

Macrophage Apoptosis by Anthrax Lethal Factor Through p38 MAP Kinase Inhibition

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The bacterium *Bacillus anthracis* causes the death of macrophages, which may allow it to avoid detection by the innate immune system. We found that *B. anthracis* lethal factor (LF) selectively induces apoptosis of activated macrophages by cleaving the amino-terminal extension of mitogen-activated protein kinase (MAPK) kinases (MKKs) that activate p38 MAPKs. Because macrophages that are deficient in transcription factor nuclear factor κ B (NF- κ B) are also sensitive to activation-induced death and p38 is required for expression of certain NF- κ B target genes, p38 is probably essential for synergistic induction of those NF- κ B target genes that prevent apoptosis of activated macrophages. This dismantling of the p38 MAPK module represents a strategy used by *B. anthracis* to paralyze host innate immunity.

Bacillus anthracis, the causative agent of anthrax, has gained notoriety as a potential bio-warfare and bioterrorism agent. During inhalation anthrax, the most lethal form of the disease, *B. anthracis* spores are engulfed by alveolar macrophages (1). The spores, however, survive phagocytosis and germinate within phagosomes, and the bacteria spread to regional lymph nodes and eventually the bloodstream. In this late stage of bacteremia, the infected individual is subjected to fatal systemic shock (2). For successful infection, *B. anthracis* must evade the host innate immune system by killing macrophages (3), a strategy used by other highly virulent bacteria (4). It is unclear how *B. anthracis* interacts with macrophages in such a contradictory manner.

Three proteins secreted by *B. anthracis* are central to its pathogenicity: protective antigen (PA), edema factor (EF), and lethal factor (LF) (5). By binding a specific cell-surface receptor, PA translocates EF and LF into the cytosol (6). EF is an adenylate cyclase that causes tissue edema (7), whereas LF is a metalloprotease that exhibits unique specificity toward MKKs, cleaving between their NH₂-terminal extension and the catalytic domain (8). Because the NH₂-terminal extension is required for interactions with both MAPKs and MKK kinases (MKKKs) (9), this cleavage prevents MAPK activation (10). Lethal toxin (LT), a complex of PA and LF, is the major factor responsible for the lethality of anthrax (1). Ex vivo, LT exhibits cytotoxicity toward macrophages, an activity likely to be important for evasion of host defenses (1, 4). Together, all three toxin compo-

nents also inhibit neutrophil migration and phagocytosis (11), further helping immune system evasion. A direct causal relation between

dismantling of MAPK signaling and LT-mediated cytotoxicity is currently lacking. Although in culture, LT was mostly described as inducing macrophage necrosis (12), necropsy of inhalation anthrax victims revealed extensive macrophage apoptosis (13). Furthermore, mouse strains whose macrophages are resistant to LT-induced necrosis are more susceptible to *B. anthracis* infection (14), thus questioning the role of the necrotic response.

We sought physiologically relevant conditions under which LT could induce macrophage apoptosis. Treatment of J774A.1 macrophage-like cells with the protein phosphatase inhibitor calyculin A was found to sensitize them to LT-induced apoptosis (12). Treatment of different cells with such phosphatase inhibitors activates numerous protein kinases, including MAPKs (15) and inhibitor of NF- κ B (I κ B) kinase (IKK) (16). Hence, calyculin A could activate J774A.1 macrophages. We therefore tested the effect of the general macrophage activator lipopolysaccharide (LPS) on the response of J774A.1 cells and bone marrow-derived macrophages (BMDMs) to LT. Titrations

