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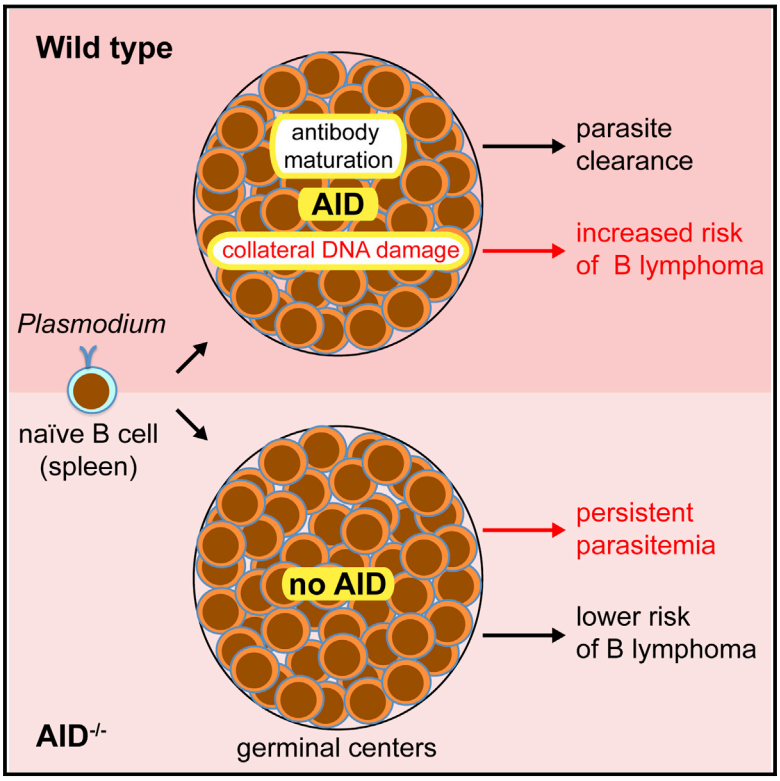
Plasmodium Infection Promotes Genomic Instability and AID-Dependent B Cell Lymphoma

Graphical Abstract



Highlights

- Capture of chromosome translocations occurring in germinal center B cells in vivo
- AID promotes *Plasmodium*-associated B cell lymphoma
- Chronic infection causes a shift toward a more mature lymphoma phenotype
- *Plasmodium*-induced lymphomas bear chromosome translocations, including *c-myc/Igh*

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In Brief

Chronic infection with *Plasmodium* parasites induces widespread genomic instability and favors mature B cell cancers by eliciting protracted AID expression in GC B cells, explaining the association between malaria and development of Burkitt's lymphoma.

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Plasmodium Infection Promotes Genomic Instability and AID-Dependent B Cell Lymphoma

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SUMMARY

Chronic infection with *Plasmodium falciparum* was epidemiologically associated with endemic Burkitt's lymphoma, a mature B cell cancer characterized by chromosome translocation between the *c-myc* oncogene and *Igh*, over 50 years ago. Whether infection promotes B cell lymphoma, and if so by which mechanism, remains unknown. To investigate the relationship between parasitic disease and lymphomagenesis, we used *Plasmodium chabaudi* (*Pc*) to produce chronic malaria infection in mice. *Pc* induces prolonged expansion of germinal centers (GCs), unique compartments in which B cells undergo rapid clonal expansion and express activation-induced cytidine deaminase (AID), a DNA mutator. GC B cells elicited during *Pc* infection suffer widespread DNA damage, leading to chromosome translocations. Although infection does not change the overall rate, it modifies lymphomagenesis to favor mature B cell lymphomas that are AID dependent and show chromosome translocations. Thus, malaria infection favors mature B cell cancers by eliciting protracted AID expression in GC B cells.

INTRODUCTION

Many pathogens have been implicated as etiologic agents in the development of human cancer. For example, individuals infected with Epstein-Barr virus (EBV), hepatitis C virus, HIV, *Helicobacter pylori*, or *Plasmodium falciparum* are all at higher than average risk of developing B cell lymphoma (de Martel et al., 2012; Epeldegui et al., 2010; Kutok and Wang, 2006; Marcucci and Mele, 2011; Molyneux et al., 2012; Zucca et al., 2000; Zur Hausen, 2009). Although viruses can promote neoplasia directly, by delivering virally encoded cancer genes to target cells, the link between most pathogens and tumor development remains obscure (Karin et al., 2006; Mesri et al., 2014). For instance, endemic Burkitt's lymphoma (eBL), a lymphoma of GC origin, is among the most common childhood cancers in Africa, and it occurs at higher incidence in areas in which *Plasmodium falciparum* infection is endemic. This epidemiologic association is poorly understood in part because eBL cells are infected with EBV, which can induce B cell malignancy, and there is little insight into how malaria might play an additional role (Burkitt, 1961; Kutok and Wang, 2006; Magrath, 2012; Molyneux et al., 2012).

Based on histologic, molecular, and gene expression analysis, it has been proposed that BL cells represent transformed GC B cells that carry t(8;14) chromosome translocations (Klein and Dalla-Favera, 2008; Küppers et al., 1999; Magrath, 1990; Shaffer et al., 2002; Victora et al., 2012). This translocation joins *c-myc* to immunoglobulin (*Ig*) gene loci and thereby deregulates *c-myc* expression. However, deregulated *c-myc* alone is not sufficient to produce lymphoma (Adams et al., 1985; Janz et al., 2003; Leder et al., 1986), and transformation requires additional lesions in genes encoding proteins, such as P53, which regulates cell-cycle checkpoints and apoptosis (Gaidano et al., 1991; Love et al., 2012). Neither p53 mutation nor EBV infection are eBL specific, as they occur broadly in lymphoid malignancies (Cesarman, 2014; Forbes et al., 2015; Koduru et al., 1997; Kutok and Wang, 2006; Saha and Robertson, 2011). In contrast, malaria is exquisitely associated with eBL (Magrath, 2012; Molyneux et al., 2012).

GC B cells are rapidly dividing cells that are unique in expressing high levels of activation-induced cytidine deaminase (AID), a mutator enzyme that deaminates cytidines and produces U:G mismatches in *Ig* gene loci to initiate somatic hypermutation (SHM) and class-switch recombination (CSR) of antibodies (Muramatsu et al., 2000; Petersen-Mahrt et al., 2002; Revy et al., 2000). Although AID has a strong preference for *Ig* gene loci, it is not entirely *Ig* specific, and it produces off-target mutations or DNA breaks in oncogenes, including *c-myc*, that lead to translocations in activated B cells in vitro and in IL-6 transgenic mice that develop polyclonal plasmacytosis (Klein et al., 2011; Pasqualucci et al., 2001; Ramiro et al., 2004; Robbiani et al., 2008).

Here, we show that in the absence of p53, *Pc* infection results in development of mature B cell lymphoma. Malaria infection alters lymphomagenesis to favor development of mature GC or post-GC origin B cell lymphomas and also destabilizes the genome in rapidly dividing AID-expressing GC B cells.

