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IL-19 Reduces VSMC Activation by Regulation of mRNA Regulatory Factor HuR and Reduction of mRNA Stability

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Abstract

Aims—While much is known about the deleterious effects of pro-inflammatory cytokines on development of vascular disease, little is reported on direct effects of anti-inflammatory cytokines on the vascular smooth muscle cell (VSMC) response to injury. Interleukin-19 (IL-19) is a recently described Th2, anti-inflammatory interleukin. We have previously reported that IL-19 is absent in normal VSMC, but induced in VSMC by inflammatory cytokines and in arteries by injury. IL-19 is anti-proliferative for VSMC. The purpose of this study is to determine the molecular mechanism of these effects.

Methods and Results—In cultured, primary human VSMC, IL-19 reduces abundance of proliferative and inflammatory gene proteins and mRNA, including Cyclin D1, IL-1 β , IL-8, and COX2. IL-19 does not inhibit NF- κ B, but does transiently reduce cytoplasmic abundance of the mRNA stability factor HuR. The mRNA stabilizing function of HuR is linked to its phosphorylation and cytoplasmic translocation. IL-19 reduces serine phosphorylation of HuR, and activation of PKC α , a known regulator of HuR translocation. Actinomycin D transcription blockade demonstrates that IL-19 treatment significantly reduces stability of proliferative and inflammatory mRNAs. Knock down of HuR with siRNA also reduces stability of these inflammatory mRNA transcripts.

Conclusions—These data indicate that IL-19 has direct effects on VSMC mRNA stability. One potential mechanism whereby IL-19 reduces the VSMC response to injury is by regulation of HuR abundance and cytoplasmic translocation, with a subsequent decrease in mRNA half-life of proliferative and inflammatory mRNA transcripts.

Keywords

Interleukin-19; vascular smooth muscle cell; mRNA stability; HuR; anti-inflammatory cytokine; actinomycin; Protein Kinase C

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CONFLICT OF INTEREST:

none declared

INTRODUCTION

Many vascular diseases are inflammatory in nature, and the deleterious effects of pro-inflammatory cytokines on vascular smooth muscle cell (VSMC) pathophysiology are well characterized [1–3]. VSMC are capable of synthesizing many pro-inflammatory immune modulators, including IL-1 β , IL-8, and MCP-1 [1–4]. Although a great deal of attention has been given to the negative effects of pro inflammatory interleukins in vascular disease, little has been reported on the potential protective effects of anti-inflammatory cytokines on the VSMC response to injury [5]. Utilizing systemic infusions, knock out, or transgenic mouse approaches, important literature reports protective effects of anti-inflammatory interleukins on the vascular response to injury [5]. Summation of this literature suggests that reduction of vascular injury attributed to these soluble factors are paracrine, and likely mediated by alteration of the T lymphocyte Th2/Th1 ratio toward a more anti-inflammatory phenotype. This dampens the host immune response and subsequently reduces the vascular response to injury [3,5,6,7]. A limitation with this approach is that since the infusion is systemic and effects every cell type, it cannot be determined if these anti-inflammatory cytokines target and directly suppress VSMC activation. A gap in our knowledge exists regarding potential direct molecular effects and mechanisms of anti-inflammatory interleukins on VSMC pathophysiology.

We have previously reported that the IL-10 family member, Interleukin-19 (IL-19) is expressed in injured, but not uninjured arteries, and in stimulated, but not quiescent VSMC [8]. IL-19 expression is constitutive in monocytes, T, and B lymphocytes, and can be up regulated in these cells by LPS and G-CSF [9–10]. IL-19 is considered to be an anti-inflammatory interleukin, as in T-lymphocytes it promotes the Th2 [regulatory], rather than the Th1 (T helper) response [9–11]. It is known that IL-19 can down-regulate Th1-like adaptive immune responses in T-cells, but the molecular mechanisms of these effects on any cell type remains uncharacterized. In an earlier study we found that IL-19 had anti-proliferative effects on cultured VSMC, and IL-19 adenoviral gene transfer significantly reduced neointimal hyperplasia and proliferation of medial and intimal VSMC in balloon angioplasty-injured rat carotid arteries [8]. This remains the only report in the literature describing IL-19 expression and function in VSMC.

