Endothelial IL-33 expression is augmented by adenoviral activation of the DNA damage machinery [1]

Running Title: Adenoviral DNA triggers endothelial IL-33 expression

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Abstract

Interleukin-33 (IL-33), required for viral clearance by cytotoxic T-cells, is generally expressed in vascular endothelial cells in healthy human tissues. We discovered that endothelial IL-33 expression was stimulated as a response to adenoviral transduction. This response was dependent on MRE11, a sensor of DNA damage that can also be activated by adenoviral DNA, and on IRF1, a transcriptional regulator of cellular responses to viral invasion and DNA damage. Accordingly, we observed that endothelial cells responded to adenoviral DNA by phosphorylation of ATM and CHK2, and that depletion or inhibition of MRE11, but not depletion of ATM, abrogated IL-33 stimulation. In conclusion, we show that adenoviral transduction stimulates IL-33 expression in endothelial cells in a manner dependent on the DNA binding protein MRE11 and the antiviral factor IRF1, but not on downstream DNA damage response signaling.

Introduction

Interleukin-33 (IL-33) is a member of the IL-1 family (1, 2) that appears crucially involved in establishing a successful anti-viral CD8 T cell response in the mouse (3). Viral infection also drives expression of IL-33 in many contexts. For example, murine lungs infected with influenza A show a dramatic increase in IL-33 expression (4), and patients with chronic viral hepatitis have elevated serum levels of IL-33 (5). These observations have triggered interest in understanding how IL-33 expression is regulated at the cellular level. For example, transcription of IL-33 in murine macrophages partially depends on activation of IRF3 (interferon regulatory factor 3) via the RNA sensor RIG-I (retinoic acid inducible gene I) (6). IL-33 synthesis can also be triggered by detection of poly I:C (a synthetic analogue of viral, double-stranded RNA) by TLR3 (toll-like receptor-3) in murine hepatocytes (7) and human fibroblasts (8). In addition, synthesis of IL-33 is strongly boosted in human fibroblasts when poly I:C acts in concert with TGF-β (transforming growth factor-β) (8).

Host recognition of viral infection involves several classes of sensors including TLRs, C-type lectins, cytosolic RNA or DNA sensors, as well as the nuclear MRN complex (consisting of MRE11, NBS1, and RAD50) (9-11). This complex is well-characterized as an initiator of the DNA damage response. The DNA damage response is crucial to prevent replication of damaged genomic host material, but it also serves to recognize foreign DNA. Human adenovirus 5 (Ad5) has a 36 kb double-stranded DNA genome that is replicated concomitant with cellular DNA. Thus, the discovery that Ad5 early proteins interfere with DNA damage response mediators excited great interest, suggesting that the cellular DNA damage response also plays an anti-viral role (discussed in reference (11)). Indeed, adenovirus targets the MRN complex for proteasomal degradation by expressing the early proteins E1b55k/E4orf6 and E4orf3, thus limiting activation of the DNA damage machinery in response to adenoviral DNA (12). In the absence of adenoviral E4 proteins, MRN associates with viral DNA and initiates repair processes that result in tethering of viral linear DNA (concatemer formation) and prevents viral replication (12, 13).

While the *in vivo* importance of IL-33 in antiviral defense has been highlighted experimentally in mice (3), significant differences in the distribution of IL-33 between mouse and human may point to species-specific functions. For example, while IL-33 is almost absent from vascular endothelial cells in the mouse, most IL-33 in healthy human tissues is

62 found in the vasculature (14-16). It is currently unclear whether this vascular pool of IL-33
63 has a function in anti-viral immune defense that cannot be accounted for in murine models.

64 We here report that non-replicative adenovirus 5 increases endothelial expression of IL-33
65 and initiates a DNA damage response. Depletion of MRE11 or IRF1 (essential transcriptional
66 regulator of the DNA damage response (17)) prevented the observed stimulation of IL-33,
67 implying that sensing of viral DNA by MRE11 boosts endothelial IL-33 expression.

Materials and Methods

Cell culture and reagents

Umbilical cords were obtained from the Department of Gynecology and Obstetrics at the Oslo University Hospital according to a protocol approved by the Regional Committee for Research Ethics (S-05152a). Human umbilical vein endothelial cells were isolated as described by Jaffe *et al* (18) and cultured in MCDB 131 medium (Life Technologies) containing 7.5% fetal calf serum (FCS), 5mM L-glutamin (Invitrogen), 10 ng/mL epidermal growth factor (R&D Systems), 1 ng/mL basic fibroblast growth factor (R&D Systems), 1 µg/mL hydrocortisone (Sigma-Aldrich), 50 µg/mL gentamicine (Lonza) and 250 ng/mL amphotericin B (Lonza), unless otherwise stated. Cells were used at passage level one to six, maintained at 37°C in 95% humidity/5% CO_2 atmosphere and split at a ratio of 1:3. The γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester, EMD Chemicals) was dissolved in DMSO (dimethyl sulfoxide, Sigma) at 25 mM and used at a final concentration of 5-25 µM. Cycloheximide and the MRN inhibitor mirin were purchased from Sigma and used at 3 µg/mL and 1-10 µM, respectively.