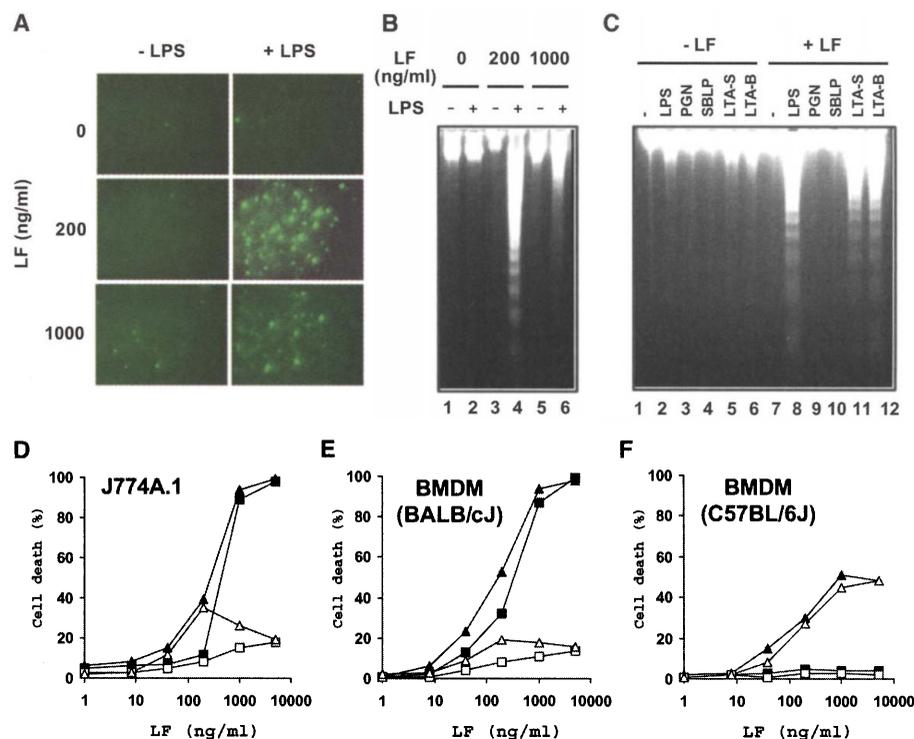


Fig. 1. Anthrax LT induces apoptosis of activated macrophages. (A) J774A.1 cells were incubated without or with LPS (100 ng/ml) in the absence or presence of purified recombinant PA₆₃ (2.5 μ g/ml) and the indicated amounts of LF. After 8 hours, the cells were analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining for presence of apoptotic bodies. (B) J774A.1 cells were treated as in (A). After 18 hours, genomic DNA was isolated and analyzed by agarose gel electrophoresis and ethidium bromide staining. (C) J774A.1 cells were incubated with LPS or other microbial components, including peptidoglycan (PGN; 10 μ g/ml), synthetic bacterial lipopeptide (SBLP; Pam₃CSK₄; 1 μ g/ml), and lipoteichoic acids from *S. aureus* (LTA-S; 10 μ g/ml) or *B. subtilis* (LTA-B; 10 μ g/ml) in the absence or presence of LF, and apoptosis was analyzed by DNA fragmentation. (D to F) J774A.1 cells and BMDMs were treated with (▲, △) or without (■, □) LPS and the indicated concentrations of LF, and the extent of total (necrotic and apoptotic) cell death was determined after 8 hours by staining with Hoechst 33258 dye (▲, ■) and compared with the extent of apoptotic cell death determined by TUNEL staining (△, □).

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tion experiments revealed that in a manner strictly dependent on PA₆₃ (17), a mature and active form of PA (18), LF caused rapid apoptosis of LPS-activated macrophages at 200 ng/ml, a suboptimal concentration for inducing necrosis in J774A.1 and BMDMs from C57BL/6 mice (Fig. 1, A, B, and F). No apoptosis was detected in resting (nonactivated) macrophages. In addition to LPS derived from Gram-negative bacteria, lipoteichoic acids (LTAs) from the Gram-positive bacteria *Staphylococcus aureus* and *B. subtilis* also induced apoptosis of LT-treated cells (Fig. 1C), suggesting that a similar component of *B. anthracis*, a Gram-positive bacterium, can activate macrophages and trigger apoptosis in the presence of LT. Apoptosis induced by LTAs was not inhibited by polymyxin B (17), indicating that it is not mediated by contaminating LPS. At 200 ng/ml, most of the LF-induced cell death in activated J774A.1 cells was apoptotic in nature, whereas in activated BALB/c BMDMs, only 50% of the observed cell death was due to apoptosis (Fig. 1, D and E). At higher concentrations, LT caused the necrotic death of both resting and activated macrophages, and the apoptotic response was attenuated. As previously

