A20 Regulates Atherogenic Interferon (IFN)-γ Signaling in Vascular Cells by Modulating Basal IFNβ Levels*

Received for publication, June 25, 2014, and in revised form, September 11, 2014. Published, JBC Papers in Press, September 12, 2014, DOI 10.1074/jbc.M114.591966

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Background: IFNγ signaling is a major culprit in occlusive vascular disease. In vivo, inadequate A20 expression in A20 heterozygote mice aggravated intimal hyperplasia following partial carotid artery ligation. This outcome uniquely associated heterozygote mice aggravated intimal hyperplasia following

Results: The anti-inflammatory protein A20 negatively regulates IFNγ signaling in human EC and SMC by modulating STAT1 transcription. In vivo, inadequate A20 expression in A20 heterozygote mice aggravated intimal hyperplasia following partial carotid artery ligation. This outcome uniquely associated with increased levels of Stat1 and super-induction of IFN-γ-dependent genes. Transcriptome analysis of the aortic media from A20 heterozygote versus wild-type mice revealed increased basal Ifnβ signaling as the likely cause for higher Stat1 transcription. We confirmed higher basal IFNβ levels in A20-silenced human SMC and showed that neutralization or knockdown of IFNβ abrogates heightened STAT1 levels in these cells. Upstream of IFNβ, A20-silenced EC and SMC demonstrated higher levels of phosphorylated/activated TANK-binding kinase-1 (TBK1), a regulator of IFNβ transcription. This suggested that A20 knockdown increased STAT1 transcription by enhancing TBK1 activation and subsequently basal IFNβ levels. Altogether, these results uncover A20 as a key physiologic regulator of atherogenic IFNγ/STAT1 signaling. This novel function of A20 added to its ability to inhibit nuclear factor-κB (NF-κB) activation solidifies its promise as an ideal therapeutic candidate for treatment and prevention of vascular diseases. In light of recently discovered A20/TNFAIP3 (TNFα-induced protein 3) single nucleotide polymorphisms that impart lower A20 expression or function, these results also qualify A20 as a reliable clinical biomarker for vascular risk assessment.

Conclusion: A20 impacts IFNγ signaling by modulating basal IFNβ and downstream STAT1 expression.

Significance: This novel function of A20 supports its promise as a therapeutic target and prognostic marker for atherosclerotic disease.

IFNγ, typically secreted by Th1 and natural killer (NK) cells, is a potent cytokine with well characterized antiviral and immunomodulatory functions (1). In addition to its immunomodulatory effects, IFNγ, which is highly expressed in atherosclerotic lesions of patients and experimental animals, is recognized as a direct driver of pathologic vascular remodeling (2–4). Sole infusion of exogenous IFNγ is sufficient to cause transplant vasculopathy in human vascular allografts implanted into severe combined immunodeficiency mice (5), whereas knockdown of IFNγ receptor (Ifngr) in atherosclerosis-prone apolipoprotein E (ApoE) knock-out mice significantly attenuates atherosclerotic lesions (6). Accordingly, tight control of IFNγ production and signaling during antiviral and immune responses is necessary to avoid unforeseen pathologic responses, in particular in the vasculature.

The ubiquitin-modifying protein A20 (7) has recognized atheroprotective properties. Those stem from its potent anti-inflammatory function in EC and SMC through inhibition of NF-κB activation (8, 9). They also rely on its NF-κB-independent anti-apoptotic, anti-oxidative, and immunomodulatory functions in EC (10–12) and anti-proliferative and pro-apoptotic (only neointimal SMC) functions in SMC (9, 13–15). However, the impact of A20 on pro-atherogenic IFNγ signaling in EC and SMC had never been explored. In this study we uncovered A20 as a physiologic regulator of IFNγ signaling in human

*The abbreviations used are: NK, natural killer; EC, endothelial cell; SMC, smooth muscle cell; α-induced protein 3; NF-κB, nuclear factor-κB; IFNGR, IFNγ receptor; apoE, apolipoprotein E; ICAM-1, intercellular adhesion molecule-1; I-TAC, IFN-inducible T cell α chemottractant; IDO, indoleamine 2,3-dioxygenase; ISG, IFN stimulated gene; I kBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α; HET, A20 heterozygote mice; IH, intimal hyperplasia; CAL, partial carotid artery ligation; I/M, intima over media; LCM, laser capture microdissection; MAP3K7, mitogen-activated protein kinase kinase kinase 7; TBK1, TANK binding kinase-1; IKKα/β, IkB-kinase-α/β; TRAF-3, rAd, recombinant adenovirus; qRT, quantitative RT.
Loss of A20 Aggravates Pathologic Vascular IFNγ Signaling

TABLE 1

| Gene   | Species | Forward (5’-3’) | Reverse (5’-3’) |
|--------|---------|----------------|----------------|
| A20    | Human   | CCCCCAGAGAGAGCAGACATCAGTTCA | TCAAGTTCCCGGCGCTTC |
| CYP4A  | Human   | CTTTGACTGACACTGTCAGAGGCTA | CACCACGTCTCCGCACTCA |
| I-TAC  | Human   | AGGGACCGCTAAGCAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| ICAM-1 | Human   | TGCTGAAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| IDO    | Human   | CTAAGTTCGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| IRF1   | Human   | GAGTTGTCTAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| IRF7   | Human   | TCTCCAGTACGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| ip-10  | Human   | CTTCTAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| MCP-1  | Human   | GATGAGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| STAT1  | Human   | CCTCCAGTACGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| 28S    | Mouse   | ATACCGGCGAGAGCAGTGAGTCGA | GCGGACCCCCACCCCCGGTCC |
| Cd37y  | Mouse   | ATCAGGGCCGAGAGCAGTGAGTCGA | GCGGACCCCCACCCCCGGTCC |
| i-Tac  | Mouse   | AGGTGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Isam-1 | Mouse   | GACCCCGAGAGAGAGGAGGCTC | CCGGGCGCCGAGAGGCTC |
| Ido    | Mouse   | GATGAGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Ifnγ   | Mouse   | AGGTGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Irf1   | Mouse   | GAAATTAGAGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Irf3   | Mouse   | GTGAGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Irf5   | Mouse   | ACGCGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Ip-10  | Mouse   | GCTGAGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Maptk7 | Mouse   | TGAGGCTGAGGAGGAGGCTC | CCGGGCGCCGAGAGGCTC |
| Mav1-1 | Mouse   | TCCTGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |
| Stat1  | Mouse   | TGAGGCTGAGGAGGAGGCTC | CCGGGCGCCGAGAGGCTC |
| Stat2  | Mouse   | TGAGGCTGAGGAGGAGGCTC | CCGGGCGCCGAGAGGCTC |
| Tbp    | Mouse   | TTTCAGGAGGAGGAGGCTC | TTTGAGATCGTTCGGTCGC |

EC and SMC cultures and in vivo in a mouse model of pathologic vascular remodeling driven by hemodynamic injury to the vessel wall.

