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The Ataxia Telangiectasia Mutated and Cyclin D3 Proteins Cooperate To Help Enforce TCRβ and IgH Allelic Exclusion

Natalie C. Steindl,*,†,‡,1 Megan R. Fisher,*,†,‡,1 Katherine S. Yang-Iott,*,† and Craig H. Bassing*,†,‡

Coordination of V rearrangements between loci on homologous chromosomes is critical for Ig and TCR allelic exclusion. The Ataxia Telangiectasia mutated (ATM) protein kinase promotes DNA repair and activates checkpoints to suppress aberrant Ig and TCR rearrangements. In response to RAG cleavage of Igk loci, ATM inhibits RAG expression and suppresses further V→D→Jk rearrangements to enforce Igk allelic exclusion. Because V recombination between alleles is more strictly regulated for TCRβ and IgH loci, we evaluated the ability of ATM to restrict biallelic expression and V→D→Jk recombination of TCRβ and IgH genes. We detected greater frequencies of lymphocytes with biallelic expression or aberrant V→D→Jk rearrangement of TCRβ or IgH loci in mice lacking ATM. A preassembled DJβ complex that decreases the number of TCRβ rearrangements needed for a productive TCRβ gene further increased frequencies of ATM-deficient cells with biallelic TCRβ expression. IgH and TCRβ proteins drive proliferation of prolymphocytes through cyclin D3 (CcnD3), which also inhibits VH transcription. We show that inactivation of CcnD3 leads to increased frequencies of lymphocytes with biallelic expression of IgH or TCRβ genes. We also show that CcnD3 inactivation cooperates with ATM deficiency to increase the frequencies of cells with biallelic TCRβ or IgH expression while decreasing the frequency of ATM-deficient lymphocytes with aberrant V→D→Jk recombination. Our data demonstrate that core components of the DNA damage response and cell cycle machinery cooperate to help enforce IgH and TCRβ allelic exclusion and indicate that control of V→D→Jk rearrangements between alleles is important to maintain genomic stability. The Journal of Immunology, 2014, 193: 000–000.

A rtention receptor diversity is generated through assembly of TCR and Ig genes from V, D, and J gene segments. The RAG1 and RAG2 proteins introduce DNA double-strand breaks (DSBs) adjacent to gene segments, forming hairpin-sealed coding ends and blunt signal ends (1). RAG proteins cooperate with Ataxia Telangiectasia mutated (ATM) to hold these chromosomal DNA ends in postcleavage complexes and facilitate their repair by nonhomologous end-joining factors, which form coding and signal joins (2). V(D)J coding joins form the second exons of Ig and TCR genes, which are transcribed with C region exons. The combination of joining events, imprecise processing of coding ends, and pairing of different Ig or TCR proteins cooperate to create AgR diversity.

Complete assembly of most Ig and TCR genes occurs only on one allele at a time, indicating the importance of mechanisms that control recombination between alleles (3–5). The ability of Ig and TCR chains expressed from one allele to signal feedback inhibition of V rearrangements on the other allele ensures their monoallelic expression (allelic exclusion) on most lymphocytes (3–5). Asynchronous initiation of V rearrangements between loci on homologous chromosomes is likely required for feedback inhibition to enforce allelic exclusion (3–5). In addition, the ability of V(D)J recombination events on one allele to activate signals that transiently suppress V rearrangements on the other allele has been hypothesized to be important for feedback inhibition to mediate allelic exclusion (6). Consistent with this notion, we recently showed that RAG DSBs induced during Igk recombination on one allele signal through ATM to downregulate RAG expression, inhibit further V→D→Jk rearrangements on the other allele, and enforce Igk allelic exclusion (7, 8).

Assembly and expression of TCRβ and IgH genes is more stringently controlled than Igk genes. TCRβ and IgH genes assemble through D-to-J recombination and then rearrangement of V segments to assembled DJ complexes on one allele at a time (9, 10). TCRβ and IgH D-to-J recombination are not controlled by feedback inhibition, whereas VB and VH rearrangements are controlled by feedback inhibition (9, 10). In one-third of prolymphocytes, assembly and expression of in-frame TCRβ or IgH genes on the first allele generate preprereceptor complexes that signal feedback inhibition of V-to-DJ rearrangements on the other allele (9, 10). These preprereceptors also signal activation of cyclin D3 (CcnD3) protein expression to drive proliferation as cells differentiate into prolymphocytes (11–13). The two-thirds of prolymphocytes that assemble out-of-frame TCRβ or IgH genes can...
initiate V-to-DJ rearrangements on the other allele in a second attempt to assemble an in-frame VDJ rearrangement required for differentiation. As a result, ~60% of cells assemble VDJ rearrangements on one allele, and ~40% assemble VDJ rearrangements on both alleles, with one of these out-of-frame in most cells (9, 10). This limits biallelic surface expression of TCRβ-chains to ~1% of mature αβ T cells and of IgH chains to ~0.01% of mature B cells (14–17). In pre-B cells, Igk genes assemble through Vκ-to-Jκ recombination on one allele at a time (18–20). Assembly of functional Igk genes in pre-B cells can generate innocuous BCRs that suppress additional Vκ-to-Jκ rearrangements and promote differentiation (19, 20). However, most BCRs are autoreactive and induce further Igk rearrangements, which occur on either allele (19–21). Therefore, ~10% of pre-B cells assemble in-frame VκJκ rearrangements on both alleles (21). Yet, this results in equal high-level expression of Igk-chains from both alleles on only ~3% of B cells because of inability of one Igk-chain to pair with the available Igk chain in many cells (21, 22).

