memory B cells (AtM) that are characterized by expression of the inhibitory Fc-receptor-like-4 (FcRL4; Weiss et al., 2009, 2010, 2011). FcRL4-positive memory B cells have originally been described in tonsils of healthy individuals (Elhrhardt et al., 2005, 2008). Circulating FcRL4-positive AtM also present in chronic HIV infection, where they show signs of functional exhaustion and hyporesponsiveness after in vitro stimulation suggesting that their memory B cell function is impaired (Moir et al., 2008). The role of AtM in immunity to malaria is speculative and it is unclear whether AtM contribute to the production of protective serum antibodies in vivo. To address these questions, we performed a molecular and functional characterization of the anti-AtM classical memory B cells (CM) and AtM response in immune adult donors from a malaria endemic region.
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associated with humoral protection against asexual blood stage parasites (Gardner et al., 2002; Fowkes et al., 2010). We therefore focused our analysis on two Pf antigens, merozoite surface protein 3 (MSP3) and glutamate-rich protein (GLURP), that have been described to induce serum IgG responses that are associated with protection from clinical malaria (Meraldi et al., 2004; Singh et al., 2009; Fowkes et al., 2010). Specifically, we studied the anti-Pf IgG B cell response to the vaccine candidate GMZ2, a fusion protein of the immune-dominant GLURP R0 nonrepeat region that is expressed at all parasitic life cycle stages in the human host, and the conserved domain of MSP3 that is critically involved in RBC invasion (de Stricker et al., 2000; Rodríguez et al., 2005; Esen et al., 2009). All individuals, including the selected donors, had high titers of serum IgG antibodies against GMZ2, MSP3, and GLURP and showed GMZ2 reactivity in CM and AtM (Fig. 1, D–F; and not depicted). In summary, the selected donors showed representative anti-Pf serum IgG and memory B cell responses as well as high frequencies of circulating AtM.

RESULTS

Pf-reactive CM and AtM in malaria immune adults

To determine the molecular and functional antibody characteristics of anti-Pf memory B cells, we set out to generate a panel of recombinant monoclonal antibodies from circulating IgG-positive CM and AtM of three asymptomatic semi-immune adults (MP036, MP070, and MP071; Table S1). The individuals were selected from a cohort of 67 healthy subjects with neutralizing serum IgG activity against asexual blood stage parasites from a highly endemic area in Gabon (Fig. 1 A; Dal-Biacono et al., 2007). All donors presented with a high frequency of circulating CD27−CD21− AtM that showed increased FcRL4 and CD19 expression as well as lower IgG surface expression compared with CM (Fig. 1, B and C).

The Pf genome is highly complex, and only a few antigens out of the >5,000 possible protein products have been

area. By single cell antibody cloning and mass spectrometry we show that CM and AtM express Pf-neutralizing antibodies and contribute to serum IgG production in vivo.

Figure 1. Memory B cell sorting. (A) Relative 3D7Luc neutralizing activity of 100 µg/ml purified serum IgG from study participants in comparison to polyclonal serum IgG preparations from nonimmune controls (0% neutralization) and 50 mM chloroquine (100% neutralization, dashed gray line). Dots represent individual samples. Selected donors for antibody cloning are indicated. (B) Gating strategy for the flow cytometric isolation of CM and AtM. Representative plots from MP070 are shown. (C) Frequency of circulating CM and AtM. (D) IgG GMZ2 reactivity in serum as measured by ELISA. The dotted line shows a Pf-naive control serum. (E) Flow cytometric analysis of GMZ2-reactive CM and AtM in peripheral blood from MP036, MP070, and MP071 compared with a malaria naive donor. (F) Frequency of circulating GMZ2-reactive CM and AtM in all donors as determined in E. Red bars indicate arithmetic mean.
**Ig gene repertoire of Pf-reactive CM and AtM**

