The Ataxia Telangiectasia Mutated Kinase Pathway Regulates IL-23 Expression by Human Dendritic Cells

Qunwei Wang, Hester A. Franks, Stephanie J. Lax, Mohamed El Refaee, Anna Malecka, Sabaria Shah, Ian Spendlove, Michael J. Gough, Claire Seedhouse, Srinivasan Madhusudan, Poulam M. Patel and Andrew M. Jackson

*J Immunol* 2013; 190:3246-3255; Prepublished online 4 March 2013;
doi: 10.4049/jimmunol.1201484
http://www.jimmunol.org/content/190/7/3246
The Ataxia Telangiectasia Mutated Kinase Pathway Regulates IL-23 Expression by Human Dendritic Cells

Quanwei Wang,* Hester A. Franks,*, Stephanie J. Lax,* Mohamed El Refaee,*, Anna Malecka,* Sabaria Shah,* Ian Spendlove,* Michael J. Gough,† Claire Seedhouse,‡ Srinivasan Madhusudan,* Poulam M. Patel,*1 and Andrew M. Jackson*,1

Little is known of the regulation of IL-23 secretion in dendritic cells (DC) despite its importance for human Th17 responses. In this study, we show for first time, to our knowledge, that the ataxia telangiectasia mutated (ATM) pathway, involved in DNA damage sensing, acts as an IL-23 repressor. Inhibition of ATM with the highly selective antagonist KU55933 markedly increased IL-23 secretion in human monocyte-derived DC and freshly isolated myeloid DC. In contrast, inhibiting the closely related mammalian target of rapamycin had no effect on IL-23. Priming naive CD4\(^+\) T cells with ATM-inhibited DC increased Th17 responses over and above those obtained with mature DC. Although ATM blockade increased the abundance of p19, p35, and p40 mRNA, IL-12p70 secretion was unaffected. To further examine a role for ATM in IL-23 regulation, we exposed DC to low doses of ionizing radiation. Exposure of DC to x-rays resulted in ATM phosphorylation and a corresponding depression of IL-23. Importantly, ATM inhibition with KU55933 prevented radiation-induced ATM phosphorylation and abrogated the capacity of x-rays to suppress IL-23. To explore how ATM repressed IL-23, we examined a role for endoplasmic reticulum stress responses by ATM inhibition with KU55933 prevented radiation-induced ATM phosphorylation and abrogated the capacity of x-rays to suppress IL-23. To explore how ATM repressed IL-23, we examined a role for endoplasmic reticulum stress responses by ATM inhibition with KU55933 prevented radiation-induced ATM phosphorylation and abrogated the capacity of x-rays to suppress IL-23. To explore how ATM repressed IL-23, we examined a role for endoplasmic reticulum stress responses by ATM inhibition with KU55933 prevented radiation-induced ATM phosphorylation and abrogated the capacity of x-rays to suppress IL-23. To explore how ATM repressed IL-23, we examined a role for endoplasmic reticulum stress responses by ATM inhibition with KU55933 prevented radiation-induced ATM phosphorylation and abrogated the capacity of x-rays to suppress IL-23.

© 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201484

Received for publication May 31, 2012. Accepted for publication January 31, 2013.

This work was supported by the Medical Research Council, Matt’s Fund for Cancer Research, and the University of Nottingham.

Q.W. undertook or supervised all experimental work and wrote the manuscript. H.A.F. conducted myeloid dendritic cell and quantitative PCR analysis. S.J.L. conducted T cell analysis by ELISA. M.E.R. undertook work with macrophages. A.M. undertook initial studies with ionizing radiation and IL-23. S.S. performed many of the ELISA assays. I.S. was involved in T cell studies. M.J.G. contributed to radiation studies and wrote the manuscript. C.S. supervised the research, and wrote the manuscript. A.M.J. conceived the original idea, supervised the research, and wrote the manuscript.

Address correspondence and reprint requests to Dr. Andrew M. Jackson, Host: Tumour Interactions Group, Academic Unit of Clinical Oncology, University of Nottingham, Nottingham NG5 1PB, United Kingdom; †Robert W. Franz Cancer Research Center, Providence Cancer Center, Portland, OR 97213; and ‡Academic Haematology, University of Nottingham, Nottingham NG5 1PB, United Kingdom

