Impact of Chk1 dosage on somatic hypermutation in vivo

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Abstract
Checkpoint signaling in the context of a functional DNA damage response is crucial for the prevention of oncogenic transformation of cells. Our immune system, though, takes the risk of attenuated checkpoint responses during immunoglobulin diversification. B cells undergo continuous DNA damage and error-prone repair of their immunoglobulin genes during the process of somatic hypermutation. An accompanying attenuation of the DNA damage response via the ATR–Chk1 axis in B cells is believed to allow for a better DNA damage tolerance and for evasion of apoptosis, so as to ensure mutations to be passed on. We sought to determine whether the downregulation of Chk1 could also directly influence the process of hypermutation in vivo by altering the relative activity of error-prone DNA repair pathways. We analyzed the humoral response and the hypermutation process in mice whose B cells express reduced levels of the Chk1 protein. We found that Chk1 heterozygosity limits the accumulation of mutations in the immunoglobulin loci, likely by impacting on the survival of B cells as they accumulate DNA damage. Nevertheless, we unveiled an unanticipated role for Chk1 downregulation in favoring A/T mutagenesis at the antibody-variable regions during hypermutation. Even though immunoglobulin mutagenesis was found to be reduced, Chk1 signaling attenuation allows for sustained mutagenesis outside the immunoglobulin loci. Our study thus reveals that a proper Chk1 dosage is crucial for adequate somatic hypermutation in B cells.

INTRODUCTION
The preservation of genome integrity is essential for organismal survival and prevention of the generation of abnormalities that could provoke disease. Therefore, evolution has shaped different genome maintenance activities, such as DNA repair and checkpoint signaling pathways. Different DNA repair pathways have evolved to deal with different types of DNA lesions. Base and nucleotide excision repair pathways excise spontaneous or induced DNA lesions that affect single or multiple bases/nucleotides, respectively, in one DNA strand. Mismatch repair (MMR) deals mainly with the misincorporation of nucleotides in the newly synthesized DNA strand during DNA replication. Lesions that affect both DNA strands, such as double-strand breaks (DSBs), are mainly repaired by nonhomologous end joining or homologous recombination (HR). The repair, though, is not necessarily faithful: template-mediated repair, such as HR and single-stranded DNA (ssDNA) repair, acts in an error-free way, but the repair in the absence of a template, as for nonhomologous end joining repair, may lead to the incorporation of errors. Furthermore, lesions that induce replication fork stalling, such as thymine dimers or apurinic/apyrimidinic sites, could be resolved by the error-prone DNA damage tolerance mechanism of translesion synthesis, where the progression of replication is achieved at the expense of fidelity. The choice of repair pathway is governed by the type of lesion, but also by the relative activity of processing factors that contribute to the individual repair pathways.

B lymphocytes of the adaptive immune system rely on intentional error-prone DNA repair for the diversification of their antibody repertoire. During somatic
hypermutation (SHM), the basis of antibody affinity maturation in germinal centers (GCs), DNA lesions are introduced in the immunoglobulin (Ig) loci by activation-induced deaminase (AID), which is expressed in B cells upon activation. AID deaminates cytosines in the ssDNA exposed upon transcription, generating uracils that in turn are processed by multiple error-prone repair pathways leading to the introduction of a wide spectrum of point mutations. These uracils may simply be replicated over, resulting in transition mutations at C/G residues (phase 1A of SHM) or, alternatively, they may be removed by uracil glycosylase. In the latter case, the resultant abasic site may be bypassed during translesion synthesis, resulting in the generation of transition and transversion mutations at C/G base pairs (phase 1B). Furthermore, the U:G mismatch generated by AID may be processed by a noncanonical MMR (ncMMR) pathway that involves the recruitment of the error-prone polymerase η (Polη) which is the main source of mutations at A/T residues during SHM (phase 2). In this case, following mismatch recognition and nucleotide excision on one DNA strand, the ssDNA patch refill is carried out by Polη recruited via monoubiquitination of the proliferating cell nuclear antigen. Furthermore, the processing of AID-induced lesions may generate DSBs whose unfaithful repair by nonhomologous end joining could lead to the switch of the antibody isotype via class switch recombination (CSR).

Checkpoint signaling is crucial for upstream regulation of DNA repair pathways. Recognition of single-stranded lesions by ataxia telangiectasia related (ATR) or recognition of DSBs by ataxia telangiectasia mutated leads to signaling via the downstream checkpoint kinases Chk1 and Chk2, respectively. These kinases may in turn phosphorylate p53 to induce cell cycle arrest or apoptosis, but also regulate components of multiple repair pathways by phosphorylation, thus adjusting the repair capacity to the task at hand.