To generate recombinant monoclonal antibodies, the Ig gene variable regions of single isolated GM2Z-reactive CM and AtM from MP036, MP070, and MP071 were cloned into expression vectors (Tiller et al., 2008). Ig gene sequence analysis demonstrated that the memory IgG response was highly diverse (Tables S2–S4). No major differences in the IGH and IGL or IGK gene segment usage or IGH CDR3 features between CM and AtM were observed (Fig. 2 A–C; and Tables S2–S4). However, AtM had, on average, higher levels of somatic hypermutations in their V gene segments than CM (Fig. 2 D; mean ± SEM: AtM, IGH 28.1 ± 8.1, IGK 20.6 ± 9.1, and IGL 20.1 ± 1.7; CM, 24.5 ± 7.7, IGK 17.2 ± 9.5, and IGL 20.1 ± 1.5). IGH and IGK/IGL gene sequence alignments showed that clonally expanded B cells with identical Ig gene rearrangements were observed within both compartments. However clonally related CM and AtM were not detected in any of the three donors (Fig. 2 E). Statistical models based on the observed distributions of clonal relatives predict that if CM and AtM were directly derived from a shared ancestor, the likelihood of a random absence of shared clusters would be <0.1% on average for each of the donors. Thus, we conclude that CM and AtM show differences in their somatic hypermutation load and lack signs of clonal relationship, suggesting that the two populations may originate from different precursors.

**Pf MSP3 and GLURP antigen specificity of CM and AtM antibodies**

To determine whether anti-Pf CM and AtM show differences in their antigen reactivity, we expressed 236 recombinant monoclonal CM and AtM antibodies and performed ELISA (Fig. 3, A, C, and D; and Tables S2–S4). On average, 91 ± 7% of antibodies showed reactivity with the sort antigen GM2Z (Fig. 3 A). For individual binders, reactivity with Pf merozoites was confirmed by fluorescence microscopy with infected RBCs (iRBCs; Fig. 3 B). We further discriminated between MSP3 and GLURP antigen reactivity by ELISA (Fig. 3, C and D). MSP3-specific antibodies were more frequent than GLURP-specific antibodies in both memory B cell compartments (Fig. 3 E; mean ± SEM: MSP3, 70.3 ± 12.7% for CM and 67.3 ± 16.0% for AtM; GLURP, 17.0 ± 11.3% for CM and 11.3 ± 10.0% for AtM). However, even in the presence of high concentrations of
The frequency of polyreactive antibodies was significantly higher in the AtM than in the CM compartment (Fig. 4B; mean ± SEM: 64.3 ± 6.2% vs. 38.7 ± 8.4%; P = 0.004), and polyreactive antibodies were enriched in the MSP3+GLURP compartment (Fig. 4C). However, independently of the level of polyreactivity, anti-Pf antibodies, but not non-Pf reactive control antibodies, bound preferentially to iRBCs and not to non-iRBCs (Fig. 4D). Thus, we conclude that CM and AtM express polyreactive and nonpolyreactive anti-Pf antibodies, and polyreactive antibodies are enriched in the AtM compartment.

CM and AtM antibodies neutralize Pf RBC invasion

Next, we tested if antibodies from Pf-reactive memory B cells neutralize asexual blood stage parasites (Fig. 5). Using an
Thus, IgG memory B cells expressing neutralizing antibodies against genetically diverse asexual blood stage parasites develop in response to Pf infection. CM and AtM contribute to the production of anti-Pf serum IgG. CM can contribute indirectly to the production of serum IgG antibodies after differentiation into antibody-secreting cells (Bernasconi et al., 2002), but whether AtM produce serum Ig in vivo is not known. To determine if the IgG antibodies that we cloned from CM and AtM could be detected in serum, we analyzed Fab2 fragments derived from protein G–purified MSP3- and GLURP-reactive serum IgG of donor MP036 by mass spectrometry (Fig. 6 A; Scheid et al., 2011). We then compared the amino acid sequences obtained from the mass spectrometric analysis to the Ig sequence database of the recombinant monoclonal CM and AtM antibodies from the same donor (Fig. 6 B and Fig. S1). We obtained amino acid sequence hits that mapped to the CM and AtM database but not to the international human protein index database, suggesting that both populations contribute to anti-Pf serum IgG production.

