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Research Paper

Follicular Helper T Cells are Essential for the Elimination of *Plasmodium* Infection

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ABSTRACT

CD4$^+$ follicular helper T (Tfh) cells have been shown to be critical for the activation of germinal center (GC) B-cell responses. Similar to other infections, *Plasmodium* infection activates both GC as well as non-GC B cell responses. Here, we sought to explore whether Tfh cells and GC B cells are required to eliminate a *Plasmodium* infection. A CD4 T cell-targeted deletion of the gene that encodes *Bcl6*, the master transcription factor for the Tfh program, resulted in complete disruption of the Tfh response to *Plasmodium chabaudi* in C57BL/6 mice and consequent disruption of GC responses and IgG responses and the inability to eliminate the otherwise self-resolving chronic *P. chabaudi* infection. On the other hand, and contrary to previous observations in immunization and viral infection models, Signaling Lymphocyte Activation Molecule (SLAM)-Associated Protein (SAP)-deficient mice were able to activate Tfh cells, GC B cells, and IgG responses to the parasite. This study demonstrates the critical role for Tfh cells in controlling this systemic infection, and highlights differences in the signals required to activate GC B cell responses to this complex parasite compared with those of protein immunizations and viral infections. Therefore, these data are highly pertinent for designing malaria vaccines able to activate broadly protective B-cell responses.

1. Introduction

Follicular helper T cells (Tfh cells) are a particular subpopulation of CD4$^+$ T cells that is critically required for the activation of follicular B cells. Since their identification as a discrete CXCR5$^+$ subset specialized in collaborating with B cells (Schaerli et al., 2000; Breitfeld et al., 2000; Kim et al., 2001), and the identification of the repressor Bcl6 as the master regulator transcription factor of this subset (Johnston et al., 2009; Nurieva et al., 2009; X. Liu et al., 2009), a large body of research has helped to understand their activation requirements and the differential signals underlying their communication with B cells (Crotty, 2014; Vinuesa et al., 2016). Both soluble factors (i.e. cytokines) and cell surface molecules have been shown to act in parallel to orchestrate this communication. Moreover, several of these signals have been proposed as candidate targets for immunotherapeutic interventions to treat diseases in which B-cell responses are relevant (Hu et al., 2013; Spolski and Leonard, 2014). However, the relative impact of these different types of signals on the outcome of infection or disease remains poorly explored.

The Signaling Lymphocytic Activation Molecule (SLAM)-Associated Protein (SAP) is a small intracellular adaptor protein, which interacts with the cytoplasmic tails of the SLAM family of cell surface receptors and mediates signaling downstream of these receptors (Cannons et al., 2011). Mutations in SAP were originally associated with most cases of X-linked lymphoproliferative syndrome (Cannons et al., 2011). One outstanding function of SAP is to mediate signaling leading to the stable long-duration contact of T and B cells (Qi et al., 2008). This physical interaction between T and B cells, tightly regulated by SAP, has been shown to be critical for the activation of germinal center (GC) B-cells (Qi, 2012), and the activation of Tfh cells in some models (Cannons et al., 2010; Deenick et al., 2010; Linterman et al., 2011).

The B-cell response to the blood stages of *Plasmodium*, the protozoan parasite that causes malaria, is thought to be important for protective immunity in human infections (Cohen et al., 1961; Conway et al., 2000; Fowkes et al., 2010; Osier et al., 2008; Sabchareon et al., 1991). B cells and antibodies are also necessary for the elimination of this stage of infection in experimental mouse models (Burns et al., 1997; von der Weid et al., 1996); thus, these models allow an examination of the relative importance of different Tfh-derived signals in the control of infection. Dysfunctional Tfh responses have been described in children exposed to *P. falciparum*, which are thought to be responsible for impaired development of protective B cell-responses (Obeng-Adjei et al., 2015). *P. berghei* infection in mice inhibits Tfh differentiation (Ryg-Cornejo et al., 2015), whereas boosting of Tfh responses in mice by therapeutic interventions has been shown to accelerate the control of chronic *P. chabaudi* infection (Butler et al., 2012). The critical signals

