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T cell help controls the speed of the cell cycle in germinal center B cells

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Abstract

The germinal center (GC) is a microanatomical compartment wherein high affinity antibody-producing B cells are selectively expanded. B cells proliferate and mutate their antibody genes in the dark zone (DZ) of the GC, and are then selected by T cells in the light zone (LZ) on the basis of affinity. Here we show that T cell help regulates the speed of cell cycle phase transitions and DNA replication of GC B cells. Genome sequencing and single-molecule analyses revealed that T cell help shortens S phase by regulating replication fork progression while preserving the relative order of replication origin activation. Thus, high affinity GC B cells are selected by a mechanism that involves prolonged dwell time in the DZ where selected cells undergo accelerated cell cycles.

Antibodies elicited during T cell-dependent immune responses undergo significant increases in affinity over time (1). This phenomenon, known as affinity maturation, takes place in the germinal center (GC), where antigen-specific B cells diversify their antibodies by somatic hypermutation (2) and undergo selective clonal expansion (3–7). Together, these events are essential to the development of effective antibody responses.

GC B cells bearing antibody variants with higher affinity are selectively expanded during iterative rounds of migration between the DZ, where they proliferate and hypermutate, and the LZ, where they capture antigen displayed on the surface of follicular dendritic cells (8–11). By binding and internalizing more antigen in the LZ, high affinity clones present more peptide-major histocompatibility complex II (MHCII) and thereby elicit greater help from $CD4^+$ T follicular helper cells (11, 12). The magnitude of T cell help determines how long B cells reside in the DZ, providing selected cells more time to proliferate and expand in between rounds of competition in the LZ (13). Whether this mechanism alone explains how high affinity B cells are selected remains unknown.

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To explore additional mechanisms that could contribute to selection, we employed an adoptive transfer model in which antigen presentation by a subset of GC B cells can be acutely and selectively increased (11, 14, 15). B cells carrying a knock-in antigen receptor specific for the hapten 4-hydroxy-3-nitrophenylacetyl (NP) (B1–8^{hi}) were transferred into ovalbumin (OVA)-primed wild-type mice that were boosted with NP-OVA. Whereas the majority of transferred B1–8^{hi} B cells were DEC$205^{-/-}$ (~85%), a subset (~15%) of the B1–8^{hi} B cells were DEC$205^{+/+}$ (10, 16). DEC205 is an endocytic receptor expressed by GC B cells that delivers antigen to MHCII processing compartments (14). Targeting DEC205 with an antibody that is fused at its C terminus to OVA (αDEC–OVA), but not the irrelevant control antigen *Plasmodium falciparum* circumsporozoite protein (αDEC-CS) (17), increases the amount of cognate peptide-MHCII displayed on the surface of B1–8^{hi} DEC$205^{+/+}$ GC B cells, leading to their selective expansion (11–13).

To determine whether B cells receiving high levels of T cell help show a specific change in gene expression, we compared DZ cells in the G1 phase of the cell cycle from αDEC-OVA and control αDEC-CS treated GCs using a fluorescent ubiquitination-based cell cycle indicator (Fucci^{tg}) (fig. S1) (18, 19). RNA sequencing revealed that T cell-mediated selection produced a statistically significant increase in gene expression programs associated with the cell cycle, metabolism, including the metabolism of nucleotides, and genes downstream of c-Myc and the E2F transcription factors (Fig. 1A and B and fig. S2). Finding an increase in expression of c-Myc target genes is in agreement with the observation that c-Myc is induced by T cell help in the GC (20, 21). E2F transcription factors are principal drivers of the cell cycle and are activated by cyclin-dependent kinase (CDK) phosphorylation of the retinoblastoma (Rb) protein (22, 23). Consistent with this, Rb was highly phosphorylated in GC B cells receiving enhanced T cell help (Fig. 1C). E2F and c-Myc are crucial drivers of cell cycle phase transitions; moreover, their activation regulates nucleotide metabolism and controls DNA replication dynamics (23–26), suggesting that T cell help might control the cell cycle dynamics of selected GC B cells *in vivo*.