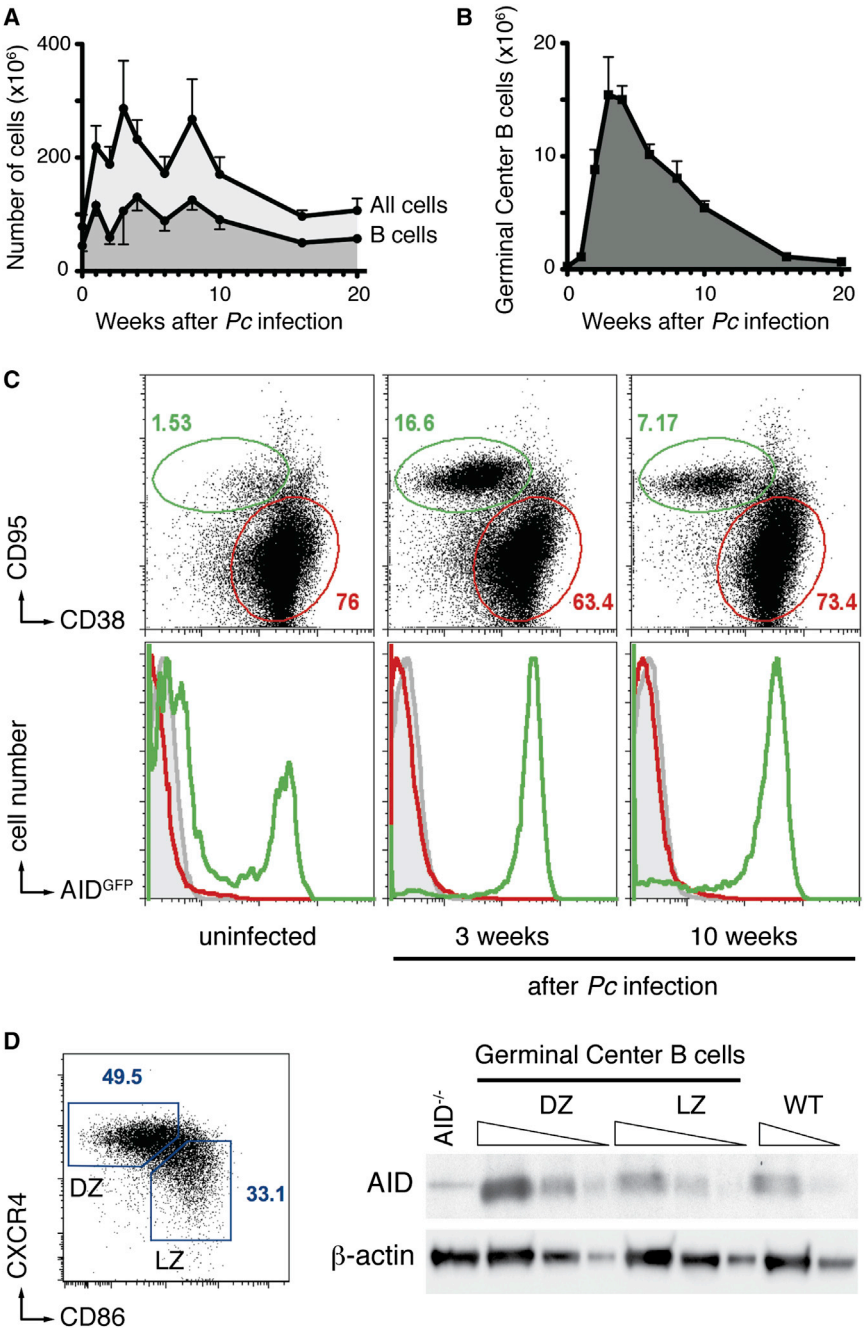

Figure 1. B Cell Responses to *Plasmodium* Infection

(A and B) The total spleen cellularity, the total B cells (A), and the germinal center B cells (B), following *Pc* infection. The mean with SD is shown; at least five mice were evaluated for each time point.

(C) AID expression is confined to germinal center B cells. Representative flow cytometry plots of splenocytes from *Plasmodium*-infected AID^{GFP} mice. The top row shows the relative expansion of germinal center (green) over non-germinal center (red gate) B cells over time (gated on $B220^{+}$). The bottom row shows the expression of AID^{GFP} in non-B cells ($B220^{-}$, gray), non-germinal center B cells ($B220^{+}CD38^{+}CD95^{-}$, red), and germinal center B cells ($B220^{+}CD38^{-}CD95^{+}$, green). At least three mice were evaluated for each time point.

(D) AID protein in malaria GC B cells. Gating strategy (left) and semiquantitative western blot analysis (right) identifying AID in both light zone (LZ) and dark zone (DZ) cells sorted 3 weeks post-inoculation. The triangles indicate 3-fold serial dilution. $AID^{-/-}$ and wild-type (WT) control lanes are from in vitro-activated B cells of the respective genotypes. One of two independent experiments is shown.

See also Figure S1.

RESULTS

Plasmodium Infection Induces AID-Expressing Germinal Centers

We used *Plasmodium chabaudi* (*Pc*) to establish chronic malaria infection in mice. In agreement with previous reports (Achtman et al., 2007; Horne-Debets et al., 2013), parasitemia peaked between 7 and 10 days after infection and was followed by an increase in total spleen cellularity (mean of 286.6 mio cells/spleen, 4-fold expansion at 3 weeks), including B lymphocytes (mean of 130.4 mio B cells/spleen, 3-fold expansion at 4 weeks; Figures 1A and S1A). Strikingly, splenic GC B cells expanded by 63-fold (mean of 15.4 mio GC B cells/spleen at 3 weeks), and this response was sustained for over 10 weeks (Figure 1B). We conclude that *Plasmodium* infection induces a robust and long-lasting expansion of GC B cells.

Ig somatic mutation and class-switch recombination are initiated by AID, a cytidine deaminase (Muramatsu et al., 2000; Petersen-Mahrt et al., 2002; Revy et al., 2000). This enzyme is typically restricted to activated B cells in germinal centers (GCs), but malaria infection produces widespread B cell activation (Scholzen and Sauerwein, 2013). To determine if AID is restricted to GCs during *Pc* infection, we examined AID^{GFP} reporter mice (Crouch et al., 2007). We found that AID expression was restricted to GC B cells and was sustained over a period of at least 10 weeks (Figures 1C and S1B). To confirm AID protein expression, we sorted GC B cells into dark- and light-zone cells and performed western blot analysis. As expected, AID was found mainly in dark-zone cells, which contained three times more AID than did light-zone cells or in vitro-activated B cells (Figure 1D). We conclude that AID is primarily expressed in *Plasmodium*-induced GC B cells.

Widespread Chromosome Translocations in Malaria Germinal Centers

AID and replication-associated stress can independently produce DNA breaks and translocations in cultured B cells (Barlow et al., 2013; Hakim et al., 2012; Klein et al., 2011). To map the DNA damage caused by *Plasmodium* infection in vivo, we adapted a previously described next-generation translocation capture and sequencing method (TC-seq; Klein et al., 2011). Mice with an I-SceI recognition site at *c-myc* (Myc^{I}) were bred to a $ROSA^{erISCEI}$ transgene (encoding for I-SceI fused to the estrogen receptor ligand binding domain, er) and to either AID-deficient or AID-overexpressing mice ($ROSA^{AIDer}$; see the Experimental Procedures; Figure S2). Compound $Myc^{I/I}$ $ROSA^{erISCEI/erISCEI}AID^{-/-}$ and $Myc^{I/I}ROSA^{erISCEI/AIDer}$ mice were infected with *Plasmodium* and treated with Tamoxifen at the peak of the GC response, to induce the nuclear shuttling of erISCEI in the absence or presence of AID, and translocations between the DNA break at Myc^{I} on chromosome 15 and lesions in the rest of the genome were captured by TC-seq (Figure 2A).

