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***Atm* deficient mice exhibit increased sensitivity to dextran sulfate sodium-induced colitis characterized by elevated DNA damage and persistent immune activation**

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Abstract

The role of ATM, a DNA double-strand break recognition and response protein, in inflammation and inflammatory diseases is unclear. We previously demonstrated high levels of systemic DNA damage induced by intestinal inflammation in wildtype mice (1). To determine the effect of *Atm* deficiency in inflammation, we induced experimental colitis in *Atm*^{−/−}, *Atm*^{+/-} and wildtype mice via dextran sulfate sodium (DSS) administration. *Atm*^{−/−} mice had higher disease activity indices and rates of mortality compared to heterozygous and wildtype mice. Systemic DNA damage and the immune response were characterized in peripheral blood throughout and after three cycles of treatment. *Atm*^{−/−} mice demonstrated increased sensitivity to levels of DNA strand breaks in peripheral leukocytes, as well as micronuclei formation in erythroblasts compared to heterozygous and wildtype mice, especially during remission periods and after the end of treatment. Markers of reactive oxygen and nitrogen species-mediated damage, including 8-oxoguanine and nitrotyrosine were present in both the distal colon and in peripheral leukocytes, with *Atm*^{−/−} mice manifesting more 8-oxoguanine formation than wildtype mice. *Atm*^{−/−} mice demonstrated greater upregulation of inflammatory cytokines, and significantly higher percentages of activated CD69⁺ and CD44⁺ T-cells in the peripheral blood throughout treatment. ATM therefore may be a critical immunoregulatory factor dampening the deleterious effects of chronic DSS-induced inflammation, necessary for systemic genomic stability and homeostasis of the gut epithelial barrier.

Keywords

Atm; dextran sulfate sodium; ulcerative colitis; DNA damage

Introduction

Long standing inflammation contributes to the development of over 20% of all human cancers, owing to increased cellular proliferation in environments favoring DNA damage and

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tumorigenesis (2). Ulcerative colitis (UC) is one such chronic inflammatory condition affecting millions of people worldwide, leading to increased risk of colorectal cancer. Duration and severity of inflammation correlate to cumulative probability of cancer development, ranging from 2% at 10 years of colitis to 18% after 30 years (3). A dysregulated immune response to commensal flora caused by transient breaks in the mucosal barrier is thought to be involved in pathogenesis of UC (4), though exact mechanisms remain to be elucidated.

The *Atm* gene codes for ataxia telangiectasia mutated (ATM), a pleiotropic kinase involved in DNA double strand break recognition, activation of DNA repair proteins, and signaling in cell cycle checkpoint control (5,6). Its deficiency leads to ataxia telangiectasia (A-T), a rare human disease involving defects in T-cell maturation, cerebellar degeneration, radiosensitivity, and increased susceptibility to lymphoma among other cancers (7). *Atm*^{-/-} mice foster similar defects as A-T patients (8–10), and have allowed for studying A-T, mechanisms of DNA damage responses, and carcinogenesis. Notably, *Atm*^{-/-} mice do not spontaneously develop colitis or colorectal cancer, however demonstrate elevated levels of oxidative stress compared to wildtype mice. Though inflammation-derived oxidative and nitrate stress leading to DNA damage have been recently implicated in colitis-associated cancers (11,12), the role of ATM in chemically induced intestinal inflammation has not been previously studied.