Antibodies

The antibodies used in this study were: Ad5 Hexon (8C4) and β-Tubulin from Abcam; ATM pSer1981 (D6H9), ATM (D2E2), ATR pSer428, Chk1 pSer345 (133D3), Chk2 pThr68 (C13C1), H2A.X pSer139 (20E3), Histone 3 (D1H2), IFI-16, IRF1 (D5E4), IRF3 (D6I4C), IRF7, MRE11, NICD1 Val1744 (D3B8), STAT1 pTyr701 (58D6), RAD50, STING (D2P2F), and TLR9 (D9MH9) from Cell Signaling Technologies; GAPDH from Santa-Cruz; IFN α/β receptor chain 2 (MMHAR-2) and STAT1 (STAT1-79) from Thermofisher Scientific; DLL4 (YW152F) and NOTCH1 (YW169.60.79) from Genentech; IL-33 (Nessy-1) from Enzo Life Sciences; Secondary antibodies were from Jackson ImmunoResearch. Further detail of antibodies can be found in Supplementary table 1.

Amplification of Ad5ΔE1ΔE3-GFP, Ad5ΔE1ΔE3, Ad5ΔE1 and wtAd5 in mammalian cells

Human embryonic kidney (HEK) 293T cells, transformed with Adeno E1 and simian virus 40 (SV40) large T antigen, were used to amplify non-replicative Ad5 (nrAd5). Ad5ΔE1ΔE3-GFP, Ad5ΔE1ΔE3 (AdEasy system, Stratagene) were transfected into 293T cells 24 hours after seeding (8.0 x 10^4 cells/cm^2), *i.e.,* at 50–70% confluence. 4 µg of nrAd5 plasmid DNA (linearized with *PacI*) was transfected using Lipofectamine 2000 (Thermo Fisher Scientific)

according to the manufacturer's recommendation. Viral plaques were observed seven to ten days after transfection and both floating and adherent cells were collected by scraping. The cells were pelleted by centrifugation at 300 x g for 10 min at 4°C and resuspended in 2 mL of the supernatant. After three cycles of freeze/thawing (dry ice/methanol bath and rapid thawing at 37°C) and vortexing, the cell debris was pelleted by centrifugation at 3000 x g for 20 min at 4°C. The viral lysates were used to infect 293T cells (70% confluent, 1mL lysate/T25 tissue culture flask). Three to five days post infection, when cytopathic effects (CPE) were observed in 30-50 % of the cells, viruses were harvested as described above. The viral titers were determined by infecting 293T cells with tenfold dilutions (from 10^{-2} to 10^{-9}) of virus stocks, culturing the cells for 48 hours and harvesting by fixation in 100% methanol (-20° C for 15 min). Endogenous peroxidase was quenched with 0.3% H_2O_2 in water for 30 min. Cells were washed in 1% BSA (Sigma-Aldrich) diluted in PBS. Plaques were stained with murine monoclonal antibody specific for the adenovirus hexon protein (Supplementary Table I, using 1 µg/mL in 1% BSA diluted in PBS) for 1 hour at 37°C. After washing, the cells were incubated with HRP-conjugated goat anti-mouse IgG (Supplementary Table I, 0.8 µg/mL in 1% BSA diluted in PBS)) for 1 hour at 37°C. The cells were washed prior to 3,3'-diaminobenzidine staining using Fast DAB tablets according to the manufacturer's recommendation (Sigma-Aldrich). Positive plaques were counted in a minimum of three fields per well per dilution in duplicated wells. The average titer was determined as plaque forming units per mL. Helper-dependent Ad5 lacking all adenoviral genes was produced as described by Dormond *et al.* (19).

Viral transduction

For transduction, HUVECs were seeded in complete medium at 3.8 or 1.9 x 10^4 cells/cm^2, 24 or 48 hours prior to infection, respectively. On the day of infection, when cells were sub-confluent (70-80%) or confluent (90-100%), the complete medium was replaced with fresh complete medium and viral stocks were added to obtain the desired multiplicity of infection (moi). Viral UV-inactivation was performed by diluting the virus stock in 150 µL complete medium in 24-well plates followed by irradiation on ice, using different doses of ultraviolet (UV) light to a maximum of 7 J (760 µW/cm^2, up to a maximum of 2 hours and 30 min).

Interferon-α/β neutralization

In vitro blocking of the interferon-α/β receptor was performed with a murine monoclonal antibody to human interferon-α/β receptor chain 2 (MMHAR-2, 10 µg/mL). A species-,

isotype- and concentration-matched monoclonal antibody against the E-tag epitope (Supplementary Table I, 10 µg/mL) was used as a negative control. 50 moi Ad5ΔE1ΔE3-GFP was then added to the culture and the cells were incubated for 24 hours. The results were compared to those of non-treated and non-transduced cells. To test the efficacy of the neutralizing antibody, CXCL10 was measured in isotype and MMHAR-2 treated cells stimulated with 1000 U/mL interferon-α.

