Complement Contributes to Protective Immunity against Reinfecion by Plasmodium chabaudi chabaudi Parasites

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We have studied the impact of deficiency of the complement system on the progression and control of the erythrocyte stages of the malarial parasite Plasmodium chabaudi chabaudi. C1q-deficient mice and factor B- and C2-deficient mice, deficient in the classical complement pathway and in both the alternative and classical complement activation pathways, respectively, exhibited only a slight delay in the resolution of the acute phase of parasitemia. Complement-deficient mice showed a transiently elevated level of gamma interferon (IFN-γ) in the plasma at the time of the acute parasitemia compared with that of wild-type mice. Although there was a trend for increased precursor frequencies in CD4+ T cells from C1q-deficient mice producing IFN-γ in response to malarial antigens in vitro, intracellular cytokine staining of spleen cells ex vivo showed no difference in the numbers of IFN-γ+ splenic CD4+ and CD8+ cells. In contrast, C1q-deficient animals were significantly more susceptible to a second challenge with the same parasite. C1q-deficient animals had increased levels of IgM and IgG2a anti-malarial antibodies 100 days after primary infection. However, following a significantly higher parasitemia, C1q-deficient mice had increased levels of IgM and IgG2a anti-malarial antibodies. In summary, this study indicates that while complement plays only a minor role in the control of the acute phase of parasitemia of a primary infection, it does contribute to parasite control in reinfection.

The cellular immune response to Plasmodium chabaudi chabaudi malaria in mice has been extensively studied in vivo. The early period of infection is associated with a strong TH1-like CD4+ T-cell response characterized by the production of high levels of gamma interferon (IFN-γ). The demonstration of exacerbated P. c. chabaudi infections in mice treated with anti-IFN-γ (24, 35) or mice lacking IFN-γ (41) or an effective IFN-γ receptor (9, 40) supports the view that IFN-γ-mediated pathways play a role in the control of acute parasitemia. Later in the infection, after the initial reduction in parasitemia, there is a switch to a TH2-like response associated with production of interleukin-4 (IL-4) and IL-10 and the provision of help to B cells for antibody responses (19). At this stage, B cells are necessary for the control and clearance of residual parasites (25, 42, 44), suggesting a requirement for antibody in the resolution of infection. More recently, B cells have also been implicated in the regulation of the switch of T cells from the initial TH1-like response to a TH2-like response (17). The mechanism by which antibody mediates its protective effect is not known. Neutralization or agglutination of parasites, inhibition of merozoite invasion (2, 8), Fc receptor phagocytosis or cytotoxicity (4), and complement-dependent lysis or uptake are all possible effector mechanisms.

Studies of human malaria suggest that the complement system, particularly the classical pathway, may play a role in host defense against malarial infection (13, 30, 33, 47). The first component of the lectin pathway, mannose binding lectin (MBL), is an acute-phase reactant which increases in serum during malarial attacks (39). However, deficiency of MBL is relatively common (36), and it does not seem to be associated with increased susceptibility to severe malaria and/or cerebral malaria (1).

Several attempts have been made to address the potential role of complement in host defense against malaria infection in vitro with varied results. Complement has been shown to be able to kill both human and rodent malaria parasites in vitro, at different stages in the life cycle, in the presence of specific antibodies (10, 11, 29). However, infected erythrocytes, in spite of their ability to activate complement, seem quite resistant to complement-mediated lysis, a phenomenon attributable in part to the presence of complement-regulatory proteins on the infected cells (14, 48). Moreover, Plasmodium berghei sporozoites have been shown to be resistant to complement from their susceptible rodent hosts but not to human serum (15). Complement has also been assigned a role in the enhancement of Plasmodium falciparum parasite killing by the monocytic cell line THP-1 and human neutrophils (16, 32).

Ward and colleagues studied the role of complement in host defense against P. berghei in vivo in rats by depletion of complement with cobra venom factor (46). They found that com-
plement-depleted rats suffered from more rapid and higher parasitemias and that 60% of the depleted animals succumbed to what in normal rats would have been a nonlethal infection.