The purpose of the present study is to extend those initial observations and identify potential molecular mechanisms for IL-19 growth-suppressive effects in VSMC. In the present study, we demonstrate that one means whereby IL-19 may attenuate VSMC proliferation is by reduction in abundance of proliferative and inflammatory proteins. One mechanism for these suppressive effects is by IL-19-mediated reduction in the abundance and cytoplasmic translocation of the mRNA stability factor HuR (Human antigen R), resulting in decreased half-life of proliferative and inflammatory mRNA transcripts. The expression and direct autocrine or paracrine effects of IL-19 on VSMC has important implications for the role of Th2 cytokines in regulation of the vascular response to injury, vascular and immune cell crosstalk, and local inflammation.

MATERIALS and METHODS

Cells and culture

Primary human coronary artery VSMC were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and subcultured in growth medium as described previously [8]. Cells from *three different male donors, aged 18–25 years*, passage 3–5 were used in the described studies. Recombinant IL-19 (100ng/ml for all studies) was from R&D, Inc.

Western blotting

Human VSMC extracts were prepared as described [8]. Membranes were incubated with a 1:2000–4000 dilution of primary antibody, and a 1:5000 dilution of secondary antibody. *Interleukin-1 beta (IL-1 β)*, *Interleukin-1 (IL-8)*, *cyclooxygenase 2 (COX2)*, *Cyclin D1*, *pan-actin*, *heterogeneous nuclear ribonucleoprotein (HnRNP)*, *Human antigen R (HuR)*, *AU-factor-1 (AUF-1)*, *Inhibitor of kappa B (I κ B)*, *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, *proliferating cell nuclear antigen (PCNA)*, and *Rac1* antibody were from Santa Cruz, Inc.; *total and phospho-serine and phospho-PKC (protein kinase C)* were from Cell Signaling, Inc. Reactive proteins were visualized using enhanced chemiluminescence (Amersham) according to manufacturer's instructions. Relative intensity of bands was normalized to GAPDH and SMC actin, and quantitated by scanning image analysis and the Image J densitometry program.

Transfection and siRNA knockdown

Gene silencing was performed using ON-TARGET plus SMARTpool HuR siRNA (30 nM) purchased from Dharmacon, Inc. SMARTpool HuR siRNA contains a mixture of four siRNAs which target HuR. Transfection of VSMC was performed using the Human AoSMC Nucleofector™ Kit (Amaxa, Inc) following the manufacturer's instructions. *Using this method transfection efficiency was between 70–90%, and >95% of the HuR protein was reduced.* Lysates were immunoblotted for HuR 72 hours post-transfection, and RNA extracted 72 hours post-transfection.

RNA extraction and quantitative RT-PCR

RNA was isolated and reverse transcribed into cDNA as we have described, and target genes amplified using an Eppendorf Realplex4 Mastercycler [8]. Multiple mRNAs (Ct values) were quantitated simultaneously by the software. The following primer pairs were used: Cyclin D1: F: TATTGCGCTGCTACCGTTGA, R: CCAATAGCAGCAAACAATGTGAAA, COX2: F:GAATCATTCACCAGGCAAATT, R: TCTGTACTGCGGGTGGAAACA, HuR: F: CCGTCACCAATGTGAAAGTC, R:TCGCGGCTTCTTGATAGTTT, GAPDH: F: CGAGAGTCAGCCGCATCTT, R: CCCCATGGTGTCTGAGCG, IL-1 β : F:TTCCCAGCCCTTTTGTGTTGA, R:TTAGAACCAAATGTGGCCGTC, IL-8: F:CCAGGAAGAAACCAGGGGA, R: GAAATCAGGAAGGCTGCCAAG, PCNA: F: TCCTGTGCAAAAGACGGAGTG R: TCTACAACAAGGGGTACATCTGC. Some samples were treated with or without 10 μ g/ml of the transcription inhibitor Actinomycin D and RNA extracted at indicated time points as described.