We recovered 191,150 and 65,905 unique rearrangements to the I-SceI site in Myc^{I} from 105 million AID-deficient or AID-proficient malaria GC B cells, respectively (see the Experimental Procedures). In agreement with previous in vitro experiments (Klein et al., 2011), malaria rearrangements were preferentially intrachromosomal (95.2% versus 94.3% of all rearrangement-associated reads, in the absence or presence of AID, respectively; Figure 2B) and enriched at the I-SceI site (Figure 2C). Regardless of AID expression, rearrangements were significantly increased in genic regions over the predicted random distribution of 40.1% (Figure 2D) and favored more actively transcribed genes (Figure 2E). Consistent with this, the asymmetric distribution of events in the direction of transcription at the I-SceI site was less pronounced in malaria GCs than in vitro-stimulated B cells, which express higher levels of *c-myc* (Figures 2C and S2E; Klein et al., 2011; Shaffer et al., 2001; Thomas and Rothstein, 1989). We conclude that the overall distribution of chromosome rearrangements induced by *Plasmodium* in vivo is similar to the one observed in retrovirally infected cells in vitro.

AID-Independent Damage Involves Sites of DNA Replication-Associated Fragility

Early replication fragile sites (ERFSs) are a set of genomic regions prone to breakage during DNA replication (Barlow et al., 2013). To assess the contribution of DNA replication to malaria-induced rearrangements, we analyzed their occurrence within hotspots of viral integration at ERFSs (M.J. and I.T.S., unpublished data; Barlow et al., 2013). The fraction of translocations in ERFSs was similar in the presence and absence of AID (1.5% and 2% of total, respectively) and significantly enriched over the random distribution as determined by Monte-Carlo simulation (Figure 3A). ERFS translocations preferred genic regions and more highly transcribed genes (Figure S3). In contrast, no enrichment was observed within common fragile sites (CFSs), which are late-replicating regions that preferentially break during mitosis (Figure 3B; Helmrich et al., 2006). We conclude that in malaria GC B cells DNA damage preferentially occurs at ERFSs, regardless of AID.

AID Damages the Genome of Malaria GC B Cells

To determine whether AID destabilizes specific regions in the GC B cell genome, we compared AID-deficient and AID-proficient TC-seq libraries for local accumulations of translocations using stringent criteria (Wang et al., 2014). As expected, hotspots of AID-dependent translocation were observed at the *Igh* locus, with accumulations involving the switch regions Sμ, Sγ2b, and Sγ2a (Figure 4A; Table S1). In contrast, AID translocations from B cells activated in vitro formed hotspots in all switch regions, and particularly at Sμ, Sγ1, and Sγ3 (Figure 4A). An additional 15 hotspots were identified in non-*Ig* regions, and off-target AID-dependent mutational activity was confirmed at *c-myc* and near *Ly6e* (Figures 4B, 4C, and S4A; Table S1). In agreement with previous reports, AID translocations were enriched in genic regions and favored more highly transcribed genes (Figures S4B and S4C; Klein et al., 2011; Wang et al., 2014). Moreover, none of the AID translocation off targets identified in vivo were shared with those from the in vitro dataset (Table S1; Klein et al., 2011). Altogether, translocations within AID-dependent hotspots, including the *Igh* locus, comprised only 0.7% of total. We conclude that AID is not limited to, but primarily destabilizes, *Ig* regions in GC B cells.

AID Promotes Late-Stage Lymphomas

In addition to deregulated *c-myc*, BLs bear frequent mutation of p53 (Gaidano et al., 1991; Love et al., 2012). To model this phenomenon, we produced mice that carry a B cell-specific deficiency in p53 ($CD19^{cre/+}p53^{lox/lox}$) and are either proficient or deficient for AID. B lymphocyte development was not significantly altered in these mice despite efficient p53 deletion (Figure S5). To mimic the chronic exposure in areas of endemic malaria, the two cohorts were repeatedly infected with *Pc* and monitored for disease. Mice developed lymphomas in an AID-dependent manner (Figure 5). Malaria infection was not required for lymphoma development but altered the phenotype to favor more mature B cell lymphomas (Figure 6).

Whereas none of 11 wild-type mice became ill after infection, the two p53-deficient groups succumbed with a median of 60 weeks for $CD19^{cre/+}p53^{lox/lox}$ and 58 weeks for $CD19^{cre/+}p53^{lox/lox}AID^{-/-}$ ($p = 0.8$ with the Mantel-Cox test; Figure 5A). However, all of 12 $CD19^{cre/+}p53^{lox/lox}$ mice developed lymphoma while only 5 out of 15 $CD19^{cre/+}p53^{lox/lox}AID^{-/-}$ mice had evidence of lymphoma (33%, $p < 0.0004$ with Fisher's exact test; Figures 5B and 5C). The majority of the $CD19^{cre/+}p53^{lox/lox}AID^{-/-}$ mice (67%) presented with splenomegaly associated with marked extramedullary hematopoiesis but no histologic evidence of cancer (Figures 5B–5D). Consistent with lack of transformation, splenocytes from these mice were unable to expand in vitro ($n = 5$), as opposed to 3 out of 4 control lymphomas (observation period of 3 weeks). Moreover, lymphoma developed only in 1 out of 4 immunodeficient mice, in which $CD19^{cre/+}p53^{lox/lox}AID^{-/-}$ splenocytes from malaria-infected mice had been transplanted (Figure S5D). Finally, all of the $CD19^{cre/+}p53^{lox/lox}AID^{-/-}$ mice analyzed showed persistent parasitemia ($n = 5$), low red blood cell counts, decreased hemoglobin levels, and increased mean cell volume in peripheral blood (Figure S5E). All together, these findings are consistent

