

# NF- $\kappa$ B Regulates MICA Gene Transcription in Endothelial Cell through a Genetically Inhibitable Control Site<sup>\*S</sup>

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**Background:** Expression of activating immune ligands promotes inflammation.

**Results:** A pro-inflammatory immune ligand (MICA) is expressed in atherosclerotic lesions and is regulated by different transcription factors (NF- $\kappa$ B and HSF1) that compete for binding to overlapping promoter sites.

**Conclusion:** Competition for overlapping promoter sites integrates input from multiple transcriptional pathways.

**Significance:** Inflammatory immune responses may be influenced by genetic targeting of overlapping promoter sites.

Endothelial cells form a barrier between blood and the underlying vessel wall, which characteristically demonstrates inflammatory damage in atherosclerotic disease. MICA is a highly polymorphic ligand for the activating immune receptor NKG2D and can be expressed on endothelial cells. We hypothesized that damaged vessel walls, such as those involved in atherosclerosis, might express MICA, which could contribute to the vascular immunopathology. Immune activation resulting from MICA expression could play a significant role in the development of vascular damage. We have demonstrated that TNF $\alpha$  up-regulates MICA on human endothelial cells. The up-regulation is mediated by NF- $\kappa$ B, and we have defined the regulatory control site responsible for this at  $-130$  bp upstream of the MICA transcription start site. This site overlaps with a heat shock response element and integrates input from the two pathways. We have shown that in atherosclerotic lesions there is expression of MICA on endothelial cells. Using lentivirus-mediated gene delivery in primary human endothelial cells, we were able to inhibit the MICA response to TNF $\alpha$  with a truncated HSF1 that lacked a transactivation domain. This highlights the potential for transcription-based therapeutic approaches in atherosclerotic vascular disease to reduce immune-mediated endothelial and vessel wall damage.

Endothelial cells on the luminal surface of blood vessels provide an active interface between blood and underlying tissues, including those of the vessel wall itself (1). Damage to endothelial cells occurs in a wide range of pathological situations, including atherosclerotic disease, infections, and autoimmune diseases (1). Endothelial damage can lead to exposure of the vessel wall and underlying tissues to blood cells and other blood

components. Potential consequences of these interactions include luminal thrombosis or further vessel wall damage with the influx of immune cells and consequent vascular inflammation (1, 2). Endothelial cell damage could arise if endothelial cells up-regulate ligands for activating innate immune receptors. We hypothesized that pro-inflammatory stimuli might promote the up-regulation of such ligands, which would promote innate immune attack and so contribute to vessel wall inflammation and damage.

Human MICA is a highly polymorphic molecule and is a ligand for the activating receptor NKG2D, which is expressed on human natural killer cells and cytotoxic T lymphocytes. The engagement of NKG2D on a human natural killer cell by MICA on a potential target cell can result in cytotoxic killing of the target cell and inflammatory cytokine release (3). The interaction of MICA with NKG2D on T cells also exerts an activating influence on the T cells (4–6). MICA is encoded in the major histocompatibility complex (MHC) and is the most highly polymorphic human gene after the canonical MHC molecules, with over 70 alleles identified to date (7). MICA has structural similarities to the canonical MHC class I molecules but does not bind peptides (8). MICA is absent from the surface of most normal cells but is expressed on stressed cells, tumor cells, and pathogen-infected cells; the molecular mechanisms regulating MICA expression are poorly understood (3, 8, 9).

Endothelial cells can be activated by a range of stimuli, including bacteria or viruses and certain cytokines such as TNF $\alpha$  and IL1 $\beta$  (10, 11). Activation is associated with the release of cytokines, which can include TNF $\alpha$  itself and with changes at the endothelial cell surface, including expression of MHC class II molecules and enhanced capacity for leukocyte adhesion (12–15). These factors contribute to local inflammation. Endothelial dysfunction arises at an early stage in the atherosclerotic process, and there is much interest in factors that might contribute to this, especially inflammation (14, 16). There is an established correlation between inflammation and cardiovascular disease, and TNF $\alpha$  levels are elevated in patients with atherosclerotic vascular disease (17, 18).

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<sup>S</sup> This article contains supplemental Tables 1 and 2, Figs. 1–5, Materials, and an additional reference.