We ascribed the molecular basis of this novel function of A20 to its regulation of the expression of STAT1, the key mediator of IFNγ signaling (16). Overexpression of A20 significantly decreased and A20 knockdown significantly increased STAT1 expression. Upstream of STAT1, A20 uniquely modulated basal sub-threshold levels of IFNβ, a prime regulator of the cross-talk between type I and type II IFNs (17). By interrupting both IFNγ and NF-κB pro-atherogenic and inflammatory signals in vascular cells, A20 qualifies as an ideal candidate for the treatment of occlusive vascular diseases, the primary cause of death in the Western world (18).

EXPERIMENTAL PROCEDURES

Cell Culture—Primary human coronary artery EC and SMC were purchased from Lonza (Allendale, NJ) and cultured in 5% CO2-humidified atmosphere using EGM2-MV (EC) and SmGM (SMC) media supplemented with insulin, 5% fetal calf serum, and respective growth factors, as recommended. In all experiments, cells between passage 5 and 7 were serum-starved 24 h before stimulation. Human recombinant IFNγ was purchased from PeproTech (Rocky Hill, NJ). Neutrophizing sheep anti-IFNα and IFNβ antisera were obtained from PBL InterferonSource (Piscataway, NJ). The monocytic cell line U937 was cultured in Dulbecco’s modified essential medium (DMEM; Sigma) supplemented with 10% FCS, penicillin, and streptomycin.

Recombinant Adenoviruses—A20 cDNA, a kind gift of Dr. Vishva Dixit (Genentech, previously University of Michigan), was cloned into pAC.CMV-plPASR* to generate a recombinant adenovirus (rAd.) (11). rAd.Inhibitor of Nr-κBα (IκBα), encoding porcine IκBα, was generated by C. J. Wrighton (19). rAd.A20 was a kind gift of Dr. Robert Gerard (University of Texas, Southwestern Medical Center). Human SMC cultures were transduced at a multiplicity of infection of 500, which achieves expression of the transgene in >95% of the cells without causing toxicity (9).

siRNA Transfection—EC (A20) and SMC (A20, STAT1, and IFNβ) were transfected with specific FlexiTube siRNA (Qiagen, Valencia, CA) at a final concentration of 20 μM, using HiPerfect transfection reagent (Qiagen) in serum-free Opti-MEM medium (Invitrogen). Four hours after transfection, the medium was replaced with regular culture medium, and cells were maintained in culture for 24 h prior to being used in selected experiments. EC and SMC transfected with AllStars negative control siRNA (Qiagen) served as controls.

Quantitative Real Time-Polymerase Chain Reaction—RNA from cells and tissue samples was extracted using RNeasy mini kit (Qiagen) and reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). cDNA was subsequently analyzed by quantitative PCR using gene-specific primers, as detailed in Table 1, and iTaq Universal SYBR Green Supermix (Bio-Rad) or TaqMan Mm00627280_m1 for A20 (Applied Biosystems, Foster City, CA) on a 7500 Fast Real Time PCR System (Applied Biosystems). Gene expression was quantified using the Pfaffl method (20), and results were normalized using cyclophilin A (CYP4A, cell cultures) and TATA box-binding protein (Tbp) or 28 S (tissue samples), previously shown to be adequate housekeeping genes (15).

Western Blot Analysis—For Western blot analysis, SMC were harvested before and after IFNγ treatment at the indicated time points using a lysis buffer containing 20 mM Tris, 100 mM NaCl, 1 mM Na2VO4, 100 mM NaF, 20 mM glycerol 2-phosphate, 2.5 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, and 1 mM PMSF, and Complete Protease Inhibitor Tablets (Roche Applied Science). Equal protein amounts were loaded on 4–15% polyacrylamide gels (Bio-Rad), separated using an SDS-containing running buffer (Boston BioProducts Inc., Ashland, MA), and transferred into polyvinylidene difluoride membranes (Mil-
lipore) by semi-dry blotting. After blocking with 5% nonfat milk in PBS containing 0.05% Tween 20, membranes were probed with primary antibodies anti-human IDO (Millipore), A20/TNFAIP3 (TNFα-induced protein 3), TNF (Abcam, Cambridge, MA), GAPDH (Calbiochem/EMD Biosciences), STAT1 (Santa Cruz Biotechnology), phospho-(p)STAT1 (Tyr-701), TANK-binding kinase-1 (TBK1), pTBK1 (Ser-172) (Cell Signaling, Danvers, MA), followed by appropriate secondary antibodies (ThermoScientific, Rockford, IL). Densitometry measurements of identified bands were performed using ImageJ software and corrected to density values of GAPDH.

**IP-10 and IFNβ Enzyme-linked Immunosorbent Assay—**IFN-induced protein-10 (IP-10) levels in supernatants of IFNγ-treated SMC were determined using the IP-10 ELISA construction kit (Antigenix America, Huntington Station, NY) according to the manufacturer’s instructions. Results were expressed in nanograms/μg of total protein of cell lysates. Basal IFNβ levels were determined in SpeedVac (20-fold) concentrated supernatants of A20-silenced SMC cultures, using the high sensitivity IFNβ ELISA from PBL InterferonSource according to the manufacturer’s instructions. Nontransfected and SMC transfected with AllStars siRNA served as controls.

**Chromatin Immunoprecipitation—**For chromatin immunoprecipitation (ChIP), chromatin of nontreated and IFNγ-treated SMC was cross-linked by adding formaldehyde to a final concentration of 1%. After cell lysis and shearing by sonication, chromatin was immunoprecipitated with anti-polymerase II antibody (Millipore, Billerica, MA). DNA was subsequently purified using the EZ-Chip kit (Millipore) according to the manufacturer’s instructions and then analyzed by PCR using primers covering the proximal region of STAT1 transcription start codon (−18 to +81), i.e. forward primer 5′-AACAGGCCGCTCTAATTG-3′ and reverse primer 5′-ACTACCGCGAGGAGAAG-3′.