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required for Tfh activation to *Plasmodium* infection have also begun to emerge. OX40, PD-1 and ICOS cell surface molecules were shown to regulate Tfh activation during non-lethal *P. yoelii* and *P. chabaudi* infections (Zander et al., 2015; Wikenheiser et al., 2016). We have recently shown that IL-21-producing $CD4^+$ T cells, of which a substantial proportion has a Tfh cell phenotype, are required to activate IgG responses to *P. chabaudi* and to control the chronic phase of this infection (Pérez-Mazliah et al., 2015). Interestingly, acute gamma herpes virus co-infection leads to loss of control of an otherwise non-lethal *P. yoelii* infection, and this is associated with a disruption of the Tfh cell response (Matar et al., 2015).

Despite these important advances in our knowledge of Tfh cell responses, a direct link between Tfh cell responses and the control of *Plasmodium* infection remains to be demonstrated, and the relative impact of the different Tfh-derived signals (i.e. cell surface molecular interactions vs soluble factors) on the control of the infection has not been explored in detail. Moreover, despite the substantial differences in infections initiated by artificial versus natural mosquito transmission (Spence et al., 2013), our knowledge of T- and B-cell responses during experimental erythrocytic malaria models has been exclusively generated using artificial injection of infected blood to initiate the infection, thus obviating the full life cycle in the mouse. Here, using both blood transmission as well as a model of natural mosquito transmission, we compared the relative requirements of Tfh responses overall, together with the individual requirements of SAP and IL-21R on the control of *Plasmodium chabaudi* AS infection, a rodent model which presents both an acute and chronic phase (Achtman et al., 2007).

We demonstrate a critical role for Tfh cells in the elimination of the chronic phase of *Plasmodium* infection initiated by both, blood transmission, and natural mosquito transmission. In addition, and contrary to previous observations in immunization studies, and virus and helminth infections (Crotty et al., 2003; Cannons et al., 2006; Kamperschroer et al., 2006; Crotty et al., 2006; McCausland et al., 2007; Moyron-Quiroz et al., 2009; Yusuf et al., 2010; Morra et al., 2005), we show that SAP-deficient mice are able to activate Tfh and GC B cells, and an IgG response to the parasite. Finally, we demonstrate a hierarchy of immune responses needed to control the magnitude of the chronic infection, with IL-21 signaling being the most significant requirement followed by Tfh cells and SAP. Our data demonstrate the need for a fully functioning Tfh response for elimination of blood-stage *Plasmodium* infection, and highlights substantial differences in the signals required to activate Tfh and GC B cell responses to this complex parasite compared to immunizations and other infection models.

2. Materials and Methods

2.1. Ethical Statements

All scientific experiments involving procedures on mice were approved by the Ethical Review Panel of the MRC National Institute for Medical Research (NIMR). They were performed accordingly to the UK National guidelines of the Animals (Scientific Procedures) Act 1986 under the license reference number PPL 70/8326 authorized and granted by the British Home Office.

2.2. Mice

C57BL/6, *Sh2d1a*$^{-/-}$ [Sh2d1a^{tm1Cpt} (Wu et al., 2001), RRID:MGI:3576735], *CD4-Cre*$^{+/-}$ [Tg(Cd4-cre)1Cwi (P. P. Lee et al., 2001), RRID:MGI:3691126], *Bcl6*$^{fl/fl}$ [Bcl6$^{tm1.1Mtto}$ (Kaji et al., 2012)], *CD4-Cre*$^{+/-}$*Bcl6*$^{fl/fl}$ (RRID:MGI:5461330) and *Rag2*$^{-/-}$ [Rag2^{tm1Fwa} (Shinkai et al., 1992), RRID:MGI:3617415] mouse strains were bred in the specific pathogen-free facilities of the Mill Hill Laboratory of the Francis Crick Institute, and were backcrossed for at least 10 generations onto NIMR C57BL/6 mice. For experimental use, 6–12 weeks old female mice were housed in conventional facilities with sterile bedding, food and water under reversed light conditions (dark: 7.00 h to 19.00 h).

2.3. Infections

Plasmodium chabaudi chabaudi (AS) was originally obtained from David Walliker, University of Edinburgh. Infections were initiated by intraperitoneal injection of 10^5 infected red blood cells, or by the bites of infected *A. stephensi* mosquitoes as previously described (Spence et al., 2012). Blood-stage parasitemias were monitored by Giemsa-stained thin blood smears (Langhorne et al., 1989).