To examine cell cycle progression, mice were pulsed sequentially with the nucleoside analog 5-ethynyl-2’-deoxyuridine (EdU) followed 1 hour later by 5-bromo-2’-deoxyuridine (BrdU) and GC B cells were then stained for DNA content (Fig. 2A and fig. S3) (13). At 0.5 hours after the BrdU pulse, early S phase cells were EdU^-BrdU^+ labeled and had replicated only a small amount of their genome, making their DNA content similar to that of G1 cells (Fig. 2A and B). By contrast, mid/late-S phase cells were EdU^+BrdU^+ labeled, and post-S phase cells (EdU^+BrdU^- labeled) were either in G2/M phase or in the G1 phase of the next cell cycle (Fig. 2A and B). Under control conditions (αDEC-CS), B1–8^{hi} DEC$205^{+/+}$ and B1–8^{hi} DEC$205^{-/-}$ post-S phase GC B cells were similarly distributed between G2/M and G1, indicating equivalent rates of progression through the G2/M phases of the cell cycle (Fig. 2C). By contrast, inducing selection by αDEC–OVA resulted in rapid progression through G2/M and return to G1 (Fig. 2C). Thus, T cell-mediated selection accelerates progression through G2/M.

To examine S phase dynamics, we followed GC B cells 2.5 and 5 hours after the EdU/BrdU double-pulse described above. At 2.5 hours, EdU^-BrdU^+ cells, which were in early S phase at 0.5 hours, had progressed into mid/late-S phase as determined by DNA content (Fig. 2D and E). With only these 2 additional hours to replicate their genomes, selected GC B cells accumulated more DNA content and had therefore replicated their genomes at a faster rate than control cells obtained from αDEC-CS treated mice (Fig. 2D and fig. S4). After 5 hours, nearly half of control cells that were labeled in early S phase ($EdU^{-}BrdU^{+}$) had completed S phase and were in G2/M or G1 (Fig. 2E). Selection significantly accelerated progression through S phase, since a far greater fraction of B1–8^{hi} $DEC205^{+/+}$ GC cells targeted with αDEC– OVA completed DNA replication in 5 hours than did co-transferred B1–8^{hi} $DEC205^{-/-}$ GC cells (Fig. 2F). We conclude that T cells induce accelerated progression of selected GC B cells through the S and G2/M phases of the cell cycle.

To determine whether accelerated progression through the cell cycle is also a feature of polyclonal GC responses, we examined the response to NP in C57BL/6 mice. High affinity clones in anti-NP GCs carry a tryptophan to leucine mutation at position 33 (W33L) or a lysine to arginine mutation at position 59 (K59R) in the V_H186.2 antibody gene (27, 28). Two weeks after immunization with NP-OVA, mice were pulsed with EdU followed 30 minutes later by a large dose of BrdU to inhibit further EdU uptake (fig. S5). After 3.25 hours, EdU^{+} GC cells were separated into 2 distinct populations based on DNA content (Fig. 3A): (1) EdU^{+} cells in S/G2/M that represent a mixed population that initiated S phase at the time of EdU injection and/or progressed slowly through S/G2/M; (2) EdU^{+} cells in G1 that were close to completing S phase during the EdU injection and/or progressed rapidly through S/G2/M. If high affinity GC B cells progress through the cell cycle at an accelerated rate, then they should be enriched in the EdU^{+} G1 population. Sequencing the V_H186.2 genes of these cells revealed that EdU^{+} G1 cells were significantly enriched in high affinity clones compared to EdU^{+} S/G2/M cells (Fig. 3B and C). Thus, affinity-enhancing mutations in the polyclonal GC are associated with rapid progression through S/G2/M.

Once a GC B cell has entered the cell cycle, S phase occupies most of the time it takes for the cell to divide (Fig. 2) (13). We therefore sought to understand how T cell help accelerates progression through S phase. Control of S phase length is a well-documented phenomenon during embryonic development (29). In this context, S phase control operates at the level of initiation of DNA replication; rapid S phases are due to an increased number of synchronously fired replication origins (30–33).