Abbreviations used in this article: ATM, ataxia telangiectasia mutated; Ct, threshold cycle; DC, dendritic cell; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; ER, endoplasmic reticulum; HPRT, hypoxanthine phosphoribosyltransferase; idc, immature dendritic cell; IFN, IFN regulatory factor; moDC, monocyte-derived dendritic cell; mTOR, mammalian target of rapamycin; myDC, myeloid dendritic cell; PARP, poly(ADP-ribose) polymerase; RA, rheumatoid arthritis; r, recombinant human; XBP-1, spliced X-box protein-1; WM, wortmannin; XBP-1, X-box protein-1; XBP-1s, spliced transcript of X-box protein-1.
is limited. In contrast, considerably more is known regarding IL-12 regulation. In TLR4-dependent signaling events, MAPK and NF-kB signaling cascades are activated by both LPS and lipoteichoic acid, which leads to differential activation of p44/p42 Erk 1/2, p38 MAPK, JNK, and IL-12 gene expression (25–27). In this setting, the p38 MAPK is generally accepted to play a positive role in IL-12 family cytokine regulation (28). Enhanced phosphorylation of Erk1/2 and p38 MAPK lead to a synergistic increase in LPS-induced IL-23 and IL-12 in cord blood–derived DC (27). We have previously shown that LPS/IFN-γ-induced IL-23 is completely blocked by a p38 MAPK inhibitor SB203580 (29). Conversely, IL-23p19 transcription was increased in LPS-triggered macrophages after p38 MAPK blockage (30). Zymosan is well known to induce secretion of IL-6, IL-10, TGF-β, and IL-23 from DC (31) by binding to the dectin-1 and TLR-2 receptors (32) and signaling through spleen tyrosine kinase (33, 34), caspase recruitment domain family member 9 (35), and NF-κB (35, 36). Furthermore, a role for PI3K in expression of the IL-12 family cytokines has been described (37, 38).

Recently (29), we described that treatment of DC with the PI3K inhibitor, wortmannin (WM), caused a 10-fold increase in TLR-dependent IL-23 secretion. However, this was not mediated through PI3K, as the alternative PI3K inhibitor LY294002 did not augment IL-23 production. In that study, WM was used at a 10-fold higher concentration than required to inhibit PI3K (39, 40). Although other studies have also used WM at higher concentrations to dissect PI3K function [up to 20 μM (39, 41, 42)], it is recognized that at these levels, WM inhibits additional kinases. Many of these alternate targets are PI3K-related kinases including smooth muscle myosin L-chain kinase (39), Na/K-ATPase (43), mammalian target of rapamycin (mTOR), DNA-dependent protein kinase (DNA-PK) (42), ataxia-telangiectasia mutated (ATM), and P4-kinase α and β (44). However, whereas none of these intracellular signaling molecules have previously been shown to play a role in regulating the IL-23/Th17 axis, indirect evidence exists to suggest a role for the DNA damage sensor ATM kinase. The canonical role of the ATM pathway is in DNA damage sensing and repair of DNA double-strand breaks (DSB), but limited evidence links ATM with immune function. An immune-regulatory role was suggested in ATM-deficient mice that in a colitis model exhibited unrepaired DNA damage and persistent immune activation (45). The ATM kinase is activated as part of the LPS-regulated phosphorylation pathways in mouse bone marrow–derived DC, but their function in this regard has not yet been demonstrated (46). ATM was recently shown to play an immune-regulatory role for human IFN responses in T cells (47). Treatment of T cells with etoposide-induced DNA damage and the ensuing ATM activation led to induction of IFN-stimulated and IFN-α and -γ genes (47).

The present study tested the hypothesis that ATM kinase pathway, canonically regarded as a genotoxic sensor, also plays a key role in immune regulation in DC. Using a highly selective ATM antagonist, we identified a novel role in regulating expression of the IL-23 p19 gene and IL-23 secretion. We showed that further activating ATM signaling with ionizing radiation suppressed IL-23 responses, and this was prevented by inhibiting ATM function. Inhibition of ATM in TLR4–triggered DC not only resulted in exacerbated IL-23 responses but also increased the level of Th17 responses generated from naive CD4+ T cells. The mechanism through which ATM kinase is regulated remains unclear; however, our initial studies suggest that ATM may act through endoplasmic reticulum (ER) stress responses as inhibition of ATM coincided with increased expression of the spliced form of X-box protein-1 (XBP-1) mRNA. This study characterizes a new function of the ATM pathway for regulation of the IL-23/Th17 axis.

Materials and Methods

Reagents

Endotoxin-free reagents were used throughout. Recombinant human (rh) GM-CSF (PeproTech, Rocky Hill, NJ), rhIL-4 and rhIFN-γ (R&D Systems Europe, Oxford, U.K.), and Ultrapure TLR agonists (Salmonella Minnesota LPS) were obtained from InvivoGen (San Diego, CA). Wortmannin was purchased from Merck (Calbiochem, San Diego, CA). KU55933 and NU1025 were obtained from Toecis (Bristol, U.K.), and KU0063794 was from Selleck Chemicals (Houston, TX). The following Abs were used to detect phospho-ATM: mouse anti-human ATM phospho-Ser1981 from Millipore (Billerica, MA), rabbit anti-human ATM phospho-Ser1981 from Cell Signaling Technology (Danvers, MA), and IRDye 800CW donkey anti-rabbit IgG (H+L) and IRDye 680 donkey anti-mouse IgG (H+L) were purchased from Li-COR Biosciences (Cambridge, U.K.). Mouse anti-human CD4+ Ab was obtained from Sigma-Aldrich (Dorset, UK). Mouse anti-human CD4+PE Ab was from BD Biosciences (Oxford, U.K.), and mouse anti-human CD4-PECY7 Ab and mouse antihuman CD45RA-FITC Ab were from eBioscience (San Diego, CA). For Th cell activation, mouse anti-human CD28 mAb was obtained from BD Biosciences.