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## NF- $\kappa$ B Regulates MICA Transcription in Endothelial Cells

The role of MICA in atherosclerosis has not been studied, but there is increasing evidence for the expression of MICA on endothelial cells in several other disease contexts. Cytomegalovirus infection of endothelial cells has been shown to up-regulate MICA (4), and MICA is expressed on circulating inflammatory endothelial cells and kidney endothelium in patients with systemic vasculitis, but it is absent from normal kidney (19). Antibodies against MICA can develop following organ transplantation and are associated with reduced transplant organ survival (20). MICA is certainly expressed on the endothelium of the transplanted organ, in the context of an allo-immune attack and likely cellular stress from ischemia and other acute changes associated with transplantation (21).

MICA could play an important role in the interaction between the endothelium and the immune system by acting as a marker in the vascular system for areas of endothelium overlying abnormal regions of vessel wall, such as those affected by atherosclerosis, or overlying abnormal tissues, such as infected or tumor tissues. The mechanisms responsible for triggering MICA expression on endothelial cells have not been identified. Up-regulation of MICA will promote an immune attack from cells expressing NKG2D, in particular from natural killer cells that constitute 5–10% of peripheral blood cells (22). This attack could lead to endothelial damage and contribute to inflammatory changes in the vessel wall.

We hypothesized that inflammatory stimuli might up-regulate MICA on human endothelial cells, thus increasing the probability of an immune attack against these endothelial cells. Such immune-mediated endothelial cell damage could play a role in the development of vascular damage such as atherosclerosis. We show that TNF $\alpha$  up-regulates MICA surface expression through the NF- $\kappa$ B pathway. This transcriptional activation is mediated by a master regulatory DNA element in the MICA promoter. MICA is up-regulated on endothelium overlying atherosclerotic lesions, and up-regulation of MICA on endothelial cells can be inhibited by genetically targeting the master regulatory DNA element.

### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—The  $-3.8$ -kb MICA promoter reporter plasmid pOC347 MICA-3756-WT was constructed by PCR amplification of a 3.8-kb MICA promoter fragment from a genomic DNA template. This was cloned into the HindIII/NcoI sites of the pGL3-Basic plasmid (pGL3B, Promega, Madison, WI). The  $-230$ -bp reporter plasmid pOC149 MICA-233-WT was constructed in a similar way. Site-directed mutagenesis was carried out by PCR with reverse complementary primers containing the mutation followed by DpnI digestion to remove template plasmid DNA. The details of mutations for luciferase plasmids are specified in Fig. 5A. The lentiviral plasmid pHR-SIN-BX-IRES-Emerald and accessory plasmids for lentiviral production pMD.G (VSV-G expression plasmid) and p8.91 (Gag-Pol, Rev, and Tat expression plasmid) are as described (23). The truncated HSF1 expression plasmid pOC352 HSF1T and lentiviral plasmid pOC1129 LV-HSF1T were constructed by PCR amplification of the region of the HSF1 cDNA corresponding to amino acids 1–379 and cloning of this fragment into pcDNA3 and pHR-SIN-BX-IRES-Emerald, respectively.

The dominant negative I $\kappa$ B $\alpha$  lentiviral expression plasmid pOC1130 LV-IKBA-DN expressing I $\kappa$ B $\alpha$  S32A/S36A was constructed by site-directed mutagenesis of a pHR-SIN-IRES-Emerald-based wild-type I $\kappa$ B $\alpha$  lentiviral expression plasmid. The empty lentiviral expression plasmid pOC845 LV-control was constructed by removal of the BamHI/XhoI insert of pHR-SIN-BX-IRES-Emerald followed by blunt-ending with T4 DNA polymerase and re-ligation. All PCRs for cloning were carried out using *Pfu* polymerase (Stratagene, La Jolla, CA), and all constructs were verified by sequencing. All coordinates are relative to the transcriptional start site that was determined experimentally as described in the supplemental material.