Reverse transcription (RT)-quantitative PCR (qPCR)

Upon harvest, total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's recommendations. 1 µg RNA was used for first-strand cDNA synthesis with the SuperScript III Reverse Transcriptase cDNA system (Thermo Fisher Scientific), Oligo(dT) primers and dNTPs (GE Healthcare). qPCR was carried out on a Stratagene Mx3005P instrument (Agilent Technologies) and analyzed by Stratagene MxPro software (Agilent Technologies). The PCR reaction comprised 5 µL of 10 times diluted cDNA in a 20 µL qPCR reaction consisting of HotStarTaq DNA polymerase, 5000U (Qiagen), dNTP (GE Healthcare), EvaGreen, 20x (Biotium) and the individual primer sets. The PCR was run up to 40 cycles and included a melting curve analysis to ensure amplification of single products. Standard curves were made from serial dilutions of cDNA to calculate primer efficiencies. HPRT (hypoxanthine guanine phosphoribosyl transferase) was used to normalize for sample-to-sample variation. The relative quantity of gene expression levels was calculated using the Pfaffl method (when primer efficiencies differed) (20) or the comparative Ct method (21) relative to non-treated controls. The primer sets used were: *HEY1*: F, 5'GCTGGTACCCAGTGCTTTTGAG'3, R, 5'TGCAGGATCTCGGCTTTTTCT'3; *HES1*: F, 5'ACGTGCGAGGGCGTTAATAC'3, R, 5'CATGGCATTGATCTGGGTCA'3 ; *HPRT*: F, 5'AATACAAAGCCTAAGATGAGAGTTCAAGTTGAGTT'3, R, 5'CTATAGGCTCAT-AGTGCAAATAAACAGTTTAGGAAT'3; *IL-33*: F, 5'GCAGCTCTTCAGGGAAG-AAATC'3, R, 5'TGTTGGGATTTTCCCAGCTTGA'3; *NOTCH1*: F, 5'CGGGTCCAC-CAGTTTGAATG'3, R, 5'GTTGTATTGGTTCGGCACCAT'3; *DLL4*: F, 5'GAAGTGG-ACTGTGGCCTGGACAAGT'3, R, 5'TCGCTGATATCCGACACTCTGGCT'3.

siRNA transfection

HUVECs were seeded at a density of 3.8 or 1.9 x 10^4 cells/cm^2 in complete medium 24 hours prior to transfection. The transfection was carried out in medium without antibiotics. The

lipofectamine/siRNA mix (Lipofectamine 2000 or Lipofectamine RNAiMAX), respectively (Thermo Fisher Scientific)) was prepared according to the manufacturer's instructions, added to the cells, and subsequently incubated for six hours. Ambion Silencer Select siRNAs were purchased from Thermo Fisher Scientific: *IRF1* s4502, *IRF-3* s7507, *IL-33* s40521, *NOTCH1* s9633, *DLL4* s29213, *JAG1* s1175, *IFI-16* s7136, , *TLR9* s28872 and s28873, MRE11 s8960, NBS1 (NLRP2) s31177, RAD50 s793, STING s50644 (STING1) and s50645 (STING2), and *ATM* s1710. Silencer select Scrambled #1 and Scrambled #2 (Thermo Fisher Scientific) were used to control for non-specific effects of transfection.

Immunoblotting

Cultured cells were washed with PBS before harvesting samples in a Tris-HCl (pH 6.8) SDS (2.5%) /glycerol (10%) lysis buffer containing a reducing agent (100 mM β-mercaptoethanol (Sigma-Aldrich) or 10 mM dithiothreitol), protease inhibitors (1 mM phenyl-methylsulfonylfluoride (Sigma-Aldrich) and cOmplete Protease Inhibitor Cocktail (Roche)), and phosphatase inhibitor (2nM sodium orthovanadate (Sigma-Aldrich)). The samples were homogenized using a QIAshredder (Qiagen) according to the manufacturer's instructions and incubated at 95˚C for 5 min before loading them onto 10% Mini-PROTEAN TGX Precast Gels (Bio-Rad), 4-20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) or 12.5% SuperSep Phos-tag gels (Wako Pure Chemical Industries). The Phos-tag gels were used according to the manufacturer´s instructions to evaluate the phosphorylation of IRF1. After loading cell lysates onto gels, they were run for 15-25 minutes at 300 V before blotting to nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) and the Turbo blotter (Bio-Rad). Blottet membranes were blocked with 5% Blotting-Grade Blocker (Bio-Rad) or 5% BSA (when using antibodies specific for phosphorylated proteins (Supplementary Table I)) and incubated with primary (4˚C, overnight) and secondary (room temperatures, 2 hours) antibodies diluted in 1% Blotting-Grade Blocker (Bio-Rad) in TBST. The protein bands were detected using Pierce Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and visualized using the ChemiDoc MP System (Bio-Rad).

Image processing

Figures and images were generated and processed in Adobe Photoshop CS6, Adobe Illustrator CS6, GraphPad Prism 6, or FlowJoVx. All adjustments were performed on the image as a whole and with equal adjustment of image series.