moDC and myDC

Peripheral blood was obtained with the approval of relevant ethical review boards. Whole blood (Buffy coat) was purchased from National Blood Service (Oxford, U.K.) and fractionated (Ficoll 1.077; Histopaque 1077; Sigma-Aldrich). myDC were isolated using the Miltenyi Biotec CD1c myeloid DC kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer’s instructions. Briefly, B cells were depleted using anti-CD19 microbeads and resuspended in RPMI 1640 plus 10% FCS plus 1% sodium pyruvate (all from Sigma-Aldrich) plus rhGM-CSF and rhIL-4 (1000 U/ml). Supernatant was collected after 48 h and the sensitivity was 9 pg/ml. IL-1α was measured with assays from R&D Systems, and assay sensitivity was 3.9, 7.8, and 161.3, respectively.

Secreted cytokine determination

Unreated iDC and drug-treated iDC were stimulated with LPS (500 ng/ml) and rhIFN-γ (1000 U/ml). Supernatant was collected after 48 h and the secretion of IL-23p19/p19 and IL-12p70 determined by commercial human IL-23 Ready-set-go ELISA (eBioscience, San Diego, CA) and human IL-12p70 ELISA kits (BD BioSciences, Oxford, U.K.). Assays did not significantly react with other proteins, and the sensitivities were 15 and 7.8 pg/ml, respectively. IL-6 was measured with assays from ImmunoTools (Friesoythe, Germany). The sensitivity was 9 pg/ml. IL-1β, IL-17, and IL-27 were measured with DuoSet assays (R&D Systems), and assay sensitivity was 3.9, 7.8, and 161.3, respectively. Absorbance was measured at 450 nM using a spectrophotometer.

Measurement of TGF-β

The TMLC coculture assay was used to detect active TGF-β secreted by DC. TMLC cells were harvested by trypsinization and resuspended in serum-free DMEM at 5 × 10^5/ml. The medium was removed from the experimental cells, and an equal volume (100 μl) of TMLC cells and medium containing experimental stimulant was added on day 3, and immature moDC were harvested at days 5 to 6. After induction, the harvested macrophages were combined with flow cytometry using the following markers: immature DC (iDC) were confirmed to be CD1a+, CD11c+, CD14-, CD40lo, CD86+, CD80lo, CD83-, CD86-, CD209+, and MHC class II (data not shown).

Quantitation of mRNA levels

RNA was isolated using Nucleospin RNA II Extraction kits (Macherey-Nagel, Düren, Germany). CDNA was prepared using the MessageAmp Reverse Transcription Kit (Ambion Austin, TX). Real-time PCR was performed using TaqMan PCR Core Reagents Kit (Applied Biosciences, Carlsbad, CA) and a Stratagene MXP 3000 (Stratagene). Primers and probe for the housekeeping gene Top1 were from Applied Biosystems. The

Downloaded from http://www.jimmunol.org/ by guest on April 22, 2017
following oligonucleotide sequences were used as sense primers, antisense primers, and probes, respectively: 5′-TACCTGGGCTTCAGCCAAC-3′, 5′-GAAGGTTTTGAAAGGCGAAA-3′, and 5′-6′Fam-CTCATCAGCCAACGTCGCCATT-5′, and 5′-6′Fam-CACAGAGAGAGAGTAGAATT-5′, and 5′-6′Fam-CAGCCCAAGGACCGTCTATAGCT(Tamra)(phosphate)-3′ for p40.

Primers and probes were used at 15 pmol/μL. Reactions (20 μL) were performed in triplicate and standardized to a Top1 control for each sample. IL-23p19 and p40 mRNA levels were expressed relative to the maximal levels following LPS stimulation using threshold cycle (Ct) and normalized according to Top1 levels as previously described (29). The CT values were calculated according to the formula relative value: 2^(-ΔΔCt) = 2^(-ΔCt) = 2^(-CtTop1 stimulated)/2^(-CtTh17 baseline) – 2 – (CtTh17 baseline).

Pharmacological dissection of intracellular signaling

Specific kinase inhibitors (dissolved in DMSO) were used to dissect the role of signaling pathways. IC50 were obtained in 96-well tissue-culture plates and exposed to inhibitors for 1 h prior to stimulation via TLR4. The PI3K inhibitor WM was used at 10 μM. The ATM inhibitor KU55933 was used at up to 10 μM, the mTOR inhibitor IN2063794 at up to 1 μM, and the poly(ADP-ribose) polymerase (PARP) inhibitor NU1025 at up to 300 nM.