**Flow Cytometry—**SMC were immunostained using antibodies to IFNγ receptor (IFNGR)-1 (Abcam), IFNGR2 (Santa Cruz Biotechnology), or respective isotype control and FITC-labeled secondary antibody. Flow cytometric analysis was performed using a FACSort flow cytometer (BD Biosciences).

**Migration Assay—**Supernatants of IFNγ-treated SMC were added to the lower chamber of a 96-transwell plate (5 μm pore), whereas 1 × 10⁵ U937 cells were seeded in the upper chamber. Six hours later, migration of U937 cells to the lower chamber was analyzed using the CytoSelect Cell Migration Assay (Cell Biolabs, Inc., San Diego) according to the manufacturer’s instructions.

**Mouse Model of Focal Arterial Stenosis—**A20 heterozygote (HET) mice (21), a kind gift of Dr. Averil Ma (University of California in San Francisco) and WT littermate mice, fed standard chow diet, were used in a model of focal arterial stenosis, as achieved by partial carotid artery ligation (CAL). Prior to surgery, mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (50 mg kg⁻¹) and xylazine (10 mg kg⁻¹). After preparing mice with betadine and alcohol and shaving the neck from thorax to jaw, we performed a midline incision from mandible to thorax. Using blunt end forceps, carotid sheath structures were dissected to mobilize the common carotid artery. A blunt 35-gauge needle (World Precision Instruments, Inc., Sarasota, FL) was placed parallel to the artery, and a 9-0 nylon suture was placed 2.5 mm proximal to the bifurcation and tied with a surgeon’s knot around both artery and needle. The needle, which served as mandrel, was then carefully removed to restore blood flow (Fig. 6A). Sham mice received identical treatment but without vessel ligation. The neck was closed with a 5-0 nylon suture. All surgical procedures were conducted aseptically, and animal body temperature was maintained at 37 °C on a heated water pad. For pain control, Meloxicam (5 mg kg⁻¹) was injected subcutaneously up to 2 days post-surgery. Sutures were removed 10 days after surgery.

For tissue harvesting, animals were sacrificed 10 days (n = 5–6 per group) or 4 weeks (n = 5–6 per group) after surgery, and their carotid arteries were recovered and either frozen in liquid nitrogen for RNA isolation (10 days) or embedded in tissue freezing medium (Triangle Biomedical Science, Durham, NC) for immunohistochemistry (4 weeks). All animal experiments were approved by the Institutional Committee for Use and Care of Laboratory Animals and were in accordance with the United States Department of Health and Human Services “Guide for the Care and Use of Laboratory Animals.”

**Histology—**For morphometric analysis, serial 6-μm tissue sections were collected up to 1500 μm proximal to the stenosis site and stained with H&E. After careful delineation of the external and internal elastic laminae, we measured media, intima, and lumen surface areas using the ImageJ software, as described (9). By immunohistochemistry, carotid sections were stained for Cd3⁺ cells using a specific antibody for mouse Cd3 (Abcam), and for NK cells using the Nkp46-specific antibody (R&D Systems, Minneapolis, MN) followed by HRP-conjugated secondary antibody (Invitrogen). Stat1 expression in vascular tissue sections was evaluated by immunofluorescence using a polyclonal anti-mouse Stat1 primary antibody (Santa Cruz Biotechnology), followed by Alexa Fluor 594-conjugated secondary antibodies (Invitrogen). Nuclear counterstain was done using 4’,6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma).

**Laser Capture Microdissection—**Mice were anesthetized and prepared as described above. The belly was shaved and harvested by using aseptic technique and RNase-free surgical instruments. The thoracic aortae were retrieved and rinsed with heparin (10 units/ml). Subsequently, aortic tissue was embedded in tissue freezing medium (Triangle Biomedical Science) and frozen in liquid nitrogen. For whole aortic lysates, tissue was snap-frozen in liquid nitrogen. Serial 6-μm sections were cut using a cryotome (Fisher), captured on RNase-free slides, and coated with RNAlater (Ambion, Austin, TX). Slides were then dehydrated sequentially in ethanol and xylene. By laser capture microdissection (LCM), performed on Arcturus XT LCM system (Applied Biosystems), we dissected out the endothelium, then dissected the medial layer off the adventitia, and captured it on a specimen cap.

**Microarray Analysis—**Medial layer samples from LCM were lysed in Arcturus PicoPure RNA lysis buffer, and RNA was isolated according to the manufacturer’s protocol (Applied Biosystems). Samples with RNA integrity numbers of >6.0, as assessed by QC bioanalyzer (Agilent, Santa Clara, CA), were
amplified and labeled using NuGEN Ovation Pico WTA System Version 2 and Encore biotin module (Fisher Scientific), respectively. After final purification using MinElute Reaction Cleanup Kit (Qiagen), hybridization to Affymetrix Mouse Gene ST2.0 microarrays (Affymetrix, Santa Clara, CA) was performed at the Microarray Core Facility of Children’s Hospital, Boston. Normalization and analysis of microarray data were carried out using R/Bioconductor statistical software packages. Canonical pathway enrichment analysis was performed using Ingenuity Pathway Analysis (IPA) tools.

Statistics—Differences between groups were analyzed by ANOVA followed by post hoc Bonferroni’s or Tukey’s multiple comparison tests using GraphPad Prism. Student’s t test was used when comparing differences between mRNA expression levels in WT versus HET aortae. p < 0.05 was considered significant.