By PCR, we were able to detect secretory IgG transcripts in AtM but not in CM, suggesting that AtM actively secrete antibodies in vivo and contribute directly to the humoral anti-Pf response (Fig. 6 C). Indeed, the IgG subclass in vitro merozoite neutralization assay with genetically modified 3D7Luc Pf parasites expressing firefly luciferase, we measured the neutralization capacity at various antibody concentrations (Fig. 5 A; Lucumi et al., 2010). CM and AtM antibodies of all three donors showed various degrees of merozoite neutralization (Fig. 5 B). We conclude that the majority of IgG-positive GMZ2-reactive memory B cells express Pf inhibitory antibodies. Of note, we did not observe significant differences in the mean neutralization activity of CM and AtM antibodies or any correlation with MSP3 or GLURP antigen specificity or polyreactivity (Fig. 5 C). Although the MSP3 and GLURP antigens analyzed in this study are relatively conserved among different Pf strains and wild isolates, antigenic variation may limit the breadth of Pf neutralizing antibodies (Manske et al., 2012). To gauge the breadth of Pf inhibition, we tested a selected set of 25 3D7Luc neutralizing antibodies for neutralization of genetically diverse Pf isolates (DD2 [Thailand], IT2 [Brazil], and HB3 [Honduras]; Fig. 5 D and Tables S2–S4; Smilkstein et al., 2004; Johnson et al., 2007). The nontransgenic 3D7 strain was included for comparison. The antibodies were diverse in their neutralization efficacy and breadth, but individual antibodies showed comparable levels of neutralization for all strains, e.g., the CM antibodies 36–0003 and 71–2339 or the AtM antibody 71–0222 (Fig. 5 D). Thus, IgG memory B cells expressing neutralizing antibodies against genetically diverse asexual blood stage parasites develop in response to Pf infection.
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reactivity, but in vivo Fc-mediated effector functions may add to parasite neutralization (Bouharoun-Tayoun et al., 1990). Humoral immunity to MSP3 and GLURP has been associated with protection from malaria, and we observed neutralization for anti-MSP3 as well as anti-GLURP memory antibodies (Singh et al., 2009; Fowkes et al., 2010). Anti-MSP3 antibodies were more frequent than anti-GLURP antibodies, suggesting that MSP3 may be more efficient in inducing IgG memory B cell responses than GLURP. However, it cannot be excluded that the use of GMZ2 as bait for the isolation of anti-*Pf* memory B cells may enrich for MSP3-specific B cells. Differences in the nature of individual *Pf* antigens may account for differences in the induction of long-term humoral memory responses that have also been observed for viral and vaccine antigens (Taylor et al., 1996; Dorfman et al., 2005; Töngren et al., 2006; Amanna et al., 2007; Akpoghenata et al., 2008; Amanna and Slifka, 2010).

The relatively low level of antigenic variation in the MSP3 and GLURP peptides analyzed in this study may facilitate the generation of broadly inhibitory serum IgG antibodies. Whether neutralizing memory B cell antibodies are generated at similar frequencies to other antigens remains to be determined. Acquisition of protective humoral immunity to *Pf* may, however, also be associated with qualitative differences in the neutralizing activity of these antibodies. Thus, future studies

**DISCUSSION**

In summary, we show that immunity to malaria is associated with the development of memory B cells that express *Pf* neutralizing antibodies and contribute to serum IgG production. In mice and humans, stable memory B cell responses against *Plasmodium* antigens have been detected, but whether the antibodies produced by memory B cells were neutralizing was not known (Wipasa et al., 2010; Nogaro et al., 2011; Ndungu et al., 2012). We found that the majority of memory B cell antibodies showed *Pf* neutralizing activity, including antibodies with broad neutralizing activity against genetically diverse *Pf* strains. Neutralization in our in vitro assays was a direct effect of antigen reactivity, but in vivo Fc-mediated effector functions may add to parasite neutralization (Bouharoun-Tayoun et al., 1990).