Cyclic administration of dextran sulfate sodium (DSS), a non-genotoxic sulfated polysaccharide, in the drinking water clinically and morphologically resembles ulcerative colitis and its progression to cancer, thus allowing the exclusive study of chronic inflammation in carcinogenesis without the use of tumor promoting carcinogens (13). Direct breaks in the epithelial barrier and the innate immune response are proposed in the pathogenesis of DSS-induced colitis (13,14). Investigators have observed oxidized DNA bases in rat and mice colonic mucosa after acute DSS treatment (15,16), microsatellite instability in colon tissue of *Msh2*^{-/-} mice with chronic DSS treatment (17), and a protective role of alkyladenine DNA glycosylase in colon tumorigenesis (11); suggesting the importance of repairing oxidative DNA damage at the site of inflammation. We have recently found that intestinal inflammation causes systemic genotoxicity in the form of DNA single- and double- stranded breaks and oxidized bases in the peripheral blood of DSS-treated wildtype mice, as well as in genetic models of mucosal inflammation (1). In order to explore the systemic role of ATM in pathogenesis of intestinal inflammation, we characterized the sensitivity of *Atm*^{-/-} mice to DSS-induced acute and chronic inflammation in terms of systemic genotoxicity and the consequentially mounted immune response.

Materials and Methods

Animals

Adult *Atm*^{-/-} mice crossed into the parental C57BL/6J *p^{un}/p^{un}* background as previously described (18), heterozygous (*Atm*^{+/-} *p^{un}/p^{un}*), and wildtype control mice (*Atm*^{+/+} *p^{un}/p^{un}*), 12 to 16 weeks old, were housed in a specific pathogen free facility fed a standard rodent chow diet, provided acidified drinking water, and 12:12 light:dark cycle. Food, bedding, and water were autoclaved. All experimental procedures were in accordance with the UCLA Animal Research Committee guidelines.

Induction of experimental colitis

Acute and chronic experimental colitis was induced by administering 3% (w/v) DSS (MP Biomedicals, MW 40,000) dissolved in sterile acidified drinking water *ad libitum* for 3 cycles. One cycle of treatment consisted of 7 days of treated water followed by 14 days of normal drinking water. Water was changed daily and symptoms including weight loss, stool consistency, and gross bleeding were also recorded for calculation of the disease activity index

(DAI), as described further elsewhere (19). Briefly, a score ranging from 0–4 was assigned for each measure (weight loss (0–15% loss), stool consistency (normal to diarrhea), and blood in stool (no blood to gross bleeding)), and the average of these scores was recorded as the DAI. Mice were monitored for 31 days after the end of treatment.

Blood Collection

Peripheral blood was collected via the mandibular vein with a 5 mm lancet (Braintree Scientific, Braintree, MA) into EDTA coated tubes (Braintree Scientific). Blood was collected before and right after each seven day treatment of DSS, for three cycles and at two and four weeks after the end of the three cycles. For the comet assay, blood was immediately diluted 1:1 in RPMI/10% DMSO and immediately frozen at -80°C until further analysis. Freshly collected blood was immediately processed for all other assays. Identical samples were used for genotoxicity endpoints as well as for cytokine expression or flow cytometry, allowing each animal to serve as its own control.

Alkaline comet assay

To detect DNA strand breaks, as well as alkali labile sites, the alkaline comet assay was performed and analyzed as described elsewhere (1,20). The olive tail moment, which represents both tail length and fraction of DNA in the tail, was used for data collection and analysis, in which apoptotic cells were excluded under previously proposed criteria (20).

Determination of oxidative DNA damage

The enzyme hOgg1-modified comet assay was used and carried out identically as previously described (1).

Immunofluorescence

Peripheral blood was incubated in Buffer EL (Qiagen, Valencia, CA) to remove erythrocytes. Samples were then processed on coverslips and stained with anti-phospho-Histone H2A.X S139(P), mouse anti-8-oxoguanine clone 413.5, or rabbit anti-nitrotyrosine (Millipore, Temecula, CA) as described previously (1,21). At least 125 cells were counted and cells with greater than four distinct foci in the nucleus were considered positive for γH2AX (21). Apoptotic cells, distinguishable due to the presence of 10-fold the number of nuclear foci in damaged cells (22), were not included in analyses.