Considering distinct features and differential regulation of V rearrangements between Igk loci and IgH/TRCf loci, it is important to determine the ability of ATM to coordinate V-to-DJ recombination between alleles and enforce allelic exclusion of IgH and TCRβ genes. We previously demonstrated that Atm−/− pro-B cells exhibit an increased frequency of γ-H2AX foci (a marker for DSBs) on both alleles (7). These data could result from loss of ATM signals that control initiation of IgH recombination between alleles or impaired DSB repair leading to V(D)J recombination on the second allele before transduction of IgH feedback signals from the first allele. Although we did not observe a profound violation of IgH allelic exclusion on mature B cells of Atm−/− mice (7), we neither determined whether the increased frequency of B cells expressing IgH chains from both alleles was significant nor considered the impact of aberrant IgH recombination on biallelic IgH expression. In this study, we monitor allele-specific TCRβ and IgH expression on and visualize TCRβ and IgH rearrangements in mature lymphocytes from Atm−/− and wild-type (WT) mice. We show that ATM helps enforce TCRβ and IgH allelic exclusion by inhibiting biallelic V-to-DJ recombination. We demonstrate that Ccn3 also helps enforce TCRβ and IgH allelic exclusion alone and in cooperation with ATM. Finally, we show that decreasing aberrant V and IgH allelic exclusion alone and in cooperation with ATM.

Materials and Methods

Mice

All mice were on a 129/C57B6 mixed background and bred and housed under specific pathogen-free conditions at the Children’s Hospital of Philadelphia (CHOP). None of the Atm−/− mice analyzed in this study showed evidence of a subclinical but emerging thymic lymphoma, as assayed Southern blotting or PCR for oligoclonal TCRβ rearrangements or by flow cytometry for increased number/frequency of TCRβ+CD4+CD8+ or TCRβ+CD8− thymocytes. All animal husbandry and experiments were performed in accordance with national guidelines and regulations and were approved by the CHOP Institutional Animal Care and Use Committee.

Preparation of single-cell suspension for flow cytometry

Single-cell suspensions for flow cytometry were isolated from the thymus, bone marrow, and spleens of 6-wk-old mice. Cells were harvested and stained in PBS containing 3% FCS and 0.25 mM EDTA. Prior to staining, suspensions were depleted of RBCs with NH4Cl lysis buffer, and FCRs were blocked using anti-CD16/CD32 (2.4G2; BD Pharmingen). Data were collected on an LSR II and analyzed with FlowJo. Single live cells were gated on the basis of forward and side scatter and DAPI exclusion (Invitrogen).

Flow cytometric analysis of Vβ surface expression

Stains were conducted using the following Abs or reagents from BD Biosciences: APC/Cy7-anti-mouse B220 (RA3-6B2), APC-anti-mouse TCRβ (H57-597), FITC-anti-mouse Vβ5 (MR9-4), FITC-anti-mouse Vβ14 (14-2), PE-anti-mouse Vβ8 (F23.1), PE-anti-mouse Vβ10b (B21.5), biotin-anti-mouse Vβ4 (KT4), biotin-anti-mouse Vβ8 (RR4-7), and PE/Cy7-streptavidin. Surface Vβ expression was assayed on single, DAPI−, B220−, and TCRβ+ cells.

Flow cytometric analysis of IgM surface expression

Stains were conducted using the following Abs or reagents: FITC-anti-mouse IgM (DS-1; BD Biosciences), PE-anti-mouse IgM (AF6-78; BD Biosciences), Biotin-anti-mouse CD23 (B3B4; BD Biosciences), PerCP/Cy5.5-anti-mouse CD21/35 (7E9; BioLegend), and PE/Cy7-BA (BD Biosciences). Surface IgM expression was assayed on single, DAPI−, TCRβ+ and B220+ cells.

Stimulation of αβ T cells for generation of hybridomas and for two-color fluorescence in situ hybridization assays

Single-cell suspensions were isolated from the spleens of 6-wk-old mice and depleted of RBCs with NH4Cl lysis buffer prior to stimulation. Each spleen was stimulated for 48 h in 40 μl of DMEM containing 15% FBS, 1% penicillin/streptomycin, and 1% RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin, 1% l-glutamate, and 30 μM 2-ME. Additional medium was added to the stimulation after 24 h.

Fusion and analysis of αβ T cell hybridomas

Hybridomas were produced by fusion of Con A/L-2–stimulated splenic αβ T cells with BW-110.129.237 thymoma cells. Southern analysis of TCRβ rearrangements was performed as described previously (23, 24).

Stimulation of splenic B cells for two-color fluorescence in situ hybridization

Single-cell suspensions isolated from the spleens of 6-wk-old mice were depleted of RBCs with NH4Cl lysis buffer prior to stimulation. Spleen cells were stimulated for 48 h in 1 μl of 10% CPG ODN1826 at 0.5 × 107 cell/ml in RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin, 1% l-glutamate, 1% nonessential amino acids, 30 μM 2-ME, 1% Hepes, and 1% oxaloacetate, pyruvate, and insulin.

Two-color fluorescence in situ hybridization assays

B and T cells stimulated for 48 h were arrested in metaphase by incubating with 0.0 μg/ml colcemid (KaryoMax) and 0.45 mM BrdU (Sigma-Aldrich) for 2 h. Metaphase-arrested cells were isolated by hypotonic treatment (40 mM KCl, 0.5 mM EDTA, and 20 mM HEPES [pH 7.4]) and fixation in methanol/acetic acid (3:1 volume). The fixed cells were then dropped on slides at 4°C and dried at 75°C for 5 min. Metaphase spreads were hybridized overnight with relevant Tcrb and Igh bacterial artificial chromosome probes: Vβ-DJβ1, RP23-203H5; Cβ, 164G11; Vh-Dh, RP24-275L15; and 3’Igh, C77-199M11. Cβ and 3’Igh probes were labeled using the DIG-NICK Translation Mix (Roche). Vβ-DJβ1 and Vh-Dh probes were labeled using the BioPrime DNA Labeling System (Invitrogen). Probes were detected using FITC-anti-digoxin Fab (Roche) and Texas Red-streptavidin (Vector Laboratories). Coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Images were captured and analyzed using Case Data Manager (Applied Spectral Imaging).