**Cell Culture and Lentivirus Infection**—Human umbilical vein endothelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal calf serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin (Sigma), supplemented with 30  $\mu$ g/ml endothelial cell growth supplement (Sigma) and 5 units/ml heparin (Sigma). Human aortic arterial endothelial cells (Invitrogen) were maintained in Medium 200 supplemented with Low Serum Growth Supplement (Invitrogen), 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin. Primary arterial and venous endothelial cells before passage 6 were used for experiments. HeLa and 293T cells were maintained in DMEM supplemented with 10% fetal calf serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin. Where appropriate, TNF $\alpha$  (R&D Systems, Minneapolis, MN) was added at 10 ng/ml. Lentivirus was generated by co-transfection of the lentiviral expression plasmid, pMD.G and p8.91, into 293T cells.

**Flow Cytometry**—Cells were detached from tissue culture plates by incubation with enzyme-free cell dissociation buffer (Invitrogen) and stained with anti-MICA monoclonal antibody (clone 2C10, Santa Cruz Biotechnology) or isotype control followed by FITC-conjugated goat anti-mouse IgG (Serotec, Oxford, UK) or Dylight649-conjugated goat anti-mouse IgG (Serotec). Lentivirally transduced cells were identified by their GFP positivity. Flow cytometry was performed using a FAC-SCanto machine (BD Biosciences), and data were analyzed using Flowjo software (Tree Star, Ashland, OR).

**Quantitative RT-PCR**—Total RNA was extracted using Tri-Reagent (Sigma), further purified using the RNeasy mini kit (Qiagen, Hilden, Germany), and reverse-transcribed into cDNA using oligo(dT) and avian myeloblastosis virus reverse transcriptase (Invitrogen) or random hexamers and Bioscript Moloney murine leukemia virus reverse transcriptase (Biolone, London, UK). Real time PCR was carried out with SYBR Green supermix (Bio-Rad) using an IQ real time PCR machine (Bio-Rad). The data were normalized to GAPDH expression using the  $\Delta\Delta C_t$  method, and all the results represent the mean of at least two replicates. Real time PCR primers are listed in supplemental Table 1.

**Reporter Assays**—For reporter assays with NF- $\kappa$ B transfection, HeLa cells were cultured in 24-well plates and co-transfected with 150 ng of each reporter construct and pCMV $\beta$  (Clontech) using FuGENE 6 (Roche Applied Science). When appropriate, cells were also co-transfected with 150 ng of p65 expression plasmid or pcDNA3 empty vector control at this stage. Cells were lysed 48 h post-transfection for luciferase

assay using the luciferase assay system (Promega) and a TD-2020 luminometer (Turner Designs, Sunnyvale, CA), as well as  $\beta$ -galactosidase assay using *ortho*-nitrophenyl- $\beta$ -galactoside (Sigma) as substrate. The luciferase activity of each sample was normalized to  $\beta$ -galactosidase activity and shown as relative luminescence units. For reporter assay with TNF $\alpha$  treatment, human arterial endothelial cells were transfected with 1  $\mu$ g of each reporter construct and 50 ng of pRL-SV40 (Promega) by nucleofection using human coronary artery endothelial cell nucleofector kit and a Nucleofector II device (Lonza BioWhittaker, Portsmouth, NH), plated to recover for 24 h, and treated with 10 ng/ml TNF $\alpha$  for 24 h. Cells were lysed for luciferase assay using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activity of each sample was normalized to *Renilla* luciferase activity and shown as relative luminescence units.

**EMSA**—EMSA was performed using nuclear extracts from endothelial cells treated with TNF $\alpha$  and [ $\gamma$ - $^{32}$ P]ATP end-labeled double-stranded DNA probes. The forward strand probe sequences are CAGCCCACTGGAATTTTCTCTTCCA (wild type), CAGCCCACTGCTTAAGTCTCTTCCA (mutant), and AGTTGAGGGGACTTTCCAGGC (NF- $\kappa$ B consensus). The mutations introduced to disrupt the NF- $\kappa$ B site are underlined. The mutations are identical to those introduced into the luciferase reporter plasmids pOC234 MICA-233P-M1 and pOC348 MICA-3756P-M1. For standard EMSA, 5  $\mu$ g of nuclear extract was incubated with 100 fmol of labeled probes in a 10- $\mu$ l binding reaction containing 1  $\mu$ g of poly(dI-dC) and 100 ng of denatured sonicated salmon sperm DNA. For EMSA with limiting probe condition, 30  $\mu$ g of nuclear extract was incubated with 2.5 fmol of labeled probes in a 20- $\mu$ l binding reaction. For supershift assay, the nuclear extract was preincubated with 1  $\mu$ g of antibody for 30 min on ice before the probe was added. The following antibodies were used for supershift assay: anti-p65 (clone F-6, Santa Cruz Biotechnology), anti-p50 (clone 4D, Biolegend, San Diego), anti-c-Rel (Calbiochem), and anti-HSF1 (clone 10H8, StressGen, Victoria, Canada). For competition assays, unlabeled probe at 100-fold excess was added to the binding mixture before the addition of labeled probes.