The activity of Chk1 was shown to be indispensable for the survival of mouse and human hematopoietic stem and progenitor cells as well as for the B cells during their development. In the absence of Chk1, the B cells undergo a block in the differentiation at the pro- to pre-B-cell transition stage, likely because of the accumulation of DNA damage during the process of V(D)J recombination. B cells are dependent on the activity of Chk1 also for the establishment of GCs: The conditional deletion of Chk1 in B cells upon activation was shown to impair the GC formation and resulted in a dampened humoral response. Because multiple repair pathways, in contrast to their error-free repair function, operate in an error-prone fashion in B cells during SHM, we have asked whether this abnormality could be a result of the altered upstream regulation of these pathways by checkpoint signaling in hypermutating cells. It was indeed shown that in normal and lymphoma B cells the ATR-Chk1 axis is downregulated by transcriptional repression mediated by the crucial GC factor Bcl-6, and it was proposed that this would allow for a better tolerance of DNA damage and prevention of cell death by apoptosis. In our previous studies we have inactivated Chk1 and Chk2 in hypermutating cell lines in order to investigate the effects of checkpoint signaling alteration on the repair pathway choice during SHM. Intriguingly, this resulted in opposing phenotypes: While SHM was found to be increased upon (partial) Chk1 inactivation, it was decreased upon Chk2 inactivation, likely because of increased Chk1 activation in Chk2-deficient cells. A defect in the activation of the error-free mechanism of HR upon Chk1 downregulation was found to be responsible for the observed effects, in line with the established role of Chk1 in promoting HR. However, we found that the complete inactivation of HR in B cells in vivo resulted in survival defects during the GC reaction.

In this study, we asked whether the downregulation of Chk1 in B cells is compatible with an efficient humoral response and analyzed the effects of its signaling attenuation on SHM in vivo.

RESULTS

In order to investigate how the attenuation of the signaling mediated by Chk1 regulates the fate of AID-induced lesions and the activity of error-prone DNA repair mechanisms during the GC reaction, we generated mice with decreased expression of Chk1 specifically in B cells. In these mice, the exon 2 of the Chk1 gene encoding for the protein kinase domain is deleted by Cre (cyclization recombination)-mediated recombination of loxP sites resulting in the inactivation of Chk1. The B-cell-specific expression of the Cre recombinase is achieved by its integration in the locus of the mb1 gene, which encodes the Ig-α signaling subunit of the B-cell receptor. Because the homozygous deletion of Chk1 has been shown to be lethal for B cells, we used heterozygous Chk1<sup>Bhet</sup>/+ Mb1<sup>Cret</sup> mice for all experiments (henceforth called Chk1<sup>Bhet</sup>) and Chk1<sup>BWT</sup> mice (Chk1<sup>Bhet</sup>+/+ Mb1<sup>Cret</sup> or Chk1<sup>BWT</sup> Mb1<sup>Bhet</sup>+) as controls. Deletion of the floxed exon occurred efficiently in Chk1<sup>Bhet</sup> mice (Figure 1a, b) and resulted in the expression of approximately half the amount of Chk1 protein in B cells in comparison to the controls (Figure 1c, d and Supplementary figure 1a).
Because the mb1 gene is expressed at the pro- to pre-B-cell transition stage,32 Chk1 deletion occurs early during B-cell development in Chk1Bhet mice. Therefore, we asked whether the early reduction of Chk1 protein levels could impair B-cell maturation and differentiation. B-cell precursor populations in the bone marrow (Supplementary figure 1b–e) and mature B-cell populations in the spleen (Supplementary figure 1f–j) of Chk1Bhet mice were found to be comparable to the controls. We therefore conclude that Chk1Bhet mice are suitable to study the effect of Chk1 signaling attenuation on B-cell responses.

We first assessed the effects of Chk1 heterozygosity on B-cell responses in vitro. It is known that B cells require signals mediated either by the interaction with T cells or by the soluble antigens themselves in order to become fully activated.33 Splenic B cells were isolated from 8–16-week-old mice and stimulated either with anti-CD40, in order to mimic a T-cell-dependent activation of the B cells, or with lipopolysaccharide, for a T-cell-independent B-cell response via TLR4. IL-4 was added to the culture to assess the effects of Chk1 heterozygosity on the CSR ability, as cells activated in the presence of IL-4 switch the Ig isotype from IgM to IgG1. We observed that viability and proliferation of in vitro-cultured B cells from Chk1Bhet mice were equivalent to the controls (Figure 2a, b and Supplementary figure 2a) for both T-cell-dependent and T-independent responses. In addition, we found that CSR to IgG1 was equally efficient (Figure 2c, d and Supplementary figure 2b, c). We conclude that Chk1 heterozygosity does not affect viability, proliferation, or CSR ability of in vitro-cultured mouse B cells.

