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A20 Haploinsufficiency Aggravates Transplant Arteriosclerosis in Mouse Vascular Allografts: Implications for Clinical Transplantation

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Abstract

Background—Inflammation is central to the pathogenesis of transplant arteriosclerosis (TA). We questioned whether physiologic levels of anti-inflammatory A20 influence TA severity.

Methods—We performed major histocompatibility complex (MHC) mismatched aorta to carotid artery interposition grafts, using wild type (WT) or A20 heterozygote (HET) C57BL/6 (H-2^b) donors and BALB/c (H-2^d) recipients, and conversely BALB/c donors and WT/HET recipients. We analyzed aortic allografts by histology, immunohistochemistry, immunofluorescence, and gene profiling (qPCR). We validated select in vivo A20 targets in human and mouse smooth muscle cell (SMC) cultures.

Results—We noted significantly greater intimal hyperplasia in HET vs. WT allografts, indicating aggravated TA. Inadequate upregulation of A20 in HET allografts after transplantation was associated with excessive NF- κ B activation, gauged by higher levels of I κ B α , p65, VCAM-1, ICAM-1, CXCL10, CCL2, TNF, and IL-6 (mostly localized to SMC). Correspondingly, cytokine-

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induced upregulation of TNF and IL-6 in human and mouse SMC cultures inversely correlated with A20 expression. Aggravated TA in HET vs. WT allografts correlated with increased intimal SMC proliferation, and a higher number of infiltrating IFN γ ⁺ and Granzyme B⁺ CD4⁺ T cells and natural killer cells, and lower number of FoxP3⁺ regulatory T cells. A20 haploinsufficiency in allograft recipients did not influence TA.

Conclusions—A20 haploinsufficiency in vascular allografts aggravates lesions of TA by exacerbating inflammation, SMC proliferation, and infiltration of pathogenic T cells. A20 single nucleotide polymorphisms (SNPs) associating with lower A20 expression or function in donors of vascularized allografts may inform risk and severity of TA, highlighting the clinical implications of our findings.

Introduction

Current immunosuppressive regimens have drastically reduced the incidence of acute allograft rejection. However, vascularized allografts continue to fail at a rate of 5% per year from chronic allograft rejection(1). Transplant arteriosclerosis (TA), the pathognomonic feature of chronic allograft rejection is the leading cause of mortality of heart transplant recipients beyond the first year of transplantation(2, 3). This mortality has not improved in 20 years, reflecting our inability to prevent and treat TA. Novel therapeutic approaches based on better characterization of the risk factors that contribute to TA may help overcome this problem.

In previous gain-of-function studies we established that the anti-inflammatory and ubiquitin-editing protein A20 prevents TA in mouse vascular allografts(4, 5). Vascular allografts are an acceptable alternative to heart allografts for studying TA, and allow easier testing of novel therapies(6). The protective effect of A20 against TA stems from its combined atheroprotective and immunomodulatory functions(5, 7). Specifically, vascular overexpression of A20 precludes the activation of inflammatory and atherogenic NF- κ B and IFN γ signaling in EC and SMC(8–10). A20 also exerts anti-apoptotic, and anti-oxidative functions in EC, inhibits SMC proliferation and uniquely promotes apoptosis of intimal SMC(9, 11, 12).

It is well documented that A20 transcription significantly increases in EC and SMC in response to inflammatory and immune injuries(8, 9). To address whether physiologic upregulation of A20 influences vascular remodeling of TA, we undertook loss-of-function studies evaluating incidence and severity of TA in A20 haploinsufficient aortic allografts from C57BL/6 mice transplanted across total MHC mismatch into BALB/c carotid arteries. A20 knockout (KO) mice are born cachectic and die within 3 weeks of age from multi-organ inflammation and therefore cannot be used as aortic allograft donors(13). Consequently, we selected A20 heterozygote (HET) mice as donors of A20-deficient vascular allografts. At baseline, HET mice are phenotypically indistinguishable from their wild type (WT) littermates(13). However, these mice do uncover a distinct phenotype when subjected to certain procedures such as liver resection(14). We hypothesized that partial loss of A20 may negatively impact TA incidence and severity.

Materials and Methods

Mouse Cervical Aortic Allograft

Aorta-to-carotid artery interposition allografts were performed as described(15). Aortic allografts, harvested from A20 HET mice bred onto the C57BL/6 (H-2^b) background(13) or WT littermates, were transplanted into the carotid artery of MHC mismatched BALB/c (H-2^d) mice (Charles River Laboratories; Wilmington, MA). Conversely, aortic allografts recovered from BALB/c mice were transplanted into A20 HET or WT littermates. Grafts were recovered 10 and 28 days (d) after transplantation. A20 HET mice are a gift of Dr. Averil Ma, UCSF. All animal procedures were conducted in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals, and approved by the Beth Israel Deaconess Medical Center Institutional Committee for Use and Care of Laboratory Animals.

Histology, Immunohistochemistry and Immunofluorescence

Morphometric intima-media (I/M) ratios were measured using NIH ImageJ software on hematoxylin/eosin (H&E) stained sections(9). For immunohistochemistry (IHC) analysis, frozen sections were incubated with antibodies to VCAM-1, ICAM-1, CD4, CD8 (BD Biosciences, San Jose, CA), NK cells (R&D Systems Inc. Minneapolis, MN) and Ki-67 (ThermoFisher, Tewksbury, MA) followed by biotinylated secondary antibodies (Vector, Burlingame, CA)(16). Adventitial CD4, CD8, and NK positive cells were scored by cell counting using Image J, and expressed as cell number per vessel section. For immunofluorescence (IF) studies, we used primary antibodies to CD4, CD8, IFN γ , Indoleamine 2,3-dioxygenase (IDO), Interleukin-6 (IL-6), TNF, and F480 from BD Biosciences, and to FoxP3 from Biolegend, San Diego, CA, and to Granzyme B (GzmB) from Abcam, Cambridge, MA, followed by Alexa Fluor 594 conjugated secondary antibodies (Invitrogen, Carlsbad, CA).

Cell Culture and Reagents

Human coronary artery smooth muscle cells (HCASMC) from Lonza, Walkersville, MD were cultured in SmGM-2 and used between passage 5 and 8 in all experiments. HCASMC were treated with a triple cytokine (TC) cocktail consisting of human recombinant TNF α (400 U/mL), IFN γ (400 U/ml) and IL-1 β (100 U/mL) from R&D Systems, Minneapolis, MN.

Mouse aortic SMC (MASMC) were isolated from aortae of WT, A20 HET and A20 KO mice(17), and cultured in DMEM supplemented with 10% fetal calf serum. MASMC cultures showed 95% purity as determined by fluorescence staining using anti-smooth muscle alpha actin FITC-coupled antibody (Sigma, Saint Louis, MO). Passage 5 cells were used in all experiments. Cells were stimulated with a TC cocktail comprising mouse recombinant TNF α (400 U/ml; R&D Systems) and IFN γ (400 U/ml; PeproTech, Rocky Hill, NJ), in addition to human recombinant IL1- β (100 U/ml).

