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

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Abstract

Little is known of the regulation of interleukin-23 secretion in dendritic cells (DC) despite its importance for human Th17 responses. Here we show for first time 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 from human monocyte-derived DC (moDC) and freshly isolated myeloid DC (myDC). In contrast, inhibiting the closely related mammalian target of rapamycin (mTOR) had no effect on IL-23. Priming naïve CD4+ T-cells with ATM-inhibited DC increased Th17 responses over and above those obtained with mature DC. Whilst ATM-blockade increased the abundance of p19, p35 and p40 mRNA, IL-12p70 secretion was unaffected. In order 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 ER-stress responses by measuring generation of the spliced form of X-box protein-1 (XBP1s), a key ER-stress transcription factor. Inhibition of ATM increased the abundance of XBP1s mRNA and this was followed 3hr later by increased peak p19 transcription and IL-23 release. In summary, ATM-activation or inhibition respectively inhibited or augmented IL-23 release. This novel role of the ATM pathway represents a new therapeutic target in autoimmunity and vaccine development.

Keywords

Dendritic Cell; IL-23; Th17; ATM

Introduction

Expression of interleukin-23 (IL-23), a heterodimeric cytokine comprising the unique p19 subunit (1) and a p40 subunit shared with IL-12p70, is tightly controlled. IL-23 secretion is...
largely restricted to APC, including monocyte-derived dendritic cells (moDC), myeloid DC (myDC) (2), macrophage (3) and microglia (4) in response to immune danger. However, in contrast to IL-12, relatively little is understood of factors involved in the regulation of IL-23 production. The differentiation of naïve CD4+ T cells into specialised T-helper (Th) effector subtypes is regulated by cytokines derived from DC. Interferon-γ (IFNγ) producing Th1 cells are generated in response to IL-12 whilst IL-4 drives the development of Th2. In contrast, the presence of IL-1 and IL-6 induces inflammatory Th17 responses whilst IL-23 promotes the survival and/or expansion of Th17 cells (5, 6). The absence of IL-23 exerts a pronounced impact on Th17 responses and IL-23p19 deficient animals have depressed Th17 numbers and fail to develop *Mycobacterium tuberculosis* and pertussis toxin induced encephalomyelitis (7).

The inflammatory role of Th17 cells is largely mediated by production of IL-17A, IL-17F, IL-21, IL-22, tumour necrosis factor (TNF) and IL-6 (8, 9), whose roles in inflammation and autoimmune diseases are established (10). Increased IL-17 levels are found in autoimmune diseases such as rheumatoid arthritis (RA) (11), multiple sclerosis (MS), inflammatory bowel disease (IBD) and psoriasis. However the role of IL-17 in malignancy remains controversial as on the one hand it can promote invasion and angiogenesis, whilst on the other Th17-cell may enhance tumour rejection (12) (13) (14) (15) (16) (17).

The role for IL-23 in human Th17 generation is established (18) whilst that of TGF-β remains controversial (19-21). IL-23 itself plays a role in immunity to infectious organisms including fungal infections (23, 24) and activates macrophages to produce TNF-α and nitric oxide (25). Transgenic mice over expressing the IL-23p19 subunit suffer from severe multi-organ inflammation, failure to thrive, infertility and premature death characterised by increased levels of TNFα and IL-1 (25).

Despite the important roles for IL-23, our understanding of factors governing its regulation and the signaling events involved is limited. In contrast, considerably more is known regarding IL-12 regulation. In TLR4-dependent signaling events, mitogen-activated protein kinase (MAPK) and nuclear factor-κβ (NF-κβ) signaling cascades are activated by both lipopolysaccharide (LPS) and lipoteichoic acid which leads to differential activation of p44/p42 extracellular signal-related kinase (Erk) 1/2, p38 MAPK and Jun N-terminal kinase (JNK) and IL-12 gene expression (26-28). In this setting the p38 MAPK is generally accepted to play a positive role in IL-12 family cytokine regulation (29). 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 (28). We have previously shown that LPS/IFN-γ-induced IL-23 is completely blocked by a p38 MAPK inhibitor SB203580 (30). Conversely, IL-23p19 transcription was increased in LPS-triggered macrophages after p38 MAPK blockage (31). Zymosan is well known to induce secretion of IL-6, IL-10, TGF-β and IL-23 from DC (32) by binding to the Dectin-1 and TLR-2 receptors (33) and signaling through spleen tyrosine kinase (Syk) (34, 35), caspase recruitment domain family member 9 (CARD9) (36) and NF-κβ (36, 37). Furthermore a role for PI3K in expression of the IL-12 family cytokines has been described (38, 39).