Paraffin sections (5 μm) of colons from *Atm*^{-/-} and wildtype controls were microwaved in 10 mM citrate buffer (pH 6) for 10 min for antigen retrieval, blocked, then incubated with anti-8-oxoguanine or anti-nitrotyrosine followed by secondary antibodies identical to procedures described above. Images were captured with CytoVision® (Applied Imaging, UK) and staining was quantified using ImageJ software(23).

In vivo micronucleus assay

Micronuclei (MN) formation was determined in peripheral blood erythrocytes to assess chromosomal instability as previously described(1). At least 4000 mature erythrocytes were counted per animal, and the frequency of MN formation was calculated as the number of micronucleated erythrocytes per 1000 normochromatic erythrocytes.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using QiaAmp RNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. 25ng/ μl of total RNA was used for reverse transcription using OligodT (Invitrogen) and Superscript III Reverse Transcriptase (Invitrogen). 10ng/ μl of cDNA was used for quantitative real time PCR using Taqman Gene Expression Assays (Applied

Biosystems, Foster City, CA) for TBP (TATA box binding protein), TNF- α (tumor necrosis factor-alpha), MCP-1 (monocyte chemoattractant protein-1), IFN- γ (interferon-gamma), TGF- β (transforming growth factor-beta), IL-4 (interleukin-4), IL-10 (interleukin-10), IL-6 (interleukin-6), IL-17 (interleukin-17), IL-23 (interleukin-23), IL-12 (interleukin-12) according to manufacturer's instructions on the ABI Prism 7500 sequence detection system (ABI). TBP was chosen as the endogenous control due to its low variability and low to medium relative abundance in expression in blood (24). Each measurement was performed in triplicate and results were analyzed using SDS 2.2.1 software (ABI). Quantification of gene expression was determined using the relative standard curve method normalized to TBP expression.

Flow Cytometry

T cell populations were characterized for activation status (CD69 and CD44) and CD4 or CD8 α expression using flow cytometry. Erythrocytes were immediately lysed with BD PharmLyse Lysis Buffer (BD Biosciences, San Diego, CA). After washing with Stain Buffer with 0.2% BSA (BD), cells were stained with FITC conjugated Hamster Anti-Mouse CD69, FITC conjugated Rat Anti-Mouse/Human CD44, R-PE conjugated Rat Anti-Mouse CD4, PerCP Rat Anti-Mouse CD8 α , or appropriate negative isotype controls (BD Biosciences) for 30 min at 4°C. Cells were then washed and analyzed using BD FACScan. Fluorescence intensity was normalized to each respective isotype control antibody and data were analyzed with CellQuest® (BD Biosciences). Dead cells were excluded by gating on forward/side scatter. Marker expression was recorded either as percent positive of the absolute count of total T cells, or by median fluorescence intensity if the control and marker populations overlapped.

Statistical Analyses

Results (error bars) are expressed as mean $\pm$ standard error of the mean (SEM) with $n=10$ mice per genotype. Statistical significance was determined by nonparametric one-way/two-way ANOVAs with Dunn's multiple comparison post test or paired Student's t -tests with log-transformed data for time point comparisons, defined as $p<0.05$. ANOVAs of linear regression models were used as appropriate. Genotoxicity assays and flow cytometry were repeated twice. Calculations were performed with GraphPad InStat 3.00 (GraphPad Software, San Diego, CA) or R: a language and environment for statistical computing (Vienna, Austria)(25).

Results

Atm^{-/-} mice show elevated sensitivity to DSS treatment

Mice were monitored daily for measurement of the disease activity index (DAI); an average score taking into account weight loss, stool consistency, and presence of blood in the stool, with a maximum score of 4. After an acute 7 day exposure to DSS, *Atm*^{-/-} mice had a mildly higher disease activity index compared to wildtype and heterozygous mice (Figure 1). Differences in symptom severity became more apparent towards the end of the second and third cycles (**: $p<0.01$), during chronic inflammation. Heterozygous and wildtype mice had similar DAIs throughout the study, indicating a lack of a gene dosage effect. In addition, *Atm*^{-/-}, *Atm*^{+/-}, and wildtype mice without DSS treatment had DAIs of 0 throughout the entire study (data not shown), demonstrating no baseline clinical symptoms. Two out of ten *Atm*^{-/-} mice died due to severe symptoms and rectal prolapse; one at the end of the second, and one at the end of the third cycle. All other mice survived the entire treatment. During remission periods, no signs of weight loss or persistent diarrhea were present in all genotypes. Surviving mice were also followed for four weeks after the end of the third cycle, however no symptoms were evident.