A recent study has shown that the in vivo downregulation of Chk1 levels in established GC B cells generated upon immunization does not affect their survival in the spleen.22 We first asked whether the reduction of Chk1 levels at an earlier stage of B-cell development could still allow for a normal GC formation; therefore, we evaluated the percentage of background splenic GC B cells in unchallenged mice. We found a comparable fraction of GC B cells in Chk1BWT and Chk1Bhet mice and observed proper distribution of the GC B cells in the dark and light zone upon reduced Chk1 levels (Figure 3a and Supplementary figure 3a). Furthermore, we observed that Chk1Bhet and Chk1Bhet mice are equally able to generate plasmablasts/cells (PBs/PCs) in the spleen (Figure 3b and Supplementary figure 3b). We concluded that Chk1Bhet mice are a suitable model for the investigation of the B-cell

![Figure 1. Efficient B-cell-specific Chk1 downregulation in Chk1Bhet mice.](image)
responses during an immune reaction in vivo. For this purpose, we immunized 8–12-week-old Chk1Bhet and Chk1BWT mice (only Chk1+/+Mb1Cre/+ were used as controls in immunization experiments) with alum-precipitated nitrophenylacetyl chicken γ globulin (NP-CGG). Immunization with NP-CGG induces a T-cell-dependent B-cell response resulting in the efficient formation and maturation of GCs in secondary lymphoid organs within 10–14 days post-immunization. We observed no significant differences between the numbers of total splenocytes and splenic B cells in immunized and nonimmunized (n.i.) mice 14 days post-immunization (Figure 3c), even though both Chk1Bhet and Chk1BWT immunized mice generate a comparable GC B-cell fraction (Figure 3d), significantly higher than the n.i. controls (Figure 3e).

We monitored the levels of NP-binding antibodies and their affinity maturation over the course of the immune reaction. We measured the levels of total (α-NP15) and high-affinity (α-NP3) NP-binding IgG1 in the sera of immunized mice (and n.i. mice as controls) at day 7 and day 14 post-immunization and found that the generation of class switched NP-binding IgG1 and its affinity maturation were normal in Chk1Bhet mice compared with the controls (Figure 3f, g). Apparently, Chk1 heterozygosity does not greatly affect the B-cell responses to NP-CGG.

After verifying that the humoral response is not impaired by Chk1 heterozygosity, we sought to determine whether Chk1 levels could influence the choice of the repair pathways involved in the resolution of AID-induced lesions during SHM. We sorted splenic GC B
cells from immunized Chk1<sup>BWT</sup> and Chk1<sup>Bhet</sup> mice 14 days post-immunization and sequenced the JH<sub>4</sub> intronic region downstream of the V186.2-D-JH<sub>4</sub> rearrangement known for dominating NP responses. We performed Sanger sequencing in order to avoid noise background that would affect the mutational spectra and
confirmed the high quality of the sequencing data by analyzing samples deriving from non-GC B cells in parallel (sorted as in Supplementary figure 3c), which rarely show mutations (data not shown). Strikingly, we found that the frequency of hypermutation was reduced in Chk1Bhet mice in comparison to the controls (Figure 4a), and observed that this effect was mostly because of a paucity of highly mutated sequences (Figure 4b). To assess the role of Chk1 levels in the repair pathway choice, we compared the pattern of mutations between Chk1Bhet and Chk1BWT mice (Figure 4c–f and Supplementary figure 4a). We focused our investigation on the unique mutations in order to rule out the overrepresentation of mutations accumulated as consequence of clonal expansion. We found that the lower mutation frequency observed in Chk1Bhet mice in comparison to the controls was because of a significant reduction of mutations at C/G residues, whereas the frequency of mutations at A/T residues was not affected (Figure 4c and Supplementary figure 4a). As percentage of total mutations, A/T base substitutions were found to be significantly higher at the expense of mutations at C/G residues in Chk1Bhet mice (Figure 4d–f and Supplementary figure 4b, c). Collectively these results indicate that Chk1 levels in GC B cells indeed influence the processing of uracils introduced in the variable region of the Ig locus during SHM without altering the proportion of mutations occurring within and outside the typical hotspots (Figure 4g and Supplementary figure 4d).

As we found that Chk1 heterozygosity reduces the accumulation of mutations and affects the repair pathway choice in the variable region of the Ig locus during SHM, we asked whether Chk1 downregulation could have effects on the repair pathway choice also for lesions induced in the switch regions during the process of CSR. AID activity at the switch regions could lead to the generation of DSBs whose unfaithful repair by nonhomologous end joining leads to the antibody isotype switch.36,37 However, not all the lesions generated in the switch regions lead to DSB generation and CSR, but some rather lead to the accumulation of mutations as during SHM.38 We analyzed mutations occurring in the pre-Sµ region of GC B cells isolated 14 days post-immunization with NP-CGG. While the overall frequency of mutations in the pre-Sµ region was also found to be decreased to the same extent as for the JH4 intronic region (1.5-fold reduction; Figure 5a and Supplementary figure 5a), interestingly the pattern concerning A/T mutagenesis was not affected likewise (Figure 5b–d and Supplementary figure 5b–e). We conclude that the attenuation of Chk1 signaling in B cells limits the accumulation of mutations throughout the Ig locus, but Chk1 heterozygosity does not seem to affect the repair pathway choice for lesions introduced in the switch regions.

We observed that the Chk1 heterozygosity in B cells impacts on the accumulation of mutations at the Ig locus. We speculate that the levels of Chk1 may regulate the threshold of DNA damage that can be tolerated in hypermutating B cells.