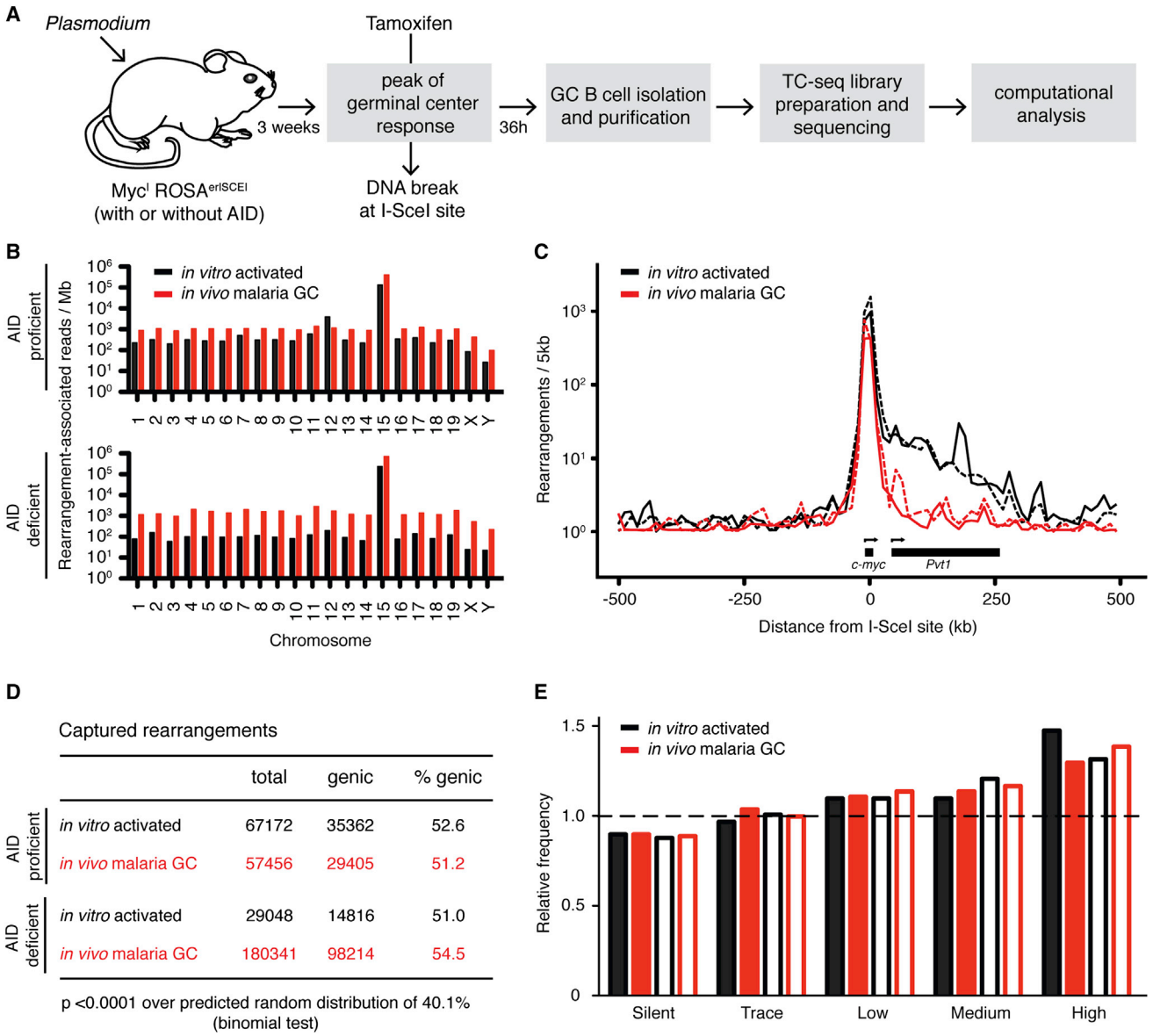

Figure 2. Translocations in Malaria GC B Cells

(A) A schematic diagram of the protocol to identify genomic rearrangements induced by *Plasmodium* in vivo.

(B) The chromosome distribution of rearrangement-associated reads captured by I-SceI breaks on chromosome 15.

(C) The profile of rearrangements around the I-SceI site. The dotted lines represent AID-deficient samples.

(D) The proportion of genic rearrangements.

(E) The frequency of rearrangements at genes with various levels of transcription. The empty bars represent AID-deficient samples. The dashed line represents the expected frequency based on random model.

For all, rearrangements from malaria GC B cells are compared to cultured B cells (Klein et al., 2011). See also Figure S2.

with the majority of $CD19^{cre/+}p53^{lox/lox}AID^{-/-}$ mice succumbing to a condition characterized by reactive extramedullary hematopoiesis in response to ongoing malaria infection. In contrast, all AID-sufficient mice developed lymphoma.

To determine whether AID is required to control malaria, we inoculated $AID^{-/-}$ mice and monitored parasitemia over time. Regardless of p53, AID deficiency did not alter the acute infection, but AID was required to quench parasitemia in the chronic phase (Figures S5F). In agreement with this finding, AID deficiency was associated with anemia, splenomegaly, extramedullary hematopoiesis, and reduced survival (Figures S5G–S5I; data not shown). We conclude that AID promotes *Plasmodium*-induced lymphomagenesis, but it is required to control chronic malaria infection.

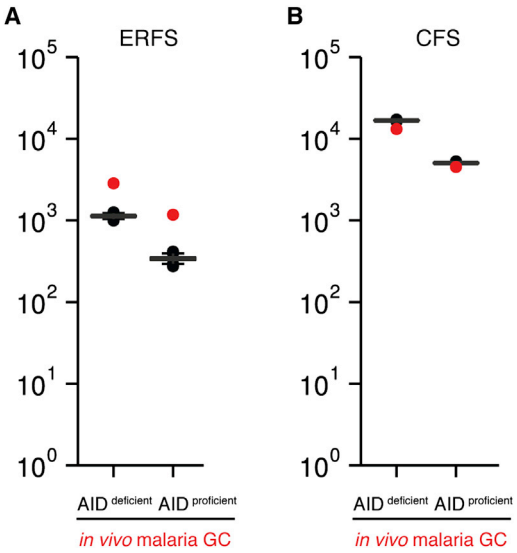

Figure 3. AID-Independent DNA Damage in Regions of DNA Replication-Associated Fragility

(A) The observed number of chromosome rearrangements within hotspots of viral integration at early replication fragile sites (ERFSs, red dot), as compared to a random Monte-Carlo simulation ($p < 0.001$ for both).

(B) The number of chromosome rearrangements within common fragile sites (CFSs, red dot), as compared to a random Monte-Carlo simulation.

See also Figure S3.

Phenotype and Karyotype of *Plasmodium*-Induced Lymphoma

We used flow cytometry to characterize the phenotype of lymphomas arising in AID-sufficient $CD19^{cre/+}p53^{lox/lox}$ mice. Infected and uninfected mice developed lymphoma with similar kinetics (median of 60 weeks for infected and 63 weeks for uninfected; $p = 0.07$ with the Mantel-Cox test; Figure S6A). However, malaria-infected mice developed B cell lymphomas, a significant majority of which showed a more mature phenotype consistent with GC origin (72.7%; n = 11; *Ig*-switched or $CD138^+$), while most uninfected controls developed pre-GC lymphomas (83.3%; n = 12; $p < 0.01$ with Fisher's exact test; Figures 6A and S6B; Tables 1, S2, and S3). As a control, we repeatedly immunized a cohort of AID-sufficient $CD19^{cre/+}p53^{lox/lox}$ mice with sheep red blood cells (SRBCs), a well-characterized antigen that induces long-lived germinal centers. Survival was similar in SRBC-immunized mice (median of 68 weeks; $p > 0.2$ with the Mantel-Cox test; Figure S6A). However, in contrast to malaria, only a minority of SRBC-associated lymphomas displayed a post-GC phenotype ($p < 0.03$, with Fisher's exact test; Figure S5C; Table S4). We conclude that *Plasmodium* infection, unlike SRBC immunization, causes a shift toward a more mature, post-GC lymphoma phenotype.