Western Blot Analysis

Cells lysates (20–40µg protein) were separated under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad Laboratories, Hercules, CA,) and transferred onto a Polyvinylidene fluoride (PVDF) membranes (PerkinElmer Life Science, Waltham, MA) by semi-dry electroblotting. Membranes were probed with rabbit anti-human A20/TNFAIP3 (Abcam), Ser-536 Phospho-P65 (Cell Signaling, Danvers, MA), Ser-32 phospho-IκBα, (Cell Signaling) and, GAPDH (Calbiochem/ EMD Biosciences, Gibbstown, NJ) followed by appropriate secondary HRP-conjugated antibodies (Thermo Scientific, Rockford, IL). Protein bands were detected with an enhanced chemiluminescence kit (PerkinElmer Life Science) followed by exposure to an autoradiography film(5, 9).

IL-6 Enzyme-linked Immunosorbent Assay

IL-6 (R&D Systems) was measured in supernatants of SMC using a quantitative sandwich immunoenzyme linked assay according to the manufacturer's instructions. Results were normalized by total protein and expressed as relative fold over nontransduced, nontreated control cells.

Quantitative Real-time Reverse-transcriptase Polymerase Chain Reaction (qPCR)

RNA from tissue samples was extracted using Norgen all-in-one purification kit (Norgen, Thorold, ON, Canada) and from cells using the RNeasy Mini Kit (QIAGEN). Complementary DNA (cDNA) was subsequently synthesized using iScript cDNA Synthesis Kit (Biorad, Hercules, CA). qPCR reactions were prepared in duplicate using iTaq Fast SYBR Green Supermix with ROX (BioRad) and gene specific primers (Invitrogen, Carlsbad, CA) (Supplementary Table I), and performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Target gene expression was determined by the relative quantification method of Pfaffl(18) using TATA-box binding protein (TBP) and 28S ribosomal RNA or Cyclophilin A (CypA) as housekeeping genes for mouse tissue and cells and HCASMC, respectively.

Silencing RNA (siRNA)—HCASMC were transfected with A20 specific FlexiTube siRNA at a final concentration of 40nM, using HiPerfect Transfection Reagent (QIAGEN) in serum-free OptiMEM medium (Invitrogen). Four hours after transfection, the medium was changed to regular culture medium. Cells transfected with AllStars negative Control siRNA (QIAGEN) served as controls.

Statistics

Statistics were performed using Prism 5 software (GraphPad, La Jolla, CA). Unpaired t-test was used for 2-group comparisons. Analysis of variance (ANOVA) followed by Duncan post-hoc test was used for 3-group comparisons. Differences between groups were rated significant at a probability error (P) < 0.05.

Results

Partial loss of A20 in aortic allografts aggravates lesions of intimal hyperplasia

WT and HET aortic allografts showed minimal intimal hyperplasia (IH) lesions 10 days following implantation. However, both developed substantial TA characterized by occlusive IH, 28 days after transplantation. Remarkably, HET allografts had significantly worse IH than WT vessels, as evidenced by higher I/M ratios in HET (1.624 ± 0.216) vs. WT (0.977 ± 0.139) allografts (Fig. 1A & B, $p < 0.001$).

We confirmed that basal A20 mRNA levels were undetectable in the aortae of KO mice, and reduced by 50% in aortae of HET as compared to WT mice (Fig. 1C). This analysis was done in 4 week-old mice to include KO mice that usually die in the first weeks of life from multi-organ inflammation(13). Basal A20 mRNA levels in the aortae of 8-10 week-old HET mice that were used as allografts remained consistently ~40% lower than those detected in aortae of WT mice (Fig. 1D). A20 mRNA levels significantly increased in both HET and WT aortic allografts 10 days after transplantation. However, despite being upregulated in both, there was a clear trend for a much lower (~40%) upregulation of A20 mRNA in HET vs. WT allografts (Fig. 1D).

Insufficient upregulation of A20 in HET aortic allografts amplifies vascular inflammation and increases proliferation of intimal SMC

We checked mRNA levels of 2 key molecules in the NF- κ B signaling pathway, the inhibitor of NF- κ B alpha ($\text{I}\kappa\text{B}\alpha$) and the transactivation subunit of NF- κ B, p65/RelA, before and 10 days after transplantation. $\text{I}\kappa\text{B}\alpha$ and p65/RelA are NF- κ B target genes, and hence indicators of the intensity of the inflammatory response that the allograft mounts (18–19). $\text{I}\kappa\text{B}\alpha$ and p65/RelA mRNA levels increased in WT and HET vascular allografts 10 days following transplantation (Fig. 2A). However, this increase was significantly greater in HET vs. WT allografts ($p < 0.001$ for $\text{I}\kappa\text{B}\alpha$ and $p < 0.05$ for p65). Higher mRNA levels of $\text{I}\kappa\text{B}\alpha$ and p65/RelA in HET vs. WT allografts corresponded with substantially higher levels of their phosphorylated protein levels, as evaluated by Western blot (WB) analysis (Fig. 2B). Phosphorylation of Ser-32 of $\text{I}\kappa\text{B}\alpha$ and Ser-536 of p65 are surrogate markers of the activation status of these proteins (19).

Similarly, mRNA levels of NF- κ B dependent VCAM-1 and ICAM-1, 2 molecules that enable vascular adhesion and transmigration of mononuclear cells that are a major source of proinflammatory cytokines (14, 20), were significantly higher in HET vs. WT aortic allografts (Fig. 2C, $p < 0.05$, and $p < 0.01$, respectively). Intensity and extent of VCAM-1 and ICAM-1 immunostaining was substantially greater in media of HET vs. WT allografts, 10 days following transplantation (Fig. 2D).

Migration of SMC from the media and their high proliferation rate in the newly formed intima are pathognomonic features of TA. Therefore, we checked for rate of SMC proliferation by staining for the proliferation marker Ki67. Our data demonstrated a significantly higher number of Ki67 positive cells per high power field (HPF) in the intima of HET vs. WT allografts (Fig. 2E, $p < 0.01$).

A20 knockdown in aortic allografts and in mouse and human SMC cultures increases inflammation-induced upregulation of pathogenic TNF and IL-6

Upregulation of proinflammatory TNF and anti-tolerogenic IL-6 in vascularized allografts are key pathogenic contributors to TA(20–25). TNF and IL-6 mRNA levels increased in vascular allografts 10 days after transplantation. This increase was substantially greater in HET vs. WT allografts (Fig. 3A and 3C, $p<0.05$). Paralleling mRNA levels, we showed by IF staining that TNF and IL-6 protein levels were also higher in HET vs. WT allografts 10 days after transplantation. TNF immunostaining colocalized with infiltrating F480⁺ monocytes/macrophages in the adventitia of the allografts, and also with medial and intimal SMC (Fig. 3B & Suppl. Fig. 1A). IL-6 immunostaining mostly localized to medial and intimal SMC and not the infiltrating monocytes and macrophages (Fig. 3D & Suppl. Fig. 1B).