In Peyer’s patches (PPs) along the gut, B cells are continually exposed to mucosa-derived antigens, therefore chronic GCs can be found.39 A recent study has shown that Chk1 downregulation affects the survival of those long-lasting GCs and the authors propose that this effect may be a result of an excessive accumulation of DNA damage over time.22 As we did not see Chk1 dosage effects on the survival of primary B cells cultured in vitro for 72–96 h (Figure 2a and Supplementary figure 2a) nor on the formation of short-lived GCs in unchallenged and immunized mice (Figure 3a, d and e), we analyzed chronic PP GCs. Even though the total cell count (Figure 6a) and the percentage of B cells (Figure 6b) were not impaired in the PPs upon Chk1 downregulation, we found that the percentage of PP GC B cells was significantly reduced in Chk1Bhet mice in comparison to the controls (Figure 6c, d). As we observed comparable distribution of PP GC B cells in the dark and light zone (Figure 6e–f), we concluded that Chk1 downregulation affects the persistence of those long-lasting GCs rather than their structure.

We asked whether the downregulation of Chk1 in B cells could limit the accumulation of mutations also at AID off-target genes. We sorted GC B cells from PPs of 24–30-week-old mice (Supplementary figure 6), as non-Ig mutagenesis is traditionally measured in these cells. We analyzed mutations occurring at the Cld83 locus, a well-known AID off-target40 and, surprisingly, the mutation frequency was not found to be affected, but rather amounted to a similar degree irrespective of Chk1 dosage (Figure 6g). In the same way as for the pre-Sµ region, the downregulation of Chk1 does not seem to affect the mutational pattern at the Cld83 locus (Figure 6h), suggesting that the contribution of Chk1 in regulating the repair pathway choice during SHM is restricted to the variable region of the Ig loci, perhaps governed by the extent of the DNA damage.

We conclude that Chk1 signaling attenuation allows for more A/T mutagenesis at the expense of C/G mutagenesis during SHM at the variable region of the Ig loci (Supplementary figure 7) but limits the overall accumulation of mutations. Our data on the percentage of GC B cells in the PPs suggest that Chk1 signaling aids the longevity of GCs as DNA damage accumulates. Interestingly, even though the percentage of chronic GC
incompatible with cell survival in adult tissues.30 It was shown that Chk1 is essential for normal B-cell development and for the establishment of GCs.20,22 Given the dependency of B cells on Chk1 activity, Chk1 heterozygosity represents the only possible scenario that could allow for the investigation of the role of Chk1 signaling attenuation in the process of SHM in vivo. Therefore, we used Chk1Bhet mice whose B cells undergo Cre-mediated deletion of only one Chk1 allele upon expression of the mbl1 gene. We found that our Chk1Bhet mice were able to mount a proper humoral response upon immunization with NP-CGG, ruling out possible alterations of mature B-cell functionality upon early Chk1 downregulation during B-cell development.

Even though Chk1Bhet mice were able to undergo a normal antibody affinity maturation and to perform

B cells was found to be reduced upon Chk1 downregulation, we observed sustained mutagenesis at the AID off-target gene Cid83 in these cells.

**DISCUSSION**

The threat to genome stability and cell survival presented by the complete absence of Chk1 has limited the exploration of its functions in vivo. Complete deletion of Chk1 is lethal at the embryonic stage41,42 and incompatible with cell survival in adult tissues.30 It was shown that Chk1 is essential for normal B-cell development and for the establishment of GCs.20,22 Given the dependency of B cells on Chk1 activity, Chk1 heterozygosity represents the only possible scenario that could allow for the investigation of the role of Chk1 signaling attenuation in the process of SHM in vivo. Therefore, we used Chk1Bhet mice whose B cells undergo Cre-mediated deletion of only one Chk1 allele upon expression of the mbl1 gene. We found that our Chk1Bhet mice were able to mount a proper humoral response upon immunization with NP-CGG, ruling out possible alterations of mature B-cell functionality upon early Chk1 downregulation during B-cell development.

Even though Chk1Bhet mice were able to undergo a normal antibody affinity maturation and to perform
efficient CSR, we observed a reduction in the accumulation of mutations at the Ig locus in comparison to the Chk1BWT mice. It is conceivable that the presence of one functional Chk1 allele limits the detection of the effects that Chk1 downregulation exerts on the process of Ig diversification in vivo with techniques other than sequencing. The mutation frequency at the Ig locus in Chk1Bhet mice was found to be 1.5-fold lower in both JH4 and pre-Sµ regions in comparison to the controls. These results are at odds with our previous findings in vitro on the chicken lymphoma B-cell line DT40 and the human Burkitt lymphoma cell line RAMOS.25,26 The reason may be that in chicken and human lymphoma cell lines, on the one hand, the lack of p53 function desensitizes the cells to DNA damage-induced apoptosis43,44 and, on the other hand, the overexpression of the proto-oncogene myc fuels proliferation, ensuring the transmission of mutations.45,46 In our previous studies on these cell lines, we found that the downregulation or the chemical inhibition of Chk1 leads to an increase of the mutation frequency in the Ig locus, while a stronger Chk1 activation in the absence of Chk2 has opposite effects. Chk1 promotes the activation of HR by phosphorylation of Rad51 and BRCA2,27,28 and the inhibition of HR was found to lead to an increase of SHM in vitro models.47 However, in vivo we found that the conditional inactivation of HR in B cells impairs their survival during the GC reaction, which in turn limits the accumulation of mutations during SHM.29 Therefore, we suggest that, in vivo, the downregulation of Chk1 signaling beyond the levels dictated by Bcl-6-mediated transcriptional repression23,24 limits the accumulation of mutations at the Ig locus, likely because of survival or proliferation defects.