Malaria-associated Burkitt's lymphoma bears characteristic chromosome translocation of *c-myc* to *Ig* genes (Adams et al., 1983; Dalla-Favera et al., 1982; Hamlyn and Rabbitts, 1983; Taub et al., 1982). To determine whether *Plasmodium*-induced lymphoma in this model was associated with translocations, we performed multicolor-FISH (M-FISH) analysis of tumor metaphase spreads. We found that all of the informative lymphomas

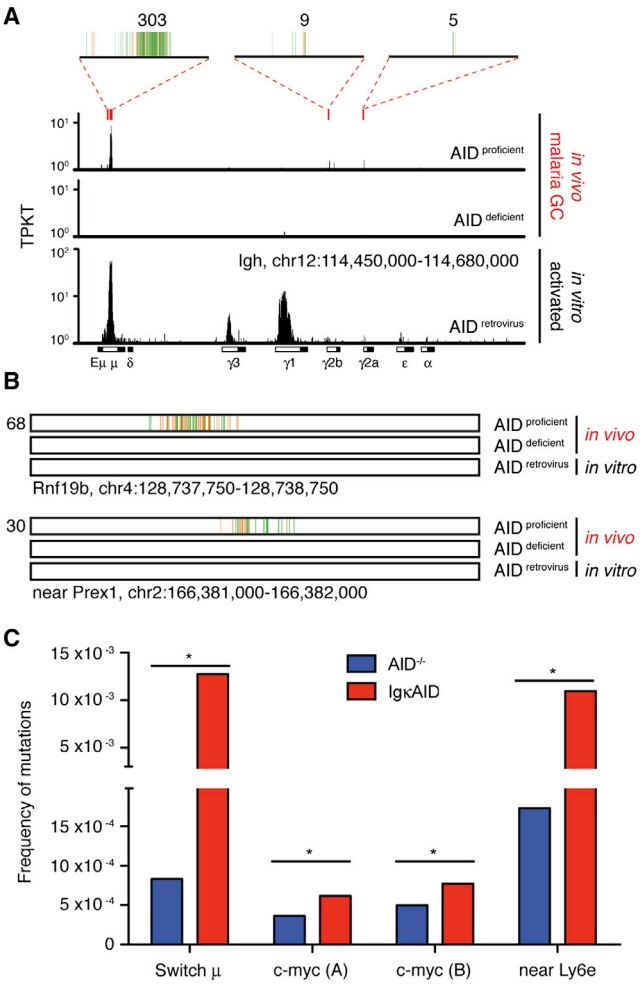

Figure 4. AID Contributes to *Plasmodium*-Induced DNA Damage

(A) Translocations at the physiologic AID target *Igh*. The red rectangles indicate the location of AID hotspots, and 4-kb regions at these hotspots are magnified on top, where vertical lines each represent a unique translocation event. The numbers on top indicate the total of translocations at each hotspot region. The rearrangements obtained in cultured B cells retrovirally expressing AID are shown for comparison (Klein et al., 2011). TPKT is the normalized number of translocations per kilobase per 1,000 translocations in the library.

(B) Examples of non-*Igh* hotspots of AID-dependent translocation induced by malaria. A 1-kb region is shown, with each vertical line representing a unique translocation. The numbers on the left indicate the total number of translocations.

(C) A mutational analysis of malaria GC B cells DNA by MutPE-seq. For *c-myc*, two adjacent regions in intron 1 were analyzed. *$p < 0.000001$ for all (one-tailed Student's t test).

See also Figure S4 and Table S1.

(n = 7) contained clonal translocations, four tumors displayed multiple translocations (L4, L23, L24, and L62) and one (L62) had a complex translocation involving three chromosomes (Figure 6B; Table 1). Lymphoma L23 had a T(12;15) between chromosomes 12 and 15, similar to the *c-myc*/*Igh* translocation found in pristane-induced plasmacytoma (Figure 6B; Potter and Wiener, 1992). To examine the L23 lymphoma at higher resolution, we sequenced its genome (see the Experimental

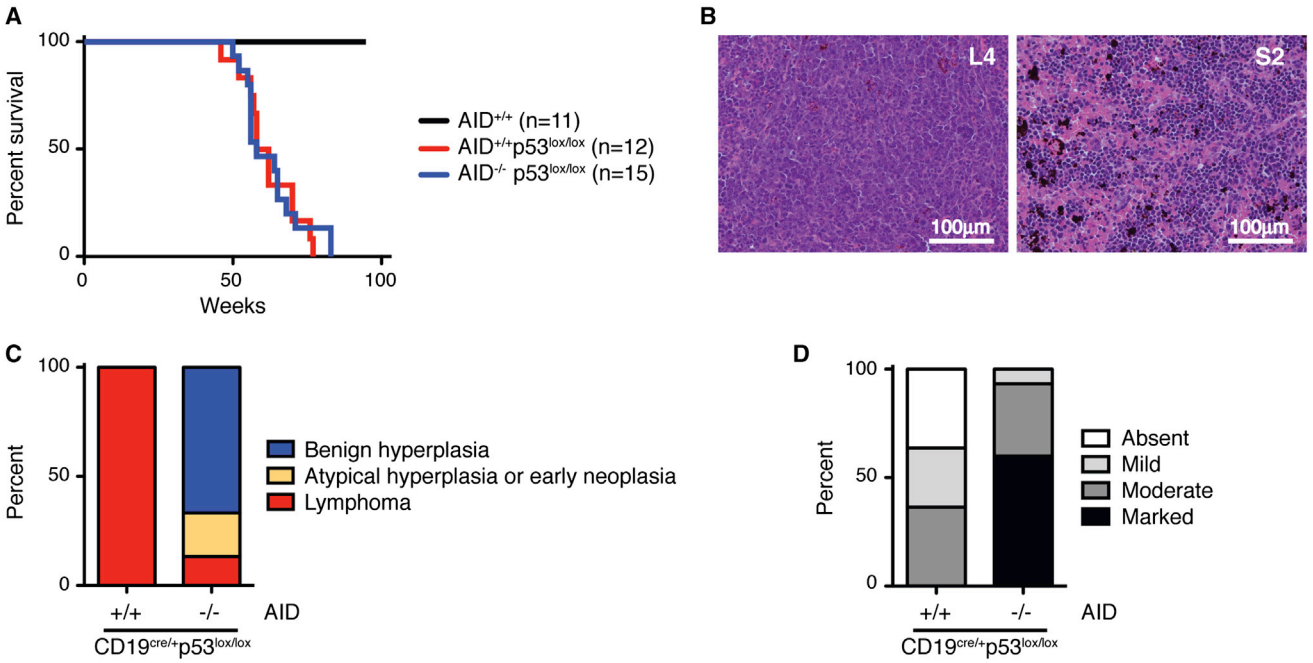

Figure 5. P53 Suppresses and AID Promotes *Plasmodium*-Induced Lymphoma

(A) Survival of *Plasmodium*-infected mice. All mice are also $CD19^{cre/+}$.

(B) Spleen histology of *Plasmodium*-infected $CD19^{cre/+}p53^{lox/lox}$ mice. L4 is AID-proficient lymphoma and S2 is AID-deficient benign B cell hyperplasia with marked extramedullary hematopoiesis.