To evaluate the dynamics of DNA replication initiation, we sorted and sequenced the genomes of B1–8^{hi} $DEC205^{+/+}$ GC B cells in G1 and S phase under control conditions or during their selective expansion (fig. S6A and B). In a population of S phase cells, genomic sites that replicate early have higher copy number than regions that are replicated later during S phase. Thus, by analyzing the relative copy number of DNA sequences along chromosomes in S phase cells compared to G1 phase cells, we obtained genome-wide profiles of DNA replication timing (34, 35). The relative timing of DNA replication among selected and non-selected GC B cells was essentially identical, with the same locations and activation times of origins throughout the genome (r = 0.98, Fig. 4A and B and fig. S6C). Thus, T cell help accelerates S phase by proportionally condensing the replication timing program, while maintaining the overall dynamics of DNA replication albeit in a shorter timescale.

Elevated c-myc, CDK activity and nucleotide metabolism can increase the speed of DNA replication fork progression in tissue culture cells, yet a physiological role for this mechanism has not been documented (26, 36, 37). Since T cell help produces similar molecular changes in selected GC B cells, we examined DNA replication forks by single-molecule analyses (38). For this purpose, αDEC-OVA or control αDEC-CS treated mice were pulsed with the nucleoside analog 5-iodo-2'deoxyuridine (IdU) (fig. S6D). GC B cells were then isolated by cell sorting and processed to measure the lengths of DNA replication tracks in individual molecules (Fig. 4C). Selection resulted in an increase in the average length of replication tracks and altered the distribution of replication track lengths, indicating that T cell help regulates DNA replication by increasing the speed of individual DNA replication forks (Fig. 4D and E, $p < 0.0001$).

The GC imparts protective immunity by selectively expanding high affinity B cells, yet the mechanisms whereby such clones come to dominate the GC population have been obscure (3, 4). T cells discern among GC B cells on the basis of cell surface peptide-MHCII levels and transduce signal(s) that regulate the amount of time selected cells spend proliferating in the DZ (8, 11–13). Thus, T cell help influences the choice that a recently divided GC B cell in G1 must make in the DZ: to either reenter another S phase or instead return to the LZ (13). Our results indicate that T cell-mediated selection extends beyond this mode of regulation, accelerating progression through the entire cell cycle in selected GC B cells. Thus, the regulatory effects transduced by T cells work in concert to induce selected GC B cells to spend a longer period of time in the DZ where they proliferate at a faster rate.