In support of this argument, we found that the overall reduced mutation frequency in the Ig locus observed in Chk1Bhet mice in comparison to the controls was a result
of the lack of highly mutated sequences. The accumulation of mutations is strictly dependent on the ability of B cells to undergo multiple rounds of proliferation and positive selection in the GCs.48–50 The paucity of highly mutated sequences in Chk1Bhet mice can be explained with the progressive slowdown of proliferation or loss of cells which may accumulate excessive DNA damage by recirculating in the GC. For instance, it was shown that genotoxic stress in GC B cells may activate the signaling via ataxia telangiectasia mutant, leading to ubiquitin-mediated degradation of the crucial GC factor Bcl-6, therefore impairing the maintenance of GCs.51 Even though we did not see differences in the percentage of B cells in acutely induced GCs upon immunization, we could observe a significant reduction of chronic GC B cells in the PPs of Chk1Bhet mice. These results were consistent with previous observations showing that chronic PP GCs but not acute splenic GCs were impaired by the depletion of one of the Chkl alleles in established GC B cells.52 It seems reasonable that the presence of one functional Chk1 allele delays the manifestation of proliferation or survival defects to later stages of the GC reaction, so that they are evident in long-lasting but not in short-lived GCs.

Even though we observed a reduced persistence of GCs in the PPs, we did not find a reduction in the mutation frequency at the Cdj83 locus, as we did for the Ig loci. Cdj83, together with Bcl6, Pim1, Pax5 and c-Myc, is a gene frequently targeted by AID because of the possibility of convergent (sense and antisense) transcription at the locus facilitated by the proximity of super enhancers.52 Mutations in these AID off-targets are associated with oncogenic transformation and lymphomagenesis.53,54 We have argued that survival or proliferation defects upon Chk1 downregulation may account for the reduced mutation frequency observed in the Ig loci of Chk1Bhet mice.
mice. In contrast to those findings, we did not find a reduced mutation frequency at the Cd83 locus, even though the effects of Chk1 heterozygosity on the survival of GC B cells in the PPs are appreciable. Therefore, it is tempting to speculate that Chk1 signaling attenuation could facilitate accumulation of mutations outside the Ig loci, perhaps by impairing error-free mechanisms of repair. It was shown that AID-dependent lesions in several off-target genes are more frequently repaired in an error-free fashion than at the Ig loci. A Chk1-dependent activation of HR may explain the observed effect at the Cd83 locus. However, this remains speculative as we did not see a significant increase of the non-Ig mutagenesis and the Chk1Het mice did not develop lymphomas during the 5–6-month observation period (data not shown).

In order to investigate the effects of Chk1 downregulation on the repair pathway choice during SHM described by Di Noia and Neuberger, we analyzed the pattern of mutations at the Ig locus. In the JH4 intronic region, A/T mutagenesis was found significantly higher at the expense of C/G mutagenesis upon downregulation of Chk1. A/T mutagenesis is mediated via ncMMR/Po1 or our results are at odds with previous studies that would rather link the activity of Chk1 to the facilitation of translesion synthesis via Po1 during DNA replication. We suggest that this discrepancy may be linked to the different cell cycle phase where the process of A/T mutagenesis takes place. AID-induced uracils at the Ig locus were found almost exclusively in G1 phase of the cell cycle. It has been proposed that, unlike mutations at C/G base pairs that may occur throughout the cell cycle, A/T mutagenesis may be limited to G1. During the processing of the U-G mismatch by MMR, an ssDNA gap intermediate is formed and needs to be filled. It seems conceivable that the paucity of dNTPs in G1 may be responsible for the polymerase stalling during the ssDNA patch refill, the subsequent monoubiquitination of the proliferating cell nuclear antigen and the recruitment of Po1. Alternatively, the ssDNA gap may be sensed by the ATR–Chk1 axis. Interestingly, it was found that the ATR–Chk1 response can be activated by MSH2–MSH6 mismatch recognition complex and that ATR–Chk1 activation mitigates the replication stress upon MMR activity. Therefore, we suggest that in hypermutating B cells the ssDNA intermediates for the A/T mutator generated in the G1 phase of the cell cycle may activate the ATR–Chk1 axis or may be refilled by Po1. We believe that, upon activation of the ATR–Chk1 axis, the ssDNA gaps may be marked for faithful repair by HR in the subsequent S phase of the cell cycle. Our study on the role of HR during the GC reaction confirms this hypothesis, as the damage induced by the A/T mutator was found to be highly threatening for cell survival in the absence of HR.