As well as the activities of complement in target cell lysis and opsonophagocytosis, complement has a well-established role in the regulation of antibody responses (5), suggesting that the effect of complement deficiency during infection may be more widespread than just the loss of complement-mediated parasite killing.

In the work described here we have investigated the role of complement in host defense against the malaria parasite *P. c. chabaudi* (AS strain) using mice rendered deficient in complement components by gene targeting. Our data show that the classical pathway of complement plays a minor role in the control of the acute phase of parasitemia. Despite elevated serum IFN-γ levels, C1q-deficient mice suffered a higher peak parasitemia. Of particular note, complement-deficient mice were more susceptible to secondary challenge with the same parasite, indicating impairment in the development of their immunity to reinfection.

**MATERIALS AND METHODS**

**Mice.** C1q-deficient (*Clqa<sup>−/−</sup>*) and factor B- and C2-deficient (*H-2b/C<sup>2−/−</sup>* ) mice, lacking the classical complement pathway and both the alternative and classical complement activation pathways, respectively, were generated as previously described (3, 38). All experimental animals were female, of the pure inbred 129/Sv genetic background and between 8 and 10 weeks of age at the start of experiments. Animal care and procedures were conducted according to institutional guidelines.

**Parasites and infection.** *P. c. chabaudi* AS parasites were maintained as described previously (27, 37). *Clqa<sup>−/−</sup>* and wild-type mice were infected by intraperitoneal injection of 10<sup>5</sup> parasitized 129/Sv females-derived erythrocytes. The course of infection was monitored by examination of Giemsa-stained blood film every 1 to 4 days throughout the experimental period. As indicated, some animals were cured of residual parasitemia 8 weeks after primary infection with three intraperitoneal injections of chloroquine (Sigma) (25 mg/kg of body weight, 48 h between injections). The absence of parasites was confirmed on blood smears before secondary infection of the animals with the same dose of *P. c. chabaudi* parasitemias 6 weeks later. Naïve *Clqa<sup>−/−</sup>* and wild-type mice were also infected at the same time and served as controls for the infection.

Infected blood from 129/Sv or C57BL/6 (H-2<sup>b</sup>) mice was used as a source of antigen in limiting-dilution assays (described below). For this purpose, blood was collected when the parasites were at the late trophozoite stage, passed over a column of CF11 powder to remove leukocytes as described previously (19), washed three times in Hanks balanced salt solution (Gibco, Paisley, United Kingdom) containing 10% fetal calf serum, and resuspended at a 0.1% (vol/vol) suspension in culture medium (described below).

**Measurement of cytokines.** (i) ELISA for cytokines. IFN-γ and IL-4, in serum and/or tissue culture supernatants, were measured by ELISA as described previously (19, 43, 44). For analysis of serum IFN-γ levels, at least four mice were studied in each group, with 5 to 12 mice in each group during the peak of infection (7 to 9 days after primary infection).

(ii) Intracellular cytokine staining. Intracellular staining was used to determine cytokine production by single cells as described previously (28). Cells were suspended at 10<sup>6</sup>/ml and stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml). Two hours after stimulation, brefeldin A was added at 10 μg/ml, and the cells were incubated for a further 2 h. Cells were harvested, washed, and stained for different surface markers using directly conjugated antibodies. At the end of the procedure, the cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS with 4% formaldehyde fixative. After incubation for 20 min at room temperature, the cells were stained for cytokines.

**RESULTS**

**Resolution of the acute phase of *P. c. chabaudi* infection in complement-deficient mice.** The initial few days of parasitemia were no different in *Clqa<sup>−/−</sup>* and wild-type mice. Parasitemia peaked in the wild-type mice at 26.0% ± 2.1% (mean ± standard error of the mean [SEM]) 8 days after infection (Fig. 1). However, parasitemia continued to rise in the *Clqa<sup>−/−</sup>* animals for another day before peaking at 28.7% ± 2.2% on day 9. The control of the acute phase of infection was mildly, but significantly, impaired in the *Clqa<sup>−/−</sup>* animals (parasitemia of 21.9% ± 1.7% in 7 *Clqa<sup>−/−</sup>* animals compared to 16.4% ± 1.5% in 11 control mice 10 days after infection; *P* < 0.05 [Mann-Whitney test]). After the initial slight delay in clearance, the parasites were cleared normally, *H-2b/C<sup>2−/−</sup>* mice (*n* = 14) exhibited parasitemia levels similar to those seen in *Clqa<sup>−/−</sup>* animals (peak parasitemia on day 9, 28.1% ± 1.8%) (Fig. 1).