Results

Endothelial IL-33 expression is enhanced by replication and transcription deficient adenovirus 5

While using a non-replicative (nr) adenoviral vector as a tool to ectopically express IL-33 in cultured human umbilical vein endothelial cells (HUVECs), we observed that transduction with a control vector (Ad5ΔE1ΔE3) markedly increased IL-33 expression 48 hours post-transduction (hpt) when compared to non-transduced cells (Figure 1A). The stimulation of IL-33 correlated positively with increasing viral titers. In contrast to non-transduced HUVECs that require contact-mediated quiescence to express IL-33 (14, 22), Ad5ΔE1ΔE3-transduced cells expressed IL-33 in both confluent and subconfluent cultures (Figure 1A). The increase in IL-33 expression was evident at 48 hpt and continued to rise until 72 hpt (Figure 1B). To establish whether the increased endothelial expression of IL-33 was due to viral gene transcription, HUVECs were transduced with a helper-dependent nrAd5 lacking all adenoviral genes, but retaining the *cis*-regulatory elements, including the viral packaging signals and the inverted terminal repeats (19, 23). We found that helper-dependent nrAd5 also enhanced IL-33 expression (Figure 1C), concluding that IL-33 can be induced by nrAd5 vectors in human endothelial cells in a manner independent of viral transcription.

IL-33 upregulation is abrogated by UV irradiation of viral particles

Although elicitation of IL-33 was independent of viral gene transcription, UV-irradiation of Ad5ΔE1ΔE3-GFP (before adding the virus to cells) dose-dependently abrogated the stimulation of IL-33 expression (Figure 2A). Detection of virally driven GFP by flow cytometry was used to control for viral inactivation, showing a steady, inverse correlation with the dose of UV light applied (Figure 2B). This shows that adenoviral entry alone is insufficient to stimulate IL-33 expression in endothelial cells, and that the host response involved in IL-33 augmentation is not triggered by UV-inactivated virus particles.

Adenoviral upregulation of IL-33 depends on Notch signaling

Our recent finding that Notch signaling drives IL-33 expression in quiescent endothelial cells (22) prompted us to ask if nrAd5 transduction might drive IL-33 expression via Notch signaling. Transcriptional analysis of HUVEC transduced with Ad5ΔE1ΔE3 revealed an increase in mRNA levels of the Notch ligand *DLL4*, the Notch receptor *NOTCH1,* and the direct Notch-target genes *HES1* and *HEY1* (Figure 3A). In addition, the levels of cleaved

NOTCH1 intracellular domain (csNICD1, the signaling mediator of activated NOTCH1) were increased after transduction (Figure 3B). Moreover, Notch signaling was required for the nrAd5-driven increase in IL-33 to take place, as IL-33 expression could be inhibited by siRNA-mediated knockdown of Notch components (Figure 3C), by the γ-secretase inhibitor DAPT (Figure 3D) or by inhibitory antibodies to NOTCH1 or DLL4 (Figure 3D) in both non-transduced and Ad5ΔE1ΔE3-transduced cells. These data demonstrate that endothelial Notch signaling is increased by nrAd5-transduction and confirms that NOTCH1 strongly supports IL-33 expression, also when enhanced by nrAd5.

Adenoviral stimulation of IL-33 depends on the antiviral transcription factor IRF1

The ability of adenoviral transduction to stimulate IL-33 expression even in subconfluent endothelial cell cultures implicated a mechanism that extends beyond the activation level of Notch signaling. We therefore embarked on assessing the involvement of transcription factors commonly involved in regulating expression of antiviral genes. We found IRF3 and IRF1 to be constitutively present in the nuclear fraction of HUVECs, whereas IRF7 was undetectable throughout the course of adenoviral stimulation (Figure 4A). We therefore depleted IRF3 and IRF1 by means of siRNA (Figure 4B and C, Supplementary Figure 1C), observing that while reduction of IRF3 did not affect IL-33 levels, reduction of IRF1 abrogated IL-33 expression in both non-transduced and Ad5ΔE1ΔE3-transduced HUVECs. We were unable to detect any change in phosphorylation status or half-life of IRF1 following transduction by Ad5ΔE1ΔE3 (Supplementary Figure 1A-B), suggesting that IRF1 either is activated by a phosphorylation-independent mechanism by Ad5ΔE1ΔE3 or that its activity is not altered and IRF1 rather plays a permissive role in IL-33 expression. The dynamics of IRF1 degradation after cycloheximide treatment was similar to that observed by others (24), indicating a halflife of approximately 30 minutes.