**ChIP Assay**—Sonicated chromatin prepared from endothelial cells treated with TNF $\alpha$  was immunoprecipitated with anti-p65 antibody or mouse IgG<sub>1</sub> isotype control using protein G-agarose beads (Millipore, Bedford, MA). ChIP samples were analyzed by PCR amplification of the *MICA* proximal promoter region containing the putative NF- $\kappa$ B site, as well as a control region at the end of *MICA* intron1 6 kb downstream. ChIP assay primers are listed in supplemental Table 2.

**Immunohistochemistry**—Formalin-fixed paraffin-embedded tissue samples of nonatheromatous aorta ( $n = 5$ ), atheromatous aorta ( $n = 4$ ), and aorta with giant cell arteritis ( $n = 3$ ), obtained with full ethical approval from the National Research and Ethics Service (Oxfordshire Research and Ethics Committee A, reference 04/Q1604/21), were immunostained for MICA with anti-MICA antibody (ab62540, Abcam, Cambridge, UK) and detected with the Novolink<sup>TM</sup> max polymer detection system (Leica Microsystems, Wetzlar, Germany), as per the manufacturer's instructions, or with detection reagents only as a negative control. Slides were mounted in Aquatex mounting

medium (Merck). Stained sections were photographed with a Nikon DS-FI1 camera with a Nikon DS-L2 control unit (Nikon UK Ltd., Kingston-upon-Thames, UK) and an Olympus BX40 microscope (Olympus UK Ltd., Watford, UK).