Cell viability

All drugs were used at concentrations in agreement with established in vitro biological systems and experimental IC50 values. Nevertheless, treatment of cells with PI3K, ATM, mTOR, or PARP inhibitors and LPS was confirmed to have no deleterious effect on DC viability as determined by dye exclusion (not shown).

Western blotting and intracellular staining for phospho-ATM

Cell lysates were prepared in RIPA buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS) containing protease inhibitors (2 mM AEBSF, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, and 0.3 μM aprotinin; Sigma-Aldrich) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich). The protein content of lysates, cleared by centrifugation (13,000 × g), were determined using a modified Bradford assay (B-acid: copper sulfate at 50:1; Sigma-Aldrich). Proteins (20 μg) were resolved on a 10% SDS-PAGE gel using Tris-glycine buffer. Following electrophoresis, proteins were transferred to nitrocellulose membrane and blocked by incubation in PBS plus 0.1% Tween 20 containing milk (5% w/v dried milk powder). For two-color infrared imaging, the membrane was incubated with primary Ab and loading control Ab and cells acquired using a Beckman Coulter FC500 (Beckman Coulter). Data was analyzed using FlowJo software (Tree Star).

Generation of Th17 responses

Naive CD4+CD45RA+ T cells were isolated using a Miltenyi Biotec naive CD4+ cell isolation kit II (Miltenyi Biotec) to a typical purity of >95%. Cells were differentiated in the presence of combinations of recombinant cytokines (IL-1β [5 ng/ml], IL-6 [5 ng/ml], IL-23 [10 ng/ml], and TGF-β [5 ng/ml]; all from R&D Systems) and costimulated with anti-CD28 (5 μg/ml) and anti-CD3 (OKT3; 1 μg/ml) Abs in the presence of low-dose IL-2 (50 IU/ml; R&D Systems) (48). Cytokines were added simultaneously at 0 h. Secretion of IL-17A was determined.

Naive CD4+CD45RA+ T cells were treated previously with KU55933 and TLR4 stimulation (LPS) for 12 h before the supernatant was discarded and replaced with fresh media to prevent ATM inhibitor from affecting T cells. We have previously shown that IL-23 is not produced until >12 h after TLR stimulation (28). After 24 h, supernatants were harvested and mixed 1:1 with fresh T cell medium (RPMI 1640, 7.5% normal human AB serum [Sigma-Aldrich], 1% sodium pyruvate, 2 mm l-glutamine, 1 mM HEPES [Life Technologies], and 1% antibiotic-antimycotic [Sigma-Aldrich]) used to stimulate naive CD4+ T cells. Following 5 d primary stimulation with anti-CD3 and anti-CD28, T cells were harvested and rested for 3–5 d before restimulating with anti-CD3 and anti-CD28 for 48 h. Secretion of IL-17A was determined.

Quantitation of mRNA levels of spliced XBP-1 with real-time RT-qPCR

RNA was isolated and reverse transcribed as previously described. Real-time PCR was performed using KAPA SYBR FAST Universal 2x qPCR Master Mix (KAPA Biosystem, Woburn, MA) and a Stratagene MXP 3000 (Stratagene). Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). The following oligonucleotide sequences were used: 5′-TGCTGACGCGAGGGTGTTG-3′ and 5′-CACCTTGGCTCTGGAAATGC-3′ for spliced XBP-1 (sXBP-1) and 5′-GACACTGGCAAACATG-3′ and 5′-AACAATGTCGGCTTAAATC (phosphate)-3′ for hypoxanthine phosphoribosyltransferase (HPRT).

Primers and probes were used at 10 pmol/μL. Reactions were performed in triplicate and standardized to an HPRT housekeeping gene control for each mRNA. sXBP-1 mRNA levels were expressed relative to the levels of resting iDC using Ct and normalized according to HPRT levels as previously described. The relative thresholds were calculated according to the formula relative value = 2^(-ΔΔCt) = 2^(-CtTh17 stimulated)/2^(-CtTh17 baseline) – 2 – (CtTh17 stimulated).