(C) Lymphoma versus benign hyperplasia in *Plasmodium*-infected mice. Lymphoid tissues were evaluated by histology, immunohistochemistry, and flow cytometry. "Benign hyperplasia" indicates mice with splenomegaly, but with normal B cell distribution and $B220^+$ cells confined to follicular areas. "Atypical hyperplasia or early neoplasia" denotes splenomegaly and $B220^+$ cells expanding into the periarteriolar lymphoid sheats (PALS). "Lymphoma" defines abnormal lymphoid tissue architecture and/or dissemination to multiple organs.

(D) Extramedullary hematopoiesis in *Plasmodium*-infected mice. Spleen sections were evaluated for the degree of extramedullary hematopoiesis.

See also Figure S5 and Table S2.

Procedures; Figure S7). Bioinformatic analysis confirmed the presence of a *c-myc/Igh* translocation at T(12;15), as well as the expected deletions at the p53 locus and at the physiologically rearranged *Igh* and $Ig\kappa$ loci (Figure 6C; data not shown). Sequence analysis also identified the breakpoint of T(18;3) and revealed additional intra- and interchromosomal rearrangements, which were not detected by M-FISH (Figures 6C, S7B, and S7C; Table S5). Notably, 5 out of 8 interchromosomal breakpoints involved known AID targets (Figure 6C). We conclude that L23 carries numerous chromosome rearrangements, including the *c-myc/Igh* translocation characteristic of human Burkitt's lymphoma, and that AID shapes this lymphoma's genome.

DISCUSSION

Burkitt's lymphoma, a mature B cell lymphoma, occurs sporadically, but at higher incidence among immunodeficient individuals and in equatorial areas endemic for malaria (Cesarman, 2014; Magrath, 2012; Molyneux et al., 2012). In addition to malaria infection, most individuals developing eBL are also infected by EBV, a virus that encodes oncogenic proteins that can promote lymphomagenesis (Cesarman, 2014; Kutok and Wang, 2006; Magrath, 2012; Mesri et al., 2014; Molyneux et al., 2012; Zhang et al., 2012). Thus, EBV is thought to play a direct role in the transformation process. In contrast, malaria's contribution to lymphoma has remained elusive. We found that *Plasmodium* does not alter the incidence of cancers arising in B cells that carry a homozygous deletion of p53. Thus, *Plasmodium* infection alone does not induce cancer, and hence the requirement for EBV and additional transforming events. Instead, the parasitic infection alters lymphoma phenotype to favor more mature B cell lymphoma by inducing chronic GCs containing AID-expressing B cells.

Mature B cell lymphomas, including eBL, are frequently associated with hallmark oncogenic translocations and display phenotypic and molecular characteristics of GC B cells. However, it is not clear whether translocations are acquired within germinal centers or at earlier developmental stages (Shaffer et al., 2002). Supporting the notion that GCs are a lymphomagenic environment, we document widespread chromosome rearrangements occurring within malaria GCs. This is consistent with the high levels of AID and DNA replication in these rapidly proliferating cells (Gitlin et al., 2014; Robbiani and Nussenzweig, 2013; Victora and Nussenzweig, 2012). Early replication fragile sites (ERFSs) map to sites of early DNA replication and are particularly vulnerable to fork collapse. ERFS DNA is GC-rich and mostly euchromatic (Barlow et al., 2013). Translocations were significantly enriched at ERFSs, but not at common fragile

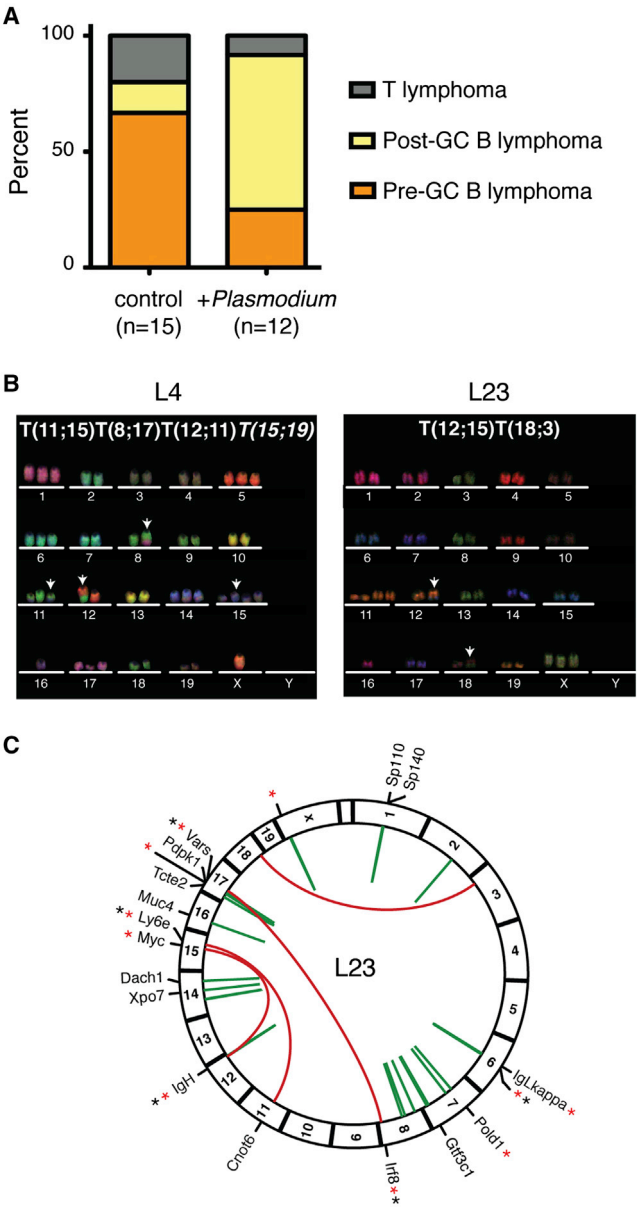

Figure 6. Genomic Rearrangements in *Plasmodium*-Induced Lymphomas

(A) Distribution of lymphoma phenotypes in *Plasmodium*-infected and control uninfected CD19$^{cre/+}$p53$^{lox/lox}$ mice.

(B) Representative M-FISH images of metaphases from *Plasmodium*-induced CD19$^{cre/+}$p53$^{lox/lox}$ lymphomas. Arrows point to chromosomes with detectable translocations.

(C) Circos diagram of the L23 genome. Red arches represent interchromosomal rearrangements, and green arches intrachromosomal ones. For genic rearrangements, the name of the gene is indicated. Asterisks indicate if the recombined site is a known AID target (red; Hakim et al., 2012; Klein et al., 2011) or within hotspots of viral integration at ERFSs (black; M.J. and I.T.S., unpublished data).

See also Figures S6 and S7 and Tables S3, S4, and S5.

sites (CFSs). This finding was unanticipated given that CFSs had been previously implicated in the genesis of lymphomagenic rearrangements (Chesi et al., 1998; Kameoka et al., 2004).