Recently (30), 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 (40, 41). Whilst other studies have also used WM at higher concentrations to dissect PI3K function (up to 20 μM (40, 42, 43)) it is recognized that at these levels WM inhibits additional kinases. Many of these alternate targets are PI3K-related kinases including smooth muscle myosin light chain kinase (smMLCK) (40), Na/K-ATPase (44),

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mTOR, DNA-dependent Protein kinase (DNA-PK) (43), ataxia-telangiectasia mutated (ATM), and PI4-kinase α and β (45). However, whilst 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 (46). The ATM kinase is activated as part of the LPS-regulated phosphorylation pathways in mouse bone marrow-derived DC (BM-DC) but their function in this regard has not yet been demonstrated (47). ATM was recently shown to play an immune-regulatory role for human IFN-responses in T-cells (48). Treatment of T-cells with etoposide induced DNA damage and the ensuing ATM-activation led to induction of IFN-stimulated, and IFNα and γ genes (48).

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 signalling with ionizing radiation suppressed IL-23 responses and this was prevented by inhibiting ATM-function. Inhibition of ATM in TLR-4 triggered DC not only resulted in exacerbated IL-23 responses but increased the level of Th17 responses generated from naïve CD4+ T-cells. The mechanism through which ATM kinase is regulated remains unclear, however our initial studies suggest that ATM may act through ER-stress responses as inhibition of ATM coincided with increased expression of the spliced form of XBP1 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, UK) and Ultrapure TLR-agonists (S. Minnesota LPS) were obtained from InvivoGen (San Diego, CA). Wortmannin was purchased from Merck (Calbiochem, San Diego, CA), KU55933 and NU1025 were obtained from Tocris (Bristol, UK) and KU0063794 was from Selleck Chemicals (Houston, TX). The following antibodies were used to detect phospho–ATM: mouse anti-human ATM phosphoSer1981 from Millipore (Billerica, MA), rabbit anti-human ATM phospho-Ser1981 from Cell Signaling Technology (Danvers, MA), IRDye 800CW donkey anti-rabbit IgG (H+L) and IRDye 680 donkey anti-mouse IgG (H+L) were purchased from Li-COR Biosciences (Cambridge, UK). Mouse anti-human β-Actin antibody was obtained from Sigma. Mouse anti-human CD4-PE antibody (BD Biosciences, Oxford, UK), mouse anti-human CD4- PECy7 antibody and mouse antihuman CD45RA-FITC antibody from eBiosciences (San Diego, CA). For Th-cell activation, mouse anti-human CD28 monoclonal antibody was obtained from BD Biosciences (Oxford, UK).

Monocyte derived (moDC) and myeloid DC (myDC)

Peripheral blood was obtained with the approval of relevant ethical review boards. Whole blood (Buffy coat) was purchased from National Blood Service, Sheffield and fractionated using Ficoll 1.077 (Histopaque 1077, Sigma, Dorset, UK). myDC were isolated using Miltenyi (Miltenyi Biotech, Bergisch Gladbach, Germany) CD1c myeloid DC kit as per manufacturer’s instructions. Briefly, B cells were depleted using anti-CD19 microbeads before positive selection of CD1c+ cells. myDC were routinely >95% pure by flow cytometry. myDC were cultured in DC medium (RPMI 1640 + 10% FCS + 1% sodium
pyruvate, all Sigma) + rhGM-CSF and rested for 2-4 hrs at 37°C before drug treatment or TLR stimulation (LPS). CD1c- cells were incubated with anti-CD14 microbeads (Miltenyi), CD14+ monocytes (>95% pure by flow cytometry) were positively selected and resuspended at 10^6/ml in DC media with rhGM-CSF (1000U/ml) and rhIL-4 (1000U/ml) for 5 days. An additional 50% of medium with cytokines was added on day 3 and immature moDC were harvested at day 5-6.

The expected phenotype of DC and macrophages was confirmed with flow-cytometry using the following markers: Immature DC (iDC) were confirmed to be CD1a+, CD11c+, CD14−, CD40lo, CD54+, CD80lo, CD83−, CD86−, CD209+, MHC class II+ (data not shown).