reported (19), LT did not cause necrosis of C57BL/6 BMDMs. Nonetheless, LT effectively induced the apoptosis of these cells (Fig. 1F). Therefore, unlike necrosis (19), LT-induced apoptosis of activated macrophages is not confined to a subset of mouse strains.

Treatment of macrophages with LPS activates extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), and p38 MAPKs (20), as well as IKK and NF-κB (21). Titration experiments revealed that LF (together with PA₆₃) inhibited ERK activation by LPS in BMDMs at a concentration as low as 40 ng/ml (Fig. 2A). Inhibition of JNK1 and p38 activation required a higher LF concentration (200 ng/ml), similar to that needed for induction of apoptosis in activated macrophages. However, LF did not inhibit JNK2 nor LPS-induced IκBα degradation, suggesting that it does not inhibit IKK activation. Using inhibitors that are selective for each MAPK cascade [PD98059, a MEK1/MEK2 inhibitor for ERK (22); SP600125 for JNK (23); SB202190 for p38 (24)], we examined the contribution of MAPK inhibition to LF-induced apoptosis of activated macrophages. Only treatment with the specific p38

inhibitor SB202190 induced apoptosis of LPS-treated BMDMs (Fig. 2B). SB202190 was not cytotoxic toward resting macrophages (17).

To determine whether cleavage of the MKKs responsible for p38 activation, MKK3 and MKK6 (25), is required for LF-induced apoptosis, we generated mutant forms of MKK3 and MKK6 that lack specific residues required for recognition by LF (26, 27). Both MKK3b^{R26Q/127G} (MKK3CR) and MKK6^{K14Q/115G} (MKK6CR) were resistant to LF cleavage (Fig. 2C). Stable expression of either mutant in RAW264.7 macrophages (which are more amenable to transfection than J774A.1) revealed that only MKK6CR partially protected p38 from inhibition by LF (Fig. 2D). Most importantly, the pooled population of MKK6CR-expressing cells exhibited considerable resistance to LF-induced apoptosis after activation (Fig. 2, E and F). Neither MKK6CR nor MKK3CR protected activated RAW264.7 cells from apoptosis induced by SB202190 (Fig. 2E). Thus, the ability of LT to induce apoptosis of activated macrophages depends on inhibition of p38 activation.