Cellular fractionation

To obtain cytoplasmic fractions VSMC were washed in PBS, scraped off the plates, and collected by low speed centrifugation for 5 min at 4°C. Cell pellets were lysed in *Lysis Buffer* (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, and 0.5% NP-40) containing protease inhibitors (1 μ M DTT, 1mM PMSF, 0.5 μ g/mL Leupeptin, 0.5 μ g/mL Pepstatin A and 0.5 μ g/mL Aprotinin) on ice for 10 minutes, centrifuged, and the supernatant isolated as cytoplasmic proteins as described [12]. One hundred μ l of Lysis Buffer was used for 5–10 $\times$ 10⁶ cells. The supernatant was saved as the cytosolic fraction.

Statistical analysis

Results are expressed as mean $\pm$ SE. Differences between groups were evaluated with the use of ANOVA, with the Newman-Keuls method applied to evaluate differences between individual mean values and by paired t tests where appropriate, respectively. Differences were considered significant at a level of P<0.05.

RESULTS

IL-19 Decreases Inflammatory and Proliferative Gene abundance

We have previously shown that IL-19 treatment significantly reduces VSMC proliferation in vivo and in culture VSMC [8]. We hypothesized that at least one mechanism for IL-19 proliferation dampening effects was an alteration of abundance in inflammatory and proliferative proteins in stimulated VSMC. VSMC were serum-starved to approximate baseline expression of target proteins, pre-treated with 100ng/ml IL-19 for 16 hours, then stimulated with 10% Fetal Calf Serum (FCS) for 24 hours (Cyclin D1, IL-8), or 48 hours (COX2, IL1 β) to elicit maximal induction of protein. These targets were chosen because they are representative markers of inflammation and proliferation, and are easily inducible by FCS. FCS was used because it contains a combination of proliferative and stimulatory cytokines as what would occur in vivo, and serves as a potent stimulatory agent for each of these multiple targets. Figure 1A illustrates that IL-19 pre-treatment significantly reduces the abundance of IL-1 β , IL-8, COX2, and Cyclin D1 proteins to varying degrees ($P < 0.05$ or 0.01 , $n = 4$, for all proteins, see supplemental data). *IL-19 can decrease abundance of proteins induced by more defined stimuli such as Tumor necrosis Factor alpha (TNF α) and Platelet Derived Growth Factor (PDGF) (please see supplemental data 2).* This is notable in that all of these proteins have demonstrated important roles in VSMC activation and development of vascular pathologies [1]. Preliminary experiments varying IL-19 pretreatment times, with target protein abundance as the readout found that 16 hour pretreatment with IL-19 was optimal for reduction.

Messenger RNA abundance for these proteins was examined by quantitative RT-PCR (qRT-PCR). IL-19 pretreatment causes a significant, but transient reduction of inflammatory and proliferative mRNA between 18.3 – 52.5% of untreated controls (Figure 1B, Table 1) ($P < 0.001$ for all). Four hour pretreatment was found to be optimal for inhibition using mRNA abundance as the readout, with the exception of Cyclin D1 mRNA, which remained significantly decreased when IL-19 was added at the same time or 2 hours post stimulation. One attribute that each of these transcripts have in common is the presence of one or more classes of AU-rich elements (ARE) stability elements in the 3' untranslated region (UTR) of their respective mRNA [13]. *We also examined abundance of Proliferating Cell Nuclear Antigen (PCNA), an important FCS-responsive protein, but one which lacks an ARE (AU-rich element) in its 3'UTR. Figures 1A and 1B show that PCNA protein and mRNA abundance are unaffected by IL-19 treatment, respectively.* Other proteins and mRNA including actin, GAPDH, NF- κ B subunit p65, MAPK p44/42 and p38, all which lack an ARE are also unaffected by IL-19, suggesting the importance of the ARE in mediation of IL-19 gene suppressive effects. This is the first demonstration of diminution of proliferative and inflammatory protein and mRNA abundance for IL-19 in any cell type.