**Secreted cytokine determination**

Untreated immature DC (iDC) and drug-treated iDC were stimulated with LPS (500ng/ml) and rhIFNγ (1000U/ml). Supernatant was collected after 48h and the secretion of IL-23p40/ p19 and IL-12p70 determined by commercial human IL-23 Ready set-go ELISA (eBioscience, San Diego, CA) and human IL-12p70 ELISA kit (BD Biosciences, Oxford, UK). Assays did not significantly react with other proteins and the sensitivities were 15pg/ml and 7.8pg/ml respectively. Interleukin-6 was measured with assays from ImmunoTools (Friesoythe, Germany). The sensitivity was 9pg/ml. IL-1β, IL-17 and IL-27 were measured with DuoSet assays (R&D Systems) and assay sensitivity was 3.9pg/ml, 7.8pg/ml and 161.3pg/ml respectively. Absorbance was measured at 450nM using a spectrophotometer.

**Measurement of TGFβ**

The TMLC coculture assay was used to detect active TGFβ 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 (100μl) of TMLC cells and medium containing experimental stimulant was added. Cells were incubated at 37°C overnight, and washed in PBS before lysis in reporter lysis buffer (Promega, Hampshire, UK). Cells were agitated with a pipette and centrifuged at 1500×g for 5min at 4°C. The supernatant was added to luciferase assay buffer (Promega, Hampshire, UK) and the luminescence measured using a MicroLumatPlus microplate luminometer (EG&G, Berthold, Hertfordshire, UK). TGFβ was quantified by comparing values obtained under experimental conditions to readings obtained from a standard curve derived from increasing concentrations of active TGFβ1.

**Quantitation of mRNA levels of p19, p35 and p40**

RNA was isolated using Nucleospin® RNA II Extraction kits (Macherey-Nagel, Dören, Germany). cDNA was prepared using MessageSensor™ 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. Primers and probe for the housekeeping gene Top1 were from Applied Biosystems. The following oligonucleotide sequences were used as sense primers, antisense primers and probes, respectively: 5’-TACTGGGCTCAGCCAAGCT-3’, 5’-GAAGGATTGTAAGCGAGAAG-3’, and 5’-(6-Fam) CCTCAGTCCACGCAAGCATG(Tamra)(phosphate)-3’ for p19; 5’-CTCCCTGGACCACTCCAGTTG-3’, 3’-GGTGAAAGGCGTGGGATCTT-5’, 5’ (6-Fam) CCAGAAACCTCCCGTGCCA(Tamra)(phosphate)-3’ for p35; 5’-CGGTCATCTTGCCGAAGCT-3’, 5’-TGCCCATTGCTGATTTT(6-Fam) CGGGCCAGGCCAGCTACTATGCT(Tamra)(phosphate)-3’ for p40.

Primers and probes were used at 15 pmol/μl. Reactions (20μl) were performed in triplicate and standardised to a Top1 control for each sample. IL-23p19 and p40 mRNA levels were expressed relative to the maximal levels following LPS stimulation using Ct threshold and
normalised according to Top1 levels as previously described (30). The relative levels were calculated according to the formula relative value = \(2 - \frac{\text{Ct } \text{IL-23p19 stimulated} - \text{CtTop1 stimulated}}{\text{Ct IL-23p19 iDC baseline} - \text{Ct Top1iDC baseline}}\).

### Pharmacological dissection of intracellular signaling

Specific kinase inhibitors (dissolved in DMSO) were used to dissect the role of signaling pathways. Immature DC were plated in 96-well tissue culture plates and exposed to inhibitors for 1h prior to stimulation via TLR4. The PI3K inhibitor Wortmannin was used at 10\(\mu\)M. The ATM inhibitor KU55933 was used at up to 10\(\mu\)M, the mTOR inhibitor KU0063794 at up to 1\(\mu\)M, and the PARP inhibitor NU1025 at up to 300nM.