Statistical analyses

All p values were generated by two-tailed Student t test using Prism (GraphPad Software).

Results

ATM inhibits biallelic expression and recombination of Vβ segments and suppresses aberrant Vβ-to-DJβ rearrangements

To determine the effect of ATM on TCRβ allelic exclusion, we used flow cytometry to quantify the percentages of αβ T cells
from Atm−/− or WT mice that express cell surface TCRβ-chains from both alleles. Because an allotypic marker has not been found or generated for mouse TCRβ-chains, only anti-Vβ Abs can identify expression of TCRβ-chains from both alleles on mouse αβ T cells. However, because of the absence of anti-Vβ Abs for all mouse Vβ peptides, this underestimates the actual frequency of biallelic TCRβ expression. We used 14 distinct combinations of available anti-Vβ Abs to monitor TCRβ expression from both alleles on αβ T cells isolated from the thymi or spleens of age-matched littermate Atm−/− or WT mice. We conducted this cellular analysis on TCRβhigh thymocytes and on TCRβ+ splenocytes. As compared with WT mice, we detected significantly higher percentages of Vβ14+Vβ8+ (p = 0.0019) and Vβ14+Vβ6+ (p = 0.0197) thymic αβ T cells and of Vβ8+Vβ12+ (p = 0.0497) and Vβ8+Vβ6+ (p = 0.0077) splenic αβ T cells from Atm−/− mice (Fig. 1A, 1B). The frequencies of these αβ T cells that express TCRβ-chains from both alleles were 1.3- to 1.6-fold higher in Atm−/− mice relative to WT mice (Fig. 1B). We also observed higher frequencies of αβ T cells expressing two different Vβ peptides for most other combinations of anti-Vβ Abs, although none of these differences reached significance with the numbers of mice analyzed (Fig. 1B). These data suggest that ATM helps enforce TCRβ allelic exclusion.

In addition to asynchronous initiation and feedback inhibition of Vβ-to-DJβ rearrangements, posttranscriptional silencing of in-frame TCRβ genes controls TCRβ allelic exclusion (25). Therefore, to determine whether ATM helps enforce TCRβ allelic exclusion by limiting the frequency of mature αβ T cells with biallelic Vβ-to-DJβ recombinination, we analyzed TCRβ rearrangements in a panel of αβ T cell hybridomas that we made from Atm−/− mice. The TCRβ locus consists of 34 upstream Vβ segments, 2 DJβ-Cβ clusters, and the downstream Vβ14 segment (Fig. 1C). All TCRβ rearrangements delete intervening sequences, except for Vβ14-to-DJββ rearrangements that occur through inversion. To analyze TCRβ rearrangements, we conducted Southern blots on EcoRI-digested hybridoma DNA using 3′Jβ2, 3′Jβ1, and 5′Dβ1 probes (Fig. 1C). Hybridization of 3′Jβ2 and 3′Jβ1 probes to nongermline fragments identifies alleles with DJββ and/or VβDββ rearrangements. Hybridization of the 5′Dβ1 probe to nongermline fragments identifies alleles with DJββ or Vβ14DJββ rearrangements, while lack of 5′Dβ1 probe hybridization reveals alleles with VβDββ rearrangements involving upstream Vβs. By this approach, we identified 76 hybridomas with VβDββ rearrangements on both alleles, and 70 hybridomas with VβDββ rearrangements on one allele and DJββ rearrangements on the other allele (Fig. 1D). At first approximation, these data suggest that 48% of Atm−/− αβ T cells contains VβDββ rearrangements on one allele and 52% contains VβDJββ rearrangements on both alleles, consistent with a role for ATM in coordinating Vβ recombination between alleles. However, we also identified 44 hybridomas with VβDJββ rearrangements on one allele, but no 3′Jβ2, 3′Jβ1, or 5′Dβ1 probe hybridization on the other allele, indicative of aberrant TCRβ rearrangements on nonselected alleles in these cells (Fig. 1D). Because ATM prevents RAG DSBs from aberrantly resolving as small chromosomal deletions (26), both DJβ-to-Jβ and Vβ-to-DJββ recombinination in Atm−/− mice could lead to loss of sequences to which the 3′Jβ2, 3′Jβ1, and 5′Dβ1 probes hybridize. Accordingly, our Southern analysis of TCRβ rearrangements in Atm−/− αβ T cell hybridomas prevents any conclusion regarding whether ATM suppresses the frequency of mature αβ T cells with biallelic Vβ rearrangements.

To determine whether ATM inhibits biallelic Vβ rearrangements, we needed approaches to isolate the Vβ-to-DJββ recombinination step and capture aberrant Vβ rearrangements that delete 3′DJβ sequences. We previously created and characterized Jb4poly.

![FIGURE 1. ATM helps control TCRβ allelic exclusion and TCRβ recombinination. (A) Representative flow cytometry analysis of Vβ14 and Vβ8 expression on the surface of TCRβ+ thymocytes or splenocytes isolated from WT or Atm−/− mice. The percentages of Vβ14+Vβ8+ αβ T cells in the upper right quadrant are shown. (B) Graphs depicting the average frequencies of TCRβ+Vβ8+ thymocytes or splenocytes isolated from WT or Atm−/− mice that express each indicated combination of surface Vβ-chains. Data are from four independent experiments conducted on a total of 8 WT and 10 Atm−/− littermate mice. Error bars indicate the SEM. (C) Diagram of the TCRβ locus illustrating the relative positions of the upstream Vβ segments, the two DJβ-Cβ clusters, and the downstream Vβ14 segment. Locations of the EcoRI sites and 5′Dβ1, 3′Jβ1, and 3′Jβ2 probes used for Southern blot analysis of TCRβ rearrangements are indicated. (D) Table depicting the frequencies of Atm−/− αβ T cell hybridomas with a normal VβDββ rearrangement on one (VDJ/DJ) or both (VDJ/VDJ) alleles or with a normal VβDββ rearrangement on one allele and an aberrant Tcrb rearrangement on the other allele (VDJ/Ab).](http://www.jimmunol.org/)