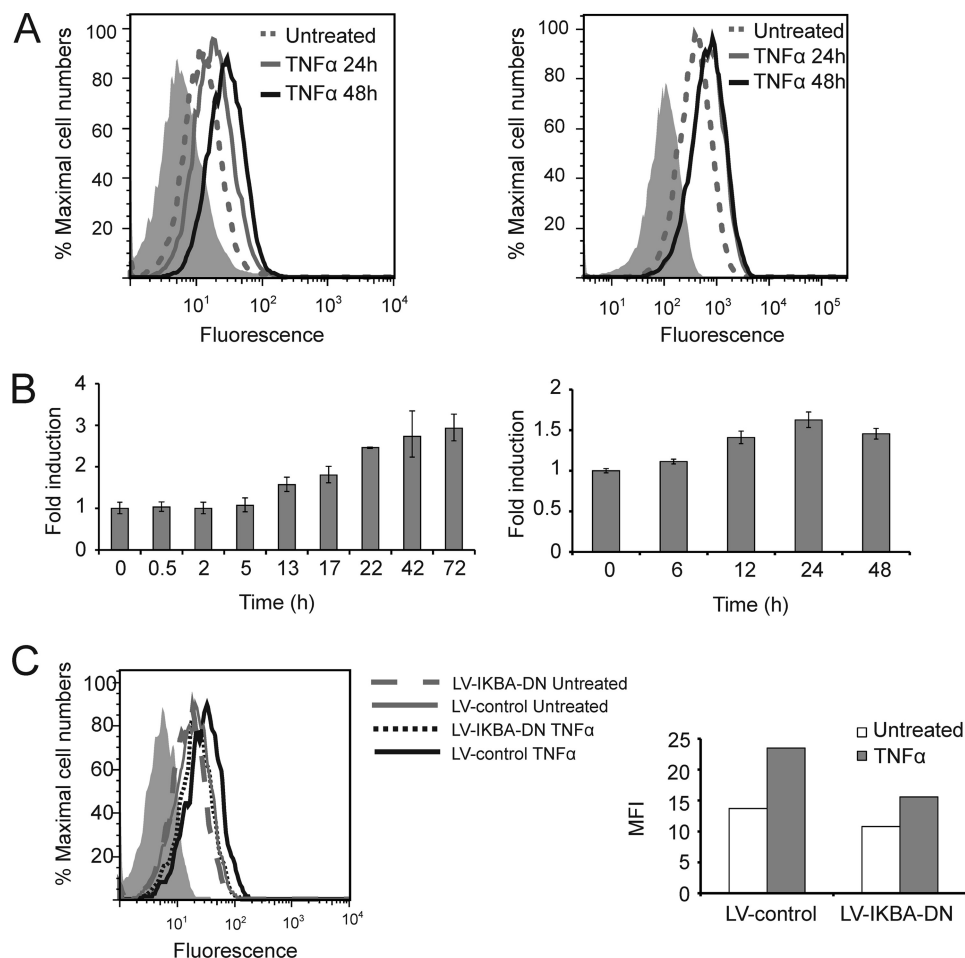
## RESULTS

**TNF $\alpha$  Induces MICA Expression on Human Endothelial Cells via NF- $\kappa$ B**—We hypothesized that MICA expression might be regulated by TNF $\alpha$  in endothelial cells. To test this hypothesis, primary human arterial and venous cells were treated with TNF $\alpha$ , and the cell surface expression of MICA was assessed by flow cytometry. Surface expression of MICA was significantly up-regulated at 24 and 48 h following TNF $\alpha$  treatment in both arterial and venous cells (Fig. 1A). Quantitative real time RT-PCR analysis demonstrated marked MICA mRNA up-regulation in response to TNF $\alpha$ , with a time course that is consistent with regulation of MICA by TNF $\alpha$  at the transcription level (Fig. 1B).

TNF $\alpha$  activates multiple pathways in endothelial cells, including the MAPK, JNK, and NF- $\kappa$ B pathways (24). The NF- $\kappa$ B pathway is activated by TNF $\alpha$  through phosphorylation-dependent degradation of I $\kappa$ B, which under normal circumstances retains NF- $\kappa$ B transcription factors in the cytoplasm (25). I $\kappa$ B degradation results in translocation of NF- $\kappa$ B transcription factors into the nucleus. Using bioinformatic analysis, we identified putative NF- $\kappa$ B response elements in the *MICA* promoter. The role of NF- $\kappa$ B in TNF $\alpha$ -induced MICA expression in endothelial cells was studied using lentivirus-mediated expression of a dominant negative I $\kappa$ B $\alpha$ . The dominant negative I $\kappa$ B $\alpha$  is mutated to prevent serine phosphorylation by the activating kinases and so specifically sequesters NF- $\kappa$ B components in the cytoplasm, thus blocking NF- $\kappa$ B-mediated transcriptional activation (26). Expression of the dominant negative I $\kappa$ B $\alpha$  inhibited the induction of MICA expression at the cell surface by TNF $\alpha$  (Fig. 1C) in primary endothelial cells. This confirms that the NF- $\kappa$ B pathway is the dominant pathway in the regulation of MICA by TNF $\alpha$  in endothelial cells.

**NF- $\kappa$ B Regulates MICA through a Master Control Site at -130 bp**—A detailed bioinformatic analysis identified three putative NF- $\kappa$ B-binding sites in the *MICA* promoter at -3140, -2850, and -130 bp upstream of the experimentally determined transcriptional start site (Fig. 2A). To determine whether any of these sites contribute to the transcriptional regulation of MICA by NF- $\kappa$ B, a series of reporter constructs were created in which *MICA* promoter regions containing the putative NF- $\kappa$ B sites drive the expression of luciferase. As Fig. 2B indicates, constructs containing the -3.8-kb and -230-bp regions displayed similar levels of NF- $\kappa$ B-mediated transactivation of the *MICA* promoter. These data are consistent with NF- $\kappa$ B acting at the predicted -130-bp site and demonstrate that there is no requirement for the predicted -3140- or -2850 bp sites. To confirm this, the constructs covering the -3.8-kb and -230-bp region were both similarly mutated within the -130-bp site to abolish NF- $\kappa$ B binding. This mutation abolished the inducibility of both the -3.8-kb and -230-bp constructs by NF- $\kappa$ B, confirming that the -130-bp site is necessary and sufficient for transcriptional transactivation of *MICA* by NF- $\kappa$ B (Fig. 2B). The role of the -130-bp regulatory element in