Even though LF does not inhibit the IKK to

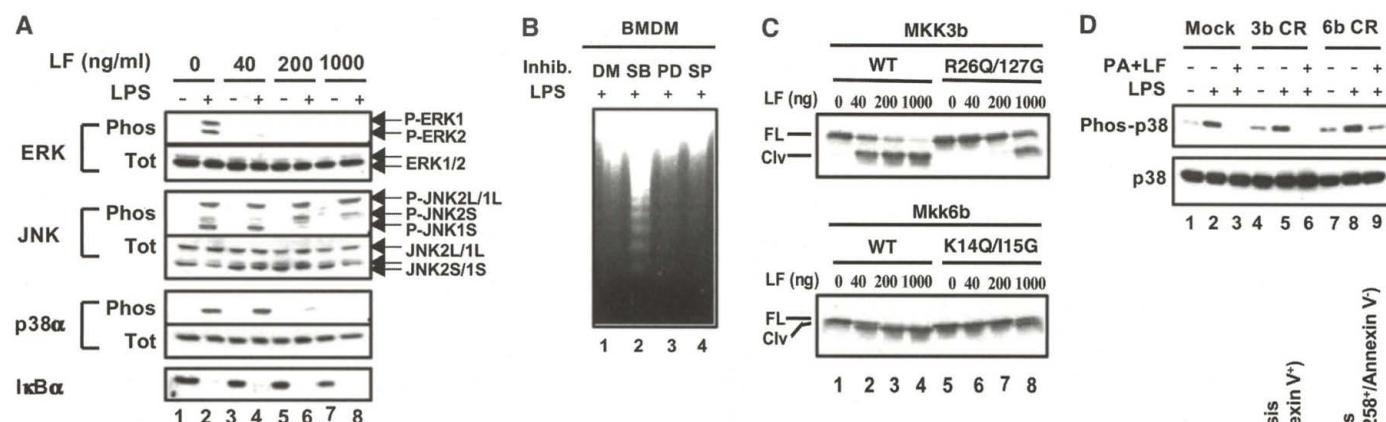
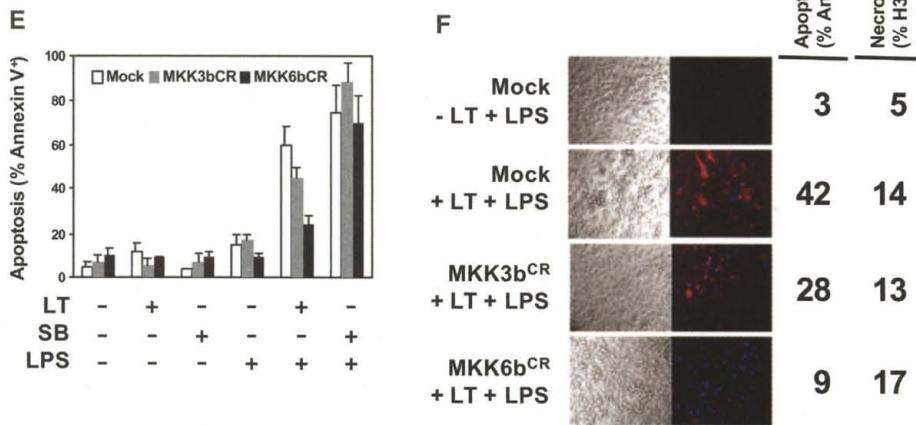


Fig. 2. Inhibition of p38 MAPK induces apoptosis of activated macrophages. (A) BMDMs (C57BL/6) were left unstimulated or stimulated with LPS in the presence of PA₆₃ (2.5 μg/ml) and the indicated amounts of LF. After 20 min, cell lysates were prepared and analyzed by immunoblotting with antibodies specific to different MAPKs (Tot) or their phosphorylated (activated) forms (Phos). (B) BMDMs (C57BL/6) were stimulated with LPS in the presence of dimethylsulfoxide (DM), SB202190 (SB; 10 μM), PD98059 (PD; 20 μM), or SP600125 (SP; 20 μM) and analyzed as in Fig. 1B. (C) ³⁵S-labeled wild-type (WT) and mutant MKK proteins were synthesized in vitro and then incubated with indicated amounts of LF for 30 min. MKK cleavage was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. FL, full length; Clv, cleavage product. (D) RAW264.7 cells were transfected with cytomegalovirus-driven expression vectors for green fluorescent protein (Mock), MKK3bCR (3b CR), and MKK6bCR (6b CR). Stable transfectants were left unstimulated or stimulated with LPS in the presence or absence of PA₆₃ (2.5 μg/ml) and LF (500 ng/ml) and analyzed for p38



activation as in (A). (E and F) The RAW264.7-derived transfected cell pools were treated with LPS, LT, and SB as indicated. After 8 hours, the number of apoptotic and necrotic cells was determined by staining with Hoechst 33258 (blue) and annexin V-Alexa568 (red), respectively.