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mice with TCRβ alleles that contain a preassembled DJβJβ1 complex, lack the DJβ2-Jβ2 cluster, and are only capable of Vβ recombination (24). Because αβ T cells from Jb1DDDJ mice exhibit normal frequencies of biallelic TCRβ expression and VβDJβJβ rearrangements (24), we generated and analyzed Atm<sup>−/−</sup>Jb1DDDJ mice to isolate the Vβ-to-DJβJβ recombination step. We used the same 14 combinations of available anti-Vβ Abs to monitor TCRβ expression from both alleles on TCRβ<sub>αβ</sub> thymocytes or splenocytes isolated from age-matched littermate Atm<sup>+/+Jb1DJ</sup> mice. As compared with Atm<sup>+/+Jb1DJ</sup> mice, we detected 1.6- to 2.6-fold higher percentages of Vβ14<sup>+</sup>Vβ8<sup>+</sup> (p = 0.0008), Vβ5<sup>+</sup>Vβ12<sup>+</sup> (p = 0.0007), Vβ14<sup>+</sup>Vβ6<sup>+</sup> (p = 0.0067), and Vβ5<sup>+</sup>Vβ6<sup>+</sup> (p = 0.004) thymic αβ T cells and of Vβ14<sup>+</sup>Vβ6<sup>+</sup> (p = 0.0001), Vβ5<sup>+</sup>Vβ12<sup>+</sup> (p = 0.0133), and Vβ5<sup>+</sup>Vβ6<sup>+</sup> (p = 0.0016) splenic αβ T cells from Atm<sup>−/−Jb1DDDJ</sup> mice (Fig. 2A, 2B). We also observed higher frequencies of cells expressing two different Vβ peptides for most other combinations of anti-Vβ Abs, with many of these differences significant (Fig. 2B). These data indicate that preventing aberrant DJβ-to-Jβ recombination increases the frequency of mature Atm<sup>−/−</sup> αβ T cells that express TCRβ-chains from both alleles.

To capture aberrant Vβ-to-DJβJβ rearrangements that lead to deletion of 3′DJβJβ sequences, we developed a two-color fluorescence in situ hybridization (2C-FISH) approach to quantify Vβ-to-DJβJβ recombination in age-matched littermate Atm<sup>−/−Jb1DDDJ</sup> and Atm<sup>+/+Jb1DDDJ</sup> αβ T cells. We conducted 2C-FISH on metaphases prepared from ex vivo stimulated splenic αβ T cells using probes that hybridize to sequences between the upstream Vβ segments and DJβ1 (Vβ-DJβ1 probe) or downstream of the preassembled DJβJβ complex (Cβ probe) (Fig. 2C). Cohybridization of both probes identifies alleles with no VβDJβJβ rearrangements or those involving Vβ14 that occur in ∼5% of αβ T cells (23). Hybridization of only the Cβ probe identifies alleles with VβDJβJβ rearrangements. The Cβ probe can hybridize to alleles with deletion of 3′DJβJβ sequences to which the 3′Jβ1 and 3′Jβ2 Southern probes cannot hybridize and therefore scores aberrant Vβ-to-DJβJβ rearrangements that could not be captured in our Southern analysis of hybridomas. Hybridization of both probes on different chromosomes identifies TCRβ translocations that also could not be captured in our hybridoma analysis. We detected a probe hybridization pattern indicative of VβDJβJβ rearrangements on both alleles in a significantly greater percent-

**FIGURE 2.** ATM enforces TCRβ allelic exclusion, inhibits biallelic Vβ-to-DJβJβ recombination, and suppresses aberrant Vβ rearrangements. (A) Representative flow cytometry analysis of Vβ14 and Vβ8 expression on the surface of TCRβ<sup>αβ</sup> thymocytes or splenocytes isolated from Atm<sup>+/+Jb1DDDJ</sup> or Atm<sup>−/−Jb1DDDJ</sup> mice. The percentages of Vβ14<sup>+</sup>Vβ8<sup>+</sup> αβ T cells are shown in the upper right quadrant. (B) Graphs depicting the average frequencies of TCRβ<sup>αβ</sup> thymocytes or splenocytes isolated from Atm<sup>+/+Jb1DDDJ</sup> or Atm<sup>−/−Jb1DDDJ</sup> mice that express each indicated combination of surface Vβ chains. Data are from two independent experiments conducted on a total of six Atm<sup>+/+Jb1DJ</sup> mice. (C) Diagram of the Jb1DJ locus illustrating the relative positions of upstream Vβ segments, the two DJβ-Jβ-Cβ clusters, and the downstream Vβ14 segment. Locations of the Vβ-DJβ1 and Cβ probes used for 2C-FISH analysis of Vβ rearrangements are indicated. (D) Representative 2C-FISH images showing the metaphase chromosome probe hybridization patterns that identify Atm<sup>+/+Jb1DDDJ</sup> or Atm<sup>−/−Jb1DDDJ</sup> αβ T cells with a normal VβDJβJβ rearrangement on one (VDJ/DJ) or both (VDJ/VDJ) alleles or with a normal VβDJβJβ rearrangement on one allele and an aberrant TCRβ rearrangement on the other allele (VDJ/Ab). (E and F) Graphs depicting the average frequencies of Atm<sup>+/+Jb1DDDJ</sup> and Atm<sup>−/−Jb1DDDJ</sup> splenic αβ T cells with TCRβ alleles of the VDJ/DJ, VDJ/VDJ, or VDJ/Ab configurations (E) or the frequencies of the types aberrant TCRβ rearrangements in Atm<sup>+/+Jb1DDDJ</sup> and Atm<sup>−/−Jb1DDDJ</sup> splenic αβ T cells (F). Data are from 388 Atm<sup>+/+Jb1DDDJ</sup> and 234 Atm<sup>−/−Jb1DDDJ</sup> metaphase analyzed among four independent experiments.
age of Atm−/−Jb1+/- cells as compared with Atm+/+Jb1+/- cells (45.0 ± 3.6 versus 35.3 ± 2.1%, p = 0.0372) (Fig. 2D, 2E). Consistent with this observation, we also detected a probe hybridization pattern indicative of V/DJ recombination on only one allele in a smaller fraction of Atm−/−Jb1+/- cells relative to Atm+/+Jb1+/- cells (52.2 ± 2.7 versus 64.5 ± 2.0%, p = 0.013) (Fig. 2D, 2E). These frequencies of V/DJ B rearrangements are underestimated because our assay cannot capture the ∼5% of alleles containing preassembled DHJH complexes are unavailable, addition, we observed probe hybridization pattern indicative of a normal V/DJ recombination on one allele and an aberrant IgH recombination (mostly Cβ deletions) on the other allele in a greater percentage of Atm−/− cells as compared with WT cells (17.2 ± 2.2 versus 1.9 ± 0.5%, p = 0.0005) (Fig. 3D–F). These data are consistent with the notion that ATM helps enforce IgH allele exclusion by limiting the frequency of mature B cells with V/DJ rearrangements on both alleles. However, because D4to-DJH and class-switch recombination can cause aberrant IgH rearrangements detected by FISH, and because mice with IgH alleles containing preassembled DHJH complexes are unavailable, these data prevent conclusions about whether ATM suppresses the frequency of B cells with biallelic V/DJ-JH rearrangement.