### Cell viability

All drugs were used at concentrations in agreement with established in vitro biological systems and experimental IC\(_{50}\) 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 (20mM Tris, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1mM EDTA, 0.1% SDS) containing protease inhibitors (2mM AEBSF, 130\(\mu\)M Bestatin, 14\(\mu\)M E-64, 1\(\mu\)M Leupeptin, 0.3\(\mu\)M Aprotinin, Sigma) and phosphatase inhibitor cocktails 1 and 2 (Sigma). The protein content of lysates, cleared by centrifugation (13,000 \(\times\) g) were determined using a modified Bradford assay (B-acid: Copper sulphate at 50:1, Sigma). Proteins (20\(\mu\)g) 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-T (PBS + 0.1% Tween 20) containing milk (5% w/v dried milk powder). For two-colour infrared imaging the membrane was incubated with primary antibody and loading control anti-\(\beta\) actin. The blot was then incubated with both IRDye 800CW donkey anti-rabbit IgG(H+L) to detect anti-p-ATM, and IRDye 680 donkey anti-mouse IgG (H+L) was used to detect anti-\(\beta\) actin. The image was analysed by Li-COR\® odyssey-v3.0 software (Cambridge, UK).

Intracellular staining for phospho-ATM was undertaken in DC that had been fixed and permeabilised in 2% formaldehyde and cold methanol. Binding of primary antibody was detected with a FITC-conjugated secondary antibody and cells acquired using a Beckman Coulter FC500. Data was analysed using FlowJo\® software.

### Generation of Th17 responses

Naïve CD4+ CD45RA+ T-cells were isolated using Miltenyi naïve CD4 T cell isolation kit II to a typical purity of >95%. Cells were differentiated in the presence of combinations of recombinant cytokines (IL-1\(\beta\) [5ng/ml], IL-6 [5ng/ml], IL-23 [10ng/ml], TGF-\(\beta\) [5ng/ml], all R&D Systems) and co-stimulated with anti-CD28 (5\(\mu\)g/ml) and anti-CD3 (OKT3, 1\(\mu\)g/ml) antibodies in the presence of low-dose IL-2 (50IU/ml, R&D Systems) (49). Cytokines were added simultaneously at 0hr. Secretion of IL-17A was determined.

DC were treated as previously with KU55933 and TLR4 stimulation (LPS) for 12h 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 >12h after TLR stimulation (29). After 24h, supernatants were harvested and mixed 1:1 with fresh T cell medium (RPMI 1640, 7.5% normal human AB serum (Sigma), 1% sodium pyruvate, 2mm L-Glutamine, 1mM HEPES (Gibco), 20\(\mu\)M mercaptoethanol (Sigma)), used to stimulate naïve CD4+ T-cells. Following 5 days primary stimulation with anti-CD3 and
anti-CD28, T-cells were harvested and rested for 3-5 days before re-stimulating with anti-CD3 and anti-CD28 for 48hrs. Secretion of IL-17A was determined.

Quantitation of mRNA levels of sXBP-1 with real time RT-PCR

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

Primers and probes were used at 10 pmol/μL. Reactions were performed in triplicate and standardised to a HPRT housekeeping gene control for each sample. sXBP-1 mRNA levels were expressed relative to the levels of resting immature DC using Ct threshold and normalised according to HPRT levels as previously described. The relative thresholds were calculated according to the formula relative value

\[ \text{relative value} = 2^{-(\text{Ct sXBP-1 stimulated} - \text{Ct HPRT stimulated})} / 2^{-(\text{Ct sXBP-1 iDC baseline} - \text{Ct HPRT iDC baseline})} \]

Note, HPRT was retained as a housekeeping gene for XBP1s PCR in order to permit direct comparison of data with the laboratory of Dr Jane Goodall and Prof Hill Gaston (University of Cambridge).

Irradiation of cells

DC were irradiated in tissue culture plates immediately prior to activation. Radiation (0-6Gy of 195kVp X-rays, 0.87Gy/min, 0.5mm Cu filter, 48.4cm FSD) was delivered using a Gulmay Xstrahl cabinet irradiation facility within the department. Cell morphology was examined by phase-contrast microscopy (x40) following radiation and after a further 24-48hrs of culture and viability determined at 24-48hrs by dye exclusion. At the doses used no impact on viability was observed (not shown).

Statistical analysis

Statistical analysis was undertaken by Student’s t-test using Graphpad Prism® software and Microsoft® Excel. (*:0.05>p value≥0.01, **:0.01>p value≥0.001, ***: p value<0.001). The normal distribution of the data was confirmed using the Kolmogorov-Smirnov test, prior to application of the t-test. Data that was abnormally distributed was tested using the Mann-Whitney U-test. Results are presented as the mean ± standard deviation (SD), unless otherwise stated.