RESULTS

A20 Knockdown Increases and A20 Overexpression Decreases IFNγ-mediated Up-regulation of Atherogenic Genes in Human Endothelial and Smooth Muscle Cells—To investigate whether A20 impacts pathologic IFNγ signaling in vascular cells, we evaluated the response of A20-deficient and A20-overexpressing human coronary artery EC and SMC cultures to IFNγ. In particular, we probed for mRNA levels of bona fide IFNγ atherogenic genes, including intercellular adhesion molecule-1 (ICAM-1) (22), the chemoattractant molecules IP-10, macrophage chemoattractant protein-1 (MCP-1), and interferon-inducible T cell α-chemoattractant (I-TAC) (23), the transcription factor interferon regulatory factor-1 (IRF1) (24), and the metabolic and immunoregulatory enzyme IDO (25). In loss-of-function studies, we silenced A20 by siRNA transfection, which decreased A20 mRNA levels by 70–80% in EC and SMC (Fig. 1, A and B). This corresponded to total blunting of the A20 protein in SMC (Fig. 1C). IFNγ-mediated up-regulation of all tested interferon-stimulated genes (ISG) was significantly greater in A20-silenced versus control (nontransfected and transfected with AllStars siRNA) EC and SMC (Fig. 1, A and B). This corresponded to total blunting of the A20 protein in SMC (Fig. 1C). IFNγ-mediated up-regulation of all tested interferon-stimulated genes (ISG) was significantly greater in A20-silenced versus control (nontransfected and transfected with AllStars siRNA) EC and SMC (Fig. 1, A and B). This corresponded to total blunting of the A20 protein in SMC (Fig. 1C). IFNγ-mediated up-regulation of all tested interferon-stimulated genes (ISG) was significantly greater in A20-silenced versus control (nontransfected and transfected with AllStars siRNA) EC and SMC (Fig. 1, A and B). This corresponded to total blunting of the A20 protein in SMC (Fig. 1C). IFNγ-mediated up-regulation of all tested interferon-stimulated genes (ISG) was significantly greater in A20-silenced versus control (nontransfected and transfected with AllStars siRNA) EC and SMC (Fig. 1, A and B).
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A20 Modulates IFNγ Signaling in SMC by Regulating Expression of STAT1 in a Non-NF-κB-dependent Manner—This significantly impacts monocyte chemotactant properties of IFNγ-stimulated SMC. Having established A20 as a physiologic regulator of IFNγ signaling in vascular cells, we probed for the molecular target(s) of A20 within the IFNγ signaling cascade that could account for this effect. After ruling out any impact of A20 on surface expression of IFNGR1 and -2 in SMC (data not shown), we determined that A20 silencing significantly increased, whereas A20 overexpression significantly decreased basal mRNA and protein levels of STAT1, the key transducer of IFNγ signaling in vascular cells.

A20 overexpression inhibits IFNγ-mediated gene up-regulation in human coronary artery SMC. Nontransduced (Ctrl), rAd.A20-, and rAd.βgal-transduced SMC were treated with 400 units/ml IFNγ. A, representative Western blot analysis of total STAT1 in control, A20 siRNA, and control (C) siRNA-transfected SMC, before and 6 and 24 h after IFNγ treatment. Immunoblotting for A20 and βgal verified transgene expression, whereas immunoblotting for GAPDH corrected for loading, and enabled semi-quantitative evaluation of IDO by densitometry, using ImageJ. B, relative ICAM-1, IP-10, MCP-1, I-TAC, IRF1, and IDO mRNA levels were determined before and 6 h after IFNγ by qRT-PCR.; #, p < 0.01; ###, p < 0.001 versus each group’s respective time 0. C, IP-10 protein levels were determined 6 and 24 h after IFNγ treatment with IFNγ (Fig. 1, A and B). At the protein level, we verified by Western blot and ELISA that A20 knockdown significantly increased IFNγ-mediated up-regulation of IDO (Fig. 1C) and IP-10 (Fig. 1D).

In gain-of-function studies, we overexpressed A20 by means of rAd.-mediated transduction, which achieves expression of the transgene in >95% of cultured cells (9). Overexpression of A20 in SMC significantly blunted IFNγ-mediated up-regulation of ICAM-1, IP-10, MCP-1, I-TAC, IRF1, and IDO mRNA (Fig. 2A). Remarkably, mRNA levels of ICAM-1, MCP-1, and I-TAC were not significantly induced by IFNγ treatment in A20-overexpressing cells. We confirmed this outcome for IDO and IP-10 at the protein level (Fig. 2, B and C). These data uncover a novel function for A20 as a physiologic regulator and a potential therapeutic inhibitor of atherosigenic IFNγ signaling in vascular cells.

FIGURE 2. A20 overexpression inhibits IFNγ-mediated gene up-regulation in human coronary artery SMC. Nontransduced (Ctrl), rAd.A20-, and rAd.βgal-transduced SMC were treated with 400 units/ml IFNγ. A, representative Western blot analysis before and 6 and 24 h after IFNγ treatment. Immunoblotting for A20 and βgal verified transgene expression, whereas immunoblotting for GAPDH corrected for loading, and enabled semi-quantitative evaluation of IDO by densitometry, using ImageJ. B, relative ICAM-1, IP-10, MCP-1, I-TAC, IRF1, and IDO mRNA levels were determined before and 6 h after IFNγ by qRT-PCR.; #, p < 0.01; ###, p < 0.001 versus each group’s respective time 0. C, IP-10 protein levels were determined 6 and 24 h after IFNγ treatment with IFNγ (Fig. 1, A and B). At the protein level, we verified by Western blot and ELISA that A20 knockdown significantly increased IFNγ-mediated IFNγ in SMC significantly blunted IFNγ-mediated up-regulation of IDO (Fig. 1C) and IP-10 (Fig. 1D).