IRF1 can be activated in response to cytosolic DNA in several cell types (25, 26), has powerful cell-intrinsic antiviral properties (25), and can also be activated by type I interferons (27). To test the involvement of interferon and possible auto/paracrine effects, we therefore assessed phosphorylation of the essential interferon-activating transcription factor STAT1, finding that it was phosphorylated at an earlier time point than IL-33 was upregulated after transduction of HUVECs with Ad5ΔE1ΔE3 (Figure 4D). Considering the possibility of interferon-signaling, we exposed cells to an antibody specific for the interferon-α/β receptor

chain 2 during infection. This reagent neutralizes the effect of seven different type I interferons (28) and in our hands reduced interferon-α-driven induction of CXCL10 in control cells (data not shown), yet it failed to reduce the viral stimulation of IL-33 expression (Figure 4E). Finally, we assessed the possible involvement of other soluble factors by exposing non-transduced cells to supernatants harvested from transduced cell cultures (Figure 4F), again observing no increase in IL-33 expression. Taken together, these findings indicate that endothelial expression of IL-33 is supported by the presence of the antiviral transcription factor IRF1, but not by IRF3 or by the auto/paracrine stimulation of soluble mediators such as type I interferons.

Nonreplicative adenovirus activates the endothelial DNA damage response

As IRF1, in addition to its role in innate immune responses, is closely linked to the DNA damage response (17, 29) we next evaluated whether the DNA damage response was activated in our system. Endothelial cells transduced with Ad5ΔE1ΔE3 responded by inducing elements of a DNA damage response 24 hours post transduction (Figure 5A). Both ATM (ataxia telangiectasia mutated) and CHK2 (checkpoint kinase 2) were phosphorylated after transduction with Ad5ΔE1ΔE3 (Figure 5A, B) correlating in time with the upregulation of IL-33. However, phosphorylation of histone H2AX was not observed, in line with a previous report showing that ATM activation by adenoviral DNA is not accompanied by an extensive amplification by pH2AX, most likely due to the limited size of the adenoviral DNA (30).

The DNA damage machinery-component and dsDNA-sensor MRE11 is required for viral IL-33 stimulation

Activation of the DNA damage response in adenovirally transduced cells is initiated by MRE11, the DNA binding component of the MRN-complex, which in the absence of the early adenoviral protein E4 (not expressed by replication-deficient viral vectors) is reported to associate with viral DNA in nuclear replication centers (13, 30, 31). We therefore targeted the MRN-components MRE11, NBS1 and RAD50 as well as the downstream kinase ATM by means of siRNA-mediated knockdown before transduction with nrAd5, observing that depletion of MRE11 abrogated stimulation of IL-33 expression, and also reduced the basal expression of IL-33 in nontransduced cells (Figure 5C). In accordance with previous studies, MRE11-depleted cells also showed reduced levels of RAD50 and pATM (32, 33). Depleting RAD50 reduced phosphorylation of ATM, but did not affect IL-33 expression. In addition,

inhibition of MRE11 nuclease activity by mirin reduced IL-33 expression, confirming the role of MRE11 in IL-33 stimulation (Figure 5D). Mirin also inhibited IL-33 expression in the absence of nrAd5 (Figure 5D). Together with the reduction of IL-33 observed in nontransduced cells when MRE11 was depleted by siRNA (Figure 5C), this suggests a low level activation of MRE11 in nontransduced confluent endothelial cell cultures that also contributes to the constitutive expression of IL-33. When MRE11 acts as a cytoplasmic sensor of dsDNA (32), it activates IRF3 via the endoplasmic reticulum-resident protein STING. However, siRNA-mediated knockdown of STING did not affect IL-33 expression in response to nrAd5 transduction (Figure 5D). Likewise, knockdown of another nuclear sensor of foreign DNA, IFI-16, did not affect IL-33 expression (Supplementary figure 1C). Taken together, our observations suggest that MRE11-mediated sensing of nuclear adenoviral DNA promotes IRF1-driven stimulation of IL-33 in primary human endothelial cells.

Discussion

This study shows that expression of IL-33 in human endothelial cells is upregulated by the nuclease activity of DNA-binding MRE11 in response to transduction of adenoviral vectors. Our observation that IL-33 expression was enhanced not only by transduction with Ad5ΔE1ΔE3, but also by helper-dependent nrAd5 that lacks all viral genes, implicates the involvement of a viral structure common to these constructs. Adenoviral entry and the cytoplasmic presence of viral capsid proteins may in itself trigger cellular responses even when viral DNA is absent (34). However, UV-irradiation of virus particles before transduction abrogated IL-33 stimulation, suggesting that viral entry is insufficient to trigger the response and that intact viral DNA is required. Interestingly, while most viral DNA has been removed from helper-dependent nrAd5, the construct still contains viral packaging signals and inverted terminal repeats (23). Such terminal repeats are also expressed by adeno-associated viral vectors and are believed to represent a favored recognition site for the MRN complex (35). The ability of helper-dependent nrAd5 to enhance IL-33 expression is therefore in line with our finding that MRE11, the DNA-binding component of MRN, is crucial for the stimulation of IL-33 production observed in endothelial cells transduced with adenoviral vectors.