There are few examples of physiological regulation of cell cycle speed in eukaryotes, and, to our knowledge, none known that involve directed signaling by one cell type to another (29). High levels of replication factors and CDK activity in the early embryo synchronize initiation from a greater number of origins to increase the rate of cell division (31, 39). By contrast, during B cell selection, T cell help induces higher levels of critical regulators of the cell cycle (c-myc and CDK) and nucleotide metabolism to accelerate DNA replication elongation while preserving the temporal order and the number of initiation events throughout the genome. We conclude that selection in the GC involves dynamic regulation of genome replication in S phase, the longest phase of the committed cell cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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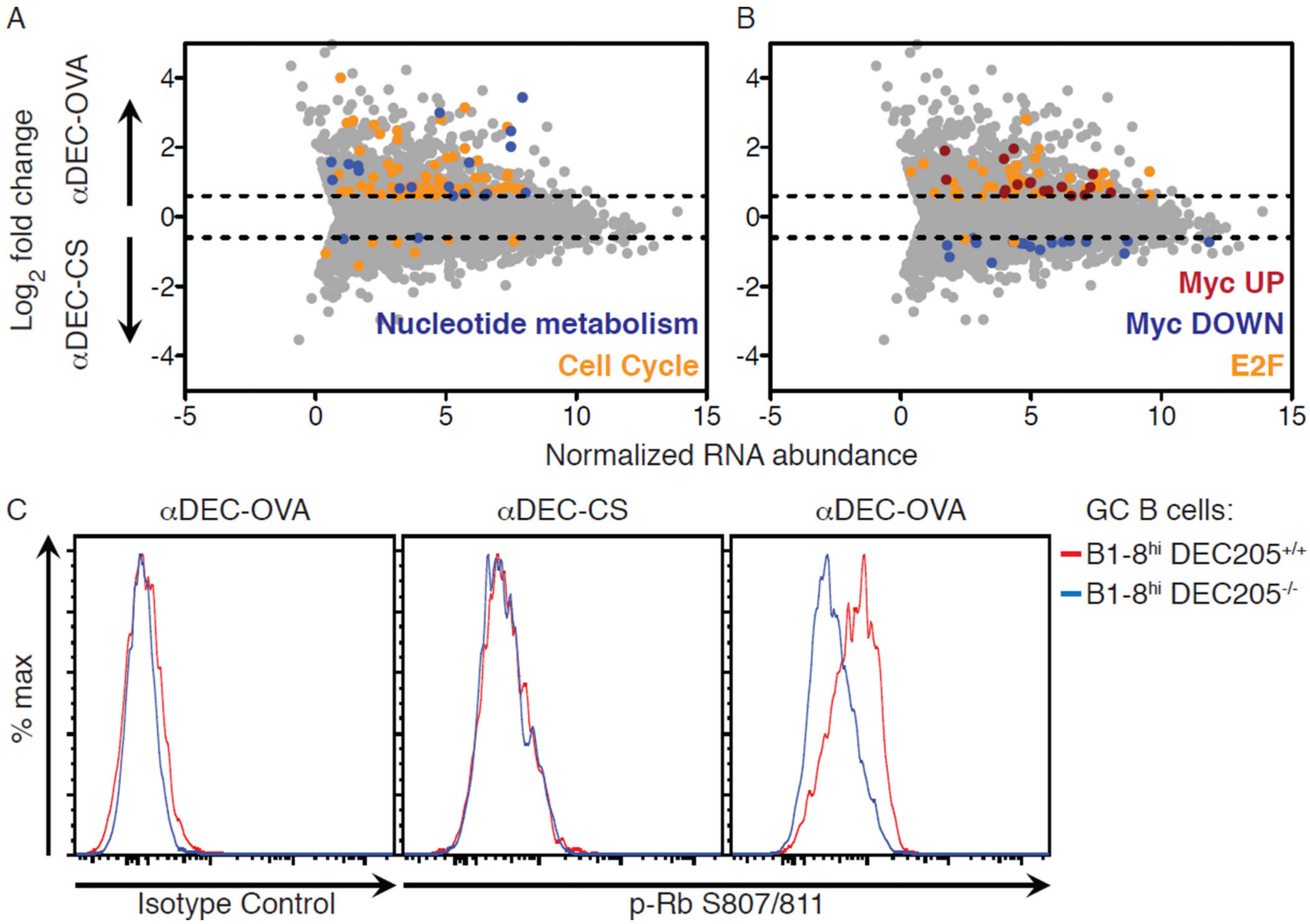

Figure 1. T cell help regulates cell cycle and metabolic gene expression programs in selected GC B cells

(A, B) RNA sequencing analysis showing genes up- or down-regulated by a fold-change of at least 0.6 (log_2) upon treatment with αDEC-OVA or αDEC-CS. For clarity, enriched gene sets according to curated reactome gene sets **(A)** and transcription factor target genes **(B)** are shown separately. **(C)** Histograms showing intracellular levels of Rb phosphorylation in B1–8^{hi} $DEC205^{+/+}$ and B1–8^{hi} $DEC205^{-/-}$ GC B cells from mice treated 2 days earlier with αDEC-OVA or αDEC-CS. Results represent two **(A, B)** or three **(C)** independent experiments with 4–5 mice per condition for each experiment.