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**FIGURE 1. TNF $\alpha$  up-regulates MICA expression on human endothelial cells via the NF- $\kappa$ B pathway.** *A*, up-regulation of MICA surface expression on endothelial cells by TNF $\alpha$ . Human primary endothelial cells were treated with TNF $\alpha$  for 24 or 48 h and analyzed for MICA surface expression by flow cytometry. The *left panel* shows venous cells, and the *right panel* shows arterial cells, and in both cases MICA expression is up-regulated by TNF $\alpha$ . *Shaded histograms* represent isotype controls. *B*, up-regulation of MICA mRNA expression by TNF $\alpha$ . Human primary endothelial cells were treated with TNF $\alpha$  for the indicated times. Cells were then harvested, and MICA mRNA expression was quantified by real time RT-PCR. The *left panel* shows venous cells, and the *right panel* arterial endothelial cells. The *error bars* represent standard deviations of two (venous) or three (arterial) replicates. *C*, dominant negative  $\kappa$ B $\alpha$  blocks the induction of MICA by TNF $\alpha$  in endothelial cells. Primary human arterial endothelial cells were infected with a lentivirus which expressed a dominant negative  $\kappa$ B $\alpha$  (LV-IKBA-DN) or empty vector control (LV-control). 24 h later, the cells were treated with TNF $\alpha$  for 24 h and then analyzed for surface expression of MICA by flow cytometry. The *shaded histogram* represents an isotype control. The *right panel* illustrates the median fluorescence intensity (MFI) of MICA staining after background isotype control subtraction.

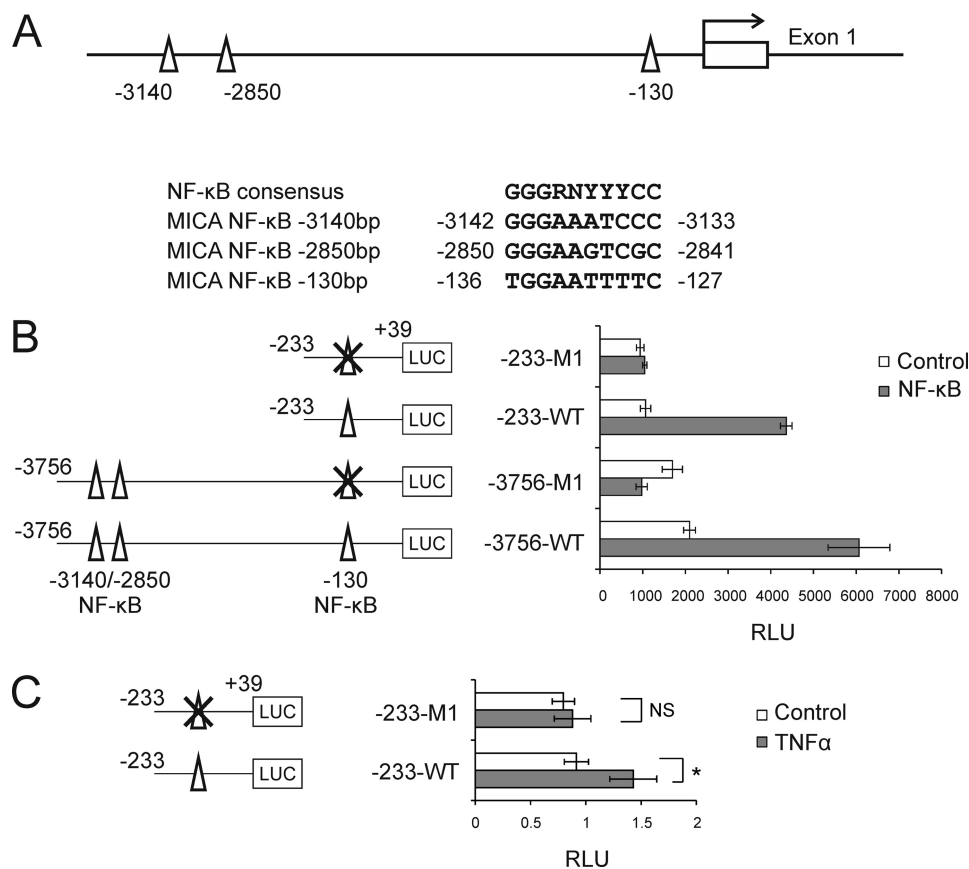
the response to TNF $\alpha$  in primary endothelial cells was also tested by reporter assay. As shown in Fig. 2C, TNF $\alpha$  significantly induces the wild-type MICA promoter in primary endothelial cells and mutation of the -130-bp site completely abolishes this induction. A previously suggested NF- $\kappa$ B site in intron 1 of MICA was not responsive to NF- $\kappa$ B activation (supplemental Fig. 1) (27).