We also discovered that the well-known antiviral transcription factor IRF1 is essential for maintaining both basal and adenovector-induced expression of IL-33 in endothelial cells. IRF1 has been shown to inhibit a wide range of viruses in a manner preserved in STAT1-deficient fibroblasts (25, 36), and the response therefore appears to be cell-intrinsic rather than driven by interferon production. Interestingly, other IRFs are capable of inducing IL-33 in non-endothelial cells: IRF3 is required for transcription of IL-33 in murine macrophages upon nucleic acid ligand transfection and viral infection (6); IRF7 is required for induction of IL-33 by serum amyloid protein in both human and murine monocytes (37); and IRF4 is essential for IL-33 induction in mice exposed to house dust mite allergen (38). Indeed, IRF4 has been shown to bind within the first intron of the IL-33 gene in murine dendritic cells (38), supporting the concept that IL-33 can be regulated by IRF binding. While all of these interferon regulatory factors have similar DNA binding properties (39) and may regulate IL-33 gene transcription in a similar manner, they appear to differ with respect to cell type specificity and milieus.

Although IRF1 was required for the IL-33 response induced by nrAd5, the total levels of IRF1 remained constant during the course of viral transduction. Furthermore, we could not detect phosphorylated forms or an increase in the half life of IRF1. It is therefore possible that IRF1 is constitutively active in endothelial cells and permits IL-33 expression without further activation. On the other hand, IRF1 activity can also be influenced by factors not addressed in this study, including antagonistic action by IRF2 (40) and posttranslational modifications different from phosphorylation, hence, our results do not fully eliminate the possibility that IRF1 activity is altered upon adenovirus transduction in endothelial cells. It should also be noted that our approach to detect phosphorylation of IRF1 lacked a positive control. The involvement of Notch signaling in stimulation of IL-33 also tempts us to speculate that IRF1 may form a transcriptional complex with the canonical Notch transcription factor RBP-jK in an "enhanceosome" similar to that described for IRF1 and NF-kB (41). Such interactions deserve further investigations.

The involvement of IRF1 and the observation that adenoviral DNA in the absence of the adenoviral protein E4 elicits a cellular DNA damage response (12, 30), led us to explore the DNA damage response pathway in endothelial cells. Indeed, we observed that transduction with nrAd5 induced activation of ATM, but prevented the phosphorylation of H2AX, in line with recent findings in small airway epithelial cells (30). Furthermore, the dynamics of IL-33 stimulation coincided with phosphorylation of ATM, which in the context of adenoviral transduction represents a downstream event to recognition of viral DNA by the MRN complex. We next depleted the DNA-binding MRN component MRE11 by siRNA and observed a reduction both in DNA damage response signaling and IL-33 expression (Figure 5C). To confirm our data in a siRNA-independent manner, we also treated cells with mirin, an inhibitor of MRE11 nuclease activity, and observed a similar attenuation of IL-33 expression to when cells were treated with siRNA targeting MRE11. In contrast to the recently described STING/IRF3-dependent MRE11-signaling in response to cytoplasmic dsDNA (32), IL-33 stimulation by adenoviral DNA required neither STING nor IRF3.

Cellular secretion of IL-33 is still not fully understood (42). Considering our findings in the light of the recent discovery that IL-33 is required for a successful host response to viral infections (3, 43) and the fact that most IL-33 in the human body is found within nuclei of vascular endothelial cells (14, 15), the question of whether IL-33 can be released to the extracellular space from vascular endothelial cells in the absence of cell death appears more

366 relevant than ever. Full-length IL-33 can be detected in supernatants of cultured endothelial
367 monolayers after scratching (44) or in response to *in vitro* cold ischemia and reperfusion (45).
368 However, both these experimental approaches presumably bring about a significant degree of
369 cell death and the detected IL-33 could be a result of passive release from necrotic cells. In
370 contrast, efforts to demonstrate active IL-33 secretion from cultured endothelial cells have so
371 far been unfruitful. Interestingly, endothelial cells appear to be an important source of
372 extracellular IL-33 in the mouse heart during pressure overload, where it engages in
373 cardioprotective mechanisms (46), thus supporting a model where IL-33 under some
374 circumstances can be released extracellularly from endothelial cells. It is therefore urgent to
375 address whether IL-33 can undergo regulated secretion from human endothelial cells in a
376 viral context.

377 The novel connection between IL-33 and the DNA damage response, together with its
378 conserved relationship with IRFs, also makes it tempting to speculate whether IL-33 may
379 possess cell-intrinsic properties that could influence the outcome of viral infections. The
380 nuclear effects of IL-33 remain ill-defined, however IL-33 is predicted to bind an acidic
381 pocket of the nucleosome that can also harbor the latency-associated nuclear antigen (LANA)
382 of Kaposi sarcoma-associated herpesvirus (47). Similar to other proteins that dock into this
383 pocket (48), IL-33 appears to modulate chromatin condensation (47, 49) and has also been
384 reported to associate with the transcriptional repressor and histone methyltransferase
385 SUV39H1 (50). Chromatin remodeling factors take active part in the fine-tuning of DNA
386 damage responses (51) and also significantly contribute to host-viral interactions that
387 ultimately determine the outcome of viral infections (52). Further experiments should
388 therefore be designed to determine if IL-33 associates with the DNA damage machinery
389 and/or viral replication centers, and whether IL-33 expression affects viral replication or
390 persistence.