**ATM cooperates with Ccnd3 to limit biallelic IgH expression and V/DJ-JH rearrangements**

To determine whether ATM inhibits the frequency of B cells with biallelic VH rearrangements, we sought to develop an approach to decrease the frequency of aberrant V/DJ recombination events that result in IgH translocations or Cβ deletions. IgH expression in pro-B cells induces expression of Ccnd3 to drive G1 progression and S phase entry (12). Because ATM suppresses aberrant V/DJ recombination in part by preventing cells with RAG DSBs from progressing into S phase (31, 32), we reasoned that inactivation of Ccnd3 would suppress translocations and Cβ deletions in Atm−/− pro-B cells by enabling time to complete D4to-DJH and V/DJ-JH rearrangements before S phase entry. Therefore, we created Ccnd3−/-Ighko and Ccnd3−/-Ighko mice and analyzed IgH expression in splenic B cells of these and Ighko and Atm−/−Ighko mice. We observed equivalent high-level expression of IgMc and IgMr on 1.34 ± 0.04% of splenic B cells from Ccnd3−/-Ighko mice and on 2.18 ± 0.27% of splenic B cells from age-matched littermate Ccnd3−/-Ighko mice (Fig. 4A, 4B). The frequencies of B cells expressing high levels of surface IgMc and IgMr were ~1.6-fold greater in Ccnd3−/-Ighko mice relative to littermate Ccnd3−/-Ighko mice (p = 0.0222) and ~2.2-fold greater as compared with age-matched Atm−/−Ighko mice (p = 0.0005) (Fig. 4B). In addition, the frequency of splenic B cells expressing both IgMc and IgMr was ~2-fold higher in Ccnd3−/-Ighko mice as compared with age-matched Ighko mice (p = 0.0001) and ~3.3-fold higher in Ccnd3−/-Ighko mice relative to age-matched Ighko mice (p = 0.0005) (Fig. 4B). Collectively, these data show that Ccnd3 helps enforce IgH allelic exclusion alone and in cooperation with ATM.

To determine whether Ccnd3 limits biallelic IgH expression by suppressing the frequency of B cells with V/DJ-JH rearrangements on both alleles, we used 2C-FISH with V/DJ and 3’IgH probes (Fig. 3C) to quantify V/DJ-JH rearrangement in splenic B cells of age-matched littermate Atm−/− and WT mice. We detected probe hybridization pattern indicative of a V/DJ-JH rearrangement on one allele and a DJH-JH rearrangement on the other allele in a smaller fraction of Atm−/− cells relative to WT cells (43.3 ± 2.4 versus 60.8 ± 4.4%, p = 0.0125) (Fig. 3D, 3E). We observed probe hybridization pattern indicative of biallelic V/DJ-JH rearrangements in similar percentage of Atm−/− cells as compared with WT cells (39.5 ± 3.4 versus 37.3 ± 4.3%, p = 0.6923) (Fig. 3D, 3E). In addition, we observed probe hybridization pattern indicative of a normal V/DJ-JH rearrangement on one allele and an aberrant IgH rearrangement (mostly Cβ deletions) on the other allele in a greater percentage of Atm−/− cells as compared with WT cells (17.2 ± 2.2 versus 1.9 ± 0.5%, p = 0.0005) (Fig. 3D–F). These data are consistent with the notion that ATM helps enforce IgH allele exclusion by limiting the frequency of mature B cells with V/DJ-JH rearrangements on both alleles. However, because DJH-JH and class-switch recombination can cause aberrant IgH rearrangements detected by FISH, and because mice with IgH alleles containing preassembled DHJH complexes are unavailable, these data prevent conclusions about whether ATM suppresses the frequency of B cells with biallelic V/DJ-JH rearrangement.
FIGURE 3. ATM helps enforce IgH allelic exclusion, inhibit biallelic V_{H1}-to-D_{H1}H_{H} recombination, and suppress aberrant V_{H} rearrangements. (A) Representative flow cytometry analysis of IgM^{a} and IgM^{b} expression on the surface of B220^{+}TCR^{b} bone marrow cells or splenocytes isolated from Atm^{-/-}Igh^{ab} or Atm^{-/-}Igh^{bb} mice. The circle gates capture B cells expressing equivalent high levels of both IgM^{a} and IgM^{b} on their surface. The percentages of cells in these gates are indicated. The position of these gates was determined from a 1:1 mix of Ig^{ab} and Ig^{bb} stained cells as shown. (B) Graphs depicting the average frequencies of B cells in the bone marrow or spleens of Atm^{+/+}Igh^{ab} or Atm^{-/-}Igh^{bb} mice that express both IgM^{a} and IgM^{b} on their surface. Data are from three independent experiments conducted on a total of six metaphases with a normal VHDHJH rearrangement on one allele and an aberrant VHDHJH rearrangement (all being C_{H} deletions) on the other allele (VDJ/DJ) allelic configurations (E) or the frequencies of the indicated types aberrant IgH rearrangements in Atm^{+/+} and Atm^{-/-} splenic B cells (F). Data are from 410 Atm^{+/+} and 410 Atm^{-/-} metaphases analyzed among four independent experiments.