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IgM memory B cells, but their role in Plasmodium infection is not clear (Ndungu et al., 2009; Nduati et al., 2010; Reynaud et al., 2012). A thorough characterization of the quality and quantity of the human anti-Pf IgM memory B cell antibody response may help to elucidate the relative contribution of IgM versus IgG B cell memory to Pf in humans.

A large fraction of the recombinant monoclonal antibodies will have to assess whether protection from clinical disease is not only associated with changes in the magnitude and overall breadth of the response but also with changes in the frequency of memory B cells that produce Pf neutralizing antibodies (Courtin et al., 2009; Nogaro et al., 2011). In addition, the role of IgM-expressing memory B cells in the response to Pf remains to be determined. Studies in mice suggest that long-term memory is predominantly the role of IgM memory B cells, but their role in Plasmodium infection is not clear (Ndungu et al., 2009; Nduati et al., 2010; Reynaud et al., 2012). A thorough characterization of the quality and quantity of the human anti-Pf IgM memory B cell antibody response may help to elucidate the relative contribution of IgM versus IgG B cell memory to Pf in humans.

Figure 6. CM and AtM contribution to anti-Pf serum IgG. (A) Representative MS/MS spectra. Observed b-type fragment ions (containing the N terminus), y-type fragment ions (containing the C terminus), and loss of water (*) are indicated. Ions corresponding to the loss of water are labeled. Observed backbone cleavages are indicated (b for b-type ions, y for y-type ions). Data shown are representative of at least three independent experiments on two different instruments. (B) IgH amino acid sequences of CM and AtM antibodies. Framework regions (FWR) and complementarity determining regions (CDR) are indicated. Blue stars indicate somatic point mutations. Mass spectrometric peptides with identical amino acid sequence are indicated in red. (C) RT-PCR amplicons of secretory and membrane IgG transcripts from the indicated B cell subpopulations. Data shown are representative of at least two independent experiments. (D) Serum IgG subclass distribution in donors from the Lambaréné area. (E) IgG subclass distribution as determined by IGH gene transcript sequence analysis. n indicates the number of tested sera. Error bars indicate SD.
AtM compartment. However, polyreactive antibodies specifically recognized iRBCs and not non-iRBCs, suggesting that the level of binding to non-Pf antigens was relatively low compared with the affinity for Pf. Polyreactivity is a frequent feature of antibody responses to viral infections and has been shown to play a protective role against HIV, but whether such promiscuous antibodies are also beneficial in the immune response against Pf remains to be determined (Haynes et al., 2005; Mouquet et al., 2010; Mouquet and Nussenzweig, 2012).

AtM showed signs of activation, proliferation, and active antibody secretion, suggesting that their development in response to Pf is associated with differentiation into antibody-secreting cells rather than with a defect in B cell memory as previously proposed for HIV (Ehrhardt et al., 2008; Moir et al., 2010). Immunity to Pf is not sterilizing, and the frequent antigenic stimulation by repeated infections may induce Pf-reactive antigen-experienced B cells to develop into antibody-secreting cells and to acquire an AtM phenotype, a process which may be facilitated by the polyreactive nature of the antigen receptors expressed by AtM.

The relative contribution of AtM to the anti-Pf serum IgG response cannot be assessed, and although we demonstrate that AtM secrete Pf neutralizing antibodies, it remains to be determined whether AtM fulfill true memory functions or if they simply represent a population of circulating short-lived antibody-secreting cells. The fact that AtM were almost negative for the plasma cell marker CD138, but showed signs of proliferative activity and expressed surface IgG as well as MHC II and various activation markers, supports the notion that AtM resemble plasma blasts rather than long-lived plasma cells that were displaced from the bone marrow by recent infection (Ndungu et al., 2009).