Elevated systemic genotoxicity in *Atm*^{-/-} mice

Since *Atm*^{-/-} mice are defective in DNA double strand break repair and have higher levels of cellular oxidative stress (26), we hypothesized that inflammation-induced DNA damage would be more pronounced. Sensitivity to treatment was therefore assessed in terms of genotoxicity to peripheral leukocytes, a systemic measure of DNA damage. DNA strand breaks as well as alkali-labile sites, represented by the olive tail moment, increased in wildtype mice after the first cycle ($p<0.001$) (Figure 2A). Damage was repaired during the first remission period, and successively increased after the second cycle until 2 weeks after the last cycle of treatment, before repair of damage was seen again. Oxidative base damage, as measured by incubation with hOgg1, was not significant in wildtype mice until after the third cycle of treatment.

On the other hand, DNA strand breaks successively increased in *Atm*^{-/-} mice with treatment, regardless of the remission periods. Olive tail moments were significantly higher in *Atm*^{-/-} mice especially after the second and third cycles of treatment compared to wildtype mice ($p<0.001$). Oxidative base damage was also more apparent in *Atm*^{-/-} mice, and more so after the end of the second cycle of treatment and up to 4 weeks after the end of the last treatment ($p<0.001$). *Atm*^{-/-} mice therefore incur more DNA damage than wildtype mice, especially in chronic inflammation.

DNA double-stranded breaks alone were confirmed in peripheral leukocytes via immunofluorescence of γ -H2AX (Figure 2B). Phosphorylation of histone 2AX, or γ -H2AX, occurs in response to double-stranded breaks, over a 2-Mbp region flanking the break site (22). ATM and other ATM-like kinases are responsible for this phosphorylation. Double-stranded breaks were generally more prevalent in lymphocytes than in other mononuclear cells types, and peaked after the second cycle and during the following remission period for all three genotypes. *Atm*^{-/-} mice had significantly higher levels of double-stranded breaks during all three remission periods than wildtype mice ($p<0.05$), also seen with the comet assay. Lack of repair of double-stranded breaks was once again evident 2 and 4 weeks after the end of treatment in *Atm*^{-/-} mice, possibly representing incomplete healing of the epithelial barrier, and prolonged effects of chronic inflammation. Heterozygous mice demonstrated similar patterns of γ -H2AX formation to wildtype mice throughout treatment and remission periods. A slight but non-significant increase in double-strand break formation, however, was seen over wildtype mice 2 weeks after the end of treatment.

Micronucleus formation in erythroblasts was measured as micronucleated mature erythrocytes in the peripheral blood. Toxicity of inflammation was evident as early as after the acute 7 day treatment of DSS, and more severely so in *Atm*^{-/-} mice (Figure 3). Micronucleus induction was significantly higher in *Atm*^{-/-} mice at every point of blood collection throughout treatment, and up to 4 weeks afterwards compared to both wildtype and heterozygous mice. Similarly to γ H2AX foci formation, heterozygous mice demonstrated higher levels of micronucleus formation only at 2 and 4 weeks after the end of treatment compared to wildtype mice, further indicating the importance of ATM during chronic inflammation. Increased sensitivity of *Atm*^{-/-} mice to chromosomal aberrations in the bone marrow may be due to continual induction of damage to erythroblasts in the bone marrow, or a defect in clearance of micronucleated erythrocytes.