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Authorship Contributions

TES and RJE designed and performed experiments, interpreted data, and wrote the manuscript; LLCP, OS, AMK, and DP designed and performed experiments, interpreted data, and critically revised the manuscript; AAK and FM provided virus and critically revised the manuscript; GH, MK, and JH designed experiments, interpreted data, wrote and critically revised the manuscript. All authors approved the final version of the submitted manuscript.

Conflict of Interest Disclosures

The authors declare no conflict of interest

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Figure Legends

Figure 1. Endothelial IL-33 expression is enhanced by replication and transcription deficient adenovirus 5. HUVECs were transduced with Ad5ΔE1ΔE3 (10 moi) (**A, B**) or Helper-dependent nrAd5 (HdAd5, low to high moi) (**C**) for 48 hours (**A, C**) or for the indicated time (**B**) before harvesting cellular lysates for immunoblotting with antibodies specific for IL-33 (Nessy-1, Enzo Life Sciences) and tubulin. Net luminescence of bands corresponding to IL-33 in A and B were quantified and normalized to the loading control. The amount of IL-33 in control cells (mock) was set to 1 and the average fraction of three independent experiments were plotted showing the mean±SD. The data shown are representative of three independent experiments.

Figure 2. IL-33 upregulation is abrogated by UV irradiation of viral particles. (**A**) HUVECs were transduced with Ad5ΔE1ΔE3-GFP (10 moi) that had been UV-irradiated before adding the viral particles to the cells, harvested after 48 hours, and analyzed by immunoblotting with antibodies specific for IL-33 and tubulin. Net luminescence of the bands corresponding to IL-33 was quantified and normalized to the loading control. The amount of IL-33 in control cells (mock) was set to 1 and the average fractions of three independent experiments were plotted showing the mean±SD. (**B**) Transduced HUVECs from wells parallel to those sampled in panel A were harvested for flow cytometry as a control for UV-inactivation of Ad5ΔE1ΔE3-GFP. Gating for HUVECs was performed according to size (forward and side scatter).

Figure 3. Adenoviral upregulation of IL-33 depends on Notch signaling. HUVECs were transduced with Ad5ΔE1ΔE3 (10 moi) for 0-72 hours (A) or for 48 hours (B, C, D) before harvest and analysis by qPCR (A) or immunoblotting (B, C, D). (A) Transcription levels of Notch components and target genes in Ad5ΔE1ΔE3-transduced HUVECs. Graphs show the mean±SEM. (B) Levels of active (cleaved) NOTCH1 (csNICD1) in Ad5ΔE1ΔE3-transduced HUVECs. (C) HUVECs were transfected with siRNA targeting NOTCH1 (N1), DLL4 and IL-33 (as a positive control) 24 hours before transduction with Ad5ΔE1ΔE3 and analyzed for csNICD1 and IL-33 to assess the effect of Notch inhibition on nrAd5 stimulated IL-33. (D) Neutralizing antibodies specific for NOTCH1 (N1) (0.3 µg/mL) and DLL4 (0.3 µg/mL), isotype-matched control IgG (0.3 µg/mL), and the gamma-secretase inhibitor DAPT (5 µM) were administered 15 min before transduction with Ad5ΔE1ΔE3, and lysates were analyzed for expression of csNICD1 and IL-33. The net luminescence of bands corresponding to

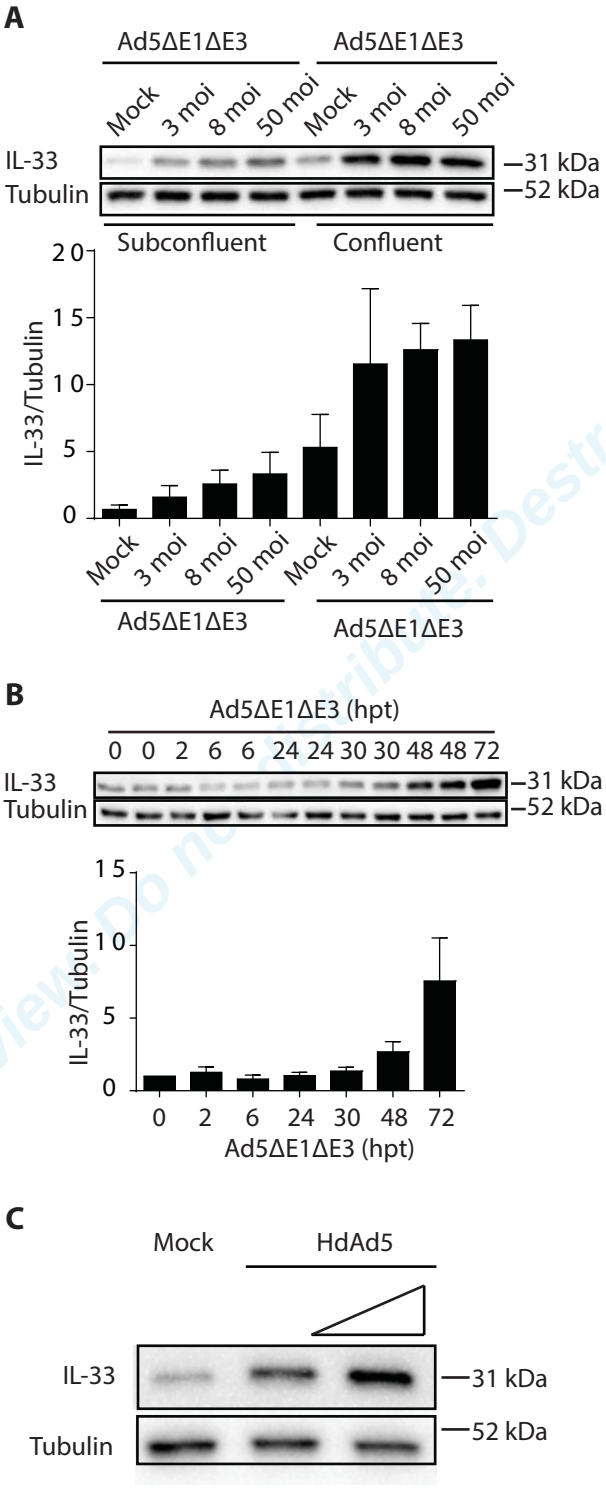
csNICD1 and IL-33 were quantified and normalized to the loading control. The amounts of csNICD1 and IL-33 in control cells (mock) were set to 1 and the average fractions of two independent experiments were plotted showing the mean. The data shown are representative of three (A, B, C) or two (D) independent experiments.