The antibody repertoire of AtM was distinct from CM, suggesting that the two populations may develop from different precursors. A deeper analysis of the Ig gene repertoire of anti-Pf CM and AtM in comparison to tissue resident memory B cells and long-lived plasma cells may help to determine the origin of circulating AtM (Ehrhardt et al., 2005, 2008; Moir et al., 2008).

The memory B cell antibodies cloned in this study are fully human and may therefore be used in experimental human malaria infection to determine their protective efficacy against Pf asexual blood stages (Roestenberg et al., 2012). Understanding the protective properties as well as the limitations...
in the memory B cell antibody response to \textit{Pf} is crucial for developing vaccination strategies that elicit neutralizing antibodies to protect from clinical malaria.

**MATERIALS AND METHODS**

**Study participants.** Healthy adults (age 19–57 yr, mean 33 ± 10 yr) were recruited in the Lambaréné area (Gabon, Africa; Table S1). Peripheral blood samples were obtained after signed informed consent during dry season. None of the donors had previously participated in any malaria vaccine study. Parasitemia was assessed by microscopy, PCR (i.e., qPCR), and rapid diagnostic test (NOW ICT Malaria Test; Binax Inc.). Ethical approval was obtained from the Comité d’Ethique Regionale Independant de Lambarène (No. 17/10).

**Flow cytometry.** Peripheral blood mononuclear cells were purified using Percoll gradient density centrifugation. Flow cytometric analyses were performed on an LSR II instrument (BD). IgG memory B cells were isolated using an ARIA II flow cytometric cell sorter (BD). \textit{Pf} GLURP, MSP3, and GMZ2 antibodies were produced and purified as previously described (Theisen et al., 2004). GMZ2 was chemically coupled to Alexa Fluor 647 according to the manufacturer’s instructions (Life Technologies). For use in flow cytometry, the following mouse antibodies were used: anti-human CD19-PE-Cy7, anti-human IgG-APC-H7, anti-human CD27-FITC, anti-human CD38-APC, anti-human IgG1-APC, anti-human CD24-biotin, anti-human CD27-biotin, anti-human CD84-biotin, anti-human HLA-DR-APC (all from eBioscience), anti-human Ki-67-PE (BioLegend), and anti-human FcRLL4-biotin (gift of G.H. Elhrhardt and M. Cooper, Emory University School of Medicine Atlanta, GA). Streptavidin-QDot605 (Life Technologies) was used to detect biotin-conjugated primary antibodies. 7-AAD (Life Technologies) was used in all experiments to exclude dead cells. For intracellular staining with anti-human Ki-67-PE (BioLegend) and 7-AAD (Invitrogen), PBMCs were permeabilized using Fix & Perm solution (BD) according to the manufacturer’s instructions.