Results

IL-23 plays an important role for human Th17 generation

Although the role of IL-1β and IL-6 in Th17 differentiation is undisputed, the importance of other cytokines is debatable, a problem exacerbated by differences between murine and human systems. Therefore, we initially determined the relative importance of IL-23 in the differentiation of human Th17 responses. Freshly isolated human naive CD4 T cells with a typical purity of at least 95% CD4+CD45RA+ (Fig. 1A) were costimulated in the presence of rIL-1β, IL-6, IL-23, and TGF-β. Naive T cells activated through CD3/CD28 alone did not secrete IL-17A or secreted very low levels of IL-17 (Fig. 1B) in all donors studied. The inclusion of IL-1β and IL-6 resulted in a pronounced increase in IL-17 secretion in all donors (p < 0.05). The addition of IL-23 to IL-1β and IL-6 markedly enhanced the secretion of IL-17A in all donors tested (p < 0.01). Further addition of TGF-β to IL-1β/IL-6 or IL-1β/IL-6/IL-23 had little effect on IL-17 secretion. Irrespective of the presence of TGF-β, IL-23 exerted a pronounced effect on CD4 polarization, leading to an increase in IL-17A levels (p < 0.001). Addition of IL-23 to IL-1β and IL-6 conditioning led to a significant increase in IL-17A (p < 0.01) over all 11 individual experiments (Fig. 1C). It is important to note that the addition of IL-23 alone to costimulated T cells did not elicit any detectable Th17 responses (data not shown).

The ATM pathway regulates IL-23 production by DC and ATM-inhibited DC prime greater Th17 responses

To define the mechanism by which WM increased the secretion of IL-23 by LPS/IFN-γ-activated DC as shown in our previous study.
FIGURE 1. The importance of IL-23 for differentiation of human Th17 responses. (A) Naive human CD4+CD45RA+ T cells were purified from peripheral blood. Typical purity was >95% CD4+CD45RA+; data are representative of eight independent donors. (B) Naive CD4+CD45RA+ T cells were costimulated for 5 d with anti-CD28 in the presence of recombinant cytokines. After resting for 2 d, cells were restimulated with anti-CD3 and IL-17A release was determined by ELISA (two representative donors from 11 experiments are shown). (C) Summary plot showing the effect of addition of IL-23 to IL-1 and IL-6-primed Th17 responses from all 11 independent experiments. **0.01 > p ≥ 0.001, ***p < 0.001.

We next determined if inhibition of ATM influenced transcription of the IL-23 genes. Treatment of moDC with KU55933 prior to stimulation resulted in a marked increase in p19 transcription compared with LPS/IFN-γ alone in all three donors tested (Fig. 3F). On average, inhibition of ATM resulted in an 8-fold increase in IL-23p19 mRNA levels compared with the peak transcription level with LPS/IFN-γ only. The addition of ATM inhibitor also resulted in increased transcription of the p40 (in two of three donors studied) and p35 genes (Fig. 3G, 3H, respectively), though the magnitude of effect on p35 was smaller than for p19 and p40.

Regulation of other cytokines by ATM kinase

We previously showed that WM (1 μM) affected cytokines other than IL-23, although to a lesser extent. In particular, IL-12p70 secretion was increased ~2-fold (29). As shown in Table I, whereas treatment of moDC with KU55933 resulted in a marked

ATM regulates IL-23 production by moDC and myDC

Our earlier work showed that WM required TLR4 triggering for DC to produce IL-23 (29). Similarly, treatment with KU55933 alone or in the presence of IFN-γ did not elicit IL-23 secretion (Fig. 3A). The increased IL-23 secretion observed following ATM inhibition was independent of IFN-γ (Fig. 3B). In the four donors, KU55933 caused a 4.2-fold increase from 1020 ± 728 pg/ml (LPS alone) to 4286 ± 1029 pg/ml (KU pretreatment with LPS) (p < 0.01) and with the addition of IFN-γ KU55933 lead to a 4.0-fold increase from 3810 ± 1202 (LPS/IFN-γ) to 15,075 ± 807 pg/ml (KU pretreatment with LPS/IFN-γ) (p < 0.01). Inhibition of mTOR, another potential WM target, did not significantly potentiate IL-23 release (p > 0.05; Fig. 3C). Furthermore, inhibition of PARP, using selective PARP inhibitor NU1025, also had no effect on IL-23 (p > 0.05; Fig. 3C).

 Although moDC are used as a model of physiological myDC, they are acknowledged to have some key differences. Therefore, we determined if the effect of ATM inhibition also occurred in myDC, freshly isolated from healthy peripheral blood. In agreement with moDC, treatment of myDC with KU55933 resulted in a marked increase in IL-23 in all four donors tested (Fig. 3D).
increase in IL-23 secretion in response to LPS/IFN-γ, it did not significantly affect IL-1β, IL-6, IL-27, or TGF-β. In contrast to the effect of WM in our previous study, KU55933 only increased IL-12p70 secretion by 1.6-fold. The median fold increase in secretion of IL-6 was 3-fold (not significant).