Figure 4. Adenoviral stimulation of IL-33 depends on the antiviral transcription factor IRF1. HUVECs were transduced with Ad5ΔE1ΔE3 (10 moi) for the indicated times (**A**, **D**) or for 48 hours (**B**, **C**, **E**, **F**) before harvest of nuclear and cytoplasmic fractions (**A**) or whole cell extracts (**B**, **C**, **D**) and analysis by immunoblotting with antibodies as indicated or qPCR (E-F). (**A**) Levels of IRF7, IRF1 and IRF3 in cytoplasmic and nuclear fractions of HUVECs transduced with Ad5ΔE1ΔE3. Lysates of HUVECs stimulated with TNF-α (10 ng/mL for 2 hours) or interferon-α (100 ng/mL for 4 hours) were included as positive controls for IRF expression. (**B-C**) Levels of IL-33 after siRNA-mediated depletion of IL-33 (as a positive control) and IRF3 (**B**) or IRF1 (**C**) 24 hours before transduction with Ad5ΔE1ΔE3. (**D**) Levels of pSTAT1 and STAT1 in Ad5ΔE1ΔE3 transduced HUVECs. Net luminescence of bands corresponding to IL-33 and pSTAT1 were quantified and normalized to the loading control. The amount of IL-33 and pSTAT1 in control cells (mock) were set to 1 and the average fractions of three independent experiments were plotted showing the mean±SD. (**E**) A neutralizing antibody specific for the INF-α/β receptor (MMHAR-2) or a negative control antibody was administered to HUVECs 30 minutes before transduction with Ad5ΔE1ΔE3. IL-33 expression was analyzed by qPCR. (**F**) Supernatants from Ad5ΔE1ΔE3-transduced HUVECs 24 hpt were transferred to non-transduced, subconfluent cells. Cells were harvested after another 24 hours and analyzed by qPCR. Fresh growth medium was used as control (The graph shows the mean±SD. The SD is not visible due to low variation). IL-33 mRNA expressions are presented relative to control cells with HPRT as a reference gene. The data shown are representative of three independent experiments.

Figure 5. nrAd5 activates a MRE11-dependent DNA Damage Response in HUVECs and MRE11 mediates the nrAd5 stimulation of IL-33. HUVECs were transduced with Ad5ΔE1ΔE3 (10 moi) for 0-72 hours (**A, B**) or for 48 hours (**C**) before harvesting and analyzing by immunoblotting as designated. (**A**) DNA damage components are activated in HUVECs in response to nrAd5. (**B**) The net luminescence of the bands in panel A was quantified and normalized to the loading control. The average amount of luminescence relative to control cells (mock) of three individual experiments was plotted. (**C**) ATM, RAD50, NBS1, MRE11 or IRF1 was depleted using siRNA 24 hours before transduction

with Ad5ΔE1ΔE3. The stippled box emphasizes the effect of MRE11 knockdown in Ad5-transduced cells. * indicates proteins that were reduced after MRE11 depletion (pATM, IL-33, RAD50, MRE11) (**D**) To inhibit MRE11 endonuclease activity HUVECs were treated with mirin at the indicated concentrations together with nrAd5 as designated. (**E**) STING was depleted using two different siRNAs (STING1 and STING2) 24 hours before transduction with Ad5ΔE1ΔE3 (10 moi). The net luminescence of the bands in panel D and E was quantified and normalized to the loading control. The average amount of luminescence relative to control cells (mock) of three individual experiments was plotted. The data shown are representative of three independent experiments.

Stav-Noraas et al - Figure 1



Stav-Noraas et al - Figure 2