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NF-κB pathway, survival of LPS-activated macrophages depends on IKK and NF-κB activation. Using a conditional *Ikkβ* allele in which

exon 3, which encodes part of the kinase domain, was flanked by binding sites (*loxP*) for the Cre recombinase, we generated IKKβ-deficient

myeloid cells by crossing *Ikkβ^{loxP/loxP}* mice with mice that express a lysozyme M promoter-driven Cre recombinase (28). The frequency of *Ikkβ* deletion in BMDMs of *Ikkβ^{loxP/loxP} LysM-Cre* mice was ~50% (Fig. 3A), resulting in only a partial decrease in IKK (Fig. 3B) and NF-κB (Fig. 3C) activation in the mixed cell population. To circumvent difficulties associated with this heterogeneity, we examined LPS-induced apoptosis in individual macrophages and correlated it with the presence or absence of the p65 NF-κB subunit in the nucleus. These experiments revealed that LPS only induced apoptosis of those cells lacking nuclear p65 (Fig. 3D). No apoptosis was detected upon incubation of *Ikkβ^{loxP/loxP}* BMDMs lacking Cre with LPS. In addition, expression of a degradation-resistant form of IκB in RAW264.7 cells sensitized them to LPS-induced apoptosis (17).

The marked sensitivity of BMDMs that lack either p38 or NF-κB activity to activation-induced death suggests that p38 may be required to activate a transcription factor or recruit a coactivator that synergizes with NF-κB to induce transcription of a gene(s) whose product inhibits apoptosis. Previous analysis of NF-κB-mediated gene expression in dendritic cells revealed that p38 is required to induce some NF-κB target genes (29). To investigate this possibility, we used real-time polymerase chain reaction (PCR) (17) to examine the requirement of p38 for the expression of known NF-κB target genes in J774A.1 cells and BMDMs. Expression of many NF-κB-regulated genes, such as IκBα, iNOS, A20, and GADD45β, was effectively induced by LPS in untreated cells as well as in cells treated with either SB202190 or LT (Fig. 4A). However, expression of other NF-κB target genes, including those encoding interleukin-1α (*IL-1α*), *IL-1β*, and *COX-2*, was induced by LPS in untreated macrophages but was inhibited by either SB202190 or LT. Expression of only one gene, that encoding tumor necrosis factor-α (TNF-α), was partially inhibited by LT but not by SB202190. Inhibition of LPS-induced TNF-α, IL-1α, and IL-1β expression by LT was observed previously (30). Because p38 is also involved in mRNA stabilization (25), we used nuclear run-off experiments to confirm that the effect of its inhibitor is transcriptional (Fig. 4B). On the basis of these results, we suggest that through phosphorylation of an as-yet unidentified target, p38 synergizes with NF-κB to induce the expression of a subset of target genes, which in macrophages includes inhibitors(s) of activation-induced death. Inhibition of either p38 or NF-κB is sufficient to sensitize macrophages to activation-induced death by preventing induction of this antiapoptotic factor.

Our results uncover a strategy by which *B. anthracis* paralyzes the innate immune system to promote its undisturbed spread toward systemic infection. By inhibiting activation of p38 MAPK, this deadly pathogen switches the sig-