To check whether higher TNF and IL-6 levels in SMC of HET allografts intrinsically resulted from partial loss of A20 or merely reflected an adapted response to higher *in situ* inflammation, we compared TNF and IL-6 mRNA and protein (ELISA) levels in A20-deficient and A20-sufficient mouse and human SMC cultures following a constant inflammatory stimulus comprised by a cocktail of cytokines (IL-1 β , TNF, and IFN γ). TNF mRNA levels were comparable at baseline in KO, HET and WT MASMCMC and moderately increased in response to TC with a non-significant trend towards higher levels in KO vs. HET and WT MASMCMC (Fig. 3E). However, there was a clear trend towards higher basal IL-6 mRNA and protein levels in KO vs. WT and HET MASMCMC, and this became highly significant following TC treatment ($p<0.01$ vs. WT, and $p<0.05$ vs. HET, Fig. 3E). Also, there was a trend for higher IL-6 mRNA and protein levels in TC-treated HET vs. WT MASMCMC (Fig. 3E). We validated these results in HCASMCMC by showing that siRNA-mediated knockdown of A20 yields significantly higher TNF and IL-6 mRNA and IL-6 protein levels than nontransfected and Allstars siRNA transfected controls, both at baseline and following TC treatment (Fig. 3F, $p<0.01$ and $p<0.001$). We verified by WB that A20 silencing decreased A20 protein levels by >90% (Fig. 3G).

A20 haploinsufficiency in aortic allografts enhances CD4⁺ T-cells and NK cell infiltration and favors a pathogenic over a regulatory immune response

HET allografts express higher VCAM-1 and ICAM-1, favoring adhesion and transmigration of immune cells. In addition, HET allografts produce higher levels of pathogenic IL-6, a chemoattractant for T and NK cells(22, 26), and also of the *bona fide* monocytes and T cell chemoattractants, CXCL10 and CCL2 (Fig. 4A). Accordingly, we noted a trend towards a higher number of infiltrating CD4⁺ and CD8⁺ T cells, as well as of NK cells in HET vs. WT allografts 10 days after transplantation (Fig. 4B). In phenotyping T cell infiltrates, we showed significantly higher levels of IFN γ mRNA in HET vs. WT allografts (Fig 5A, $p<0.05$), which correlated with a higher number of IFN γ ⁺ cells in these allografts, as evidenced by IF staining (Fig. 5A). IFN γ , the pathognomonic cytokine of CD4⁺ TH1-cells and NK cells, is a critical mediator of TA through direct deleterious effects on the vasculature(27, 28). Downstream of IFN γ , in addition to higher levels of IFN γ -induced CXCL-10, we demonstrated significantly higher mRNA levels of IFN γ -dependent IDO in HET vs. WT vascular allografts 10 days after transplantation (Fig. 5B, $p<0.01$). IDO

expression mostly localized to medial and intimal SMC, as demonstrated by IF staining (Fig. 5B). Granzyme B (GzmB), the serine protease that is typically released from cytotoxic CD8⁺ T cells and NK cell granules to destroy their target, was shown to positively correlate with severity of arteriosclerotic lesions(29, 30). The number of GzmB⁺ cells was initially comparable between HET and WT allografts 10 days post-transplantation but became substantially higher in HET vs. WT allografts 28 days after (Fig. 5C). Whereas GzmB IF staining expectedly localized to CD8⁺ cytotoxic T cells 10 days after transplantation, GzmB⁺ cells were mostly CD4⁺ T cells in HET but not WT allografts 28 days after transplantation (Fig. 5C & Suppl. Fig. 2A&B). Importantly, we also noted by IF staining a lower number of Forkhead boxP3 (FoxP3)⁺ regulatory T cells (Treg) in HET vs. WT allografts 10 days after transplantation (Fig. 5D). We confirmed this by showing significantly lower levels of FoxP3 mRNA levels in HET vs. WT allografts (Fig. 5E). This further qualifies the reduced ability of these allografts to counterbalance the deleterious effects of their heightened number of pathogenic T cells(31, 32). GzmB and FoxP3 IF staining did not co-localize in the same cells, indicating that each corresponded to a different subset of CD4⁺ T-cells (Suppl. Fig. 2C).

Partial loss of A20 in C57BL/6 (H2^b) recipients of fully mismatched BALB/c (H2^d) aortic allografts does not impact TA incidence and severity

Since expression of A20 in both the allograft and in the immune cells has been implicated in modulating the immune response through seemingly unrelated mechanisms(5, 33), we questioned whether partial loss of A20 in the recipient, rather than the donor impacts the incidence and severity of TA. Accordingly, we transplanted fully mismatched aortic allografts from BALB/c mice (H2^d) into the carotid arteries of HET or WT mice (C57BL/6, H2^b). As for the opposite scenario, we recovered the vascular allografts 10 and 28 days later and evaluated TA by morphometry analysis of their I/M ratio. Our results indicate minimal IH lesions 10 days following implantation. However, we noted substantial and comparable TA in both groups 28 days later. I/M ratio in vascular allografts implanted into WT or HET mice were not substantially different, even though slightly greater in HET vs. WT recipients, reaching 1.629 ± 0.03 , and 1.852 ± 0.02 , respectively (Fig. 6). These results likely indicate that partial loss of A20 in the recipient is not sufficient to drastically impact lesions of TA.

Discussion

TA is the primary cause of death in heart transplant patients after the first year of transplantation. A number of molecular culprits are linked to the pathogenesis of TA, with NF- κ B activation and IFN γ signaling leading the charge(27, 28, 34–36). Accordingly, individual genetic variations that modulate NF- κ B and IFN γ signaling, likely influence severity of TA.

We extensively documented in gain-of-function studies that A20's vasculoprotective functions, including to prevent TA, largely rely on its ability to simultaneously interrupt NF- κ B and IFN γ signaling in the vasculature, in addition to inhibiting SMC proliferation and promoting the demise of synthetic intimal SMC(5, 9, 10). Given that A20/TNFAIP3 (tumor necrosis factor alpha-inducible protein 3) single nucleotide polymorphisms (SNPs)

associating with approximately 40% decrease in A20 mRNA levels are highly predictive of an increased risk for coronary artery disease in diabetic patients, we questioned whether such SNPs could also modulate TA incidence and severity(37). Accordingly, we performed loss-of-function experiments using A20 haploinsufficient (HET) vascular allografts in total MHC mismatch with their mouse recipients. Vascular allografts from HET mice that expectedly have half the A20 levels of WT vessels better resonate with 40% decreased A20 expression in humans bearing deleterious A20 SNPs(37–39). A20 was significantly upregulated in both WT and HET vascular allografts following transplantation. However, not as robustly in HET compared to WT allografts. Inadequate upregulation of A20 was remarkably sufficient to significantly aggravate lesions of TA in HET allografts. This novel result places A20 as a key regulator of the pathologic remodeling of TA and suggests that A20 SNPs that negatively influence A20 expression or function could serve as predictive biomarkers of TA risk in human allografts.

Notably, neither haploinsufficient HET mice that breed normally and are phenotypically undistinguishable from WT mice nor cachectic A20 KO mice that suffer multi-organ uncontrolled inflammation and premature death(13) demonstrate any overt vascular phenotype (unpublished data). The vascular phenotype of A20 HET vessels only uncovers when these vessels are subjected to an insult such as an alloimmune response.