Therefore, we uncover a new unanticipated role for the ATR–Chk1 signaling attenuation in facilitating A/T mutagenesis and we propose a model where the increased activity of the ncMMR pathway in G1 reduces the amount of uracils that persist in the DNA until S phase, leading to less C/G mutations and in particular replication-associated transitions.

In the pre-Sμ region, we did not observe such effects of Chk1 dosage on the repair pathway choice. This may reflect the intrinsic differences between mutagenesis at the variable and at the switch regions, and in particular the differential activity of the ncMMR pathway. It was shown that the knockout of MSH6 and Po1 results in a reduced mutation frequency in the JH4 intron but not in the pre-Sμ region, hence indicating a minor involvement of the ncMMR pathway via Po1 at the switch regions. Here, the presence of overlapping, highly repetitive and palindromic motifs S'-AGCT-3' facilitates the generation of R-loops during transcription and it has been suggested that this could reduce the activity of the MSH2–MSH6 complex. The processing of U-G mismatches by MMR at the switch regions may facilitate CSR over A/T mutagenesis: MMR excises nucleotides on one strand of the DNA and, when reaching a nick on the opposite strand, its activity would terminate creating DSBs. Furthermore, the targeting of AID at the pre-Sμ region seems to be an early event upon B-cell activation, as the completion of CSR seems to precede the formation of the GCs. At this stage, the expression levels of Po1 are lower than in mature GCs as they gradually increase following B-cell activation.

Altogether, the results in this study show that Chk1 dosage regulates the threshold of DNA damage that can be tolerated by hypermutating B cells in vivo and unveil a novel role for Chk1 signaling attenuation in facilitating A/T mutagenesis during SHM at the variable regions. These findings underline the importance of keeping Chk1 levels in check, to ensure a weaker but still functional signaling. Therefore, this work contributes to the understanding of the peculiar calibration of Chk1 amount within the GCs, as the transcription activity at the Chk1 gene is repressed in the dark zone and restored in the light zone. Ultimately, these findings have important implications for the comprehension of potential effects of anticancer therapies based on ATR/Chk1 inhibition on the humoral immunity.

**METHODS**

**Mice**

Chk1Het mice, generated as previously described, were obtained from The Jackson Laboratory (Charles Rivers,
Sulzfeld, Germany). Mb1\textsuperscript{Cre/+} mice were obtained from Dr C. Kosan with permission of M. Reth.\textsuperscript{31} Chk1\textsuperscript{fl/+} and Mb1\textsuperscript{Cre/+} mice are on a C57BL/6 background, were bred in specific pathogen-free conditions and were intercrossed to generate Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/+}, Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/-} and Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/-} mice for experiments. Genotyping for Chk1\textsuperscript{fl} and Mb1\textsuperscript{Cre} alleles was performed on genomic DNA isolated from mouse tails, as described previously.\textsuperscript{30,31} B-cell-specific recombination of the Chk1 locus in Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/+} mice was verified by PCR on genomic DNA from tails and sorted splenic B cells: The primer Chk1-R3 (sequence in Supplementary table 1) was used in combination with the genotyping primers described in Lam et al.\textsuperscript{30} (63°C, 35 cycles) in order to detect the Cre-mediated deletion through the amplification of a 718-bp PCR product.

Mice were sacrificed at 6–30 weeks (for bone marrow, spleen and PP collection). Immunization was performed by peritoneal injection of 100 μg of alum-precipitated NP-CGG (BIOCat, Heidelberg, Germany). Five Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/+} and five Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/-} mice between 8 and 12 weeks of age were immunized, and five Chk1\textsuperscript{fl/+}Mb1\textsuperscript{Cre/-} mice were used as n.i. controls. At days 7 and 14 post-immunization, blood samples were collected from immunized and n.i. mice. At day 14 post-immunization, mice were sacrificed for spleen collection and GC B-cell sorting.

Female and male littermates were used without discrimination in all experiments but the immunization experiments; in each immunization experiment, littermates of the same gender were used. All animal experiments were approved by the Thüringer Landesamt für Verbraucherschutz.

**B-cell isolation and in vitro stimulation**

Spleens were collected from 8–16-week-old mice and homogenized into a single-cell suspension. Total splenocytes were counted; splenic B cells were isolated using MACS depletion with α-CD43 beads (Miltenyi Biotec, Bergisch Gladbach, Germany), counted and seeded at the density of 5 × 10\textsuperscript{5} cells mL\textsuperscript{-1}. B cells were cultured in Roswell Park Memorial Institute Medium 1640 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal calf serum (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Invitrogen) 50 mM beta-mercaptoethanol (Sigma-Aldrich) and 0.2 U mL\textsuperscript{-1} penicillin/streptomycin (Invitrogen). Lipopolysaccharide (10 μL mL\textsuperscript{-1}; Sigma-Aldrich), α-CD40 (1 μg mL\textsuperscript{-1}; ebioscience, Thermo Fisher Scientific) and IL-4 (20 ng mL\textsuperscript{-1}; ebioscience) were added to the culture medium 1 h after seeding.