IL-19 decreases HuR abundance and cytoplasmic abundance

Many suppressive effects ascribed to IL-10 are mediated by inhibition of NF- κ B activation [14,15]. In contrast to IL-10, we observed that *there is no difference in I κ B abundance between IL-19 treated and control samples*, (Figure 2A) or does IL-19 reduce NF- κ B DNA binding (not shown). A secondary mechanism ascribed to IL-10 anti-inflammatory effects in immune cells is through reduction in mRNA stability of inflammatory cytokines [16,17]. Considering our data suggesting the importance of the ARE in mediation of IL-19 suppressive effects, we investigated if IL-19 could reduce expression of deleterious proteins by reduction in mRNA stability. We examined if IL-19 could modify the abundance of HuR, a member of the ELAV family of mRNA stability proteins which regulates mRNA abundance in stimulated VSMC [18,19]. VSMC were serum-starved to approximate basal conditions, and HuR detected at various times post-IL-19 addition. HuR is constitutively expressed in many cell types, yet four hour incubation of VSMCs with IL-19 causes a rapid, but transient reduction of HuR protein

(Figure 2B). *IL-19 treatment results in significant (59.1±9%) decrease in HuR abundance at 4 hours (P<0.05 n=4).* IL-19 also caused a moderate, but significant and transient reduction in HuR mRNA abundance (Figure 2C). IL-19 has no effect on abundance of any isoform of AU Factor-1 (AUF-1), an mRNA destabilizing protein [13]. This is the first report showing that an anti-inflammatory cytokine can reduce HuR abundance in VSMC. The transient nature of IL-19 led us to investigate the effects of chronic IL-19 exposure on HuR abundance. Figure 2D shows that VSMC infected with IL-19 Adenovirus maintain a sustained decreased in HuR 72 hours post infection.

The ability of HuR to stabilize mRNA corresponds with its translocation from a predominate nuclear location to the cytoplasm, which in VSMC peaks between 6 and 8 hours of FCS or PDGF stimulation [18,19]. We tested if IL-19 pre-treatment of VSMC could inhibit FCS-driven HuR cytoplasmic abundance. Twenty four hours of pretreatment was necessary because at this time point, total HuR levels return to baseline (Figure 2). Figure 3 shows that in quiescent VSMC, HuR is predominantly nuclear, and as expected, FCS stimulation drove a portion of HuR into the cytoplasm, which peaked at 8 hours. *We observed a significant increase in cytoplasmic HuR abundance at 6 and 8 hours stimulation (51.2 and 59.1%, for 6 and 8 hours, respectively P<0.05, n=4).* It has previously been estimated that approximately 10% of nuclear HuR translocates into the cytoplasm in response to FCS, which is consistent with our results (18). Twenty four hour pretreatment with IL-19 significantly inhibited HuR translocation by 79.4% and 90.7% at 8 and 24 hours post-FCS stimulation, respectively (P<0.05 for both). This is not a result of reduced HuR abundance at these times, as determined by total HuR amounts, which stayed constant due to the presence of serum. These data indicate that not only does IL-19 decrease total HuR accumulation, but also its cytoplasmic abundance, indicating a complex mechanism of HuR regulation by IL-19.

IL-19 inhibits HuR serine phosphorylation and PKCα activation

HuR cytoplasmic translocation is associated with its serine phosphorylation [20,21]. To determine if IL-19 reduction of HuR translocation was due to a decrease in HuR phosphorylation, VSMC were serum starved, then stimulated with 10% FCS for various times. Time course studies determined that 45 minutes stimulated resulted in maximal HuR phosphorylation [please see Supplemental data 3]. In the next series of experiments, serum-starved VSMC were pre-treated with IL-19, then stimulated with 10% FCS for 45 minutes, at which point HuR was immunoprecipitated with HuR antibody, then blotted with phosphoserine antibody. *Reverse immunoprecipitation was also performed in which proteins were precipitated with anti-phosphoserine antibody, then blotted with HuR antibody. The results of both directions of immunoprecipitation are presented in Figure 4A and show that IL-19 pre-treatment significantly reduces FCS-induced HuR serine phosphorylation (39.1±5, and 84.5 ±7% for forward and reverse immunoprecipitations, P<0.05 n=3 for both directions).* Studies in mesangial cells have implicated the involvement of PKCα in the serine phosphorylation of HuR [21,22]. To determine if IL-19 decreases activation of PKCα, VSMC were serum-starved, pre-treated with IL-19, then stimulated with 10% FCS for 45 minutes, then immunoblotted with antibody specific for the activated (phospho-specific form) PKCα. Figure 4B shows that IL-19 pre-treatment reduces FCS-induced PKCα phosphorylation. Quantitation by densitometry of three independent experiments shows significant reduction of PKCα phosphorylation by IL-19 (P<0.05 at 45 minutes stimulation n=3) (Figure 4C). This is the first report of inhibition of PKCα by IL-19.