aberrant IgH rearrangement (all being C_{H} deletions) on the other allele in 8.4 ± 0.7% of cells (Fig. 4C–E). The frequency of metaphases with V_{H}D_{H}J_{H} rearrangements on both alleles was significantly higher in Ccnd3^{−/−}/Atm^{−/−} cells as compared with Ccnd3^{−/−} (p = 0.0184), Atm^{−/−} (p = 0.0396), and WT (p = 0.0413) cells (Fig. 4C). This was associated with a significantly lower percentage of cells as compared with Atm^{−/−} and Ccnd3^{−/−} B cells when hybridized with biotin-labeled DNA probes and imaged with anti-biotin Abs conjugated to Texas Red or FITC. Original magnification ×40. (E and F) Graphs depicting the average frequencies of Atm^{+/+} and Atm^{-/-} splenic B cells with IgH alleles of the VDJ/DJ, VDJ/VDJ, or VDJ/Ab configurations (E) or the frequencies of the indicated types aberrant IgH rearrangements in Atm^{+/+} and Atm^{-/-} splenic B cells (F). Data from 410 Atm^{+/+} and 410 Atm^{-/-} metaphases analyzed among four independent experiments.

Ccnd3 also cooperates with ATM to limit biallelic TCRβ expression

To determine whether Ccnd3 also cooperates with ATM to limit biallelic TCRβ expression, we analyzed allele-specific TCRβ expression on splenic αβ T cells of Ccnd3^{−/−} and Ccnd3^{−/−}/Atm^{−/−} mice. As compared with Ccnd3^{−/−} mice, we detected 1.7- to 2.2-fold significantly higher percentages of VB14^{+}/VB8^{+} (p = 0.0236), VB8^{+}/VB12^{+} (p = 0.0238), VB14^{+}/VB6^{+} (p = 0.0118), VB5^{+}/VB6^{+} (p = 0.0344), VB14^{+}/VB12^{+} (p = 0.0301), VB8^{+}/VB6^{+} (p = 0.0103), VB14^{+}/VB4^{+} (p = 0.0456), and VB5^{+}/VB4^{+} (p = 0.0001) cells in Ccnd3^{−/−}/Atm^{−/−} mice (Fig. 5). Relative to WT mice, we found 1.8- to 2.9-fold significantly higher frequencies of VB14^{+}/VB6^{+} (p = 0.008), VB5^{+}/VB6^{+} (p = 0.0035), VB14^{+}/VB12^{+} (p = 0.0298), VB5^{+}/VB4^{+} (p = 0.0001), and VB10^{+}/VB4^{+} (p = 0.0166) cells in Ccnd3^{−/−}/Atm^{−/−} mice (Fig. 5). These data demonstrate that Ccnd3 cooperates with ATM to help enforce TCRβ allelic exclusion.

Discussion

In this study, we have used flow cytometry to monitor allele-specific TCRβ or IgH expression and FISH to quantify biallelic...
VDJ rearrangements in WT and ATM-deficient mice. Our analyses of TCRβ and IgH expression and rearrangements in Atm<sup>2/2</sup> and WT mice demonstrate that ATM helps enforce TCRβ and IgH allelic exclusion by inhibiting the frequencies of mature T and B lymphocytes with biallelic VDJ rearrangements. Although we detected elevated intracellular expression of TCRβ and IgH allelic exclusion by inhibiting the frequencies of mature T and B lymphocytes with biallelic VDJ rearrangements. Although we detected elevated intracellular expression of TCRβ and IgH Allelic exclusion, inhibit biallelic V<sub>H</sub>-to-D<sub>H</sub>-J<sub>H</sub> recombination, and suppress aberrant V<sub>H</sub> rearrangements. 

**FIGURE 5.** ATM and Ccnd3 cooperate to enforce TCRβ allelic exclusion. (A) Representative flow cytometry analysis of Vβ14 and Vβ8 expression on the surface of splenic αβ T cells isolated from Ccnd3<sup>2/2</sup> or Ccnd3<sup>2/2</sup>Atm<sup>2/2</sup> mice. Our analyses of TCRβ and IgH expression and rearrangements in Atm<sup>2/2</sup> and WT mice demonstrate that ATM helps enforce TCRβ and IgH allelic exclusion by inhibiting the frequencies of mature T and B lymphocytes with biallelic VDJ rearrangements. Although we detected elevated intracellular expression of TCRβ and IgH allelic exclusion, inhibit biallelic V<sub>H</sub>-to-D<sub>H</sub>-J<sub>H</sub> recombination, and suppress aberrant V<sub>H</sub> rearrangements. 