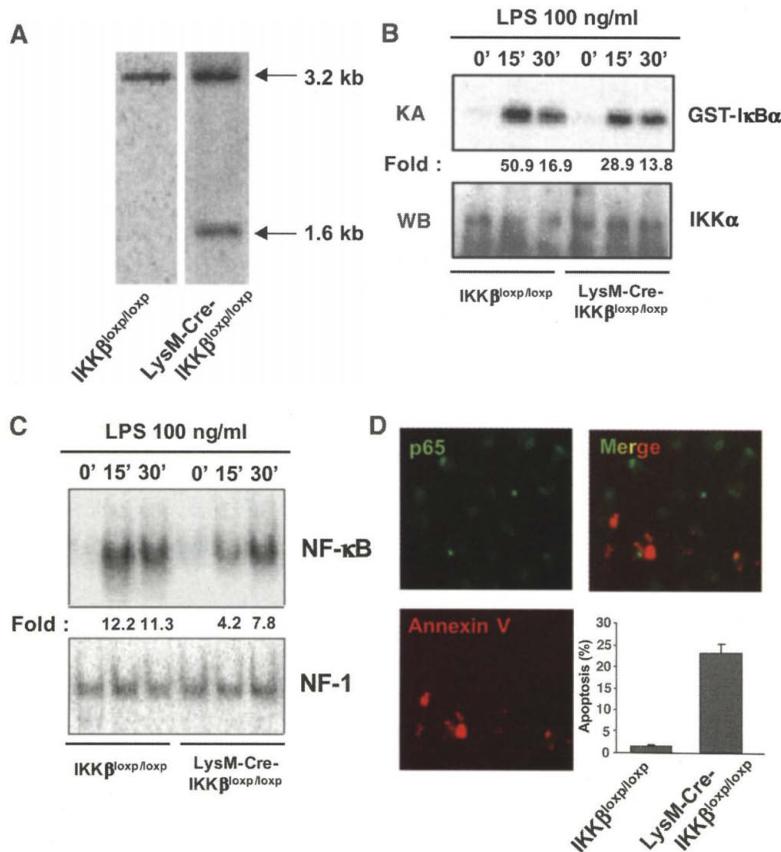
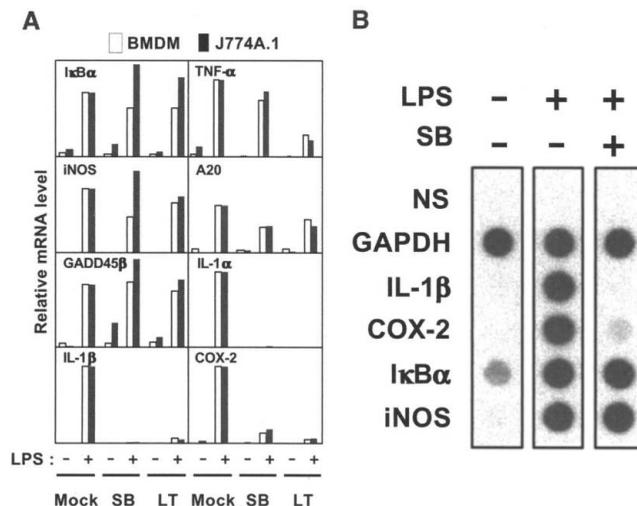


Fig. 3. Defective NF-κB activation sensitizes macrophages to activation-induced death. (A) Southern blot analysis of Hind III-digested genomic DNA from BMDMs of *Ikkβ^{loxP/loxP}* or *LysM-Cre-Ikkβ^{loxP/loxP}* mice. The 3.2- and 1.6-kilobase (kb) fragments correspond to the floxed and deleted alleles, respectively. (B and C) BMDMs of the two genotypes were treated with LPS for the indicated times, and IKK (B) and NF-κB (C) activation was assayed. Gel loading and extract preparation were controlled by Western blotting (WB) for IKKα (B) or a mobility shift assay for NF-1 (C). Fold increase in IKK and NF-κB activities was determined by densitometry. (D) BMDMs were stimulated with LPS (100 ng/ml) for 8 hours and stained with annexin V to visualize apoptotic cells. Nuclear p65 was identified by immunofluorescence, using a rabbit polyclonal antibody against p65 (Santa Cruz Biotechnology, Santa Cruz, CA).

Fig. 4. Induction of certain NF-κB target genes during macrophage activation is p38 dependent. (A) C57BL/6 BMDMs (white bars) and J774A.1 cells (black bars) were stimulated or not with LPS in the absence or presence of SB202190 (10 μM) or LT (500 ng/ml LF and 2.5 μg/ml PA₆₃). After 4 hours, cellular RNA was isolated, and relative expression of the indicated genes was determined by real-time PCR. The results shown are averages of three determinations. (B) The transcription rates of the indicated genes in J774A.1 cells that were or were not treated with LPS and SB202190, as indicated, were determined by nuclear run-off assays.