IL-19 decreases Inflammatory and Proliferative gene mRNA Stability

The ARE of the transcripts decreased by IL-19 are all predicted to be targets of HuR [13]. We hypothesized that since IL-19 decreased HuR abundance and translocation, then it would also decrease mRNA stability of these transcripts. To test this hypothesis, VSMC were serum

starved 48 hours, stimulated with 10% FCS for 4 hours, one group was pre-treated with IL-19 for 4 hours (time needed for HuR reduction, Figure 3). After 0, 2 and 4 hours exposure to the transcription inhibitor Actinomycin D, RNA was isolated and target mRNA accumulation was assessed by quantitative RT-PCR normalized to GAPDH. Figure 5 shows that with IL-19 treatment, mRNA stability of Cyclin D1, IL-1 β , IL-8, and Cox2 are significantly decreased ($P < 0.02$ or 0.01 for all). The half life levels of these mRNAs are significantly reduced compared with untreated controls (Table 1) ($P < 0.05$ or 0.01 for all). *Similar to what we found in Figure 1, IL-19 has no effect on PCNA mRNA stability. This suggests that IL-19 decreases inflammatory and proliferative gene mRNA stability of selected ARE containing mRNA transcripts.*

To further define a role for HuR in this process, VSMC were transfected with HuR siRNA or scrambled control, and treated with Actinomycin D as described in the previous section. Figure 6F shows that HuR siRNA was effective in reducing HuR protein abundance. Figures 6A–E show that stability IL-1 β and IL-8, Cyclin D1, and COX2 mRNA are significantly reduced when HuR is knocked down ($P < 0.01$ for all). The half-life levels of these transcripts are reduced 62%, 36%, 45%, and 16% for IL-1 β , IL-8, COX2, and Cyclin D1, respectively. As expected, depletion of HuR has no effect on PCNA mRNA stability. Together, this associates HuR abundance with stability of these transcripts in human VSMC.

DISCUSSION

We previously reported that VSMC could express IL-19 and that IL-19 could significantly reduce proliferation of VSMC [8]. To identify mechanisms for these effects, we began the present study with an investigation of IL-19 treatment of VSMC on abundance of proliferative and inflammatory mRNA and protein. There are 5 novel features of the present study. The first unique finding is that IL-19 could reduce both protein and mRNA abundance of several inflammatory and proliferative genes, all of which play a pertinent role in vascular pathology. To our knowledge, this is the first report of the effect of IL-19 on abundance of regulatory proteins. *Optimal IL-19 pre-treatment times differed between readouts of protein or mRNA abundance. One reason may be because temporally, mRNA expression precedes protein expression. An additional reason could be that mRNA abundance represents transcription and stability, whereas protein accumulation involves many more endpoints, including mRNA processing, translation, and protein turnover.*

IL-10 has been shown to inhibit VSMC proliferation, and it was proposed that these observed growth inhibitory effects are due to inhibition of NF- κ B -dependent inflammatory gene expression [22,23]. IL-19 had no inhibitory effect on NF- κ B, as assayed by I κ B degradation or EMSA. While this does not completely rule out regulation of transcriptional mechanisms by IL-19, it does eliminate the contribution of NF- κ B, a major regulator of inflammatory gene transcription. This is an interesting distinction which contrasts IL-19 from IL-10, and also prompted us to investigate other mechanisms of IL-19 effects. In immune cells, one means whereby IL-10 can dampen the inflammatory response is by modification of post-transcriptional processing of inflammatory proteins [16,17,23]. One hundred ng/ml of IL-19 was used in these studies, which is the same concentration of IL-10 used to decrease mRNA half life in monocytes [16]. The 3' UTR of many labile transcripts associated with inflammation contain AU-rich elements (ARE) which are target sites for these mRNA-binding stability factors. Different classes of ARE have been identified based on their nucleotide sequence, which is generally a repeat of an AUUUA motif, or a variation thereof [24]. The transcripts for IL-1 β , IL-8, COX2, and Cyclin D1 all contain an ARE element and are predicted or have been shown to bind to HuR [13]. In this study mRNA and protein of these transcripts are reduced, whereas PCNA and other mRNAs, though an important regulators of VSMC activation, do not contain these elements and are unaffected by IL-19.