**Malaria-specific antibody responses.** Plasma samples were collected from at least five *Clqa<sup>−/−</sup>* and wild-type mice before infection, at intervals during the primary infection, and just prior to and 18 days after secondary challenge. The levels of malaria-specific antibodies were measured by ELISA as described previously (18). Briefly, a lysate of *P. c. chabaudi* parasites was used as a source of antigen. In addition to the test plasma, hyperimmune plasma from mice that had survived more than five challenges of *P. c. chabaudi* infection was used as a positive control and standard and was given an arbitrary value of 1,000 U/ml for each of the isotypes. Goat anti-mouse immunoglobulin M (IgM), IgG1, IgG2a, IgG2b and IgG3 antibodies conjugated to alkaline phosphatase (Seralab, Leicester, United Kingdom) were used to detect specifically bound mouse Ig of the respective isotypes.

The relative amounts of malaria-specific antibody of the different isotypes are expressed as values of arbitrary ELISA units (AEU) as calculated from the standard hyperimmune serum. Expression of antibody amounts in arbitrary units allows a direct comparison of the amounts of a single isotype produced by *Clqa<sup>−/−</sup>* and wild-type mice but does not allow a comparison of the amounts of different isotypes.

**Limiting-dilution assay.** Splenic CD4<sup>+</sup> T cells were positively selected using a magnetic cell sorting separation system described elsewhere (44). The cells were labeled with biotinylated anti-CD4 monoclonal antibody followed by streptavidin- and/or tissue culture supernatants, were measured by ELISA as described previously (19, 43, 44).

Antibody levels were determined in the culture supernatants after 7 days of culture, and cytokines and proliferative responses were measured after the cultures had been incubated for a further 2 days with irradiated normal C57BL/6 mouse spleen cells and antigen. Precursor frequencies were determined from the zero-order term of the Poisson distribution using regression analysis. Cultures were considered positive when either proliferation or antibody or cytokine production exceeded the background response (without T cells) by more than 3 standard deviations.

**Statistics.** Statistics were calculated using GraphPad Prism version 2.0 (GraphPad Software, San Diego, Calif.). Nonparametric tests were applied throughout unless otherwise stated.
Increased susceptibility of C1qa<sup>−/−</sup> mice to secondary infection with P. c. chabaudi. Previously infected C1qa<sup>−/−</sup> and wild-type mice were challenged a second time with P. c. chabaudi 14 weeks after primary infection as described in Materials and Methods (Fig. 2). Six days after secondary infection, all the wild-type and C1qa<sup>−/−</sup> animals had detectable parasitemias (>0.01%). The mean percentage of blood cells that were parasitized was significantly greater at this point in 9 C1qa<sup>−/−</sup> mice (0.94% ± 0.36% compared to 0.13% ± 0.02% in 11 control animals) (P < 0.05 [Student’s t test on log-transformed data]). Parasitemia levels as high as 3.14% were observed among the C1qa<sup>−/−</sup> mice, but they did not exceed 0.26% in the wild-type controls.