AID was not absolutely required for malaria-associated lymphomagenesis since a small number of the AID$^{-/-}$ p53-deficient mice also developed lymphoma. Moreover, only a fraction of rearrangements found in malaria GC B cells in vivo could be attributed to AID. Nevertheless, AID strongly promoted lymphomagenesis possibly because in addition to causing translocations (Chiarle et al., 2011; Klein et al., 2011; Ramiro et al., 2004; Robbiani et al., 2008, 2009), it can mutate cancer genes directly (Hakim et al., 2012; Liu et al., 2008; Pasqualucci et al., 2001).

In the absence of *Plasmodium* superinfection EBV is associated with B cell cancers other than eBL. For example, EBV is commonly detected in plasmablastic and Hodgkin's lymphoma, and approximately one-third of immunodeficiency-associated diffuse large B cell lymphoma is EBV positive (Cesarman, 2014). Moreover, when immune surveillance is impaired, the EBV gene LMP1 alone directly promotes B cell transformation (Zhang et al., 2012). Thus, EBV is oncogenic in B cells, but alone cannot account for the specific phenotype of eBL. Similarly, mutations in p53 and other cancer genes are not exclusive for eBL, as they occur broadly in B cell malignancy (O'Shea et al., 2008; Young et al., 2008). Instead, the distinctive phenotype of eBL is specifically associated with malaria. Essentially all eBLs bear *c-myc* translocation to *Ig* regions, and AID is accountable for the DNA lesions at both sites, leading to their translocation (Robbiani et al., 2008). By eliciting protracted, high levels of AID in GCs, malaria acts as a disease modifier and predisposes B cells to acquire *c-myc* translocation at a stage in B cell development that mirrors the molecular and phenotypic characteristics of eBL.

EXPERIMENTAL PROCEDURES

Mice and Parasites

All experiments were performed in agreement with protocols approved by the Rockefeller University Institutional Animal Care and Use Committee. Mutant mice used in this study include CD19$^{cre/cre}$ (*Cd19$^{tm1(cre)Cgn}$*;Rickert et al., 1997), p53$^{lox/lox}$ (Trp53^{tm1Brn};Marino et al., 2000), AID$^{-/-}$ (Muramatsu et al., 2000), AID^{GFP} (Crouch et al., 2007), IgH$^{I-96k/+}$ (Bothmer et al., 2010), Myc$^{I/I}$ and IgH$^{I/I}$ (Robbiani et al., 2008), and IgκAID (Robbiani et al., 2009). The ROSA^{AIDer} and ROSA^{erISCEI} alleles were generated in-house by gene targeting into the ROSA26 locus of C2J embryonic stem cells, as previously described (Srinivas et al., 2001), and the loxP-flanked transcriptional stop was removed from the germline by crossing to EIIA^{cre} mice (B6.FVB-TgN[EIIa-Cre]C5379Lmgd; Lakso et al., 1996). With the exception of the immunodeficient-NRG strain (NOD.Cg-*Rag1^{tm1Mom} Il2rg^{tm1Wjl}*/SzJ; Pearson et al., 2008), mice used in this study were either generated in C57Bl/6 background or backcrossed into it for at least 11 generations. *Plasmodium chabaudi chabaudi* ASS (*Pc*; MRA-429; Carter and Walliker, 1975; Peters and Robinson, 2000) was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH.

Plasmodium Infection

Parasites were maintained as frozen stocks and passaged in wild-type mice. For the analysis of B cell responses, 6- to 12-week-old mice were injected intraperitoneally (i.p.) with 10^5 *Pc*-infected erythrocytes from a donor mouse. In tumor experiments, 6- to 8-week-old mice were used for primary infection and secondary and tertiary infections were performed at 8-week intervals using the same dose and route. Parasitemia was monitored by flow cytometry

Table 1. Karyotype and Phenotype of Lymphomas in *P. chabaudi*-Infected CD19$^{cre/+}$p53$^{lox/lox}$ Mice

Tumor ID	Karyotype	Phenotype	S	MLN	sbLN	oLN	L
L21	nd	CD3$^+$	+	–	–	–	+
L4	T(11;15) T(8;17) T(12;11) T(15;19)[a]	CD19$^+$ IgM$^-$ IgD$^-$ IgK$^+$ CD138$^-$ CD38$^{+/-}$ FAS$^-$ GL7$^-$	+	+	+	+	–
L9	no metaphases	CD19$^+$ IgM$^-$ IgD$^-$ IgK$^+$ CD138$^-$ CD38$^+$ FAS$^+$ GL7$^-$	+	+	+	+	–
L23	T(12;15) T(18;3)	CD19$^-$ IgM$^-$ IgD$^-$ IgK$^-$ CD138$^+$ CD38$^-$ FAS$^-$ GL7^{dim}	+	–	–	–	+
L24	T(3;11) T(18;1)	CD19$^+$ IgM$^-$ IgD$^-$ IgK$^+$ CD138$^+$ CD38$^+$ FAS^{dim} GL7$^-$	+	+	+	+	–
L33	T(2;16)	CD19$^+$ IgM$^+$ IgD$^-$ IgK$^+$ CD138$^+$ CD38$^+$ FAS$^+$ GL7$^-$	+	+	+	+	–
L52	no metaphases	CD19$^+$ IgM$^-$ IgD$^-$ IgK$^+$ CD138$^-$ CD38$^{+/-}$ FAS$^-$ GL7$^+$	+	–	–	+	–
L62	T(8;13)[b] T(3;17;3;4)	CD19$^+$ IgM$^+$ IgD$^-$ IgK$^+$ CD138$^+$ CD38$^+$ FAS$^+$ GL7^{dim}	+	+	+	+	–
L68	nd	CD19$^-$ IgM$^-$ IgD$^-$ IgK$^-$ CD138$^+$ CD38$^+$ FAS^{dim} GL7$^-$	–	+	+	+	–
L31	T(2;10)	CD19$^+$ IgM$^+$ IgD$^+$ IgK$^+$ CD138$^-$ CD38$^+$ FAS^{dim} GL7$^-$	+	+	+	+	+
L39	T(7;13)	CD19$^+$ IgM$^+$ IgD$^-$ IgK$^+$ CD138$^-$ CD38$^+$ FAS$^+$ GL7$^+$	+	–	–	+	+
L69	nd	CD19$^+$ IgM$^+$ IgD$^+$ IgK$^+$ CD138$^-$ CD38$^+$ FAS$^-$ GL7$^-$	+	–	–	–	–

Translocations are clonal if present in all metaphases. At least 20 metaphases were analyzed for each tumor. The extent of macroscopic tumor dissemination at necropsy is as follows: S, spleen; MLN, mesenterial lymph nodes; sbLN, submandibular lymph nodes; oLN, axillary, thoracal, or inguinal (other) lymph nodes; L, liver or other metastasis. nd, not determined. See also Figure S6 and Table S2.

[a]Sporadic translocation.

[b]Clonal, reciprocal translocation.

after staining 1 μl of blood with anti-Ter119 (eBiosciences) and Hoechst33342 (Invitrogen) for 30 min at room temperature, and confirmed by blood smear analysis.

Immunization with Sheep Red Blood Cells

1 ml of sheep red blood cells (SRBCs) (Colorado Serum Company) was diluted with 4 ml PBS, and 0.2 ml of the mix was injected intraperitoneally. Mice were immunized five times at 4-week intervals.