Results

IL-23 plays an important role for human Th17 generation

Whilst 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 naïve CD4 T cells with a typical purity of at least 95% CD4+CD45RA+ (Figure 1A) were co-stimulated in the presence of recombinant IL-1β, IL-6, IL-23 and TGF-β. Naïve T cells activated through CD3/CD28 alone did not secrete IL-17A, or secreted very low levels of IL-17 (Figure 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-polarisation leading to an increase in IL-17A levels (p<0.001). Addition of IL-23 to IL-1β and IL-6 conditioning lead to a significant increase in IL-17A (p<0.01) over all 11 individual experiments (Figure 1C). It is important to note that the addition of IL-23 alone to co-stimulated 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/IFNγ-activated DC as shown in our previous study (30), we examined potential candidates (identified from the known off-target effects of WM) by using inhibitors with high selectivity. Notably, the highly-selective ATM inhibitor KU55933 resulted in a pronounced (4-8 fold) induction of IL-23 over and above the levels elicited by LPS/IFNγ alone (Figure 2A). This effect was dose dependent up to the accepted maximum-inhibitory concentration of KU55933 (10μM). Whilst baseline levels of IL-23 secretion varied by donor, treatment with KU55933 enhanced IL-23 secretion in all 32 donors tested, median 5-fold increase above levels obtained with LPS/IFNγ with significance of p=6x10−10 (Figure 2A).

To analyse the consequences of ATM-blockade on Th17 polarization naïve CD4+CD45RA+ T-cells were primed in the presence of supernatant from ATM-competent or ATM-inhibited DC. As previously observed when KU55933 treated moDC were stimulated with LPS/IFNγ, IL-23 secretion was markedly increased in the supernatants transferred into T cell cultures (Figure 2B left panel). When naïve CD4+ T-cells were co-stimulated with anti-CD3/CD28 antibody in the presence of supernatant from iDC there was little detectable secretion of IL-17 (Figure 2B right panel). Activation of naïve CD4+ T-cells in the presence of supernatant from matured moDC increased IL-17 secretion significantly. When naïve CD4+ T-cells were primed in the presence of supernatant from ATM-inhibited DC there was a further, and significant increase in IL-17 secretion above levels obtained with mature moDC alone (p<0.05) (Figure 2B left panel). Additionally, KU55933 treated TLR4 stimulated myDC secreted IL-23 whereas TLR4 stimulation of myDC alone gave undetectable levels of IL-23 (Figure 2D). In two donors tested, the supernatant from KU55933-treated myDC significantly elevated IL-17A production from Th-cells compared with supernatant from myDC matured in the absence of KU55933 (Figure 2C). Note: there was no detectable IL-17A in the conditioned medium from DC cultures. ATM inhibited moDC supernatant gave a significant increase in IL-17A level (p<0.05) compared with LPS/IFNγ only triggered supernatant in all 8 individual experiments (Figure 2C left panel) with a mean fold changes about 2 fold overall (Figure 2C right panel).

ATM regulates IL-23 production by moDC and myDC

Our earlier work showed that WM required TLR4 triggering for DC to produce IL-23 (30). Similarly, treatment with KU55933 alone or in the presence of IFNγ did not elicit IL-23 secretion (Figure 3A). The increased IL-23 secretion observed following ATM inhibition was independent of IFNγ (Figure 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 IFNγ KU55933 led to a 4.0 fold increase from 3810±1202 pg/ml (LPS/IFNγ) to 15075±807 pg/ml (KU pretreatment with LPS/IFNγ) (p<0.01). Inhibition of mTOR, another potential WM target, did not significantly potentiate IL-23 release (p>0.05, Figure 3C). Furthermore inhibition of poly (ADP-ribose) polymerase (PARP), using a selective PARP inhibitor NU1025, also had no effect on IL-23 (p>0.05, Figure 3C).
Whilst 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 (Figure 3D).

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 to LPS/IFNγ alone in all three donors tested (Figure 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/IFNγ only. The addition of ATM-inhibitor also resulted in increased transcription of the p40 (in 2/3 donors studied) and p35 genes (Figure 3G and 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 (1μM) affected cytokines other than IL-23, although to a lesser extent. In particular IL-12p70 secretion was increased approximately 2-fold (30). As shown in Table 1, whilst treatment of moDC with KU55933 resulted in a marked increase in IL-23 secretion in response to LPS/IFNγ, it did not significantly affect IL-1β, IL-6, IL-27 or TGFβ. 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 (ns).