Aggravated TA in A20 HET allografts corresponded with their inability to sufficiently contain NF- κ B. In consequence, NF- κ B-dependent genes including the NF- κ B subunit p65, I κ B α , VCAM-1, and ICAM-1 were significantly higher in A20 HET vs. WT allografts. By promoting mononuclear leukocyte trafficking through the allograft vasculature, upregulation of VCAM-1 and ICAM-1 in EC and SMC is key to both acute rejection and TA pathogenesis(8, 15, 40–43). Accordingly, A20-deficient allografts showed a greater number of infiltrating T and NK cells, a phenomenon further amplified by the higher levels of CXCL10 and CCL2 chemokines in these vessels. Heightened NF- κ B activation in SMC is also involved in their phenotypic switch from contractile to synthetic and proliferative(44–46). Inhibiting SMC proliferation is therefore a sound therapeutic strategy to counteract TA, and we had convincingly reported that overexpression of A20 inhibits SMC proliferation both in vitro(9), and in rodent models of IH, including TA(5, 9, 16, 47, 48). Conversely, our data indicate that A20 knockdown in allografts associates with a significant increase in the proliferation rate of SMC. Altogether, these data ascertain the key role that A20 plays in modulating the inflammatory and proliferative responses of SMC, 2 key contributors to TA(49). Notably, we found a trend for higher incidence of thrombosis in HET vs. WT allografts (data not shown). Higher thrombotic events reflected an imbalance between protective vs. inflammatory EC responses. We documented significantly lower mRNA levels of protective endothelial nitric oxide synthase (eNOS)(50), and higher mRNA levels of the inflammatory EC-specific adhesion molecule, E-selectin, in HET vs. WT allografts 10 days after transplantation (Suppl. Fig. 3).

Pro-inflammatory TNF and IL-6 have extensively been implicated as key pathogenic determinants of TA(21, 23, 51, 52). IL-6 deficiency in donor allografts significantly prolongs survival in a mouse heart transplant model(53). We detected significantly higher levels of TNF and IL-6 mRNA and protein in HET vs. WT vascular allografts 10 days after

transplantation. As in our gain-of-function studies(5), we identified vascular allograft SMC (medial and intimal) as the main source of IL-6 production, while TNF was produced by both SMC and infiltrating monocytes/macrophages. In vitro results showing that cytokine-induced upregulation of IL-6 is greater in SMC from A20 HET and KO mice than in SMC from WT mice indicate that both an excessive susceptibility of HET SMC to inflammation and the greater inflammatory milieu in the HET allograft contribute to higher production of IL-6 by these allografts. Discrepancy between significantly higher IL-6 mRNA levels in HET vs. WT allografts, and the mere trend for higher IL-6 mRNA levels in TC-treated HET vs. WT SMC cultures likely indicates that the TC cocktail we used in vitro fails to reproduce the intensity of the inflammatory milieu that the HET vessel encounters in the setting of an alloimmune response. Our validation of these results in HCASMC highlights the clinical relevance of this finding.

IL-6 is the prototypical proinflammatory and anti-tolerogenic cytokine through its chemoattractive effects on T and NK cells(22, 26) and its impact on amplifying NK cell activity(54). Accordingly, the combination of higher IL-6 and higher chemokine levels in HET vs. WT allografts resulted in a greater number of T and NK cell infiltrates in these allografts. Qualitatively, increased T and NK cells in HET allografts correlated with significantly higher levels of IFN γ . IFN γ , the archetypal cytokine of pathogenic TH1 and NK cells is a key culprit in the development of TA(27, 28, 34, 55–57). In patients, IFN γ producing CD4⁺ TH1 cells dominate all other T-cell phenotypes in TA-ridden cardiac allografts(58). Mere IFN γ infusion causes TA in human vascular allografts transplanted into immunodeficient mice(59) by directly engaging atherogenic signals to EC and SMC(34). Accordingly, the exclusively IFN γ -dependent gene IDO, which is induced in medial SMC of vascular allografts and maintains immune privilege of the vessel media, was significantly higher in HET vs. WT allografts.

Higher IDO levels in HET vs. WT allografts could simply reflect greater IFN γ levels, but it could also relate to heightened IFN γ signaling in SMC. Indeed, we showed in gain-of-function studies that A20 overexpression conversely down-regulates IFN γ signaling in EC and SMC by decreasing expression of the transcriptional regulator of IFN γ signaling, signal transducer and activator of transcription (STAT1)(10).

As part of phenotyping the cellular infiltrate of A20 haploinsufficient vascular allografts, we checked for GzmB expression. GzmB, a serine protease primarily expressed by activated memory CD8⁺ and CD4⁺ T cells and NK cells, is implicated in the pathogenesis of TA(30), in part by promoting EC apoptosis(60). HET and WT vascular allografts had a substantial number of GzmB⁺ cells 10 and 28 days posttransplantation. At 10 days posttransplantation, the majority of GzmB⁺ cells were CD8⁺ T cells in both WT and HET allografts. At 28 days posttransplantation, GzmB⁺ staining remained restricted primarily to CD8⁺ T cells in WT allografts. However, this was not the case in HET allografts where GzmB⁺ staining localized primarily to CD4⁺ T cells. To the best of our knowledge, this is the first report indicating that A20 haploinsufficiency in vascular allografts may favor the development of this subset of GzmB⁺/CD4⁺ T cells. Once thought an anomaly associated with long-term T cell cultures, this subset of CD4⁺ cytotoxic helper T cells initially reported in chronic viral infections, has also been identified in the settings of acute viral infections, leishmaniasis,

and lymphopenia(61, 62). Unlike cytotoxic CD8⁺ T cells that recognize antigen in the context of Class I, cytotoxic CD4⁺ T cells are Class II restricted and their secretion of GzmB is reportedly less strictly regulated than that of CD8⁺ T cells(63). GzmB expression in CD4⁺/CD25⁺ Treg has also been reported as a key component of Treg-mediated suppression, in part through inducing apoptosis of effector T cells including anti-tumoral CD8⁺ T cells and NK cells(64, 65). Our data showing that GzmB and FoxP3 do not co-localize in the HET allograft infiltrating CD4⁺ T cells rules out that these GzmB⁺ T cells are also Treg. The mechanism(s) that favor(s) the generation of this subset of GzmB⁺/FoxP3⁻/CD4⁺ T cells and by which they aggravate vascular remodeling of TA in A20 haploinsufficient allografts remains to be elucidated. One plausible scenario is that GzmB⁺/CD4⁺/T cells may be induced by the heightened IL-6 levels in these allografts, akin GzmB⁺/CD8⁺/T-cells(66). To address this question, future studies using allografts from A20 HETxIL-6 KO donors, and GzmB KO mice as recipients of HET allografts should elucidate these questions.

A20 haploinsufficiency in vascular allografts also impeded the generation of Treg, as identified by their signature transcriptional repressor FoxP3. FoxP3⁺ Treg promote allograft tolerance, reduce the incidence of TA and improve survival of experimental cardiac allografts(67–69). In a remarkable study, Nadig et al showed that the injection of *ex vivo* expanded human Treg totally prevents TA in a humanized mouse model of TA(70). Accordingly, their decreased number in HET allografts is likely an aggravating contributor to TA. Notably, there was no difference in Treg counts in spleen, ipsilateral or contralateral cervical lymph nodes in recipients of WT vs. HET allografts (Suppl. Fig. 4). This supports our hypothesis that the relative enrichment of Treg in WT allografts occurs in the graft. IL-6 being one of the key cytokines that counteracts TGFβ-mediated FoxP3 transcription(71), we surmised that impaired generation of FoxP3⁺ Treg is related to significantly higher intra-graft production of IL-6 by HET vs. WT allograft SMC(20, 22, 72).