Anti-malaria specific antibody responses in C1q-deficient mice. During the primary infection, anti-malaria specific antibody responses (days 7 and 40) were no different in C1qa<sup>−/−</sup> and wild-type mice. One hundred days after the primary infection (immediately prior to reinfection), the C1qa<sup>−/−</sup> mice had lower levels of IgG2a anti-malarial antibodies (median, 33 AEU; range, 12 to 74 AEU; n = 5) than the wild-type mice (median, 82 AEU; range, 78 to 170 AEU; n = 5; p < 0.01 [Mann-Whitney test]) (Fig. 3). The mice were reinfeected on day 102, and antibody levels were again measured on day 120. After the second challenge, the C1qa<sup>−/−</sup> animals (n = 9) had significantly greater levels than the wild-type mice (n = 11) of IgM (median, 1,457 AEU [range, 790 to 3,380 AEU] and 1,014 AEU [range, 260 to 1,390 AEU], respectively; P < 0.05 [Mann-Whitney test]) and of IgG2a (median, 3,910 AEU [range, 936 to 6,290 AEU] and 1,430 AEU [range, 229 to 4,880 AEU], respectively; P < 0.05 [Mann-Whitney test]) (Fig. 3).

Augmented serum IFN-γ response in C1qa<sup>−/−</sup> mice during the acute phase of infection. We have shown previously that C1qa<sup>−/−</sup> mice immunized with a conventional antigen have lower levels of IFN-γ produced by antigen-specific CD4<sup>+</sup> T cells (6). Since IFN-γ has been shown to be important in the control of the acute phase of malarial infection (9, 24, 35, 40, 41), the circulating levels of IFN-γ in C1qa<sup>−/−</sup> and wild-type mice during a P. c. chabaudi infection were compared. IFN-γ was detectable in the sera of wild-type mice only during the peak of infection (days 7 to 9), as previously reported (Fig. 4). However, circulating IFN-γ was detectable in the C1qa<sup>−/−</sup> animals at significantly elevated levels throughout the acute phase (Fig. 4A). By day 5, the median concentration of IFN-γ in the sera of C1qa<sup>−/−</sup> mice (n = 6) was 4.46 ng/ml (range, 0.86 to 23.12 ng/ml) compared to <0.04 ng/ml (range, <0.04 to 0.34 ng/ml) in five wild-type mice (P < 0.01 [Mann-Whitney test]). Seven days after infection, the median concentration in 10 C1qa<sup>−/−</sup> mice was 18.66 ng/ml (range, 2.83 to 188 ng/ml) compared to 7.34 (range, 4.20 to 17.62 ng/ml) in 12 wild-type mice (P < 0.01 [Mann-Whitney test]). Serum IFN-γ was still significantly higher in the C1qa<sup>−/−</sup> mice (n = 8) 9 days after infection (median, 46.91 ng/ml; range, 1.32 to 74.50 ng/ml) than in the six wild-type mice (median, 6.14 ng/ml; range, 1.32 to 21.46 ng/ml) (P < 0.05 [Mann-Whitney test]). After day 9, IFN-γ was no longer detectable in the serum, consistent with previous reports. H2-Bf/C2<sup>−/−</sup> mice also exhibited increased circulating levels of IFN-γ during the acute phase of parasitemia, with significantly greater amounts of protein detectable on days 6, 8, and 9 (Fig. 4B). Six days after infection, five H2-Bf/C2<sup>−/−</sup> mice had a median concentration of IFN-γ in circulation of 0.81 ng/ml (range, 0.65 to 2.55 ng/ml) compared to 0 ng/ml (range, 0 to 0.38 ng/ml) in five control mice (P = 0.0079 [Mann-Whitney test]). By days 8 and 9, the median concentration of IFN-γ was still significantly higher in the five H2-Bf/C2<sup>−/−</sup> mice (median, 3.21 ng/ml; range, 0.86 to 4.04 ng/ml) compared to 0.34 ng/ml (range, 0 to 0.34 ng/ml) in five control mice (P < 0.01 [Mann-Whitney test]).
Anti-malarial IgG1, IgG2b, and IgG3 titers were similar in C1qa−/− mice against a primary blood stage malaria infection in mice. This study of P. c. chabaudi infection in C1qa−/− mice demonstrated that only a minor role was played by either the alternative or classical pathway of complement activation in the early stages of malaria infection. The relatively small contribution of complement to this early stage of infection by the blood stage parasite is perhaps a consequence of the inefficiency of complement in mediating the lysis of parasite-infected cells (14, 48). These data, taken together with studies demonstrating that antibody-mediated protection in nonlethal or lethal malaria models does not require Fe receptors (31, 45), suggest that there is not a prominent role for either of the major opsonophagocytic systems in host defense against a primary blood stage malaria infection in mice.