**FIGURE 4.** ATM and Ccnd3 cooperate to help enforce IgH allelic exclusion, inhibit biallelic V<sub>H</sub>-to-D<sub>H</sub>-J<sub>H</sub> recombination, and suppress aberrant V<sub>H</sub> rearrangements. (A) Representative flow cytometry analysis of IgM<sup>+</sup> and IgM<sup>+</sup> expression on B220<sup>+</sup>TCRβ<sup>+</sup> splenocytes isolated from Ccnd3<sup>2/2</sup>Igh<sup>α/β</sup> or Ccnd3<sup>2/2</sup>Atm<sup>2/2</sup>Igh<sup>α/β</sup> mice. The circle gates capture splenic B cells expressing equivalent high levels of both IgM<sup>+</sup> and IgM<sup>+</sup> on their surface. The percentages of cells in these gates are indicated. (B) Graphs depicting the average frequencies of B cells in the spleens of Atm<sup>+/+</sup>Igh<sup>α/β</sup>, Atm<sup>2/2</sup>Igh<sup>α/β</sup>, Ccnd3<sup>2/2</sup>Igh<sup>α/β</sup>, or Ccnd3<sup>2/2</sup>Atm<sup>2/2</sup>Igh<sup>α/β</sup> mice that express both IgM<sup>+</sup> and IgM<sup>+</sup> on their surface. The data for Atm<sup>+/+</sup>Igh<sup>α/β</sup> and Atm<sup>2/2</sup>Igh<sup>α/β</sup> mice is the same from Fig. 3. Data are from three independent experiments conducted on a total of six Atm<sup>+/+</sup>Igh<sup>α/β</sup> and eight Atm<sup>2/2</sup>Igh<sup>α/β</sup> littermate mice. Error bars indicate SEM. (C–E) Graphs depicting the average frequencies of Atm<sup>+/+</sup>, Atm<sup>2/2</sup>, Ccnd3<sup>2/2</sup>, or Ccnd3<sup>2/2</sup>Atm<sup>2/2</sup> splenic B cells with IgH alleles of the VDJ/DJ, VDJ/VDJ, or VDJ/Ab configurations (C and D) or the frequencies of the types of aberrant IgH rearrangements in Ccnd3<sup>2/2</sup> or Ccnd3<sup>2/2</sup>Atm<sup>2/2</sup> splenic B cells (E). Data for Atm<sup>+/+</sup>Igh<sup>α/β</sup> and Atm<sup>2/2</sup>Igh<sup>α/β</sup> mice are from Fig. 3. Data are from 404 Ccnd3<sup>2/2</sup> and 309 Ccnd3<sup>2/2</sup>Atm<sup>2/2</sup> metaphases analyzed among three independent experiments. Error bars indicate SEM. Error bars are not displayed in (C) but are in (D) because the same data are shown in both (C) and (D).
chains from both alleles in the prelymphocyte population of Atm-deficient mice as compared with Atm-deficient mice, we could not detect intracellular TCRβ or IgH allelic inclusion in prolymphocytes from either strain (Supplemental Fig. 1; data not shown), likely because of the small numbers of prolymphocytes and the insensitivity of intracellular staining. Alternatively, the joining of persistent RAG DSBs during pro- to prelymphocyte differentiation could be a major means by which dual-Vβ/IgH-expressing lymphocytes are generated. In this regard, such persistent RAG DSBs are observed at a low level in W7 mice and at a higher level in Atm<sup>-/-</sup> mice (32, 33). Asynchronous initiation of V-to-DJ recombination between alleles is required for TCRβ/IgH-mediated feedback inhibition to enforce allelic exclusion (6, 7). In the absence of any other means of V-to-DJ recombination control, the ability of feedback inhibition to enforce allelic exclusion would require efficient repair and expression of VDJ rearrangements on the first allele. Because ATM promotes coding join formation (29), the increased frequencies of Atm<sup>-/-</sup> cells with biallelic VDJ rearrangements could simply arise from inefficient repair of RAG DSBs on the first allele leading to V-to-DJ recombination on the second allele before activation of feedback inhibition signals. However, we previously used nonhomologous end-joining-deficient pro-B cells to distinguish between ATM functions in DSB repair and signaling, thereby demonstrating that RAG DSBs induced on one allele during Igk recombination signal through ATM to suppress V<sup>k</sup>-to-J<sup>k</sup> rearrangements on the other allele (8). Accordingly, the increased frequencies of Atm<sup>-/-</sup> lymphocytes with biallelic VDJ rearrangements could arise from loss of ATM signals that suppress additional V<sub>H</sub> and Vβ rearrangements in response to RAG DSBs induced during V-to-DJ recombination on the first allele. We have not been able to assess contributions of these two nonmutually exclusive mechanisms using existing mouse models and in vitro systems of thymocyte and pro-B cell development. Thus, determining how ATM helps enforce TCRβ and IgH allelic exclusion by limiting biallelic VDJ rearrangements will require development of mouse models and/or systems of prolymphocyte development that distinguish between ATM functions in DSB repair versus signaling during the V-to-DJ recombination step.