Collectively, our results prove the critical role of physiologically upregulated vascular A20 in modulating TA. Indeed, mere A20 haploinsufficiency associating with reduced upregulation of A20 after implantation significantly aggravates lesions of TA in MHC mismatched vascular allografts. Increased TA severity is multifactorial and involves exaggerated NF-κB and IFNγ signaling in the vasculature, heightened SMC proliferation, and modulation of the alloimmune response to promote infiltration of pathogenic TH1 and GzmB⁺ cytotoxic CD4 T cells over that of immunoregulatory FoxP3⁺ T cells, in part through increased IL-6 expression in the vessel wall. The translational relevance of these findings is paramount. Indeed, A20/TNFAIP3 SNPs that impart decreased A20 expression or function could inform an allograft's risk for developing TA and guide therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	Analysis of variance
CypA	Cyclophilin A
CCL2	Chemokine C-C Ligande 2
CXCL10	C-X-C motif Chemokine 10
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GzmB	Granzyme B
H&E	Hematoxylin/eosin
HCASMC	Human coronary artery smooth muscle cells
HET	A20 heterozygote
I/M	Intima over media
ICAM-1	Intercellular Adhesion Molecule 1
IDO	Indoleamine 2,3-dioxygenase
IF	immunofluorescence
IFNγ	Interferon gamma
IH	intimal hyperplasia
IHC	immunohistochemistry
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
IκBα	inhibitor of NF- κ B alpha
KO	A20 Knockout

MASMC	mouse aortic smooth muscle cells
MHC	major histocompatibility complex
NF-κB	Nuclear factor kappa B
NK	Natural killer
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Silencing RNA
SMC	Smooth muscle cells
SNP	Single nucleotide polymorphisms
STAT-1	Signal transducer and activator of transcription-1
TA	Transplant arteriosclerosis
TBP	TATA-box binding protein
TC	Triple cytokine
TGFβ	Transforming growth factor beta
TH1	T helper cell type 1
TNFAIP3	Tumor Necrosis Factor Alpha-Induced Protein 3
TNF	Tumor Necrosis Factor Alpha
Treg	Regulatory T cells
VCAM-1	Vascular cell adhesion protein 1
WT	Wild type

References

1. Stehlik J, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: 29th official adult heart transplant report--2012. *J Heart Lung Transplant*. 2012; 31(10):1052. [PubMed: 22975095]
2. Dhaliwal A, Thohan V. Cardiac allograft vasculopathy: the Achilles' heel of long-term survival after cardiac transplantation. *Curr Atheroscler Rep*. 2006; 8(2):119. [PubMed: 16510046]
3. Libby P, Pober JS. Chronic rejection. *Immunity*. 2001; 14(4):387. [PubMed: 11336684]
4. Shembade N, Harhaj EW. Regulation of NF-kappaB signaling by the A20 deubiquitinase. *Cellular & molecular immunology*. 2012
5. Siracuse JJ, Fisher MD, da Silva CG, et al. A20-mediated modulation of inflammatory and immune responses in aortic allografts and development of transplant arteriosclerosis. *Transplantation*. 2012; 93(4):373. [PubMed: 22245872]
6. Ensminger SM, Billing JS, Morris PJ, Wood KJ. Development of a combined cardiac and aortic transplant model to investigate the development of transplant arteriosclerosis in the mouse. *J Heart Lung Transplant*. 2000; 19(11):1039. [PubMed: 11077220]

7. McGillicuddy FC, Moll HP, Farouk S, Damrauer SM, Ferran C, Reilly MP. Translational studies of A20 in atherosclerosis and cardiovascular disease. *Adv Exp Med Biol.* 2014; 809:83. [PubMed: 25302367]
8. Cooper JT, Stroka DM, Brostjan C, Palmetshofer A, Bach FH, Ferran C. A20 blocks endothelial cell activation through a NF-kappaB-dependent mechanism. *J Biol Chem.* 1996; 271(30):18068. [PubMed: 8663499]
9. Patel VI, Daniel S, Longo CR, et al. A20, a modulator of smooth muscle cell proliferation and apoptosis, prevents and induces regression of neointimal hyperplasia. *Faseb J.* 2006; 20(9):1418. [PubMed: 16816117]
10. Moll HP, Lee A, Minussi DC, et al. A20 Regulates Atherogenic Interferon (IFN)-gamma Signaling in Vascular Cells by Modulating Basal IFNbeta Levels. *J Biol Chem.* 2014; 289(45):30912. [PubMed: 25217635]
11. Daniel S, Arvelo MB, Patel VI, et al. A20 protects endothelial cells from TNF-, Fas-, and NK-mediated cell death by inhibiting caspase 8 activation. *Blood.* 2004; 104(8):2376. [PubMed: 15251990]
12. Longo CR, Arvelo MB, Patel VI, et al. A20 protects from CD40-CD40 ligand-mediated endothelial cell activation and apoptosis. *Circulation.* 2003; 108(9):1113. [PubMed: 12885753]
13. Lee EG, Boone DL, Chai S, et al. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. *Science.* 2000; 289(5488):2350. [PubMed: 11009421]
14. Studer P, da Silva CG, Revuelta Cervantes JM, et al. Significant lethality following liver resection in A20 heterozygous knockout mice uncovers a key role for A20 in liver regeneration. *Cell Death Differ.* 2015; 22(12):2068. [PubMed: 25976305]
15. Dietrich H, Hu Y, Zou Y, et al. Mouse model of transplant arteriosclerosis: role of intercellular adhesion molecule-1. *Arterioscler Thromb Vasc Biol.* 2000; 20(2):343. [PubMed: 10669629]
16. Damrauer SM, Fisher MD, Wada H, et al. A20 inhibits post-angioplasty restenosis by blocking macrophage trafficking and decreasing adventitial neovascularization. *Atherosclerosis.* 2010; 211(2):404. [PubMed: 20430393]
17. Ray JL, Leach R, Herbert JM, Benson M. Isolation of vascular smooth muscle cells from a single murine aorta. *Methods Cell Sci.* 2001; 23(4):185. [PubMed: 12486328]
18. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29(9):e45. [PubMed: 11328886]
19. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annual review of immunology.* 2000; 18:621.
20. Zhao X, Boenisch O, Yeung M, et al. Critical role of proinflammatory cytokine IL-6 in allograft rejection and tolerance. *Am J Transplant.* 2012; 12(1):90. [PubMed: 21992708]
21. Diaz JA, Booth AJ, Lu G, Wood SC, Pinsky DJ, Bishop DK. Critical role for IL-6 in hypertrophy and fibrosis in chronic cardiac allograft rejection. *Am J Transplant.* 2009; 9(8):1773. [PubMed: 19538487]
22. Liang Y, Christopher K, Finn PW, Colson YL, Perkins DL. Graft produced interleukin-6 functions as a danger signal and promotes rejection after transplantation. *Transplantation.* 2007; 84(6):771. [PubMed: 17893611]
23. Zhang ZX, Huang X, Jiang J, et al. Natural killer cells play a critical role in cardiac allograft vasculopathy in an interleukin-6-dependent manner. *Transplantation.* 2014; 98(10):1029. [PubMed: 25286056]
24. Densem CG, Ray M, Hutchinson IV, Yonan N, Brooks NH. Interleukin-6 polymorphism: a genetic risk factor for cardiac transplant related coronary vasculopathy? *J Heart Lung Transplant.* 2005; 24(5):559. [PubMed: 15896753]
25. Wollin M, Abele S, Bruns H, et al. Inhibition of TNF-alpha reduces transplant arteriosclerosis in a murine aortic transplant model. *Transplant Int.* 2009; 22(3):342.
26. Weissenbach M, Clahsen T, Weber C, et al. Interleukin-6 is a direct mediator of T cell migration. *Eur J Immunol.* 2004; 34(10):2895. [PubMed: 15368306]
27. Mitchell RN, Libby P. Vascular remodeling in transplant vasculopathy. *Circ Res.* 2007; 100(7):967. [PubMed: 17431198]