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A20 Modulates IFNγ Signaling in SMC by Regulating Expression of STAT1 in a Non-NF-κB-dependent Manner—This significantly impacts monocyte chemotactant properties of IFNγ-stimulated SMC. Having established A20 as a physiologic regulator of IFNγ signaling in vascular cells, we probed for the molecular target(s) of A20 within the IFNγ signaling cascade that could account for this effect. After ruling out any impact of A20 on surface expression of IFNGR1 and -2 in SMC (data not shown), we determined that A20 silencing significantly increased, whereas A20 overexpression significantly decreased basal mRNA and protein levels of STAT1, the key transducer of IFNγ signaling in vascular cells. We confirmed STAT1 as the principal mediator of IFNγ signals in SMC by showing that STAT1 silencing, akin to A20 overexpression, inhibits IFNγ-mediated up-regulation of all atherogenic genes analyzed above (Fig. 3E). We obtained similar results in EC. Indeed, A20 silenc-
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FIGURE 4. A20-mediated decrease in basal STAT1 expression and subsequent inhibition of IFNγ signaling in SMC cannot be recapitulated by overexpression of the standard NF-κB inhibitor IκBα. A, STAT1 and select ISG (ICAM-1, IDO, and IP-10) mRNA levels before and 6 h after IFNγ treatment, respectively, in nontransduced (Ctrl), rAd.IκBα-, and rAd.IκBβ-gal-transduced SMC, as determined by qRT-PCR and normalized by mRNA levels of the housekeeping gene cyclophilin A (CYP A). B, STAT1 and IDO protein expression in nontransduced, rAd.A20-, rAd.IκBα-, or rAd.IκBβ-gal-transduced SMC before and after 24 h stimulation with IFNγ (100 units/ml). C, STAT1 protein expression in IκBα (rAd.IκBα) and control β-galactosidase (rAd.IκBβ-gal) overexpressing SMC that were transfected with A20 siRNA or control (C) siRNA. In both B and C, IκBα and βgal transgene expression was confirmed by immunoblotting. Also, GAPDH immunoblots were used to correct for loading and enable quantitative evaluation of STAT1 and IDO by densitometry using the ImageJ software. D, supernatants of SMC were recovered 24 h after stimulation with IFNγ (100 units/ml) for ELISA measurements of IP-10. Bars represent mean ± S.D. of 3–4 independent experiments using SMC derived from three different donors. (*, p < 0.05; **, p < 0.01).

ing resulted in a significant increase in STAT1 protein levels in these cells (Fig. 3F). Paralleling STAT1 protein levels, levels of Tyr-701 phospho-STAT1 (a proxy for STAT1 activation) following IFNγ treatment were significantly higher in A20-silenced and significantly lower in A20-overexpressing SMC than in control cells (Fig. 3, B and D).

The biologic importance of A20-dependent modulation of STAT1 expression to contain IFNγ signaling is highlighted by our data showing a significantly higher migration rate of monocytic U937 lymphoma cells in response to supernatants from IFNγ-treated (24 h) A20-silenced SMC versus controls (Fig. 3G). Conversely, the migration rate of U937 cells was significantly lower than controls when using supernatants recovered from A20 overexpressing or STAT1-silenced SMC treated with IFNγ for 24 h (Fig. 3G).

Despite well described synergy between STAT1 and NF-κB pathways (26, 27), overexpression of IκBα in SMC failed to alter basal STAT1 mRNA and protein levels or IFNγ-mediated up-regulation of STAT1 (protein), ICAM-1 (mRNA), and IDO (mRNA and protein) (Fig. 4, A and B). This result indirectly rules out the contribution of A20’s NF-κB inhibitory function to its modulation of STAT1 and selected ISG expression in vascular cells. The inability of IκBα overexpression to reduce heightened STAT1 levels in A20-silenced SMC further supports this conclusion (Fig. 4C) Notably, overexpression of IκBα in SMC still significantly reduced IFNγ-induced up-regulation of IP-10 (Fig. 4, A and D), which implied that certain ISG still required NF-κB activity to be expressed in SMC (28).

Aggravated Pathologic Remodeling Following Focal Carotid Artery Stenosis in A20 Heterozygote Mice Associates with Increased Expression of Atherogenic Genes, in Particular a Unique Subset of IFNγ-dependent Genes—To validate in vivo this novel function of A20 as a regulator of IFNγ signaling in vascular cells and to gauge its implication in modulating the severity of occlusive vascular disease, we subjected A20 HET to a model of hemodynamic injury of the carotid artery that causes significant intimal hyperplasia (IH). In this model, vascular injury is achieved by partial CAL, which by altering blood flow and shear stress results in florid IH and lumen reduction within 4 weeks of the procedure (Fig. 5A). We confirmed this outcome in A20-competent WT mouse arteries (Fig. 5B). H&E-stained tissue sections of WT carotid arteries showed significant lumen narrowing, as evaluated by the ratio of lumen area over lumen and neointima areas, at stenosis site and up to 300 μm proximal to it. Narrowing of arterial lumen lessened by 600 and 1000 μm and was totally absent by 1500 μm proximal to the stenosis (Fig. 5C). Paralleling luminal narrowing, intima over media (I/M) ratio was highest at the site of stenosis and receded by 1500 μm proximal to the stenosis (Fig. 5, B and D). Although lumen narrowing was similar at the stenosis site in HET and WT carotid

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arteries, it was significantly worse in HET carotids at all other analyzed sites and remained substantial by 1500 μm proximal to the stenosis (Fig. 5, B and C). I/M ratios were higher in HET versus WT carotid arteries at all analyzed sites, and this difference was significant at 300 and 600 μm proximal to the stenosis (Fig. 5, B and D). Altogether, these data indicated that partial loss of A20 aggravates IH following focal stenosis by increasing lesion size and length. We confirmed A20 knockdown by demonstrating ~30% lower A20 mRNA in carotid arteries of HET versus WT mice before stenosis (Fig. 5E). HET carotids also failed to adequately up-regulate A20 mRNA 10 days after CAL, which resulted in 50% lower A20 mRNA in HET versus WT carotid arteries at this time point (Fig. 5E).

In exploring the molecular signature of aggravated vascular remodeling in HET carotid arteries, we checked for Ifnγ mRNA levels 10 days following CAL. Ifnγ mRNA levels were substantially increased in carotid arteries following CAL, confirming IFNγ as part of the molecular response to hemodynamic injury (Fig. 6A). However, Ifnγ levels were not significantly different between HET and WT. We next investigated the presence of cytotoxic Th1 and NK cells, the main source of Ifnγ (29), and showed heightened Cd3+ T cell infiltration (but not NK cells, data not shown) in media and neointima of HET and WT carotid arteries (Fig. 6B). This suggested that T cells were the likely source of Ifnγ in this model.