Increased 8-oxoguanine formation in peripheral blood and colon tissue

The presence of inflammation-derived reactive oxygen and nitrogen species potentially causative for the observed DNA strand breaks as well as micronucleus formation was measured in the form of 8-oxoguanine in DNA and nitrotyrosine in proteins of peripheral leukocytes and in the distal colon (Figure 4). 8-oxoguanine is a DNA lesion caused by the reaction of oxidative reactive species such as hydroxyl radicals with DNA causing G:C to T:A transversions during

replication (27), and nitrotyrosine is formed from NO-induced peroxynitrite reacting along with other reactive species to tyrosine residues of proteins (28). Wildtype mice alone demonstrated significant increases after an acute 7 day exposure to DSS in both 8-oxoguanine and nitrotyrosine formation in peripheral leukocytes ($p < 0.01$). *Atm*^{-/-} mice also demonstrated significant increases in 8-oxoguanine ($p < 0.05$) and nitrotyrosine formation ($p < 0.05$) after 7 days of DSS treatment, however, only 8-oxoguanine formation was significantly higher in *Atm*^{-/-} compared to wildtype mice at the end of treatment ($p < 0.05$). Both 8-oxoguanine and nitrotyrosine were also evident in surface epithelial cells proximal to and in the villous crypts closest to the intestinal lumen and in inflammatory cells of the distal colon (Figure 4E-H). Staining for 8-oxoguanine localized in the nucleus while nitrotyrosine was evident in both the nucleus and cytoplasm of damaged cells. Staining for 8-oxoguanine was more prominent in the *Atm*^{-/-} compared to wildtype mice ($p < 0.01$), while nitrotyrosine levels were similar in both genotypes (Figure 4I).

Persistent immune response in *Atm*^{-/-} mice

As a possible explanation for the severe systemic genotoxicity displayed by *Atm*^{-/-} mice, the immune response at each point of blood collection was characterized and compared to wildtype mice. Though the innate response primarily drives DSS-colitis and potentially the observed genotoxicity, we hypothesized the adaptive immune response would be also modulated and play a role in driving genotoxicity. Transcript levels of Th1, Th17/23, and Th2 cytokines in the peripheral blood, where genotoxicity was measured, were quantified via quantitative real-time PCR (Figure 5). *Atm*^{-/-} mice displayed greater upregulation of TNF- α (Tnf1) and MCP-1 (Ccl2) during the second remission period and after the third cycle of treatment ($p < 0.05$) than wildtype mice, indicative of a chronically activated innate immune response. Levels of IL-6, IL-12, and IL-23 were also significantly upregulated in *Atm*^{-/-} compared to wildtype mice after treatment cycles and during remission periods, indicative of T-cell mediated proinflammatory responses. Interestingly, IL-17 transcripts were not detected in both genotypes (not shown). Similarly, lower levels of IL-17, and increased levels of Th12/23 and Th1 cytokines have been previously observed in DSS treated C57BL/6 mice (29).

Although levels of IFN- γ (Ifng), also an indicator of a T-cell response, were modulated in *Atm*^{-/-} mice, no significant differences were seen compared to wildtype mice. The Th2 response was more pronounced in *Atm*^{-/-} mice in chronic phases of treatment, characterized by increased expression of IL-4 (Il4), IL-10 (Il10), and TGF- β (Tgfb). A defect in tolerance mechanisms associated with anti-inflammatory cytokines are therefore most likely not the cause of increased sensitivity of *Atm*^{-/-} mice to chronic inflammation.