**Ionising radiation activates ATM resulting in suppression of IL-23**

As inhibition of ATM was observed to increase IL-23 expression, we hypothesised that ATM activation would suppress IL-23 production. Therefore we exposed DC to low doses of ionizing radiation (2-6Gy), sufficient to cause activation of ATM in a variety of cell types (50, 51). 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 (Figure 4A and B). Treatment of DC with KU55933 prior to irradiation reduced phospho-ATM expression to levels similar to those observed in non-irradiated cells (Figure 4A and B). Irradiation of immature moDC with as little as 2Gy resulted in significant suppression in their IL-23 response to TLR4 agonist, p<0.05 for 6/6 donors (Figure 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 (Figure 4C). It should be noted that whilst 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 48hr later (data not shown). Pre-incubation of DC with KU55933 significantly (p<0.001) prevented the reduction in IL-23 production caused by irradiation in all donors (Figure 4D).

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

Previous studies (52) found that tunicamycin induced ER stress was enhanced in ATM deficient human lung fibroblast cells compared to the wide type controls characterized with increased level of XBP-1 splicing. More recently, Goodall et al. (53) stated that activation of ER stress in combination with TLR ligands leaded to a marked increase of IL-23p19 transcription as a result of increased level of ER stress-induced CHOP transcription. Whilst 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 XBP1s spliced
mRNA species, a key early signaling component of ER-stress responses, in the presence of functional or inhibited ATM. Messenger-RNA for XBP1s was induced 6-fold following treatment of DC with LPS/IFN-γ and the levels of this transcript started to decline after 2-6hr returning to baseline levels after 20hr (Figure 5A). However, following inhibition of ATM with KU55933 the level of LPS-induced XBP1s transcripts were consistently higher. ATM-inhibition resulted in a 2-fold increase in peak levels of XBP1s mRNA compared with LPS/IFN-γ alone. In some donors there was sustained elevated XBP1s mRNA in the presence of ATM-inhibitor however in all instances the levels of XBP1s returned to baseline after 20hr. As expected, elevated XBP1s transcription was associated with increased expression of IL-23p19 gene (Figure 5B). The peak transcriptional changes in IL-23p19 occurred at 9-12hr post activation which followed the peak in XBP1s mRNA levels in all 3 donors. As expected, this was followed by a substantial increase in IL-23 release (Figure 5C).

Discussion

Interleukin-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 MS 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 upstream 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 whilst 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 cell cycle control and activation of DNA repair systems (54). 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 (55, 56). Several lines of evidence support our findings of an immune-regulatory role for ATM. Westbrook et al (46) 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 where both naive and memory CD4+ T-cells carry substantial damaged DNA due to insufficient repair (57). Furthermore Weintz and colleagues (47) 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 (58-60). It is a potent, highly selective and competitive inhibitor with an IC50 for ATM of 13nM. In contrast, other targets require substantially greater concentrations to inhibit function e.g. DNA-PK, mTOR, PI3K, PI4K and ATR are inhibited with IC50 of 2500, 9300, 16000, >100000 and >100000nM 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 4nM, whilst inhibiting other targets, smMLCK), Na/K-ATPase, mTOR, DNA-PK, ATM, and ATR at 200nM, 130nM, 200nM, 16nM, 150nM, 1800nM respectively (40, 41). In addition, WM also inhibits members of the polo-like kinase family at similar concentrations to PI3K (61). It is critical to appreciate “off-target” effects when using kinase inhibitors to dissect function. Alternative approaches include the use of siRNA and in this regard we made repeated attempts to knock-down ATM expression using a panel of siRNA (data not shown). However, to date we have not been able to achieve complete knock-down 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