Flow Cytometry

Single-cell suspensions of lymphatic or tumor tissues were stained after erythrocyte lysis with fluorophore-conjugated anti-mouse antibodies (from BD PharMingen or eBiosciences) to detect CD19 (clone 1D3), B220 (RA3-6B2), GL-7, FAS (Jo2), CD38 (90), CD86 (GL1), immunoglobulin M (IgM) (AF6.78, II/41), immunoglobulin D (IgD) (11-26c.2a), immunoglobulin K (IgK) (187.1), CD138 (281.2), CD43 (S7), CD3 (145-2C11), or biotin-conjugated antibody for CXCR4 (2B11), followed by streptavidin-APC. Samples were acquired on a FACSCalibur or Fortessa instrument (Becton Dickinson) and analyzed with FlowJo software.

Western Blot

Germinal center light- and dark-zone (LZ and DZ) B cells were purified from spleens of *Pc*-infected mice at 3 weeks post-inoculation. Upon magnetic enrichment with anti-CD19 microbeads (Miltenyi Biotech), B cells were stained with antibodies to sort DZ (B220$^+$CD38$^-$GL7$^+$FAS$^+$CXCR4^{hi}CD86^{lo}) and LZ (B220$^+$CD38$^-$GL7$^+$FAS$^+$CXCR4^{lo}CD86^{hi}) B cells with a FACSVantage SE with Diva option or FACSAria instruments (Becton Dickinson, >95% purity). Resting B lymphocytes from wild-type and AID$^{-/-}$ mice were isolated from spleens by immunomagnetic depletion with anti-CD43 MicroBeads (Miltenyi Biotech) and cultured for 4 days in the presence of LPS (25 μg/ml) and IL-4 (5 ng/ml). AID protein was detected as previously described (McBride et al., 2008).

Purification of Malaria GC B Cells and TC-Seq Library Preparation

3–5 weeks after *Plasmodium* infection, Myc$^{l/l}$ROSA$^{erISCEI/AIDer}$ or Myc$^{l/l}$ ROSA$^{erISCEI/erISCEI}$AID$^{-/-}$ mice were injected intraperitoneally with 0.8 mg Tamoxifen in corn oil and euthanized 36 hr later. Upon ACK lysis, single-cell suspensions of splenocytes were incubated with biotinilated antibodies to mouse CD43 (clone S7), CD11c (N418), F4/80 (BMO), Gr-1 (RB6-8C5), and IgD (11-26c) for 20 min on ice (all from BD PharMingen or eBiosciences). Upon washing, cells were incubated with anti-biotin microbeads and negatively depleted with magnetic columns (Miltenyi). This was followed by incubation with anti-CD19 microbeads and positive magnetic selection. GC B cells purity was monitored by flow cytometry and ranged between 88%–95%. Two TC-seq libraries for each genotype were independently generated as previously described, with capture from the I-SceI site at *c-myc* (Klein et al., 2011), with the exception of using an improved linker (5′-GCA GCG GAT AAC AAT TTC ACA CAG GAC GTA CTG TGC GGC CGC T and 5′-/5Phos/GCG GCC GCA CAG TAC TTG ACT GAG CTT TA/3 ddC/). Deep sequencing was performed with Illumina HiSeq, 100 cycles, paired-ends.

TC-Seq and Computational Analysis

Pooled data from two independent libraries were analyzed as previously described (Klein et al., 2011; Wang et al., 2014), with minor modifications. Sequencing reads were first trimmed for quality with seqtk (error rate threshold of 0.01; Broad Institute). Reads containing primer sequences from the first PCR reaction were discarded from subsequent analysis. Finally, reads were mapped with SMALT (v.0.7.6; Sanger Institute), and only reads uniquely aligning at least 28 base pairs (bp) were included. To determine enrichment at genic regions (Figure 2D), the following portions of the genome were excluded: 1 Mb surrounding the I-SceI site, *Igh*, *Igκ*, and *Igλ* regions, 2-kb regions surrounding cryptic I-SceI sites (according to the consensus [TCA][AT]GGGATA[AC]CAGG [GCT][TC][ATC][AG][TAC]), AID-coding exons (likely representing retroviral insertions), and 3 Mb at each centromere. The portion of DNA from 2 kb upstream of the first transcription start site to the end of the last exon was considered as genic. For the role of transcription (Figure 2E), RNA-seq data (Hogenbirk et al., 2013) were mapped with STAR aligner (v.2.3.0.1) (Dobin et al., 2013), using the mouse genome (mm9) and removing multiple alignments. Transcripts were quantified and annotated using cufflinks (v.2.2.1) (Trapnell et al., 2013) and Ensembl annotation (release 66), and the following regions were excluded from downstream analysis: 1 Mb surrounding the I-SceI site, 2-kb regions surrounding cryptic I-SceI sites, and AID-coding exons. For the analysis of enrichment within hotspots of viral integration at ERFSs (M.J. and I.T.S., unpublished data) or CFSs (Figure 3), and of hotspots of AID-dependent translocation (Figure 4; Table S1), rearrangements within 1 Mb surrounding the I-SceI site, within 2 kb surrounding cryptic I-SceI sites, and within AID-coding exons were excluded from analysis. Translocation histograms (Figure 4A) were normalized for the size of the library and represented in log scale as TPKT (translocations per kilobase per 1,000 translocations in the library): TPKT = N/(B * S * 10^{-6}), where N is the number of translocations

in each bin, B is the bin size in nucleotides (in this case B = 100), and S is the total number of translocations in the library. See also the Supplemental Experimental Procedures.

M-FISH

To analyze chromosome rearrangements, metaphase spreads were obtained from primary tumor cells upon treatment with 100 ng/ml colcemid for 1 hr and were hybridized with the 21× mouse probe cocktail (Metasystems). Imaging was performed on a Zeiss AxioImager M1 equipped with a motorized scanning stage, and Isis software was used for the analysis.

Deep-Sequencing and Computational Analysis of Lymphoma L23

Whole-genome sequencing and data analysis were performed as previously described (Jankovic et al., 2013). In brief, 5 μg of genomic DNA was sonicated to an average fragment size of 500 bp and processed in accordance with the Paired-End (PE) Sample Preparation Kit protocol (Illumina). Upon quality assessment by Bioanalyzer (Agilent), the library was subjected to paired-end sequencing. Data were analyzed for structural variation (SV) discovery using the following programs: Hydra-SV (Quinlan et al., 2010), BWA aligner (Li and Durbin, 2009), Novoalign, the intersectBed software from BEDtools suite (Quinlan and Hall, 2010), RepeatMasker, and RefSeq track from the UCSC Genome Browser. In order to clear artifacts resulting from genetic variation, we subtracted SVs that were similarly found in the genomes of C57BL/6 and 129/Sv mice (Jankovic et al., 2013), or of 17 other inbred strains of laboratory mice (Keane et al., 2011). Further, SVs were removed from final analysis if any of the two reads matched to repeats for >50%, if deletions were <1 kb, or if their FinalWeightedSupport value was <2. Circular plots of genomic rearrangements were generated using Circos software (Krzywinski et al., 2009).

Supplemental Experimental Procedures are available online.

ACCESSION NUMBERS

The accession number for the TC-seq and lymphoma L23 sequencing data reported in this paper is SRA: SRP053308.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.07.019.

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