One report has described the down-regulation of HuR mRNA binding activity by IL-10 in monocytes [17]. HuR is a constitutively expressed mRNA stability protein which binds to AREs found in the 3' UTR of many labile mRNA transcripts, prompting us to investigate the effects of IL-19 on HuR abundance. The second novel finding of this study is that IL-19 treatment transiently reduces HuR abundance at both the protein and mRNA level. It is noteworthy that a single administration of IL-19 to VSMC results in a rapid, transient, and rather dramatic decrease in abundance of HuR protein. The decrease in protein is transient, and rather dramatic, considering the modest decrease in mRNA, suggesting that additional mechanism[s] may decrease HuR protein abundance. HuR is not ubiquitinated upon IL-19 treatment [data not shown]. Based on its cDNA sequence, human HuR 3'UTR does not contain any classical ARE binding sequences, but does contain two AUUUUA heptanucleotides within proximity to a U-rich region. This may classify the HuR 3'UTR as a Class III ARE [13]. It has been shown that decay kinetics of mRNAs containing class III AREs are only moderately affected by Actinomycin D [25], and in our investigation, mRNA stability experiments were not informative (data not shown). HuR is not induced by serum in VSMC. Further studies may be necessary to identify mechanisms responsible for the observed decrease in HuR protein.

The transient nature of HuR attenuation observed in cultured VSMC challenged with a single administration of IL-19 may not mirror the in vivo situation. Chronic expression of adenoviral delivered IL-19 results in a more sustained reduction in HuR protein abundance. This would be more akin to the in vivo scenario in which infiltrating lymphocytes and/or activated VSMC are present at the vascular lesion, and would synthesize IL-19 in a more chronic fashion. Figure 2 shows that chronic synthesis of IL-19 by VSMC results in a prolonged decrease in HuR abundance, and may more directly identify a molecular mechanism for IL-19 anti-inflammatory effects. To our knowledge, no other study has reported a decrease in HuR abundance in response to any cytokine or drug. IL-19 does not effect protein abundance of AUF-1, an mRNA destabilizing protein. This is the first report showing that an anti-inflammatory interleukin can reduce HuR abundance.

HuR resides in the nucleus in unstimulated cells, and its cytoplasmic shuttling is requisite for its mRNA stabilizing effects [20,26,27]. In addition to an IL-19 decrease in HuR protein abundance, a third original and important finding of this study is that IL-19 reduces HuR serine phosphorylation and cytoplasmic translocation. Serine phosphorylation of HuR is the target of many signaling pathways, and is essential for nuclear to cytoplasmic shuttling induced by serum or inflammatory stimuli [27]. We have previously determined that IL-19 treatment of VSMC inhibits activation of both p38 and p44/42 MAPKs [8]. However, HuR does not contain consensus phosphorylation sites for these MAPKs, and pharmacological inhibitors of p44/42, p38, or MEK had no effect on inhibition of cytoplasmic shuttling of HuR [20,21]. Amino acid motif analysis identifies 7 consensus PKC phosphorylation sites on HuR (ProSite database), and studies in non-VSMC have implicated the involvement of PKC α in this process [20,21]. Thus, a fourth novel observation of this study is that IL-19 pretreatment reduces both serine phosphorylation of HuR, as well as PKC α activation by FCS, providing a potential mechanism for IL-19 attenuation of HuR cytoplasmic shuttling. While this study does not show that PKC α is directly responsible for HuR serine phosphorylation, it does place both PKC α and HuR as part of an IL-19-sensitive signaling pathway. The association between PKC α , HuR phosphorylation, and HuR translocation in VSMC has not been reported. Together, these data indicate that not only does IL-19 decrease HuR accumulation, but also its cellular localization, indicating a complex mechanism of HuR regulation by IL-19.