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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 (62). Ro52 restricts the production of IL-23 and IFNβ and Ro52-loss increases cytokine secretion. The action of Ro52 is mediated by IRF3 (63, 64) and the IL-23p19 gene has IRF3/7 binding sites. Activation of IRF3 is regulated by TANK binding kinase-1 (TBK-1) that is phosphorylated upon TLR3 or RIG-I ligation (65, 66). At present we do not known 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 (67). We used typical therapeutic fractional doses of X-rays (<6Gy). In contrast, other studies have investigated doses of radiation up to 30Gy (67, 68). 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 occurred beyond 8Gy. 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 non-irradiated 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 generalised stress-sensing system whose 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 (69). Furthermore, ATM has recently been shown to interact with NF-κB essential modulator (NEMO) thus promoting NF-κB dependent signaling by increasing nuclear translocation (70). One candidate pathway is the interaction of ATM with intracellular stress-sensing systems in the endoplasmic reticulum (ER). Studies of ER-stress in macrophages show a pronounced increase in cytokines following TLR4-triggering (71) 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 (72). More recently a role for ER-stress in TLR responses has emerged (73). Importantly, ER-stress in macrophages only activated a restricted profile of cytokine genes (74). When stress was induced by pharmacologic agents and misfolded HLA it resulted in IL-23 (but not IL-12) and Th17 responses. We used the generation of a spliced transcript (XBP1s) as an indicator of ER-stress activation. XBP1s is a key early component of the ER-stress response (75) generated when IRE1 acts on X-box-binding protein 1 (XBP1) to splice the 26-bp intron from XBP1 mRNA. In doing so IRE1 removes a premature stop codon allowing transcription of the alternate spliced form, XBP1s. Our observation of increased XBP1s mRNA followed by coordinated increase in p19 mRNA in cells treated with ATM-inhibitor 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 (CHOP) as a key component of ER stress was responsible for a marked increased level of IL-23p19 in U397 cells (53).

Several studies also indicate a role for ATM in stress responses. Guo and colleagues (55, 56) highlighted an alternate ATM-activation pathway (oxidative stress) in the absence of DNA damage. Importantly the structure of ATM under these conditions was different to that observed in response to DNA damage. Upon activation by DNA-damage, monomeric ATM initiates DNA-repair (76). However under oxidative stress, but in the absence of DNA damage, ATM formed covalent dimers.

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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.

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Contribution to the study

AMJ Conceived the original idea, supervised the research and wrote the manuscript.
QW Undertook or supervised all experimental work, and wrote the manuscript.
PMP Guided the research programme and wrote manuscript.
HAF Myeloid DC and Q-PCR analysis.
SF T-cell analysis by ELISA.
IS Involved in T-cell studies.
ME Undertook work with macrophage.
MJG Contributed to radiation studies and wrote manuscript.
SM Advised on ATM inhibition strategies and reviewed manuscript.
CS Advised on ATM and reviewed manuscript.
AM Undertook initial studies with ionizing radiation and IL-23.
SS Performed many of the ELISA assays.