28. Nagano H, Libby P, Taylor MK, et al. Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. *Am J Pathol.* 1998; 152(5):1187. [PubMed: 9588888]
29. Chamberlain CM, Granville DJ. The role of Granzyme B in atheromatous diseases. *Can J Physiol Pharmacol.* 2007; 85(1):89. [PubMed: 17487248]
30. Choy JC, McDonald PC, Suarez AC, et al. Granzyme B in atherosclerosis and transplant vascular disease: association with cell death and atherosclerotic disease severity. *Mod Pathol.* 2003; 16(5):460. [PubMed: 12748253]
31. Shan J, Guo Y, Luo L, et al. Do CD4+ Foxp3+ Treg cells correlate with transplant outcomes: a systematic review on recipients of solid organ transplantation. *Cell Immunol.* 2011; 270(1):5. [PubMed: 21640985]
32. Zuber J, Grimbert P, Blanco G, et al. Prognostic significance of graft Foxp3 expression in renal transplant recipients: a critical review and attempt to reconcile discrepancies. *Nephrol Dial Transplant.* 2013; 28(5):1100. [PubMed: 23262436]
33. Coornaert B, Carpentier I, Beyaert R. A20: central gatekeeper in inflammation and immunity. *J Biol Chem.* 2009; 284(13):8217. [PubMed: 19008218]
34. Tellides G, Pober JS. Interferon-gamma axis in graft arteriosclerosis. *Circ Res.* 2007; 100(5):622. [PubMed: 17363708]
35. Yu L, Qin L, Zhang H, et al. AIP1 prevents graft arteriosclerosis by inhibiting interferon-gamma-dependent smooth muscle cell proliferation and intimal expansion. *Circ Res.* 2011; 109(4):418. [PubMed: 21700930]
36. Autieri MV. Allograft-induced proliferation of vascular smooth muscle cells: potential targets for treating transplant vasculopathy. *Curr Vasc Pharmacol.* 2003; 1(1):1. [PubMed: 15320847]
37. Boonyasrisawat W, Eberle D, Bacci S, et al. Tag polymorphisms at the A20 (TNFAIP3) locus are associated with lower gene expression and increased risk of coronary artery disease in type 2 diabetes. *Diabetes.* 2007; 56(2):499. [PubMed: 17259397]
38. Musone SL, Taylor KE, Lu TT, et al. Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. *Nat Genet.* 2008; 40(9):1062. [PubMed: 19165919]
39. Mele A, Cervantes JR, Chien V, Friedman D, Ferran C. Single nucleotide polymorphisms at the TNFAIP3/A20 locus and susceptibility/resistance to inflammatory and autoimmune diseases. *Adv Exp Med Biol.* 2014; 809:163. [PubMed: 25302371]
40. Raisanen-Sokolowski A, Glysing-Jensen T, Russell ME. Donor and recipient contributions of ICAM-1 and P-selectin in parenchymal rejection and graft arteriosclerosis: insights from double knockout mice. *J Heart Lung Transplant.* 1999; 18(8):735. [PubMed: 10512519]
41. Braun M, Pietsch P, Schror K, Baumann G, Felix SB. Cellular adhesion molecules on vascular smooth muscle cells. *Cardiovasc Res.* 1999; 41(2):395. [PubMed: 10341839]
42. Sai S, Iwata A, Thomas R, Allen MD. Vascular cell adhesion molecule-1 up-regulation and phenotypic modulation of vascular smooth muscle cells predate mononuclear infiltration in transplant arteriopathy. *J Thorac Cardiovasc Surg.* 2001; 122(3):508. [PubMed: 11547303]
43. Ferran C, Peuchmaur M, Desruennes M, et al. Implications of de novo ELAM-1 and VCAM-1 expression in human cardiac allograft rejection. *Transplantation.* 1993; 55(3):605. [PubMed: 7681227]
44. Bourcier T, Sukhova G, Libby P. The nuclear factor kappa-B signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *J Biol Chem.* 1997; 272(25):15817. [PubMed: 9188479]
45. Chung CH, Lin KT, Chang CH, Peng HC, Huang TF. The integrin alpha2beta1 agonist, aggrexin, promotes proliferation and migration of VSMC through NF-kB translocation and PDGF production. *Br J Pharmacol.* 2009; 156(5):846. [PubMed: 19239475]
46. Chan J, Prado-Lourenco L, Khachigian LM, Bennett MR, Di Bartolo BA, Kavurma MM. TRAIL promotes VSMC proliferation and neointima formation in a FGF-2-, Sp1 phosphorylation-, and NFkappaB-dependent manner. *Circ Res.* 2010; 106(6):1061. [PubMed: 20150555]