T-cell populations in the peripheral blood were also characterized by flow cytometry for CD4, CD8 α , CD69, an early activation marker of all T-cells including NK-cells (30), and CD44, expressed on leukocytes and involved in recruitment, activation, and effector functions (31) (Figures 6A-D). Spontaneously, *Atm*^{-/-} mice have significantly lower counts of mature CD4⁺ T-cells than wildtype and heterozygous mice, in agreement with previous findings (Figure 6C) (32–34). However, a significantly larger proportion of these T-cells are activated in response to DSS treatment compared to wildtype mice as shown by positive staining for CD69 and CD44 (Figures 6A and B). Heterozygous mice demonstrated similar levels of positive staining compared to wildtype mice (Figure 6D). Numbers of CD4 and CD8 α positive T-cells were also significantly modulated throughout treatment, most likely representing the dynamic influx and efflux of cells between the site of inflammation and the peripheral blood (data not shown). Percent activated T-cells remained significantly elevated especially in remission periods in *Atm*^{-/-} compared to wildtype mice until the end of the study, indicating a persistent immune response.

Discussion

Atm^{-/-} mice have decreased numbers of circulating T-cells due to intrinsic defects in T-cell progenitors and consequential developmental abnormalities of single positive thymocytes (9, 32). However, though lower in number, mature T-cells from A-T patients have been shown to be functionally normal; demonstrating the capability of mounting a competent immune response (35). *Atm*^{-/-} mice also do not develop spontaneous colitis or other inflammatory disorders of the gastrointestinal tract (36). However, when challenged with DSS causing a disruption in the integrity of the intestinal epithelial barrier, we demonstrated that *Atm*^{-/-} mice exhibit greater severity of clinical symptoms and mortality rates, DNA damage to peripheral leukocytes and erythroblasts, and mount an even stronger immune response characterized by inflammatory cytokines and circulating activated T-cells compared to wildtype mice. A significant gene dosage effect was not seen in terms of disease activity or percent activated T-cells, though a small increase in genotoxicity over wildtype mice was seen after the third cycle; indicating potential compensatory mechanisms for heterozygosity of *Atm*. Similarly, *Atm* heterozygosity does not increase tumor susceptibility in mice after γ -irradiation compared to wildtype mice (37), though increased susceptibility to mammary tumorigenesis is seen in a *Brcal* mutant background, compared to *Atm* sufficient mice (38).

The observed systemic DNA damage can be assumed to be inflammation mediated since DSS itself is not directly genotoxic (39,40). Reactive species derived from inflammatory cells through oxidative burst may cause oxidative and nitrative damage both locally and systemically measured by 8-oxoguanine and nitrotyrosine formation. Localization of this damage to the villi, surrounding epithelial cells, and infiltrating inflammatory cells may be due to DSS-induced villous atrophy and extensive epithelial turnover. Though ATM does not manifest a protective role in terms of protein damage, 8-oxoguanine levels were found to be higher in *Atm*^{-/-} mice, demonstrating lack of repair of oxidative DNA damage in addition to strand breaks. Interestingly, although DNA damage remained elevated, clinical symptoms of colitis were not present during remission periods and after the end of treatment, emphasizing the role of sub-clinical inflammation in the induction of DNA damage and the lack of repair of previously incurred damage. High levels of inflammation-associated oxidative stress, in addition to inherent deficiencies in repair of the resultant DNA damage, and partial suppression of DNA damage response-dependent apoptosis (41,42) may explain the extreme sensitivity of the *Atm*^{-/-} mice. An accumulation of DNA damage over the entire treatment period amidst slow DNA repair and cell turnover is therefore a probable explanation for increasing levels of DNA damage in *Atm*^{-/-} mice, taking into account the relatively long lifespan of lymphocytes. Differentiation of naive T cells into Th1 and Th17 effector cells could cause proliferation (43), in which accumulated DNA damage can lead to fixation of mutations.

Accumulation of double strand breaks can lead to chromosome breaks and micronuclei formation (44). Damage to erythroblasts in the bone marrow may be a humoral effect of inflammation-associated DNA damage, as with the peripheral leukocytes. Pro-inflammatory cytokines are preferentially released by cells that have migrated to the sites of inflammation rather than by resident macrophages (4). A recirculating pool of activated monocytes may recruit and activate effector cells, coming into contact with erythroblasts in the bone marrow, causing the observed clastogenicity.