We next evaluated mRNA levels of the Ifnγ inducible proatherogenic genes previously screened in vitro. Agreeing with heightened Ifnγ levels in carotid arteries following CAL, Icam-1, Ip-10, and Mcp-1 mRNA levels were significantly increased in both WT and HET versus Sham treated vessels 10 days after the procedure (Fig. 6C). However, CAL-induced increase in Icam-1 and Ip-10 mRNA levels was significantly greater in HET than in WT carotid arteries (Fig. 6C). Interestingly, I-Tac, Irf1, and Ido mRNA levels also significantly increased in A20 HET but not in WT carotid arteries 10 days following CAL (Fig. 6C). Together, these results indicated that aggravated IH in HET carotid arteries associates with both quantitative and qualitative differences in expression of atherogenic ISG when compared with WT vessels. Remarkably, expression of ISG whose up-regulation either requires synergy between IFNγ and NF-κB (Ip-10 and I-Tac) or only depends on IFNγ (Irf1 and Ido) occurred only in HET and not in WT

FIGURE 5. Partial A20 knockdown aggravates vascular remodeling after CAL. A, carotid artery focal stenosis model. The left carotid artery was ligated 2.5 μm proximal to the bifurcation using a 35-gauge needle as mandrel. The mandrel was then removed to restore blood flow. B, 4 weeks after CAL, the vessel was retrieved and sections at 300, 600, 1000, and 1500 μm proximal to CAL were analyzed for neointima formation by H&E staining. Arrows on representative photomicrographs indicate media (M) and intima (I). The degree of lumen occlusion ratio (1 – (lumen)/(lumen + neointima)) (C) and I/M ratios at different sites from the stenosis (D) were determined by morphometric analysis of vascular sections using ImageJ software (n = 6–7 mice/group). E, A20 mRNA levels in carotid arteries 10 days after CAL were determined by qRT-PCR. n = 5–6. (**, p < 0.01; ***, p < 0.001 versus 1500-μm section in each given group, and *, p < 0.05; **, p < 0.01) WT, wild-type mice; HET, A20 heterozygote mice. SHAM-treated carotid arteries served as controls.
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Microarray Analysis of Aortic Medial SMC from A20 HET Versus WT Mice Implicates Increased Ifnβ Levels and Signaling in Promoting Higher Stat1 Expression Levels in HET Vessels—To check the mechanism(s) involved in A20-mediated regulation of STAT1, we evaluated the rate of STAT1 mRNA decay in A20 overexpressing SMC treated with actinomycin D, an inhibitor of mRNA synthesis, and we showed it was comparable with control cells (data not shown). This ruled out any impact of A20 on degradation rate or half-life of STAT1 mRNA. Also, we excluded any impact of A20 on STAT1 proteasomal degradation (30, 31) by showing that addition of the proteasome inhibitor MG132 fails to increase STAT1 protein levels in A20 overexpressing SMC (data not shown). Rather, we showed that A20 regulates STAT1 expression by influencing its transcription. Indeed, we demonstrated by ChiP assay that polymerase II was recruited less to the STAT1 transcriptional start site in A20-overexpressing versus control SMC (Fig. 7A).

To gain further insights into the molecular basis of A20-mediated regulation of STAT1 transcription in vascular cells, we isolated aortic medial SMC by LCM from WT and A20 HET mice and performed mRNA expression analysis using Affymetrix mouse gene 2.0 ST array (>24,000 coding transcripts). Canonical pathway enrichment using IPA tools showed significant enrichment in type II and unexpectedly type I (predominantly IFNβ) IFN-associated genes in the medial SMC of HET versus WT aortae. All 19 differentially expressed Ifnβ-associated genes, using a cutoff of 1.5-fold, were higher in HET versus WT aortae (Fig. 7B). We validated by quantitative PCR higher mRNA levels of two of these genes in HET versus WT aortae as follows, mitogen-activated protein kinase kinase 7 (Map3k7) and Stat2, respectively, implicated in increasing IFNβ transcription and signaling (Fig. 7C) (16, 32). We also validated that A20 HET aortae had significantly higher mRNA levels of Stat1 and Irf1 (Fig. 7D).

To evaluate whether heightened IFNβ signaling in HET media contributed to increased STAT1 expression, we checked whether antibody-mediated neutralization of IFNβ- or siRNA-induced knockdown of IFNβ reduces STAT1 levels in A20-silenced SMC. Anti-IFNβ but not anti-IFNα antisera significantly decreased STAT1 mRNA in A20-silenced SMC to levels of control cells (Fig. 7E). A similar decrease in STAT1 mRNA levels occurred upon silencing IFNβ in A20-silenced SMC (Fig. 7F). Treatment of A20-silenced SMC cultures with neutralizing anti-IFNβ antisera, or co-silencing IFNβ in these cells also significantly lowered IFNγ-induced up-regulation of STAT1-dependent genes ICAM-1, IP-10, and IDO (Fig. 7, E and F). These results highly suggested that A20 regulates STAT1 expression and subsequently IFNγ-triggered signal transduction in vascular cells by modulating basal IFNβ levels. Despite the technical difficulties to measure basal IFNβ protein levels, we demonstrated using a hypersensitive IFNβ ELISA that A20 knockdown substantially increased basal IFNβ levels in supernatants of SMC (Fig. 7G). Basal IFNβ levels remained undetectable (i.e. sub-threshold) in control cells.

A20 Modulates Sub-threshold IFNβ Levels in SMC by Regulating Expression and Activation Status of Its Transcriptional Activators IRF3 and IRF7—IFNβ transcription relies on activation of IRFs, namely IRF3 and IRF7. Hence, we measured IRF3
and IRF7 mRNA levels in WT and HET aortae. Although IRF3 is constitutively expressed in most cell types, including SMC, IRF7 is transcriptionally induced by type I IFN signaling and is possibly part of a positive feedback loop aimed at enhancing IFNα/β expression (33). Whereas IRF3 mRNA levels were comparable in HET and WT aortae, IRF7 mRNA levels were significantly higher in HET versus WT aortae (Fig. 8A). We obtained concordant results in SMC cultures and showed that A20 silencing significantly increased, whereas A20 overexpression significantly decreased IRF, mRNA levels as compared with control cells, but again IRF3 mRNA levels were not affected by A20 knockdown or overexpression (Fig. 8, B and C). Beyond mRNA levels, transcriptional activity of IRF3 and IRF7 ultimately depends on their phosphorylation by activated noncanonical kinases TBK-1 and IκB-kinase-ε (IKKe) (34). Interestingly, we detected a significant increase in basal levels of Ser-172 phosphorlated (not total) TBK1 protein in A20-silenced versus control SMC (Fig. 8D), precluding an increase of IRF3 and IRF7 activation, and subsequently (as previously verified) to an increase of basal transcription of IFNβ. Similar to SMC, A20-silenced EC also had higher basal levels of Ser-172-phosphorlated TBK1 than control EC (Fig. 8D).