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Figure 1.
The importance of IL-23 for differentiation of human Th17 responses. A: Naïve human CD4+ CD45RA+ T-cells were purified from peripheral blood. Typical purity was >95% CD4+CD45RA+, data are representative of 8 independent donors. B: Naïve CD4+CD45RA+ T-cells were co-stimulated for 5d with anti-CD28 in the presence of recombinant cytokines. After resting for 2d, cells were re-stimulated with anti-CD3 and IL-17A release determined by ELISA (2 representative donors from 11 experiments are shown). C: Summary plot showing the effect of addition of IL-23 to IL-1, IL-6 primed Th17 responses from all 11 independent experiments. In all graphs, *:0.05>p value≥0.01, **:0.01>p value≥0.001, ***: p value<0.001.
Figure 2.
ATM-inhibited DC secrete IL-23 and prime increased Th17 responses. A left panel: moDC were activated through TLR4/IFNγ in the presence of KU55933. IL-23 was measured by ELISA. Data shows 1 representative donor of 6. A right panel: Comparison of the effect of ATM-inhibition on IL-23 release by moDC from 32 donors. DC were activated with LPS/IFNγ +/- KU55933 (10μM). Significance was determined by Mann-Whitney U test. B: moDC or D: myDC were treated as before but cells were washed after 12h to prevent carry-over of LPS and KU55933 to T-cells. The levels of IL-23 were determined by ELISA, and the cell-free supernatants used to activate Th responses. Purified (>95%) naive CD4+ T-cells were co-stimulated with anti-CD3/anti-CD28 antibodies in the presence of supernatant from activated ATM-competent or ATM-inhibited DC (50% v/v) for 5d. After resting, equal numbers of T-cells were re-stimulated with anti-CD3. Th-17 responses determined by IL-17A ELISA. Error bars indicate SD of triplication biological determinations. C left panel: Summary showing the IL-23 response of moDC from 8 donors in the presence or absence of ATM-inhibition. C right panel: Corresponding summary of the same 8 donors showing fold change in IL-17 secretion following priming of CD4+ T-cells in the presence of the indicated DC supernatant.
Figure 3.
Role for the ATM pathway in IL-23 secretion by DC. A: Induction of IL-23 requires TLR4-triggering. moDC were stimulated as indicated following treatment with WM (1μM) or KU55933 (10μM). Data are representative of 6 donors. B: IL-23 induction in ATM-inhibited DC occurs in the absence and presence of IFNγ. 4 independent donors are shown and the line indicates the mean value. C: IL-23 induction is specific to ATM. moDC were treated with the indicated inhibitors of ATM, or the alternate WM target (mTOR), or an irrelevant target (PARP) at the indicated concentrations prior to activation with LPS/IFNγ. IL-23 was measured as before (representative data from 3 donors). D: Inhibition of ATM in myDC induces IL-23. Freshly isolated myDC (4 donors) were treated as before and IL-23 secretion determined. For each donor, white bar: LPS/IFNγ myDC, grey bar: WM (1μM) pre-treated before LPS/IFNγ, black bar: KU55933 (10μM) pre-treated before LPS/IFNγ. E: ATM transcriptionally regulates the p19, p40 and p35 genes. moDC from 3 donors were treated with KU55933 (10μM) prior to activation with LPS/IFNγ. Relative abundance of mRNA was determined at 12hr with qPCR and expressed as fold-change relative to matured DC (treated with LPS/IFNγ alone). Error bars indicate SD of triplicate biological determinations.
Figure 4.
X-rays activate ATM in DC resulting in depressed IL-23. A: Ionising radiation activates ATM in DC. ATM-inhibited or competent moDC were exposed to the conditions including LPS and X-rays and phospho-ATM expression determined by Western blot (representative donor of 3). B: The phosphorylation status of ATM was further confirmed by intracellular staining. DC were exposed to 6Gy of X-rays +/- pretreatment with KU55933. The phosphorylation of ATM was examined by indirect intracellular staining with a phospho-ATM-specific antibody, dotted line: iDC, dashed line: X-ray exposed DC, thick solid line: KU55933 pretreated DC after X-ray exposure. C: Representative data from moDC of 1 of 6 donors exposed to KU55933 (10μM) or left un-treated prior to exposure to X-rays. Cells were subsequently activated with LPS/IFNγ and IL-23 secretion determined by ELISA. Error bars indicate the SD of triplicate biological determinations. D: Summary of 6 donors showing that ATM-inhibition negates the inhibition of IL-23 following exposure to X-rays (6Gy). Line indicates mean response.
Figure 5.
ATM-inhibition increases LPS-dependent sXBP-1 mRNA. RNA from iDC, LPS-matured DC (square symbol) and KU55933 (10μM) pre-treated matured DC (triangle) was extracted and analysed for XBP-1s and IL-23p19 mRNA. A: qPCR results were calculated relative sXBP-1 level normalised to HPRT and plotted as a fold change compared to sXBP-1 expression by iDC. Data is given for 3 donors. B: Expression of IL-23p19 gene C: secretion of IL-23 (determined in parallel wells by ELISA at 24hr) from the corresponding donors shown in A/B.
Table 1

Effect of ATM inhibition by KU55933 on cytokines production in human MoDC

The effect of ATM inhibition on cytokine production. The role of ATM for other cytokines was determined using ELISA for IL-23, IL-1β, IL-6, IL-12, IL-27 and TGFβ. Data from 5-32 donors is shown for moDC treated with LPS/IFNγ alone, or in the additional presence of 10μM KU55933. The fold change for individual donors is given and the p value is obtained from biological triplicate experiments. NS – not significant.

<table>
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<tr>
<th>Cytokine</th>
<th>LPS/IFN-γ</th>
<th>KU10μM+ LPS/IFN-γ</th>
<th>n=</th>
<th>P value</th>
<th>Mean Fold change</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td></td>
</tr>
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<td>6 8</td>
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<td>77096 596623</td>
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<tr>
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<tr>
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<td>1072 295</td>
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<tr>
<td>TGF-β (pg/ml)</td>
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