The persistently activated immune response mounted by *Atm*^{-/-} mice demonstrate a possible role of ATM in immunoregulation during DSS treatment. The recruitment of myeloid-derived cells to the site of inflammation, along with resident dendritic cells, allow for phagocytosis of DSS particles and activation of the adaptive response involving differentiation of naive T-cells into activated effector cells (45). The prolonged presence of a larger percentage of activated T-cells in *Atm*^{-/-} mice, which harbor much lower total counts of CD4⁺ T cells, represents the

capacity of these mice to mount a successful yet damaging immune response despite this deficiency. An increase in messenger levels of inflammatory cytokines especially during remission periods is in itself evidence for systemic distribution, which also corresponded to levels of activated T-cells and genotoxicity in the peripheral blood. This persistent activation of T-cells and upregulation of cytokines can result in increased activity of macrophages and oxidative bursts, which may be a potential explanation for the observed direct genotoxicity to peripheral leukocytes. Further mechanisms may be investigated by administration of enzymatic inhibitors or anti-proliferative agents.

Recent evidence has pointed to the role of DNA damage response involving ATM in modulating an immune response. Genotoxic insult and activation of the ATM/ATR pathway was shown to upregulate ligands for the NKG2D receptor in mice and in humans, present on all NK cells, $\gamma\delta$ -T cells, and activated CD8⁺ T cells (46). This serves as a link between genotoxic stress and immune activation. Therefore, not only can the immune response potentially cause DNA damage via oxidative burst, but DNA damage itself can further activate NK-cells, potentially causing further damage if not properly repaired, as in *Atm*^{-/-} mice. Nuclear ATM has also been shown to directly bind NF- κ B essential modulator (NEMO), a modulator of NF- κ B, leading to cytoplasmic translocation and activation of NF- κ B, resulting in transcription of inflammatory and prosurvival response genes specifically in response to tolerable DNA damage (47). These varying modes of activation signify a coupling of stress response and cell survival.

The lack of ATM in these aspects could result in abnormal signaling in terms of response to genotoxic stress in the setting of chronic inflammation. Transcriptional repression of ATM has recently been found selectively in naive T-cells of rheumatoid arthritis patients, in which there is increased DNA damage thought to be independent of inflammation; indicating alternative modes of increased DNA damage in cells deficient in ATM (48). Aside from ATM deficiency, FEN1 deficiency, a multifunctional endonuclease, leads to incomplete digestion of DNA in apoptotic cells and results in chronic inflammation and autoimmunity (49). Similarly, increased levels of DNA damage resultant from chronic inflammation, due to an inherent deficiency in double strand break repair in *Atm*^{-/-} mice, may actually further promote inflammation and cause further DNA damage in a positive feedback loop. ATM may play therefore a protective role, not only as a DNA damage sensor, but also as an immunoregulator. The increased sensitivity to DSS treatment was not only present as clinical symptoms from localized inflammation in the colon, but manifested itself as a systemic insult characterized by genotoxicity and activation of immune responses.

In summary, *Atm*^{-/-} mice are more sensitive to DSS-induced acute and chronic inflammation than heterozygous or wildtype mice, especially during remission and up to four weeks after the final round of treatment, demonstrating lack of repair of incurred damage. Increased sensitivity was characterized by higher incidence of mortality, clinical symptoms, systemic genotoxicity to peripheral leukocytes and erythroblasts, and an activated immune response including increased transcripts of inflammatory cytokines in the peripheral blood. Systemic genotoxic stress induced by byproducts of inflammation may be able to further promote inflammatory responses and pro-survival mechanisms, via the intricate involvement of ATM. The lack of this protein causes further DNA damage and genetic instability, along with a more potent immune response, possibly due to other pathways alerting and further activating the immune response, or by defects in resolution of activated effector cells. ATM therefore can be inferred to play a role in immunoregulation and maintenance of genetic stability during inflammation, and be considered as a potential target for not only chronic inflammatory diseases but also for cancer therapy and prevention.