Cytokine production by T cells during a P. c. chabaudi infection in C1qa−/− mice. Limiting-dilution assays of CD4+ T cells were performed 7 and 28 days after primary infection of C1qa−/− mice to measure the precursor frequencies of IFN-γ and IL-4-producing cells responding to malarial antigens. While there was a trend toward an increase in the frequency of IFN-γ-producing antigen-specific CD4+ T cells in the C1qa−/− mice, the numerical difference in precursor frequencies was small (Table 1). There was no significant difference in the frequency of antigen-specific CD4+ T cells producing IL-4 or providing help for antibody production at these times. Assessment by intracellular cytokine staining of the numbers of splenic CD4+ and CD8+ T cells producing IFN-γ and IL-4 showed no differences between C1qa−/− and wild-type mice at any time point measured during the infection (data not shown).

DISCUSSION

This study of P. c. chabaudi infection in complement-deficient mice demonstrated that only a minor role was played by either the alternative or classical pathway of complement activation in the early stages of malaria infection. The relatively small contribution of complement to this early stage of infection by the blood stage parasite is perhaps a consequence of the inefficiency of complement in mediating the lysis of parasite-infected cells (14, 48). These data, taken together with studies demonstrating that antibody-mediated protection in nonlethal or lethal malaria models does not require Fe receptors (31, 45), suggest that there is not a prominent role for either of the major opsonophagocytic systems in host defense against a primary blood stage malaria infection in mice.

Complement, however, did play a role in immunity to a second challenge, since after reinfection with the same dose of parasites used in the initial infection, the mean peak parasitemia in C1qa−/− mice was sevenfold greater than that in the control mice. The role of complement as an adjuvant to low doses of antigen is well established (7), and complement is known to play a significant role in the acquired immune response to T-cell-dependent antigens, which results in high titers of class-switched antibody and the development of immunological memory (5). In order to determine whether any defect in antibody response in C1qa−/− mice during a P. c. chabaudi infection could have contributed to their subsequent susceptibility to reinfection, the isotype and subclass levels of the malaria-specific antibodies in the sera of the infected animals were measured throughout the primary and secondary infections. In general, there was very little difference between the antibody responses of the C1qa−/− and wild-type mice during primary infection. However, after 3 months of the primary infection, there were significantly smaller amounts of malaria-specific IgG2a antibody remaining in the C1qa−/− mice. After a second challenge infection, the levels of IgG2a and IgM anti-malaria antibodies were both significantly increased in the C1qa−/− animals compared with control mice. We have previously reported that C1qa−/− mice produce significantly less antigen-specific IgG2a and IgG3 in response to low doses of T-cell-dependent antigens (6). The inability to sustain an IgG2a response when the parasite load is low may reflect a similar mechanism. The increase in antibody titer after secondary infection to levels comparable with or higher than in wild-type mice might be related to the larger parasite dose endured by the C1qa−/− animals at this time.

The experiments described here appear to contradict our previous study where, using low doses of T-cell-dependent antigens, C1q-deficient antigen-specific CD4+ T cells produced lower IFN-γ levels than the control cells (6). During P. c. chabaudi infections, IFN-γ production was not reduced in C1qa−/− mice compared with that in wild-type mice. By contrast, the numbers of IFN-γ–positive CD4+ and CD8+ T cells detected by intracellular cytokine labeling and the precursor frequencies of malaria-specific cells producing IFN-γ were
comparable, and the amount of IFN-γ transiently present in the plasma early in infection was significantly higher in the complement-deficient mice. Despite the greater amounts of IFN-γ in the plasma, and the role of IFN-γ as a switch factor for IgG2a (34), there was no concomitant rise in IgG2a titers in C1qa−/− mice. The location and cellular source of the IFN-γ observed in the plasma are not known, and this is likely to be important for B-cell switching. Markine-Goriaynoff et al. showed that antigen-specific IgG2a responses during parasitic and viral infections could be relatively normal in the absence of IFN-γ (23), suggesting that alternative mechanisms for the regulation of IgG2a exist in vivo. The relationship between complement and IFN-γ or regulation of IgG2a antibodies is not understood, and therefore the reasons for these discrep-