Whereas many inflammatory cytokines, particularly IL-1, and TNF α have been shown to stabilize inflammatory gene transcripts, very few have been shown to destabilize mRNA. Use of the transcription inhibitor Actinomycin D shows that IL-19 treatment can decrease stability of inflammatory and proliferative transcripts. We have previously reported that IL-19 induces

rapid and transient expression of suppressor of cytokine signaling 5 (SOCS5) [8]. While the SOCS family of proteins inhibit signal transduction by binding to tyrosine phosphorylated residues on receptor chains, resulting in an attenuation of signaling, there are no reports of involvement of these proteins in regulation of mRNA stability. While this study does not definitively rule out other transcriptional, translational, or degradative mechanisms for IL-19 dampening of protein accumulation, this is the first to show that IL-19 can reduce mRNA stability. While little has been reported on expression and function of HuR in VSMC, one important manuscript does show that enhanced proliferation of cultured hVSMC is linked to increases in HuR activity, and that many transcripts encoding proliferation proteins were reduced when VSMC were treated with HuR siRNA [18]. In this study we extended that report and were able to show that a decrease in HuR abundance by siRNA emulated IL-19 treatment in that stability of IL-10 β and IL-8 were both decreased.

IL-19 is a recently described immunomodulatory Th2 interleukin, the expression of which had been ascribed to be restricted to inflammatory cells. In addition to the unexpected finding that IL-19 is expressed and has suppressive effects in VSMC, this is the first report to indicate that IL-19 can reduce the abundance of inflammatory and proliferative proteins in any cell type. A probable mechanism of these effects is by regulation of HuR abundance and cytoplasmic translocation, leading to a decrease in mRNA half-life of deleterious transcripts. At least one mechanism for the decrease in HuR translocation is by inhibition of PKC α activity. Our working hypothesis is that IL-19 induces suppressive effects on VSMC pathophysiological processes by attenuation of proliferative and inflammatory gene abundance. Expression of Th2 cytokines by activated VSMC may represent a negative auto-regulatory autocrine or paracrine feed back mechanism to promote resolution of the local vascular response to injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. IL-19 pretreatment decreases proliferative and inflammatory protein expression. Human VSMC were serum-starved, pre-treated with 100ng/ml IL-19, then stimulated with 10% FCS. Extracts were blotted with the indicated antibody. Western blot shown is representative of at least 3 independent experiments, with identical results. Differences in IL-19 pretreated Vs controls are significant ($P < 0.05$, $n = 4$, see supplemental material). Actin and GAPDH were used as a loading control, and PCNA is a non-ARE-element control. **B.** IL-19 decreases mRNA abundance. VSMC were serum-starved, pre-treated with IL-19, for the times shown, then stimulated with 10% FCS. Total RNA was reverse transcribed and target mRNA quantitated by qRT-PCR. Differences in IL-19 pretreated Vs. no-IL-19 treated controls are significant

($P < 0.05$ or 0.01 , $n=3$) for all at 4 hours pre-treatment and same time treatment, at 16 hour pretreatment for Cox2, IL-1 β , and IL-8, and 2 hours post-treatment for Cyclin D1. mRNA was normalized to Actin and GAPDH, and *PCNA* is a non-ARE-element control.

Figure 2.

IL-19 reduces HuR protein and mRNA abundance. **A.** IL-19 does not activate NF- κ B. VSMC were serum-starved, then stimulated with 10% FCS for the indicated times. IkB degradation was assessed by western blot. **B.** Human VSMC were serum-starved, then stimulated with IL-19 for the indicated times, and blotted with the indicated antibody. **C.** Quantitative RT-PCR. VSMC treated as for western, with RNA extracted at the times indicated. Values reported as % of stimulated samples. $P < 0.05$ or 0.01 , $n = 3$ independent experiments. **D.** Chronic expression of IL-19 in VSMC results in sustained decrease in HuR abundance. Serum-starved VSMC were infected with AdLacZ or AdIL-19 at 30MOI. At 72 hours post infection, extracts

were made and immunoblotted with IL-19, HuR, or GAPDH antibody. All Western blots shown are representative of at least 3 independent experiments, with identical results.