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Fig. 1. Disease activity indices (DAIs) of *Atm*^{-/-}, *Atm*^{+/-}, and wildtype mice. *Atm*^{-/-} mice exhibit higher DAIs (**: p<0.01) by Student's unpaired *t*-test compared to *Atm*^{+/-} and wildtype mice. Two *Atm*^{-/-} mice died; one at end of cycle 2, and one at end of cycle 3. Non-treated mice of all genotypes had DAIs of 0 throughout the entire study (not shown).

Fig. 2.

A. Mean olive tail moments of peripheral leukocytes with and without hOgg1 incubation. A portion of cells were treated with H₂O₂ for 20min as a positive control. Two-way ANOVA with Dunn's multiple comparison test demonstrate significant ($p < 0.001$) differences between genotypes. **B.** Percent positive cells for γ H2AX in peripheral leukocytes of *Atm*^{-/-}, *Atm*^{+/-}, and wildtype mice. A portion of cells were treated with H₂O₂ for 20min before staining as a positive control. Two-way ANOVA with Dunn's multiple comparison test demonstrated significant treatment effects. Genotype differences are shown *: $p < 0.05$, **: $p < 0.01$.

Fig. 3. Micronucleated normochromatic erythrocytes (MN-NCEs) per 1000 NCEs. ANOVA of a linear regression model for all three genotypes and treatment cycle effects were **: $p < 0.01$, *: $p < 0.05$ for *Atm*^{-/-} versus *Atm*^{+/-} and wildtype mice unless indicated otherwise.

Fig. 4. 8-oxoguanine and nitrotyrosine formation in peripheral leukocytes and the distal colon. **A.,B.** 8-oxoguanine (green) and nitrotyrosine (red) staining in peripheral leukocytes, respectively (x100). **C., D.** Percent positive cells for 8-oxoguanine and nitrotyrosine, respectively, in peripheral leukocytes of *Atm*^{-/-} and wildtype mice. *: $p < 0.05$, **: $p < 0.01$ by Student's unpaired *t*-test. **E., F.** Staining in the distal colon of wildtype mice for 8-oxoguanine and nitrotyrosine, respectively, treated with DSS for 7 days. (x10) **G., H.** Staining in the distal colon of *Atm*^{-/-} mice for 8-oxoguanine and nitrotyrosine, respectively, treated with DSS for 7 days. (x10) **I.** Quantification of 8-oxoguanine and nitrotyrosine staining in wildtype and

Atm^{-/-} mice expressed in pixel area with brightness value above a set threshold (arbitrary units).
**: p<0.01 by Student's unpaired *t*-test.

Fig. 5. Cytokine panel in peripheral blood by quantitative real-time PCR. **A., B., C.** Th1 cytokine panel of TNF- α , MCP-1, and IFN- γ , respectively. **D., E., F.** IL-12, IL-23, and IL-6, respectively. **G., H., I.** Th2 cytokine panel of TGF- β , IL-10, and IL-4, respectively. Data are mean expression of gene over expression of TBP, the internal control gene. *: p<0.05, **: p<0.01 by two-way ANOVA for genotype comparisons.

Fig. 6.

Flow cytometric analysis of peripheral leukocytes. **A.,B.** Percent gated CD69⁺ T-cells (CD4 or CD8α positive) and CD44⁺ T-cells, respectively, in peripheral blood. 15,000 cells were counted per mouse. *: p<0.05, **: p<0.01 by Student's unpaired *t*-test with Welch correction for genotype comparisons. **C.** Baseline CD4⁺ and CD8α⁺ peripheral blood T-cells of *Atm*^{-/-}, *Atm*^{+/-}, and wildtype mice. **D.** Mean fluorescent intensities of CD44⁺ T-cells in *Atm*^{-/-}, *Atm*^{+/-}, and wildtype mice after cycle 2 and before cycle 3, respectively. Filled line represents isotype control.