**DISCUSSION**

Despite the initial controversy (35, 36), IFNγ, highly expressed in atherosclerotic lesions of patients and experimental animals, is now a recognized culprit of pathologic vascular remodeling (2–4). Infusion of exogenous IFNγ induces atherosclerotic lesions in human vascular allografts implanted in mice with severe combined immunodeficiency (5), and Ifngr knock-

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**FIGURE 7. A20 knockdown activates basal Ifnγ signaling in mouse medial aortic SMC.** A, ChIP using the anti-polymerase II (Pol II) antibody, in nontransduced, rAd.A20-, or rAd.βgal-transduced SMC before and 1 h after IFNγ treatment (100 units/ml). Data shown are representative of two independent experiments. B, heat map of differentially expressed Ifnγ pathway-related genes in media of A20 HET versus WT mouse aortae, after isolation by LCM. Columns represent samples, and rows represent genes. Gene expression is shown with a pseudo-color scale (−2 to 2), with red indicating increased and green indicating decreased expression. Relative mRNA expression of Stat2 and Map3k7 (C) and Stat1 and Ifngr (D) in the aorta of WT versus HET mice was analyzed by qRT-PCR (n = 9–11). Basal STAT1- and IFNγ (100 units/ml, 6 h)-induced ICAM-1, IP-10, and IDO mRNA levels were evaluated by qRT-PCR in nontransfected (Ctrl), A20 siRNA, and control (C) siRNA-transfected SMC treated with neutralizing anti-IFNγ or anti-IFNβ antiserum for 24 h (E), and in SMC co-transfected with a combination of C and A20 siRNA or A20 and IFNγ siRNA (F). SMC co-transfected with a double dose of C siRNA served as control. Graphs represent mean ± S.D. of three independent experiments. G, basal IFNγ protein levels were determined in supernatants of nontransfected (Ctrl), A20 siRNA, and control (C) siRNA-transfected SMC by ELISA. Graphs depict mean ± S.D. of three independent experiments using SMC derived from three different donors. *, p < 0.05; **, p < 0.01; ***, p < 0.001. NS, not significant; N.D., not detectable.

**FIGURE 8. Increased STAT1 expression and sensitivity toward IFNγ signaling after A20 knockdown is dependent on basal IFNβ expression.** A, mRNA expression of Ifnγ and Ifnβ in aortae of WT versus A20 HET mice were analyzed by qRT-PCR (n = 9–11). Relative mRNA levels of basal IRF3 and IRF7 mRNA in nontransfected (Ctrl), A20 siRNA, and control (C) siRNA-transfected SMC (B) and control, rAd.A20-, and rAd.βgal-transduced SMC, as evaluated by qRT-PCR (C). Graphs represent mean ± S.D. of 4–6 independent experiments. D, representative Western blot analysis of basal phospho-Ser-172 and total TBK1 in control and A20 siRNA or C siRNA-transfected SMC and EC. Immunoblotting for GAPDH was corrected for loading and enabled semi-quantitative evaluation of phospho-Ser-172-TBK1 by densitometry, using ImageJ (n = 3). **, p < 0.01; ***, p < 0.001.
down attenuates atherosclerotic lesions in atherosclerosis-prone ApoE knock-out mice (6). In this study, we noted significant super-induction of ISG in A20-deficient human EC and SMC in response to exogenous IFNγ. Conversely, overexpression of A20 in SMC significantly inhibited IFNγ-induced up-regulation of these ISG, highlighting the translational promise of A20-based therapies to curb IFNγ-driven vascular pathologies.

Interestingly, A20 impacted IFNγ signaling by uniquely modulating expression levels of its signaling transducer STAT1 (17). A20 knockdown significantly increased STAT1 mRNA and protein levels, both in vitro (EC and SMC) and in vivo (HET mouse aorta). This transpired in higher levels of phosphorylated/active STAT1 levels, paralleling total STAT1 protein levels, in A20-silenced versus control SMC after IFNγ treatment. Conversely, overexpression of A20 in SMC significantly inhibited STAT1 mRNA and protein levels. These results are in keeping with recent data demonstrating that A20-deficient astrocytes also have increased Stat1 expression and signaling (37). However, although increased Stat1 expression in A20 KO astrocytes was linked to heightened NF-κB activation, in these cells, we showed that it was independent from A20’s NF-κB inhibitory function in vascular cells. Indeed, inhibition of NF-κB by overexpression of IκBα (rather than A20) totally failed to decrease STAT1 in SMC. Furthermore, overexpression of IκBα was unable to decrease heightened STAT1 levels in A20-silenced SMC. The discrepancy in mechanism(s) engaged by A20 to impact STAT1 expression in astrocytes versus SMC may relate to cell type specificity and/or potentially result from unforeseen effects of the chemical IKK inhibitor used in astrocytes, although we used genetic means to inhibit NF-κB (37). ChIP assays and proteasome inhibition experiments supported transcriptional regulation as the main mechanism of A20-dependent modulation of STAT1 in SMC.

Using monocyte migration in response to the chemoattractant properties of IFNγ-treated SMC as an in vitro biologic surrogates for this cytokine’s pro-inflammatory/pro-atherogenic effects, we demonstrated that STAT1 silencing in SMC was as protective as A20 overexpression in limiting monocyte migration. This implied that STAT1 targeting by A20 was key to its ability to harness pathologic vascular remodeling (26).

We confirmed this in vivo by showing that a mere partial loss of A20 in carotid arteries of A20 HET mice significantly increased Stat1 expression following CAL, a model that approximates hemodynamic perturbations associated with vascular stenosis in patients. HET carotid arteries, which start with 30–50% lower baseline levels of A20 and fail to up-regulate it following CAL, displayed a pro-atherogenic profile characterized by the remarkable up-regulation of strict IFNγ-dependent ISG (I-Tac, Irf1, and Ido) (38) together with amplified inflammation (Icam-1 and Ip-10), all of which correlated with aggravated IH. These results not only implied a pathogenic role for Irf1 in CAL-induced pathologic vascular remodeling but also confirmed A20 as a novel physiologic modulator and potential therapeutic target of atherogenic Irf1/stat1 signaling in the vessel wall.