2.4. Flow Cytometry

Spleens were dissected and mashed through 70 μm filter mesh in Hank's Balanced Salt Solution (HBSS, Gibco, Invitrogen) to generate single cell suspensions. Spleens were treated in RBC lysis buffer (Sigma) and remaining cells resuspended in complete Iscove's Modified Dulbecco's Medium (IMDM supplemented with 10% fetal bovine serum (FBS) Serum Gold (PAA Laboratories, GE Healthcare), 2 mM L-glutamine, 0.5 mM sodium pyruvate, 100 U penicillin, 100 mg streptomycin, 6 mM Hepes buffer, and 50 mM 2-ME (all from Gibco, Invitrogen). Viable cells were counted based on trypan blue (Sigma) exclusion in a hemocytometer. 2×10^6 viable cells were distributed to each well of a 96-well plate (Nunc) and incubated in the presence of a monoclonal anti-mouse CD16/32 antibody (Unkeless and Unkeless, 1979, BioLegend Cat# 101318 RRID:AB_2104156) to block Fc-mediated binding of antibodies for 20 min at 37 °C, 20 min at 4 °C.

To identify Tfh cells, cells were first incubated with biotin anti-CXCR5 (BD Biosciences Cat# 551960 RRID:AB_394301) in complete IMDM (BD Pharmingen), washed twice with a staining buffer (1× PBS (pH = 7.2–7.4), 2% FBS, 0.01% Sodium azide), resuspended in 1× PBS and incubated with appropriate dilutions of PE streptavidin, APC-Cy7 anti-CD4 (BioLegend Cat# 100414 RRID:AB_312699), PE-Cy7 anti-PD-1 (BioLegend Cat# 109110 RRID:AB_572017), Pacific Blue anti-CD3, PerCP-Cy5.5 or FITC anti-CD44 (BioLegend) for 30–40 min at 4 °C. A commercial kit (eBioscience) was used for intra-nuclear detection of Bcl6, following manufacturer's instructions, and using the anti-human and mouse Bcl-6 antibody conjugated to Alexa Fluor 647 (BD Biosciences Cat# 561525 RRID:AB_10898007).

To identify germinal center Tfh and B cells, spleen cells were incubated with appropriate dilutions of PerCP-Cy5.5 anti-CD3 (BioLegend Cat# 100328 RRID:AB_893318), PE anti-CD4 (BioLegend Cat# 100408 RRID:AB_312693), PE-Cy7 anti-CD45R/B220 (BioLegend Cat# 103222 RRID:AB_313005), APC-Cy7 anti-CD19 (BioLegend Cat# 115530 RRID:AB_830707), APC anti-CD38 (BioLegend Cat# 102712 RRID:AB_312933) FITC anti-GL-7 (BioLegend Cat# 144604 RRID:AB_2561697) and BV421 anti-IgD (BioLegend Cat# 405725 RRID:AB_2562743) for 30–40 min at 4 °C. Germinal center B cells were identified by combined expression of CD19 and B220, low expression of IgD and CD38, and high expression of GL-7. Tfh cells were identified by the combined expression of CD3, CD4 and GL-7. T-B cell conjugates were studied ex vivo by flow cytometry as previously described (Reinhardt et al., 2009). Conjugates were identified by the combined expression of CD3, CD4, CD19 and B220, and identified as doublets based on FSC-A vs FSC-W.

IL-21 detection by flow cytometry was done by intracellular staining as previously described (Pérez-Mazliah et al., 2015).

All cells were fixed with 2% paraformaldehyde for 15 min at 4 °C, washed and stored in the staining buffer at 4 °C until acquisition. Cells were acquired on BD FACSVerse, BD LSRII or BD LSRFortessa X-20 (BD Biosciences) flow cytometers. Dead cells were excluded by staining with LIVE/DEAD Aqua (Invitrogen) prior fixation. Fluorescence-minus-one (FMO) controls were used to set the thresholds for positive/negative events. Analysis was performed with FlowJo software version 9.6 or higher (Tree Star). Doublets based on side scatter light and forward

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