Enhanced Tumor Formation in Mice Heterozygous for *Blm* Mutation

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nal for macrophage activation to a trigger of rapid cell death. Selective killing of activated macrophages prevents the secretion of chemokines and cytokines that alert the remainder of the immune system to the presence of the pathogen. This may explain why anthrax infections proceed undetected until the terminal stage, when vast bacteremia occurs. Future research should focus on the balance between macrophage activation and apoptosis, as it seems to play a key role in the pathogenesis of anthrax and other deadly infections.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S4

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Persons with the autosomal recessive disorder Bloom syndrome are predisposed to cancers of many types due to loss-of-function mutations in the *BLM* gene, which encodes a recQ-like helicase. Here we show that mice heterozygous for a targeted null mutation of *Blm*, the murine homolog of *BLM*, develop lymphoma earlier than wild-type littermates in response to challenge with murine leukemia virus and develop twice the number of intestinal tumors when crossed with mice carrying a mutation in the *Apc* tumor suppressor. These observations indicate that *Blm* is a modifier of tumor formation in the mouse and that *Blm* haploinsufficiency is associated with tumor predisposition, a finding with important implications for cancer risk in humans.

Bloom syndrome (BS) is characterized by small stature, immunodeficiency, male infertility, and predisposition to cancer of many tissue types (*1*). Cells from persons with BS show increased somatic recombination, chromosome breakage, and site-specific mutations (*1–3*). The BS locus, *BLM*, encodes BLM, an adenosine triphosphate-dependent, 3'-5' helicase with homology to the recQ DEXH-box-containing DNA and RNA helicases (*4*); loss of BLM helicase activity is responsible for the genomic instability of BS cells (*5, 6*). BLM resolves Holliday junctions, suppresses recombination in vitro, and is required for the fidelity of DNA double-strand break repair (*7–9*).

We have used gene targeting by homologous recombination to disrupt the mouse *Blm* gene to simulate *BLM*^{As^h}, a BS-causing mutant allele of *BLM* carried by approximately 1% of Ashkenazi Jews (*4, 10, 11*). *BLM*^{As^h} contains a frameshift mutation in exon 10 of *BLM* that results in premature translation termination (*4*). In contrast to work with two mouse models of BS previously reported (*12, 13*), we used a gene-targeting construct in which exons 10, 11, and 12 of *Blm* were replaced with a hypoxanthine phosphoribosyltransferase (*Hprt*)

cassette (fig. S1A). Germ line transmission of this mutant allele, *Blm*^{Cin}, followed blastocyst injection of targeted embryonic stem cells to generate heterozygous mice (fig. S1B). Crosses to generate *Blm*^{Cin/Cin} mice were unsuccessful, indicating that homozygous disruption of *Blm* results in embryonic lethality (*14*). Western blots of protein lysates from *Blm*^{+/+} testes, an abundant source of *Blm* RNA and BLM protein (*12, 13*), displayed a specific band of approximately 190 kD when probed with a COOH-terminal antiserum to BLM (fig. S1C). Lysates from *Blm*^{Cin/+} testes had an approximately 50% reduction in BLM in comparison to *Blm*^{+/+} testes. Lysates of heterozygous tissues were similarly evaluated with an NH₂-terminal antibody to BLM and revealed no smaller immunoreactive proteins (fig. S1D). This reduction of full-length BLM and the absence of truncated BLM in *Blm*^{Cin/+} mice confirm that we had generated a null allele. This allele allowed us to examine the biological consequences of *Blm* haploinsufficiency, that is, a reduction in wild-type (WT) *Blm* gene dosage and its gene product.

BS somatic cells exhibit increases in chromosome aberrations, sister chromatid exchanges (SCEs), homologous chromatid interchanges, and micronuclei that are a consequence of chromosome breakage (*1, 15, 16*). Although the cytogenetic analysis of somatic cells from human *BLM* heterozygotes remains to be completed (*17*), spermatogonia from two of three obligate heterozygotes have been shown to display excess numbers of chromosome breaks and rearrangements (*18*). To learn whether *Blm* haploinsufficiency affects genomic stability, we cultured primary lung fibroblasts from

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