FIG. 4. (A) Scatter plot showing the circulating quantities of IFN-γ during the acute phase of infection. In the infected wild-type mice (solid circles), IFN-γ was detected only at the peak of parasitemia (days 7 to 9); however, in the C1qa−/− mice (open circles), IFN-γ was detectable much earlier in the infection and the levels were significantly higher on days 5, 7, and 9 (P < 0.01, **), P < 0.01, and P < 0.05 [*], respectively; Mann-Whitney tests). ND, undetectable levels of IFN-γ in the serum. (B) H2-Bi/C2−/− animals (open squares) exhibited kinetics in the production of IFN-γ similar to those of wild-type mice (solid circles), but the levels of protein in the serum were significantly greater in the H2-Bi/C2−/− mice on days 6, 8, and 9 (P < 0.01, P < 0.001 [***], and P < 0.05, respectively; Mann-Whitney tests).
ELISAs. used in these studies and Pearline Benjamin for her assistance with the  

number W0554), the MRC (J.L. and E.S.), and the Wellcome Trust  

parasite. The inability of pronounced secondary infection after rechallenge with the same  

cells are the source of larger amounts.  

through complement receptors on B cells or in antigen trap-  

response to rechallenge may reflect a basic defect in signaling  

antibody.  

the major sources of early IFN-  

are able to activate dendritic cells to produce IL-12, which is a  

simply reflect the increased number of parasites in these mice.  

ables are not known. Since plasma IFN-γ was increased in Clqα−/− mice without any obvious increase in the number of T cells producing this cytokine, it may be that NK cells and not T cells are the source of larger amounts. * P. c.  

chabaudi schizonts are able to activate dendritic cells to produce IL-12, which is a  

differentiation factor for both NK cells and Th1 cells (12, 22), the major sources of early IFN-γ in this infection (21, 26).  

Dependency on complement components, for example, for increased uptake of antigen and activation of dendritic cells to initiate the IL-12–IFN-γ pathway may be circumvented by the large number of replicating parasites. The increased IFN-γ levels in the plasma of the complement-deficient mice may simply reflect the increased number of parasites in these mice.  

In summary, complement-deficient mice exhibited a slightly increased acute-phase parasitemia after infection with *P. c.  

chabaudi*, which was accompanied by significantly greater IFN-γ production. Clqα−/− mice suffered from a more pronounced secondary infection after rechallenge with the same parasite. The inability of Clqα−/− animals to mount a full response to rechallenge may reflect a basic defect in signaling through complement receptors on B cells or in antigen trapping on follicular dendritic cells, which have been implicated in the maintenance of antibody levels and in B-cell memory.  

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663.  


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**TABLE 1. Precursor frequencies of IFN-γ and IL-4-producing cells responding to malarial antigens**

<table>
<thead>
<tr>
<th>Day of infection</th>
<th>Genotype of mouse</th>
<th>CD4⁺ T-cell precursor frequency (per 10⁶ cells)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IFN-γ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Help for Ab production</td>
</tr>
<tr>
<td>7</td>
<td>Clqα−/−</td>
<td>758.2 ± 111.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>154.0 ± 77.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>102.6 ± 4.00</td>
</tr>
<tr>
<td>28</td>
<td>Clqα−/−</td>
<td>545.5 ± 46.0</td>
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<tr>
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<td></td>
<td>151.5 ± 12.5</td>
</tr>
<tr>
<td>28</td>
<td>Clqα−/−</td>
<td>326.8 ± 22.7</td>
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<tr>
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<td>Clqα−/−</td>
<td>147.0 ± 16.7</td>
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<td></td>
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</table>

*The data shown represent the mean ± SEM of two separate assays conducted on cells pooled from three mice in each group at each time point. Ab, antibody.*