In seeking the molecular basis for A20-mediated regulation of Stat1 transcription, we uncovered, thanks to transcriptional profiling of HET versus WT medial aortic SMC, that A20 knockdown also promoted Irfβ signaling and expression. Even in the absence of a viral insult, sub-threshold concentrations of Irfβ accumulate in tissues, providing local immune surveillance by engaging type I IFN signaling to support basal Stat1 expression. Basal expression of Stat1 is prerequisite for enabling secondary Irfγ and full-blown Irfα/β signaling, typically in response to viral infections (39). Accordingly, basal Stat1 levels are reduced in macrophages and mouse embryonic fibroblasts of Irfβ and Irfα receptor (Ifrar)-1 KO mice (17, 40). Also, defective Irfγ antiviral activity in Irfβ1 KO mice recovers upon restoring adequate Stat1 levels.

Messenger RNA levels of Stat2, which form together with Stat1 and Irf9 the type I IFN transcriptional complex interferon-stimulated gene factor (ISGF3) (16), were also significantly increased in HET versus WT aortae. This result agrees with data showing increased Stat1 and Stat2 levels in cells with chronic elevation of Irfβ levels (41). Interestingly, nonphosphorylated Stat1 and Stat2 can still bind Irf9 to form ISGF3 and hence drive, in a feedback loop, their own transcription (41). This maintains cells in a primed state, ready for full-scale antiviral responses. Our data showing that antibody-mediated neutralization of Irfβ or siRNA-induced Irfβ knockdown decreased basal Stat1 and blunt IFNγ-mediated hyperinduction of Icam-1, Ip-10, and Ido mRNA levels in A20-silenced SMC confirm the link between Irfβ and Stat1 in our system. To our knowledge these data are the first showing that loss of A20 modulates Stat1 and Stat2 expression in vascular cells by enhancing Irfβ levels.

The Irfβ promoter consists of four positive regulatory domains (PRD-I to PRD-IV), representing binding sites for IRF, CREM, SP1, and c-Jun (42, 43). p65 binds to PRD-II, and its activity was reported to be crucial for basal Irfβ expression in mouse embryo fibroblasts (44). However, there is also strong evidence that IRFs can outweigh p65 deficiency and enable adequate Irfβ transcription in p65 knock-out mouse embryo fibroblasts (45). In a similar vein, our data strongly argue that A20’s impact on the expression and function Irfβ supersedes its effect on NF-κB in modulating basal Irfβ and hence STAT1 levels in SMC. Indeed, as discussed previously, overexpression of IκBα to retain p65 in SMC cytosol and therefore block NF-κB activation did not affect STAT1 expression nor downstream STAT1/IFNγ-mediated expression of ISG. However, levels of the key transcriptional regulator of Irfβ, Irf7, were significantly higher in HET versus WT mouse aortae and in A20-silenced versus control SMC. Conversely, Irf7 mRNA levels were significantly lower in A20 overexpressing SMC. Because Irf7 transcription depends on type I IFN signaling, it engages in a feed-forward loop that would amplify Irfβ transcription and subsequently type I IFN responses, including its own up-regulation (33, 39).

IRF3, the other transcriptional activator of Irfβ, is constitutively expressed, and its activation, together with that of IRF7, is mostly regulated by IKKe/TBK1-mediated phosphorylation (46). TNF receptor-associated factor-3 (TRAF3)-dependent Lys-63-linked polyubiquitination of TBK1 is required for its dimerization and autophosphorylating activation at Ser-172 (47). Our data revealed that A20 knockdown in SMC signifi-
cantly increased Ser-172 basal phosphorylation of TBK1, and it was therefore likely to enhance IRF3 and IRF7 activation. This result resonates with earlier work showing that myeloid-specific A20 knock-out mice exhibit enhanced IRF3 activation, which protected them from influenza A viral infection (48). It is also in keeping with gain-of-function studies demonstrating that A20 precluded virus-induced up-regulation of type I IFN in HEK293 cells by inhibiting IRF3/7 activation (49–52).

The mechanism(s) by which A20 modulates TBK1 phosphorylation in vascular cells remain(s) to be explored. We surmise that it may relate, as in mouse embryo fibroblasts and 293T cells, to the disruption of the TRAF3 and IKK/H9280-TBK1 complex by A20 and its partner T cell leukemia virus type I-binding protein 1 (TAX1BP1) (53). This would preclude ubiquitination and secondary phosphorylation of IKK/H9280-TBK1 and hence the ability of these kinases to activate IRF3/IRF7, upstream of IFN/H9252 transcription (53). Alternatively, A20 may modulate expression or activity of signaling molecules upstream of TBK1. One such molecule could be the kinase MAP3K7/TAK1 (32), whose mRNA levels were significantly higher in HET versus WT aorta. Additional experiments are needed to address these hypotheses. Regardless of the mechanism(s), our data strongly support an atherogenic (54) rather than an atheroprotective (55) role for IFN/H9252.

In summary, our study uncovers a novel atheroprotective function of A20 in vascular cells through modulation of IFNγ/STAT1 signaling in an IFNβ-dependent, but NF-κB-independent, manner. Partial loss of A20 increases basal IFNβ levels and signaling, and subsequently STAT1 transcription, thereby amplifying IFNγ signaling. In contrast, overexpression of A20 reduces basal IFNβ signaling and hence STAT1 expression in EC and SMC, thereby hampering IFNγ signaling. We ascribed heightened IFNβ levels in A20-silenced vascular cells to increased phosphorylation of the noncanonical I KK, TBK1, upstream of IRF3 and IRF7, the direct transcriptional activators of IFNβ (Fig. 9).

Clinically, tag polymorphisms at the A20/TNFAIP3 locus that associate with 30–45% decrease in A20 mRNA levels elicit 2–3-fold higher risk for coronary artery disease in diabetic patients carrying minor versus major alleles (56). Whether these alleles also associate with heightened levels of basal IFNβ and STAT1 and with amplified IFNγ responses in the vessels of these patients needs to be determined. Such single nucleotide polymorphisms at the A20/TNFAIP3 locus may prove highly informative of patients’ risk for vascular disease.

From a therapeutic standpoint, our data strongly support the promise of A20-based therapies for the prevention/treatment of atherosclerotic vascular diseases, based on its ability to not only intercept the NF-κB pathway but also another major atherogenic pathway, i.e. the IFNγ/STAT1 pathway. To that end, our discovery that A20 modulates TBK1 phosphorylation and activation in vascular cells could represent a “druggable” target that could recapitulate the ability of A20 to interrupt IFNγ-driven atherogenesis (57). Future studies will explore this hypothesis.

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