A. **B.** **Figure 3.**

Inhibition of cytoplasmic translocation of HuR by IL-19. **A.** HuR protein abundance in cytoplasm of VSMC stimulated with 10% FCS, and pre-treated with IL-19 prior to 10% FCS stimulation. Actin was used as a cytoplasmic fraction control. HnRNP was absent from this fraction [not shown]. Representative blot shown is representative of at least 4 independent experiments. **B.** Densitometric analysis of HuR cytoplasmic abundance. Amount of cytoplasmic HuR is significant at 6 and 8 hours post-FCS stimulation, and is significantly inhibited by IL-19 ($P < 0.05$, $n = 4$, for all).

Figure 4.

IL-19 reduces HuR serine phosphorylation and PKC α activation. **A.** VSMC were serum starved, then stimulated with 10% FCS for 45 minutes, or, pre-treated with IL-19 prior to 45 min stimulation with 10% FCS. HuR was immunoprecipitated with HuR antibody, then blotted with phosphoserine or HuR antibody as a loading control. In a reverse-direction IP, VSMC were treated as described, but anti-phosphoserine antibody was used to immunoprecipitate, and proteins blotted with anti-HuR antibody. **B.** IL-19 pre-treatment inhibits FCS-induced PKC α phosphorylation. VSMC pretreated with IL-19 prior to 45 minute (peak) stimulation with 10% FCS. Lysates were blotted with anti-phospho-specific PKC α . Both images shown are representative of at least 4 independent experiments. **C.** Densitometry was performed and

values normalized to total protein. Values and means from at least 3 experiments. Asterisk indicates significant difference from control ($P<0.05$). Figures shown are representative of at least 3 performed.

Figure 5.

IL-19 decreases mRNA stability. After serum starvation, VSMC were stimulated with 10% FCS for 4 hours, one group was treated with IL-19 for 4 additional hours, at which time Actinomycin D was added. At 0, 2 and 4 hours of Actinomycin D exposure, RNA was isolated and target mRNA accumulation was assessed by quantitative RT-PCR normalized to GAPDH. All values are normalized as % of time 0. Differences in IL-19 pretreated Vs no-IL-19 controls are significant ($P < 0.05$ or 0.01 , $n = 3$).

Figure 6.

HuR knock down decreases IL-1 β and IL-8 mRNA stability. **A–E.** VSMC were transfected with HuR siRNA or scrambled control, then stimulated for 3 hours, at which time Actinomycin D was added. At 0, 2 and 4 hours of Actinomycin D exposure, RNA was isolated and target mRNA accumulation was assessed by quantitative RT-PCR normalized to GAPDH. All values are normalized as % of time 0. Differences in IL-19 pretreated Vs no-IL-19 controls are significant ($P < 0.05$ or 0.01 , $n = 3$). **F.** Seventy-two hours post transfection with 4 μ M HuR siRNA, HuR protein was examined by western blot. Representative of at least 4 experiments.

Table 1
mRNA half life in the presence and absence of IL-19 treatment (hours)

Effect of IL-19 on mRNA stability. Serum-starved VSMC were treated with or without IL-19 for four hours, then stimulated with 10% FCS for four hours, then treated with actinomycin D. RNA was extracted and transcripts quantitated at the times shown. For each mRNA, stability was significantly less when treated with IL-19, compared with untreated controls ($P<0.05$ for each mRNA shown). Results are mean $\pm$ SE from three independent experiments.

	control	IL-19
Cyclin D1	15.9 $\pm$ 5.5	5.47 $\pm$ 0.7
IL-1 β	4.4 $\pm$ 0.4	2.4 $\pm$ 0.1
IL-8	7.2 $\pm$ 2.3	2.5 $\pm$ 0.2
Cox2	5.9 $\pm$ 0.8	2.9 $\pm$ 0.3
PCNA	4.8 $\pm$ 0.1	4.6 $\pm$ 0.2