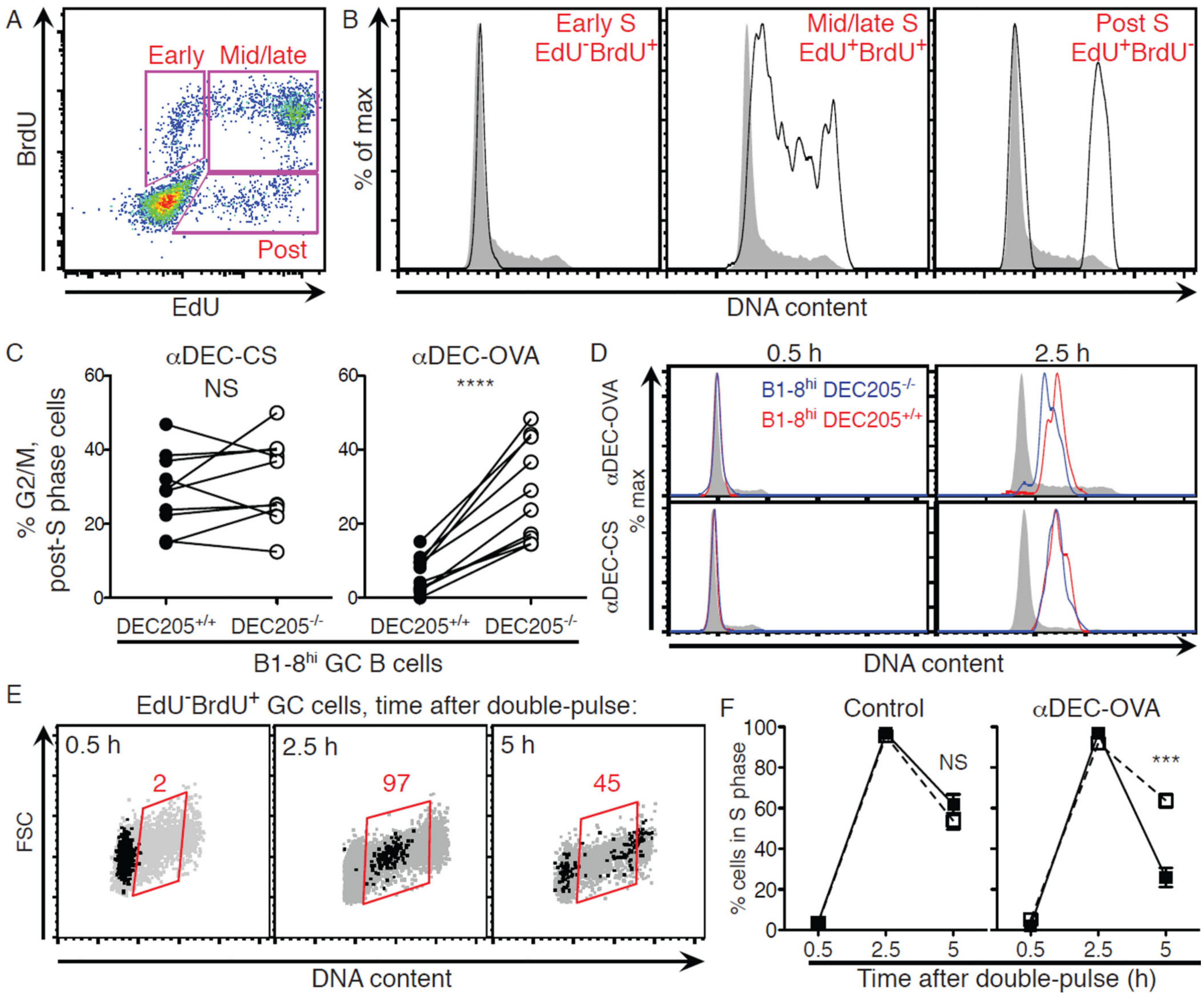

Figure 2. T cell help regulates progression through S and G2/M phases during selection

(A) Mice were injected with EdU intravenously followed 1 hour later by BrdU and then analyzed by flow cytometry 0.5 hours later. **(B)** Representative histograms displaying DNA content among all GC B cells (grey) and gated populations (black) shown in **(A). (C)** Mean fraction of cells in G2/M as determined by DNA content among $EdU^{+}BrdU^{-}$ (post-S phase) B1–8^{hi} $DEC205^{+/+}$ and B1–8^{hi} $DEC205^{-/-}$ GC B cells treated with αDEC-OVA or αDEC-CS (control) 2 days earlier. Lines connect indicated cell populations from the same animal. **(D)** Histograms showing progressive accumulation of DNA content among $EdU^{-}BrdU^{+}$ GC B cells from 0.5 to 2.5 hours after EdU/BrdU double-pulse. **(E)** Flow cytometry plots showing time course of cell cycle progression of $EdU^{-}BrdU^{+}$ cells (black dots) at 0.5, 2.5, and 5 hours following double-pulse labeling. Grey dots represent all GC B cells in the same mice. Red values represent fraction in S phase gate. **(F)** Mean fraction of GC B cells in S phase among $EdU^{-}BrdU^{+}$ B1–8^{hi} $DEC205^{+/+}$ (black squares) and B1–8^{hi} $DEC205^{-/-}$ (white squares) cells as determined by DNA content at 0.5, 2.5 and 5 hours after double-pulse labeling in mice treated 2 days earlier with αDEC-OVA or either PBS or αDEC-CS (control). Error bars = SEM. Two-tailed paired t-test was used in **(C)** and two-tailed Mann-Whitney test in **(F)**. **** $p < 0.0001$. *** $p = 0.0002$. Experiments represent 2–3 independent experiments with 7–10 mice total for each time point and condition.