**Western blot**

Fresh MACS-isolated splenic B cells were lysed in 20 mM HEPES (pH 7.9, Invitrogen), 350 mM NaCl (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 20% glycerin (Carl Roth GmbH + Co. KG), 1 mM MgCl\textsubscript{2} (Carl Roth GmbH + Co. KG), 0.5 mM EDTA (AppliChem GmbH, Darmstadt, Germany), 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Fluka Chemie, Buchs, Switzerland) and 1% NP-40 (AppliChem GmbH) in the presence of protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein quantification was performed using the Bio-Rad DC Protein Assay (Bio-Rad GmbH, Feldkirchen, Germany). The protein samples were denatured in the appropriate amount of sample buffer [120 mM Tris (pH 6.8; Carl Roth GmbH + Co. KG), 4% sodium dodecyl sulfate (Carl Roth GmbH + Co. KG), 20% glycerol (MP Biomedicals, Eschwege, Germany) and 0.1% bromophenol blue (Carl Roth GmbH + Co. KG), 0.1 mM Dithiotreitol (Invitrogen)]. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide gel), samples were transferred to a polyvinylidene fluoride membrane (Carl Roth GmbH + Co. KG) and efficient protein transfer was visualized using Ponceau S solution (Sigma-Aldrich). The following primary antibodies diluted in 5% powdered milk (Carl Roth GmbH + Co. KG) and 1% Tween (Carl Roth GmbH + Co. KG) Tris-buffered saline were used: α-Chk1 (sc-8408; Santa Cruz Biotecnology, Heidelberg, Germany) and α-vinculin (BLZ-03106; BIOZOL, Eching, Germany). Horseradish peroxidase-conjugated α-mouse IgG (W402B; Promega GmbH, Walldorf, Germany) diluted in 5% milk, 1% Tween Tris-buffered saline was used as secondary antibody. Proteins were detected by chemoluminescence using ECL Western Blotting Reagents (GE Healthcare, Garching, Germany). Intensity of the bands was quantified using ImageJ 1.52a.\textsuperscript{72}

**Flow cytometric analysis and sorting**

Viability and CSR of in vitro-cultured splenic B cells were assessed by staining with Fixable Viability Stain 780 (FVS780; 565388, BD Biosciences, Heidelberg, Germany) and α-IgG1-DyLight 405 (409109; BioLegend, Koblenz, Germany) after 72 h and 96 h in culture. For analysis of proliferation, the ex vivo-isolated splenic B cells were stained with 1 μM carboxyfluorescein succinimidyl ester (Invitrogen) before seeding; after 24 and 72 h in culture, cells were stained with α-B220-PE (553090; BD Biosciences) and FVS780. Analysis of the percentage of B cells and B-cell subsets in the bone marrow and in the spleen of 6–18-week-old mice was performed by staining fresh ex vivo-isolated samples. Bone marrow cells were counted after isolation and stained with α-B220-BV785 (103246; BioLegend), α-CD43-FTTC (501785, BD Biosciences), α-IgM-APC (551062, BD Biosciences) and 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Total splenocytes were counted; the percentages of T and B cells in the spleen were calculated by staining spleen samples with α-B220-FTTC (553088; BD Biosciences), α-CD3-PE (130-109897; Miltenyi Biotec) and DAPI; B-cell subsets ( marginal and follicular zone B cells) were analyzed by staining spleen samples with α-B200-BV785, α-CD21-FTTC (501785; BD Biosciences), α-CD23-PE (51773; BD Biosciences) and DAPI. The percentages of GC B cells and GC B-cell subsets (dark zone and light zone) in unchallenged 16–24-week-old mice were determined by staining spleen samples with α-B220-BV785, PNA-FTTC (Vector Laboratories, Burlingame, CA, USA), α-B220-FTTC (553088, BD Biosciences), α-IgM-APC (551062, BD Biosciences) and 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Total splenocytes were counted; the percentages of T and B cells in the spleen were calculated by staining spleen samples with α-B220-FTTC (553088; BD Biosciences), α-CD3-PE (130-109-897; Miltenyi Biotec) and DAPI; B-cell subsets ( marginal and follicular zone B cells) were analyzed by staining spleen samples with α-B200-BV785, α-CD21-FTTC (501785; BD Biosciences), α-CD23-PE (51773; BD Biosciences) and DAPI. The percentages of GC B cells and GC B-cell subsets (dark zone and light zone) in unchallenged 16–24-week-old mice were determined by staining spleen samples with α-B220-
USA), α-CD95-PE (554258, BD Biosciences), α-CXCR4-PerCP-eFluor710 (46-9991-80; eBioscience), CD86-APC (561964; BD Biosciences) and DAPI. The percentages of PBs/PCs and PB/PC subsets in unchallenged 16–24-week-old mice were determined as described in Pracht et al.23 by staining spleen samples with α-B220-FITC, α-CD19-BV421 (115549; BioLegend), α-CD138-PE-Cy7 (142514; BioLegend), α-TACI-APC (17-5942-81; Thermo Fisher Scientific) and 7-aminoactinomycin D (420404; BioLegend).