Our analyses of TCRβ and IgH expression and rearrangements in Ccnd3<sup>−/−</sup> and WT mice show that Ccnd3 helps enforce TCRβ and IgH allelic exclusion. V(D)J recombination is restricted to G<sub>1</sub> phase cells by CyclinA/Cdk2-mediated phosphorylation and resultant degradation of RAG2 protein (34–36). In the 1990s, it was proposed that the ability of TCRβ-chains to initiate signals that drive cells through G<sub>1</sub> and into S phase, and thus inactivate RAG activity, is important to inhibit additional Vβ rearrangements and enforce TCRβ allelic exclusion (37, 38). TCRβ and IgH expression in pro-T/B cells induces expression of Ccnd3, which complexes with Cdk4 or Cdk6 kinases to drive cells through G<sub>1</sub> and into S phase (11, 12). We detected increased TCRβ and IgH allelic inclusion in Ccnd3<sup>−/−</sup> mice that correlated with an elevated frequency of biallelic V<sub>H</sub>D<sub>β</sub>l<sub>H</sub> rearrangements. Although the latter was not significant from the numbers of cells analyzed, the ability to analyze orders of magnitude more cells by flow cytometry than by FISH renders detection of biallelic IgH expression more sensitive than biallelic V<sub>H</sub>-to-D<sub>β</sub>I<sub>H</sub> rearrangement. Consequently, our data are consistent with the notion that, after assembly of in-frame VDJ rearrangements on the first allele, Ccnd3<sup>−/−</sup> prolymphocytes have more time in G<sub>1</sub> phase to initiate V rearrangements on the second allele. Because Ccnd3 represses germline V<sub>H</sub> transcription in pro-B cells (39), Ccnd3 also could help enforce IgH allelic exclusion through downregulation of V<sub>H</sub> accessibility. Different domains of Ccnd3 drive proliferation and inhibit V<sub>H</sub> transcription (39). Thus, generation and analysis of mice expressing specific Ccnd3 mutations will determine the contribution of each Ccnd3 function to IgH and TCRβ allelic exclusion. Considering that neither Ccnd3/Cdk4 nor Ccnd3/Cdk6 complexes was tested for ability to control RAG2 protein stability (34, 35), Ccnd3 also could regulate allelic exclusion through phosphorylation of RAG2 in G<sub>1</sub> phase cells. We previously showed that Ccnd3 inactivation had no effect on TCRβ-mediated feedback inhibition in mice expressing a preassembled functional TCRβ gene (40). Therefore, our current data that Ccnd3<sup>-/-</sup> mice exhibit increased TCRβ allelic inclusion provides further evidence that using preassembled functional genes/transgenes to study feedback inhibition and allelic exclusion has limitations (4, 41, 42).

Our analyses of TCRβ and IgH expression and rearrangements in Atm<sup>-/-</sup> and Atm<sup>-/-</sup>Ccnd3<sup>-/-</sup> mice show that ATM and Ccnd3 cooperate to help enforce TCRβ and IgH allelic exclusion. Although the mechanisms that enforce TCRβ/IgH allelic exclusion have not yet been fully elucidated, it is well documented that TCRβ/IgH chains expressed from one allele signal permanent feedback inhibition of Vβ/V<sub>H</sub> rearrangements on the other allele (3–5). Lymphocyte development-stage specific changes in TCRβ/IgH locus topology and accessibility likely prevent the reinitiation of Vβ/V<sub>H</sub> rearrangement in pre-T/B cells (3–5); however, evidence suggests that additional distinct mechanisms downregulate Vβ/V<sub>H</sub> recombination prior to TCRβ/IgH-signalized differentiation of pro-T/B cells (5). Our data indicate that activation of Ccnd3 is one mechanism by which TCRβ/IgH-mediated signals suppress Vβ/V<sub>H</sub> rearrangements in pre-T/B cells. For TCRβ/IgH-mediated feedback inhibition to enforce allelic exclusion, the field recognizes that additional mechanisms must promote asynchronous initiation of Vβ/VH rearrangements between alleles (3–5), although debate exists regarding the nature of these mechanisms because of inability to identify molecules that control this level of regulation (5). Our prior findings indicate that RAG cleavage during V<sub>k</sub>-to-J<sub>k</sub> recombination on one Igk allele signals through ATM to transiently prevent RAG cleavage of the other Igk allele (8). Our data in this study suggest a similar ATM-dependent mechanism helps enforce TCRβ/IgH allelic exclusion. Because of the many mechanisms that cooperate to enforce allelic exclusion, the absence of ATM and/or Ccnd3 would be expected to result in much less than the theoretical maximum of TCRβ/IgH allelic inclusion (in 20% of lymphocytes and corresponding with biallelic V rearrangements in 100% of lymphocytes). In this context, our data showing biallelic V rearrangements in ~60% of Atm/Ccnd3-deficient cells, relative to ~40% of normal cells, indicates that asynchronous initiation and TCRβ/IgH-mediated feedback inhibition of V rearrangement cooperates with ATM-dependent and Ccnd3-dependent mechanisms to help enforce TCRβ/IgH allelic exclusion at normal levels. The theoretical maximum of TCRβ/IgH allelic inclusion also assumes that every TCRβ and IgH chain can functionally pair with every TCRα or Igλ chain. Yet, the literature shows that this assumption is not valid (21, 43). Moreover, the increased frequency of V(D)J coding end deletions that occur in ATM-deficient cells also would prevent achievement of the theoretical maximum level of TCRβ/IgH allelic inclusion in mice lacking ATM alone or both ATM and Ccnd3. Although the effects of the loss of ATM and Ccnd3 on TCRβ/IgH allelic exclusion are very small as expected, our data convincingly establish that ATM and Ccnd3 cooperate to help control monoallelic Vβ/VH recombination.

Our data also demonstrate that inactivation of Ccnd3 increases the frequency of Atm<sup>-/-</sup> B cells with biallelic IgH expression by suppressing aberrant V<sub>H</sub>-to-D<sub>β</sub>I<sub>H</sub> rearrangements that delete C<sub>H</sub> genes. Impaired G<sub>1</sub>-S and G<sub>2</sub>-M checkpoints in Atm<sup>-/-</sup> cells enable RAG DSBs on nonselected alleles to persist unrepaired or become aberrantly repaired as cells proliferate (26, 31, 32). Ac-
cordingly, the simplest explanation for our data are that inactivation of Cend3 provides Atm/cell body cluster rearrangements on and off: more questions than answers. Immuno. Rev. 209: 129–141.