47. Wang AB, Li HL, Zhang R, et al. A20 attenuates vascular smooth muscle cell proliferation and migration through blocking PI3k/Akt signaling in vitro and in vivo. *J Biomed Sci.* 2007; 14(3):357. [PubMed: 17260188]
48. Shrikhande GV, Scali ST, da Silva CG, et al. O-glycosylation regulates ubiquitination and degradation of the anti-inflammatory protein A20 to accelerate atherosclerosis in diabetic ApoE-null mice. *PLoS One.* 2010; 5(12):e14240. [PubMed: 21151899]
49. Libby P. Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2012; 32(9):2045. [PubMed: 22895665]
50. Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. *J Pathol.* 2003; 199(1):8. [PubMed: 12474221]
51. Booth AJ, Csencsits-Smith K, Wood SC, Lu G, Lipson KE, Bishop DK. Connective tissue growth factor promotes fibrosis downstream of TGFbeta and IL-6 in chronic cardiac allograft rejection. *Am J Transplant.* 2010; 10(2):220. [PubMed: 19788504]
52. Ternstrom L, Jeppsson A, Ricksten A, Nilsson F. Tumor necrosis factor gene polymorphism and cardiac allograft vasculopathy. *J Heart Lung Transplant.* 2005; 24(4):433. [PubMed: 15797745]
53. Ge F, Yuan S, Su L, et al. Alteration of innate immunity by donor IL-6 deficiency in a presensitized heart transplant model. *PLoS One.* 2013; 8(10):e77559. [PubMed: 24147024]
54. Luger TA, Krutmann J, Kirnbauer R, et al. IFN-beta 2/IL-6 augments the activity of human natural killer cells. *J Immunol.* 1989; 143(4):1206. [PubMed: 2787359]
55. Tellides G, Tereb DA, Kirkiles-Smith NC, et al. Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature.* 2000; 403(6766):207. [PubMed: 10646607]
56. Tellides G. Th1 adaptive immune responses in cardiac graft arteriosclerosis: deleterious or beneficial? *Circulation.* 2006; 114(15):1561. [PubMed: 17030702]
57. Ranjbaran H, Sokol SI, Gallo A, et al. An inflammatory pathway of IFN-gamma production in coronary atherosclerosis. *J Immunol.* 2007; 178(1):592. [PubMed: 17182600]
58. van Loosdregt J, van Oosterhout MF, Bruggink AH, et al. The chemokine and chemokine receptor profile of infiltrating cells in the wall of arteries with cardiac allograft vasculopathy is indicative of a memory T-helper 1 response. *Circulation.* 2006; 114(15):1599. [PubMed: 17015796]
59. Wang Y, Burns WR, Tang PC, et al. Interferon-gamma plays a nonredundant role in mediating T cell-dependent outward vascular remodeling of allogeneic human coronary arteries. *FASEB J.* 2004; 18(3):606. [PubMed: 14734640]
60. Choy JC, Cruz RP, Kerjner A, et al. Granzyme B induces endothelial cell apoptosis and contributes to the development of transplant vascular disease. *Am J Transplant.* 2005; 5(3):494. [PubMed: 15707403]
61. Marshall NB, Swain SL. Cytotoxic CD4 T cells in antiviral immunity. *J Biomed Biotechnol.* 2011; 2011:954602. [PubMed: 22174559]
62. Naouar I, Boussoffara T, Ben Ahmed M, et al. Involvement of different CD4(+) T cell subsets producing granzyme B in the immune response to Leishmania major antigens. *Mediators Inflamm.* 2014; 2014:636039. [PubMed: 25104882]
63. Lin L, Couturier J, Yu X, Medina MA, Kozinetz CA, Lewis DE. Granzyme B secretion by human memory CD4 T cells is less strictly regulated compared to memory CD8 T cells. *BMC Immunol.* 2014; 15:36. [PubMed: 25245659]
64. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol.* 2005; 174(4):1783. [PubMed: 15699103]
65. Cao X, Cai SF, Fehniger TA, et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity.* 2007; 27(4):635. [PubMed: 17919943]
66. Bottcher JP, Schanz O, Garbers C, et al. IL-6 trans-signaling-dependent rapid development of cytotoxic CD8+ T cell function. *Cell Rep.* 2014; 8(5):1318. [PubMed: 25199826]
67. Burrell BE, Nakayama Y, Xu J, Brinkman CC, Bromberg JS. Regulatory T cell induction, migration, and function in transplantation. *J Immunol.* 2012; 189(10):4705. [PubMed: 23125426]
68. Ali JM, Bolton EM, Bradley JA, Pettigrew GJ. Alloreognition pathways in transplant rejection and tolerance. *Transplantation.* 2013; 96(8):681. [PubMed: 23715047]

69. Turnquist HR, Zhao Z, Rosborough BR, et al. IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *J Immunol.* 2011; 187(9):4598. [PubMed: 21949025]
70. Nadig SN, Wieckiewicz J, Wu DC, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med.* 2010; 16(7):809. [PubMed: 20473306]
71. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Euro J Immunol.* 2010; 40(7):1830.
72. Wang S, Jiang J, Guan Q, et al. Reduction of Foxp3-expressing regulatory T cell infiltrates during the progression of renal allograft rejection in a mouse model. *Transplant Immunol.* 2008; 19(2):93.

Figure 1. Partial loss of A20 aggravates lesions of intimal hyperplasia in mouse aortic allografts implanted across total MHC mismatch from A20 heterozygous C57BL/6 (H2^b) donors to BALB/c (H2^d) recipients.

A. Representative photomicrographs of H&E stained whole (100X) and partial (400X) cross-sections of vascular allografts 10 and 28 days after transplantation showing lesions of TA. A-adventitia, I-intima, M-media. Magnification 100X, scale bar = 100 μ m (top panel) and 400X, scale bar = 20 μ m (lower panel). Results are representative of 5 to 8 mice per group. **B.** Morphometric analysis of intima/media (I/M) ratios demonstrates a significant increase in intima formation in A20 heterozygous (HET) allografts, as compared to wild-type (WT) controls 28 days after transplantation. 6-10 sections per vessel were measured using Image J. Results represent mean $\pm$ SEM of 5 to 8 mice per group. **C.** By qRT-PCR, we noted the absence of A20 mRNA in aortae of 4 week-old A20 KO mice, while these levels were 40-50% lower in HET vs. WT aortae. **D.** A20 mRNA levels were also lower in aortic vascular allografts retrieved from 8-10 week-old A20 HET mice as compared to WT, both at baseline and 10 days following transplantation. The graph shows relative A20 mRNA levels after normalization by the mRNA levels of the house keeping genes 28S and β actin, respectively. Results are expressed as mean $\pm$ SEM of 3 to 5 mice or vascular allografts per genotype. ** p <0.01, *** p <0.001.

Figure 2. Inadequate upregulation of A20 HET aortic allografts following implantation injury amplifies vascular inflammation and increases proliferation of intimal SMC.

By qRT-PCR, partial loss of A20 in aortic allografts led to significantly greater upregulation of NF- κ B dependent **A**. $I\kappa B\alpha$ and p65 as well as **C**. VCAM-1 and ICAM-1 mRNA levels in A20 heterozygous (HET) vs. wild-type (WT) aortic vascular allografts 10 days (d) following transplantation. The graph depicts relative $I\kappa B\alpha$ and p65 (n=3 WT allografts and 5 HET allografts), as well as VCAM-1 and ICAM-1 (n=6 WT and 10 HET allografts) mRNA levels normalized by the house keeping gene TATA-box binding protein (TBP). Results are expressed as mean $\pm$ SEM of 3-10 vascular allografts per genotype. *p<0.05, **p<0.01, ***p<0.001. **B**. Higher $I\kappa B\alpha$ and p65 mRNA levels in HET vs. WT allografts corresponded

at the protein levels with higher phospho- I κ B α (P-I κ B α) and phospho-p65 (P-p65) 10 days after transplantation, as depicted by WB analysis (n=3/group). **D.** Higher VCAM-1 and ICAM-1 expression in HET vs. WT vascular allografts was confirmed at the protein level by immunohistochemistry (IHC). Representative IHC photomicrographs of HET and WT vascular allografts mostly show medial VCAM-1 and ICAM-1 immunostaining (brown). Pictures shown are representative of 3 vascular allografts per genotype. Magnification=100X, scale bar = 100 μ m. White boxes delineate the area of the vascular allograft section that we show as close-up image to better depict the intensity of VCAM-1 and ICAM-1 immunostaining. **E.** By IHC, we also demonstrated significantly higher number of proliferating medial and intimal SMC in HET vs. WT allografts 10 days after transplantation. This was evidenced by immunostaining for the proliferation marker Ki67 (brown nuclei). A-adventitia, I-intima, M-media. Magnification 100X (top panel), scale bar = 100 μ m. Bottom panels are close-up images of the area delineated by the white boxes in the above photomicrographs. The graph represents mean $\pm$ SEM of the number of Ki67⁺ cells/high power field as determined in 4 WT and 6 HET allografts. **p<0.01.