Ionizing radiation activates ATM, resulting in suppression of IL-23

As inhibition of ATM was observed to increase IL-23 expression, we hypothesized that ATM activation would suppress IL-23 production. Therefore, we exposed DC to low doses of ionizing radiation (2–6 Gy), sufficient to cause activation of ATM in a variety of cell types (49, 50). Exposure of immature moDC to x-rays resulted in a pronounced increase in the phosphorylation state of ATM, as demonstrated by intracellular staining with Western blotting and flow cytometry (Fig. 4A, 4B). Treatment of DC with KU55933 prior to irradiation reduced phospho-ATM expression to levels similar to those observed in nonirradiated cells (Fig. 4A, 4B). Irradiation of immature moDC with as little as 2 Gy resulted in significant suppression in their IL-23 response to TLR4 agonist: \( p < 0.05 \) for six of six donors (Fig. 4C, showing one representative). Blockade of ATM-activity by treatment with KU55933 prior to irradiation abrogated the inhibitory effect of x-rays on IL-23 production (Fig. 4C). It should be noted that whereas in KU55933-treated DC, the baseline levels of IL-23 secretion were substantially greater than in control cells, the absence of ATM function prevented the suppressive effect of radiation. Importantly, exposure of DC to x-rays had no deleterious effect on their viability either immediately following radiation or 48 h later (data not shown). Preincubation of DC with KU55933 significantly \( (p < 0.001) \) prevented the reduction in IL-23 production caused by irradiation in all donors (Fig. 4D).

ATM inhibition activates ER stress responses, leading to increased XBP-1s transcription

Previous studies (51) found that tunicamycin-induced ER stress was enhanced in ATM-deficient human lung fibroblast cells compared with the wild-type controls characterized with increased...
level of XBP-1 splicing. More recently, Goodall et al. (52) stated that activation of ER stress in combination with TLR ligands lead to a marked increase of IL-23p19 transcription as a result of increased level of ER stress–induced C/EBP homologous protein transcription. Although the precise molecular mechanisms by which ATM regulates IL-23 expression are beyond the scope of this study, we wished to determine if there was a potential role for ER stress responses in this regard. Therefore, we investigated the generation of the XBP-1s spliced mRNA species, a key early signaling component of ER stress responses, in the presence of functional or inhibited ATM. mRNA for XBP-1s was induced 6-fold following treatment of DC with LPS/IFN-γ, and the levels of this transcript started to decline after 2–6 h, returning to baseline levels after 20 h (Fig. 5A). However, following inhibition of ATM with KU55933, the level of LPS-induced XBP-1s transcripts was consistently higher. ATM inhibition resulted in a 2-fold increase in peak levels of XBP-1s mRNA compared with LPS/IFN-γ alone. In some donors, there was sustained elevated XBP-1s mRNA in the presence of ATM inhibitor; however, in all instances, the levels of XBP-1s returned to baseline after 20 h. As expected, elevated XBP-1s transcription was associated with increased expression of the IL-23p19 gene (Fig. 5B). The peak transcriptional changes in IL-23p19 occurred at 9–12 h postactivation, which followed the peak in XBP-1s mRNA levels in all three donors. As expected, this was followed by a substantial increase in IL-23 release (Fig. 5C).

Discussion
IL-23 is involved in Th17 responses, and understanding the mechanisms of its regulation has ramifications for human biology, pathology, and therapeutics. IL-23 is currently an important target for chronic diseases such as RA and multiple sclerosis, and clinical inhibitors have been developed (e.g., ustekinumab, briakinumab, secukinumab). However, in contrast to IL-12, there is a limited understanding of IL-23 regulation, and little is known of the up-
stream mechanisms controlling its expression. Our study identifies ATM as a novel regulator for IL-23 in human DC, including ex vivo myDC. Inhibition of ATM increased IL-23 expression, whereas ATM activation suppressed IL-23 responses.

ATM is a serine/threonine kinase containing a C-terminal PI3-kinase–related domain where the catalytic site resides. It is the key protein coordinating cellular responses to DSB. Many proteins are phosphorylated by ATM, resulting in cell-cycle control and activation of DNA repair systems (53). In addition to the canonical route of activation through ionizing radiation, ATM is activated by oxidative stress, in the absence of DSB, and so may also regulate global cellular responses to other forms of stress (54, 55). Several lines of evidence support our findings of an immune-regulatory role for ATM. Westbrook and Schiestl (45) showed dextran-induced colitis was more severe in Atm−/− mice, and this was associated with increased p19 mRNA. The persistent activation of immune responses observed in Atm−/− mice supports our concept of an immune-regulatory role of ATM. ATM deficiency occurs in RA patients in whom both naive and memory CD4+ T cells carry substantial damaged DNA due to insufficient repair (56). Furthermore, Weintz and colleagues (46) showed ATM inhibition associated with IL-10, CCL2, and CXCL10 expression in mouse bone marrow.