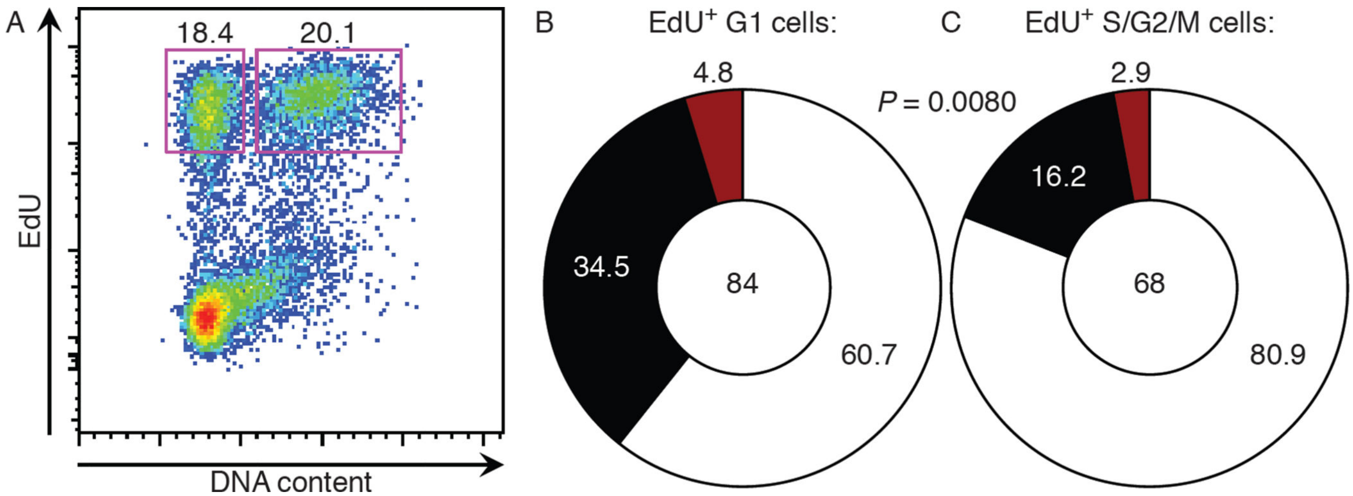

Figure 3. Affinity-enhancing mutations in polyclonal GCs are associated with accelerated S/G2/M progression

(A) WT mice were immunized with NP-OVA and 14 days later were administered EdU for 0.5 hours before BrdU. At 3.25 hours after BrdU administration, EdU^+ G1 cells and EdU^+ S/G2/M GC B cells were sorted and analyzed for affinity-enhancing mutation in VH186.2 genes. **(B, C)** Pie charts show frequency of $W33L^+$ (black) and $K59R^+$ (red) clones among VH186.2 sequences within EdU^+ G1 **(B)** and EdU^+ S/G2/M **(C)** GC B cells. One sequence was doubly positive for the W33L and K59R mutations and was counted within the black slice in **(C)**. Total number of clones analyzed is shown in center. P value was determined using Fisher's exact test. Results are pooled from 2 independent experiments with 10 mice each.