Figure 3. A20 knockdown in aortic allografts and in mouse and human SMC cultures increases expression levels of inflammatory TNF and pathogenic IL-6 in response to inflammatory stimuli. Higher TNF and IL-6 mRNA (qRT-PCR) and protein (immunofluorescence/IF staining) levels in HET vs. WT aortic vascular allografts 10 days following transplantation. **A & C.** Graphs depict relative TNF and IL-6 mRNA levels after normalization by the house keeping gene TATA-box binding protein (TBP). Results are expressed as mean $\pm$ SEM of 4 WT and 5 HET allografts. Representative coIF photomicrographs of 3 WT and 5 HET allograft sections show **B.** higher TNF (red) expression in adventitial monocytes and macrophages

(F4/80/green), and in the media and intima of HET vs. WT, 10 days after transplantation, and **D.** higher IL-6 expression (red) mostly in the media and intima of HET vs. WT, 10 days after transplantation. Nuclear blue staining corresponds to 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining. Magnification=400X, scale bar = 20µm. **E.** Mouse aortic smooth muscle cells (MASMC) isolated from A20 knockout (KO), heterozygous (HET), and wild-type (WT) mice have comparable TNF mRNA (qRT-PCR) both at baseline and after addition of a triple cytokine (TC) cocktail, comprising TNF, IL-1 β and IFN γ . However, KO MASMC demonstrate higher basal IL-6 mRNA (qRT-PCR) and protein (ELISA in supernatant) levels than WT and HET. This trend becomes significant 6 hours following addition of TC. **F.** A20 silencing in human coronary artery smooth muscle cells (HCASMC) cultures, as achieved by transfection with A20 silencing RNA (A20 siRNA) significantly increases TNF mRNA levels 6h following treatment with a TC cocktail comprising TNF, IL-1 β and IFN γ as compared to control nontransfected cells (Ctrl), and to HCASMC transfected with All-star control siRNA (Control siRNA). A20 siRNA also significantly increased IL-6 mRNA and protein levels, both at baseline and 6h following treatment with TC. Data shown in **E.** and **F.** represents the mean $\pm$ SEM of 4 to 8 independent experiments. **G.** Representative WB of HCASMC confirms significant A20 knockdown in A20 siRNA transfected cells as compared to nontransfected (Ctrl) and to Allstar siRNA transfected HCASMC. Cell lysates were immunoblotted with an anti-human A20 antibody and also with anti-GAPDH antibody to correct for loading. *p<0.05, **p<0.01 and ***p<0.01.

Figure 4. Partial loss of A20 in aortic allografts corresponds with higher mRNA levels of CXCL10 and CCL2, and increases the number of infiltrating CD4⁺, CD8⁺ and NK cells A. Higher mRNA levels of the chemokines CXCL10 and CCL2 in HET vs. WT aortic vascular allografts 10 days following transplantation, as analyzed by qRT-PCR. Graphs depict relative CXCL10 and CCL2 mRNA levels after normalization by the house keeping gene TATA-box binding protein (TBP). Results are expressed as mean±SEM of 4 (WT) and 5 (HET) vascular allografts. **B.** Representative Photomicrographs of vascular allograft sections immunostained (IHC) with antibodies to CD4⁺ and CD8⁺ T cells as well as NK cells obviate

a trend towards higher number of infiltrating CD4 and CD8 T cells, and mostly NK cells (brown) in the adventitia of HET as compared to WT allografts, 10 days following transplantation. Infiltrating CD4⁺ and CD8⁺ T cells, as well as NK cells were scored by automated cell counting using Image J and expressed as number of positive cells per adventitia of a vascular section. Photomicrographs are representative of 3-6 vascular allografts/genotype. Magnification 200X, scale bar = 50µm. The graphs represent mean $\pm$ SEM of the number of positive CD4 (n=5 vascular allografts/genotype), CD8 (n=5 WT and 4 HET allografts) and NK (n=3 WT and 6 HET allografts) cells in the adventitia of vascular allografts per tissue section.

Figure 5. A20 haploinsufficiency in aortic allografts modulates the immune response to promote intra-graft infiltration of pathogenic IFN γ producing TH1 and Granzyme B⁺ cytotoxic CD4⁺ T cells while precluding that of FoxP3⁺ immunoregulatory CD4⁺ T cells.

IFN γ expression is significantly higher in HET vs. WT aortic vascular allografts 10 days following transplantation as analyzed at the mRNA (qRT-PCR) and protein (immunofluorescence, IF) levels respectively. **B.** Downstream of IFN γ , we noted significantly higher mRNA (qRT-PCR) and protein (IF) levels of IFN γ -dependent Indolamine 2,3 dioxygenase (IDO) in HET vs. WT allografts 28 days after transplantation. Graphs in **A.** and **B.** depict relative IFN γ and IDO mRNA levels after normalization by

mRNA levels of the house keeping gene TATA-box binding protein (TBP). Results are expressed as mean $\pm$ SEM of 5 to 10 vascular allografts per genotype, * p <0.05, ** p <0.01. IF photomicrographs show representative sections of HET and WT vascular allografts (n=3), demonstrating a higher number of (A) IFN γ positive (red) cells in the adventitia and (B) higher IDO expression (red) in the media of HET vs. WT allografts, at 10 and 28 days post transplantation, respectively. **A. and B.** Top panels magnification= 200X, scale bar = 50 μ m Bottom panels are close-up images of the area delineated by the white boxes in the above photomicrographs **C.** Double IF staining for CD4 (green)/Granzyme B (GzmB, red), and CD8 (green)/GzmB (red) positive cells in aortic allografts reveals that most GzmB⁺ cell infiltrates in the adventitia of WT and HET allografts are CD8⁺ T cells 10 days after transplantation. GzmB⁺ cells become mostly CD4⁺ T cells in HET allografts whereas they remain CD8⁺ T cells in WT allografts, 28 days after transplantation. Colocalization of CD4 or CD8 with GzmB is evidenced by the yellow overlay. **D.** Double IF staining for CD4 (red) and FoxP3 (green) positive T cells in the adventitia of vascular allografts demonstrates greater number of FoxP3/CD4⁺ Treg in WT vs. HET allografts 10 days following transplantation. Colocalization of CD4 and FoxP3 is evidenced by the yellow overlay. In all photomicrographs, nuclei were depicted in blue following staining with 4',6-diamidino-2-phenylindole (DAPI). All photomicrographs in **C.** and **D.** are representative of 3 vascular allografts per genotype. **C.** Top panels magnification= 400X, scale bar = 20 μ m **D.** Top panels magnification= 200X, scale bar = 50 μ m. Bottom panels are close-up images of the area delineated by the white boxes in the above photomicrographs. **E.** We confirmed lower FoxP3⁺ T cell infiltrates in HET vs. WT allografts by demonstrating significantly lower FoxP3 mRNA (qRT-PCR) levels in HET vs. WT allografts 10 days after transplantation. Graphs depict relative FoxP3 mRNA levels after normalization by TBP. Results are expressed as mean $\pm$ SEM of 2 WT and 5 HET vascular allografts. * p <0.05.

Figure 6. Partial loss of A20 in C57BL/6 (H2^b) recipients of fully mismatched BALB/c (H2^d) aortic allografts does not impact TA incidence and severity.

A. Representative photomicrographs of H&E stained whole (100X) and partial (400X) cross-sections of vascular allografts 28 days after transplantation showing lesions of TA. A-adventitia, I-intima, M-media. Magnification 100X, scale bar = 100μm (top panel) and 400X, scale bar = 20μm (lower panel). Photomicrographs are representative of 3 mice/group.

B. Morphometric analysis of intima/media (I/M) ratios in vascular allografts implanted into

wild-type (WT) or heterozygous (HET) recipients. 6-10 sections per vessel were measured using Image J. Results represent mean $\pm$ SEM of 3 mice per group.