The KU55933 compound is the inhibitor of choice for ATM studies (57–59). It is a potent, highly selective, and competitive inhibitor with an IC50 for ATM of 13 nM. In contrast, other targets require substantially greater concentrations to inhibit function (e.g., DNA-PK, mTOR, PI3K, PI4K, and ATM and Rad3-related are inhibited with IC50 of 2500, 9300, 16,000, >100,000, and >100,000 nM, respectively). The 200-fold difference in IC50 between ATM and the next target makes KU55933 very useful. As described in our previous work, WM inhibits PI3K with an IC50 of 4 nM, whereas it inhibits other targets, such as smooth muscle myosin L-chain kinase, Na/K-ATPase, mTOR, DNA-PK, ATM, and ATM and Rad3-related at 200, 130, 200, 16, 150, and 1800 nM respectively (39, 40). In addition, WM also inhibits members of the polo-like kinase family at similar concentrations to PI3K (60). It is critical to appreciate off-target effects when using kinase inhibitors to dissect function. Alternative approaches include the use of small interfering RNA, and in this regard, we made repeated attempts to knock down ATM expression using a panel of small interfering RNA (data not shown). However, to date, we

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LPS/IFN-γ Mean</th>
<th>SD</th>
<th>KU 10 μM + LPS/IFN-γ Mean</th>
<th>SD</th>
<th>n</th>
<th>p Value</th>
<th>Mean Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-23 (pg/ml)</td>
<td>2,758</td>
<td>2,160</td>
<td>11,115</td>
<td>6,796</td>
<td>32</td>
<td>&lt;0.00001</td>
<td>4.9</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>NS</td>
<td>1.0</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>29,508</td>
<td>24,940</td>
<td>77,096</td>
<td>596,623</td>
<td>5</td>
<td>NS</td>
<td>3.4</td>
</tr>
<tr>
<td>IL-12 (pg/ml)</td>
<td>52,096</td>
<td>25,894</td>
<td>83,555</td>
<td>21,266</td>
<td>6</td>
<td>0.045</td>
<td>2.0</td>
</tr>
<tr>
<td>IL-27 (pg/ml)</td>
<td>720</td>
<td>152</td>
<td>1,072</td>
<td>295</td>
<td>5</td>
<td>NS</td>
<td>1.5</td>
</tr>
<tr>
<td>TGF-β (pg/ml)</td>
<td>480</td>
<td>260</td>
<td>1,097</td>
<td>802</td>
<td>7</td>
<td>NS</td>
<td>2.2</td>
</tr>
</tbody>
</table>

The role of ATM for other cytokines was determined using ELISA for IL-23, IL-1β, IL-6, IL-12, IL-27, and TGF-β. Data from 5–32 donors are shown for moDC treated with LPS/IFN-γ alone or in the additional presence of 10 μM KU55933. The fold change for individual donors is given, and the p value is obtained from biological triplicate experiments.
have not been able to achieve complete knockdown of ATM in human DC. In addition to incomplete knockdown, one of the key issues we encounter is the susceptibility of DC activation by manipulation. Achieving the balance between adequate knockdown without activation in primary human DC presents a substantial challenge.

Although relatively little is known concerning regulatory pathways for IL-23, the Ro52 E3 ubiquitin ligase is implicated (61). Ro52 restricts the production of IL-23 and IFN-β, and Ro52 loss increases cytokine secretion. The action of Ro52 is mediated by IFN regulatory factor (IRF) 3 (62, 63), and the IL-23p19 gene has IRF3/7 binding sites. Activation of IRF3 is regulated by TANK binding kinase-1 that is phosphorylated upon TLR3 or RIG-I ligation (64, 65). At present, we do not know if Ro52 plays any role in the function of ATM as a regulator of IL-23 expression by DC. Furthermore, a role for IRF3 in permitted transcription following ATM blockade remains the subject of ongoing investigations.

The effect of x-rays on IL-23 production has not previously been reported. However, it has been shown to inhibit IL-12 secretion (66). We used typical therapeutic fractional doses of x-rays (<6 Gy). In contrast, other studies have investigated doses of radiation up to 30 Gy (66, 67). Interestingly, in these studies, IL-12p70 secretion was inhibited at similar levels of irradiation to our observations with IL-23 and no additional suppression of IL-12p70 occurring beyond 8 Gy. The accumulation of phosphorylated ATM upon X-ray exposure clearly indicated the sensing of this insult by DC. Importantly, prevention of ATM activity using KU55933 restored IL-23 levels to those of nonirradiated cells, indicating a role of ATM in IL-23 regulation.