**Single-cell sorting and antibody cloning.** Single-cell sorting and antibody cloning was performed as previously described with the following modifications: cells were sorted into 384-well plates and volumes of RT-PCR reactions were scaled by a factor of 0.25 (Tiller et al., 2008; Schedel et al., 2011). In brief, single cells were isolated using flow cytometric cell sorting. cDNA was generated in the original 384-well sort plates. Ig gene transcripts were amplified by nested RT-PCR, and sequenced and cloned into IgG1 heavy and Ig \textit{\gamma} light chain expression vectors, respectively. For in vitro antibody production, HEK293T cells were cotransfected with Ig heavy and matching Ig light chain plasmids. Recombinant monoclonal antibodies were purified from culture supernatant under sterile conditions using protein G beads. Antibodies were eluted from protein G beads using 0.1 M glycine, pH 3, and neutralized in 10× PBS. Secretory and membrane IgG PCR. CM, AtM, naive B cells (CD19+IgM+IgD+), and plasmablasts (CD19 +CD27+CD38+IgG+) were bulk isolated by flow cytometric cell sorting. Secretory and membrane IgG transcripts were amplified by RT-PCR using an \textit{Igh}\textit{\gamma} gene forward primer and a reverse primer designed to amplify a region of \textit{Igh}\textit{\gamma} isotype genes that are preferentially expressed in these cell types. The peptides were resuspended in 0.5% acetic acid and injected onto a PicoTip emitter (New Objective) with integrated 360 µm emitter tip. The peptides were covalently attached to a nanoliter tip packed with 10 cm of reverse-phase ProteoPepII (New Objective) and 10 µm tip), packed with 10 cm of reverse-phase ProteoPepII, and eluted using 200 ng trypsin (Promega). The resulting peptides were isolated using reverse phase resin (PORS 20 R2; Applied Biosystems) and eluted using 40% acetonitrile in 0.5% acetic acid, followed by 80% acetonitrile in 0.5% acetic acid. Acetonitrile was removed using a speedvac (Thermo Fisher Scientific). The peptides were resuspended in 0.5% acetic acid and injected onto a PicoFrit column (New Objective) with integrated emitter tip (360 µm O.D., 50 µm I.D., 10 µm tip), packed with 10 cm of reverse-phase ProteoPepII (New Objective) using a Proxeon nLC 1000 (Thermo Fisher Scientific). The HPLC column was interfaced to either an Orbitrap Velos or a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The peptides were eluted using the following gradient: 0% B in 5 min, 40% B in 125 min, 60% B in 150 min, 100% B in 165 min (A = 0.1 M acetic acid, B = 90% acetonitrile in
0.1 M acetic acid, flow rate 200 nl/min). Both instruments were operated in a data-dependent mode. For analysis on the Orbitrap Velos, a full scan was followed by a 20 MS/MS scans on the 20 most abundant ions in that full scan. The peptides (only charge states >1 and <5) were isolated with a 2D window, target window of 2e5 ions, dissociated with normalized collision energy of 35, activation Q = 0.25, activation time = 30 ms) and mass analyzed in the LTQ. For analysis on the Q Exactive Orbitrap, a full scan (resolution 70,000 at m/z 200) was followed by a 10 MS/MS scans on the 10 most abundant ions from the preceding full scan. The peptides (only charge states >1 and <5) were isolated with a 2D window, target window of 2e4 ions, dissociated with normalized collision energy of 27, and mass analyzed with a resolution of 17,500 (at m/z 200). For either instrument, the ions selected for MS/MS were set on an exclusion list for 30 s. The resulting MS/MS spectra were searched against the Human IPI and in-house-specific IgG database using Xandem! Peptide hits corresponding to GMZ22-reactive CM or AtM IgG sequences were manually confirmed.

SNP analysis and stochastic calculation. MSP3 (PF10_0345) and GLURP (PF10_0344) SNP analysis was performed using next-generation sequencing data of 222 Pf isolates supplied by the MalariaGen Genomic Epidemiology Network (Manske et al., 2012; Data Release 1.0). The mean number of clusters of clonally related cells was determined by maximum likelihood estimations using a multivariate hypergeometric distribution model and the singlet/doublet frequencies observed over all donors/populations while assuming a uniform size of the individual clusters. If CM and AtM cells differed in their surface phenotype but were derived from the same population, the likelihood of not observing any shared clusters within an individual donor can be approximated as (1-c/c)^2, where c is the calculated overall number of clusters, a the number of unique clusters sampled from the first population, and b the number of unique samplings from the second population.

Online supplemental material. Fig. S1 provides peptide data for the mass spectrometric analysis of purified anti-GMZ22 serum IgG from MP036. Table S1 provides clinical information on blood samples from MP036, MP070, and MP071. Tables S2, S3, and S4 provide IgG and antibody reactivity information for antibodies from MP036, MP070, and MP071, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20121970/DC1.

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