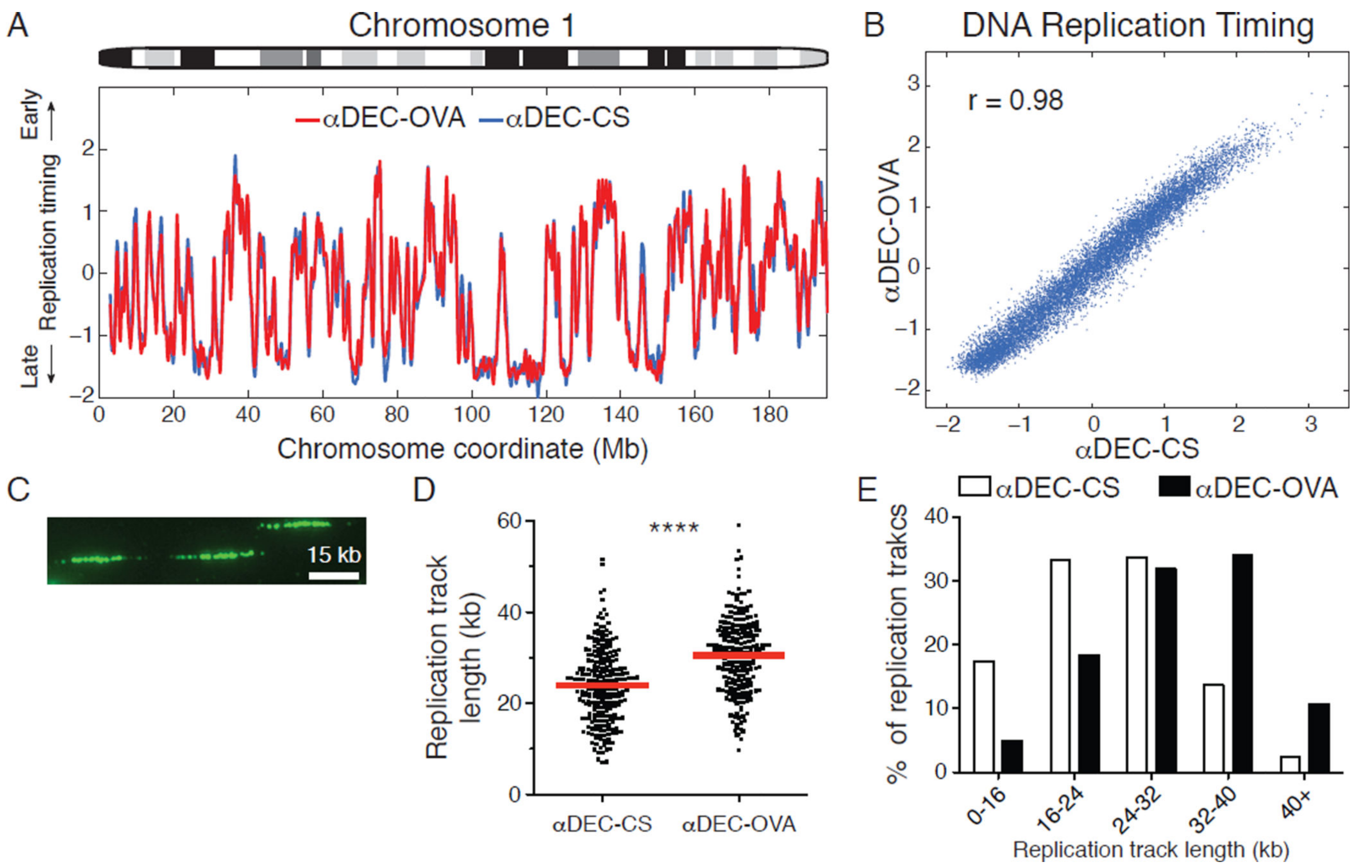

Figure 4. T cell help accelerates S phase by regulating the speed of DNA replication elongation
(A) Replication timing profile of chromosome 1 was determined by analyzing the ratio of DNA copy number between sorted S and G1 phase B1–8^{hi} DEC205$^{+/+}$ GC B cells treated with αDEC-CS or αDEC-OVA 2 days earlier. **(B)** Genome-wide correlation between replication timing in the two conditions. **(C)** Representative IdU-labeled DNA fibers are shown. **(D)** Replication track lengths from αDEC-CS or αDEC-OVA treated B1–8^{hi} DEC205$^{+/+}$ GC B cells. Each data point represents an IdU-labeled track, and red lines represent mean values. Results are pooled from 2 independent experiments and involve over 220 measured fibers in total for each condition. **(E)** Distribution of replication track lengths. **** $p < 0.0001$, Two-tailed Mann-Whitney test.