We propose that ATM is not just a sensor of DNA damage and oxidative stress, but also serves as a more generalized stress-sensing system for which activation has immune sequelae. We are currently investigating exactly how ATM mediates immune regulation, and in this regard, ATM has been described to associate with several proteins not involved with DNA repair (68). Furthermore, ATM has recently been shown to interact with NF-κB essential modulator, thus promoting NF-κB–dependent signaling by increasing nuclear translocation (69). One candidate pathway is the interaction of ATM with intracellular stress-sensing systems in the ER. Studies of ER stress in macrophages show a pronounced increase in cytokines following TLR4 triggering (70); however, little is known concerning its role in DC. In contrast to other cells, the high rates of protein turnover in DC create a state of elevated ER stress, and this may play a role in development and survival (71). More recently, a role for ER stress in TLR responses has emerged (72). Importantly, ER stress in macrophages only activated a restricted profile of cytokine genes (73). When stress was induced by pharmacologic agents and misfolded HLA, it resulted

![FIGURE 5. ATM inhibition increases LPS-dependent sXBP-1 mRNA. RNA from iDC, LPS-matured DC (square symbol), and KU55933 (10 μM) pretreated matured DC (triangle) was extracted and analyzed for XBP-1s and IL-23p19 mRNA. (A) Quantitative PCR results were calculated relative to sXBP-1 level normalized to HPRT and plotted as a fold change compared with sXBP-1 expression by iDC. Data are given for three donors. (B) Expression of IL-23p19 gene. (C) Secretion of IL-23 (determined in parallel wells by ELISA at 24 h) from the corresponding donors shown in (A) and (B). **0.01 > p ≥ 0.001, ***p < 0.001.](http://www.jimmunol.org/Downloadedfrom)
in IL-23 (but not IL-12) and Th17 responses. We used the generation of a spliced transcript (XBPs-1s) as an indicator of ER stress activation. XBPs1 is a key early component of the ER stress response (74), generated when IRE1 acts on XBPs-1 to splice the 26-bp intron from XBPs-1 mRNA. In doing so, IRE1 removes a premature stop codon, allowing transcription of the alternate spliced form, XBPs-1s. Our observation of increased XBPs-1s mRNA followed by coordinated increase in p19 mRNA in cells treated with ATMediator suggests that ATM is to some extent regulating IL-23 through the ER stress response. This agrees with the increased level of C/EBP homologous protein, as a key component of ER stress was responsible for a marked increased level of 23p19 in U397 cells (52).

Several studies also indicate a role for ATM in stress responses. Guo and colleagues (54, 55) highlighted an alternate ATM-signaling pathway, the regulation of IL-23 through the ER stress response. This agrees with the ER stress activation. XBP1s is a key early component of the ER stress response to TLR agonists. In doing so, IRE1 removes a 26-bp intron from XBP-1 mRNA. In doing so, IRE1 removes a premature stop codon, allowing transcription of the alternate spliced form, XBP-1s. Our observation of increased XBP-1s mRNA followed by coordinated increase in p19 mRNA in cells treated with ATMediator suggests that ATM is to some extent regulating IL-23 through the ER stress response. This agrees with the increased level of C/EBP homologous protein, as a key component of ER stress was responsible for a marked increased level of 23p19 in U397 cells (52).

We showed the ATM pathway regulated IL-23 in human DC, and this impacted on Th17 responses. In contrast, ATM activation repressed IL-23, and pharmacological inhibition of ATM enhanced these responses. Manipulating the activation of ATM in the immune system creates an opportunity to enhance or repress Th17 responses, and this has implications for therapeutic approaches based on immune modulation in vaccines and in autoimmunity.

Acknowledgments

We thank Dr. Monica Pallis for generous assistance with phospho-ATM flow cytometry and Dr. Stewart Martin for assistance in irradiating cells. The TMLC reporter assay was kindly conducted by Jo Porte (Respiratory Medicine, University of Nottingham). We also thank Dr. Jane Goodall and Prof. Hill Gaston (Department of Medicine, University of Cambridge) for their help with discussions regarding ER stress responses and XBPs-1 and the Wellcome Trust for the provision of a state-of-the-art tissue culture cell irradiation facility.

Disclosures

The authors have no financial conflicts of interest.

References

19. Guo and colleagues (54, 55) highlighted an alternate ATM-signaling pathway, the regulation of IL-23 through the ER stress response. This agrees with the ER stress activation. XBP1s is a key early component of the ER stress response to TLR agonists. In doing so, IRE1 removes a 26-bp intron from XBP-1 mRNA. In doing so, IRE1 removes a premature stop codon, allowing transcription of the alternate spliced form, XBP-1s. Our observation of increased XBP-1s mRNA followed by coordinated increase in p19 mRNA in cells treated with ATMediator suggests that ATM is to some extent regulating IL-23 through the ER stress response. This agrees with the increased level of C/EBP homologous protein, as a key component of ER stress was responsible for a marked increased level of 23p19 in U397 cells (52).

We showed the ATM pathway regulated IL-23 in human DC, and this impacted on Th17 responses. In contrast, ATM activation repressed IL-23, and pharmacological inhibition of ATM enhanced these responses. Manipulating the activation of ATM in the immune system creates an opportunity to enhance or repress Th17 responses, and this has implications for therapeutic approaches based on immune modulation in vaccines and in autoimmunity.