For analysis and sorting of splenic GC and non-GC B cells of immunized mice, spleens were collected 14 days post-immunization. Total splenocytes were counted and were enriched via MACS depletion of CD43-positive cells, counted and stained with α-B220-PerCP (553093; BD Biosciences), PNA-FITC, α-CD95-PE and DAPI. For analysis of GC B cells and GC B-cell subsets (dark zone and light zone B cells) from PPs of unchallenged 16–30-week-old mice, PPs were homogenized into a single-cell suspension. Total cells of GC B cells and GC B-cell subsets (dark zone and light zone B cells) from PPs of unchallenged 16–30-week-old mice, PPs were homogenized into a single-cell suspension. Total cells were counted and stained with α-B220-BV786, PNA-FITC, α-CD95-PE, CXCR4-PerCP-eFluor 710, CD86-APC and DAPI. For sorting of PP GC B cells from 24–30-week-old mice, PP cells were stained with α-B220-BV786, PNA-FITC, α-CD95-PE and DAPI.

Flow cytometry was performed with an LSRFortessa (BD Biosciences) and sorting was performed with a FACSaria (BD Biosciences). Flow cytometry data were analyzed in FlowJo (FlowJo LLC v10.7.1, Ashland, Oregon).

ELISA

To assess the titers of circulating NP-binding IgG1 in immunized and n.i. mice, serum was extracted from blood samples collected at days 7 and 14 post-immunization. ELISA plates (Thermo Fisher Scientific) were coated with NP3 and NP15 (BioCat). Serum samples were diluted 1:100 (day 7) and 1:500 (day 14) in 1% powdered milk in phosphate-buffered saline. As standard, a pooled serum control from NP-CGG-immunized mice was used and diluted 1:400. For each sample, serial dilutions of the initial dilution were applied to the plates. NP-binding antibodies were detected using an α-IgG1-biotin (535441; BD Biosciences) primary antibody, streptavidin–horseradish peroxidase (405210; BioLegend) and an o-phenylenediamine substrate (Sigma-Aldrich). The reaction catalyzed by horseradish peroxidase was stopped adding 3 N HCl (Carl Roth GmbH + Co. KG) and the absorbance was measured at 492 nm. NP-binding IgG1 titers were expressed as mean values of absorbance measured for each serial dilution relative to the standard.

Analysis of JH4, pre-Σμ and Cd83 mutagenesis

Genomic DNA from B cells sorted from spleens and PPs was isolated. The JH4 intron and pre-Σμ region were amplified from splenic GC and non-GC B cells of immunized mice and Cd83 was amplified from PP GC B cells isolated from unchallenged mice. The JH4 intron was amplified with the primers V186.2-fwd and JH4-rev (sequence in Supplementary table 1); the pre-Σμ region was amplified with the primers pre-Σμ-fwd and pre-Σμ-rev (sequence in Supplementary table 1); Cd83 was amplified with Cd83-fwd and Cd83-rev (sequence in Supplementary table 1). All PCRs were performed using Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific). PCR products were resolved by electrophoresis and the bands of the expected size were excised and purified. A-tailing and subsequent purification were performed on the PCR products prior to cloning into the pGEM-T vector (Promega). DH5α electrocompetent E. coli (Invitrogen) were electroporated with purified ligated products and plated on LB 1.5% Agar (Invitrogen) plates with 10 µg mL⁻¹ ampicillin (Roche), 1 mshow isopropyl β-d-thiogalactopyranoside (Thermo Fisher Scientific) and 20 µg mL⁻¹ X-gal (VWR International GmbH, Darmstadt). DNA from single colonies grown overnight in LB with 10 µg mL⁻¹ ampicillin (Roche) was isolated and sequenced by the Sanger method. JH4 sequences were sequenced with JH4-rev, pre-Σμ sequences with pre-Σμ-rev and Cd83 with Cd83-fwd. JH4 and pre-Σμ sequences were thus reversed prior to analysis. All identical sequences but one were removed from the analysis in order to prevent the overrepresentation of sequences deriving from the same B-cell clone. Sequences were aligned with the software Geneious Pro 5.5.6 (Biomatters, Auckland, New Zealand) and the analysis of mutagenesis was performed using the SHMTool (http://shmtool.montefiore.org/cgi-bin/p1).24

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

Amanda Bello: Conceptualization; Data curation; Investigation. Berit Jungnickel: Conceptualization; Data curation; Funding acquisition; Supervision.

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