**TNF $\alpha$  Induces NF- $\kappa$ B Binding to the Regulatory Control Site in Endothelial Cells**—To establish whether the -130-bp NF- $\kappa$ B site is involved in TNF $\alpha$ -mediated up-regulation of MICA in endothelial cells, EMSAs were performed (Fig. 3A). Following TNF $\alpha$  treatment of human arterial endothelial cells, a nuclear species was induced that bound specifically to the -130-bp site. Binding of the induced species persists for at least 24 h after the initiation of TNF $\alpha$  treatment (supplemental Fig. 2). This species was competed off by an excess of an unlabeled oligonucleotide corresponding to the wild-type -130-bp site but not by an equivalent excess of an unlabeled oligonucleotide corresponding to the mutated form of the -130-bp site, which was inactive

in the luciferase reporter assay. It was also competed off by an excess of an oligonucleotide corresponding to a consensus NF- $\kappa$ B site. Therefore, the specific DNA-binding species has the same sequence specificity for binding as that identified for regulatory activity at the -130-bp site using reporter constructs. To identify the bound species, supershift assays were undertaken using antibodies against specific NF- $\kappa$ B subunits. The bound species was supershifted by either anti-p65 or anti-p50 antibody but not by anti-c-Rel antibody (Fig. 3B). These results demonstrate that TNF $\alpha$  acts on human endothelial cells to induce binding of NF- $\kappa$ B p65/p50 heterodimers to a probe corresponding in sequence to the -130-bp site of the MICA gene. Similar experiments using nuclear extracts from venous endothelial cells confirmed that TNF $\alpha$  induces binding of p65/p50 heterodimers and also some p65/p65 homodimers to the MICA -130-bp site in these cells (supplemental Fig. 3).

To confirm that NF- $\kappa$ B binds to the -130-bp site in primary endothelial cells, ChIP assays were undertaken. Following treatment with TNF $\alpha$  or otherwise, sheared chromatin was

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**FIGURE 2. NF-κB regulates MICA expression through a control site at –130 bp.** *A*, locations and sequences of the three predicted NF-κB sites. Numbers indicate locations in base pairs with respect to the experimentally determined *MICA* transcriptional start site. *B*, mutation of the –130-bp NF-κB abolishes the induction of *MICA* promoter by NF-κB. HeLa cells were co-transfected with the indicated luciferase (*LUC*) reporter constructs plus either an NF-κB p65 expression vector (*NF-κB*) or control vector (*Control*). Data were normalized against β-galactosidase activity. *C*, treatment of primary arterial endothelial cells with TNFα regulates transcriptional activity of the *MICA* promoter through the –130-bp control site. Cells were transfected with the reporter constructs shown, treated with TNFα for 24 h, and harvested for luciferase activity. Data were normalized against *Renilla* luciferase activity. The error bars represent standard deviations of three replicates. Statistical significance was assessed using Student's *t* test: NS, not significant; \*, *p* < 0.05; RLU, relative luminescence units.

immunoprecipitated with an anti-p65 antibody and analyzed by PCR for the presence of the –130-bp site (Fig. 3, *C* and *D*). As shown in Fig. 3*D*, TNFα treatment of primary endothelial cells rapidly induced binding of NF-κB to the –130-bp promoter element but not to a control region in intron 1 of the *MICA* gene.

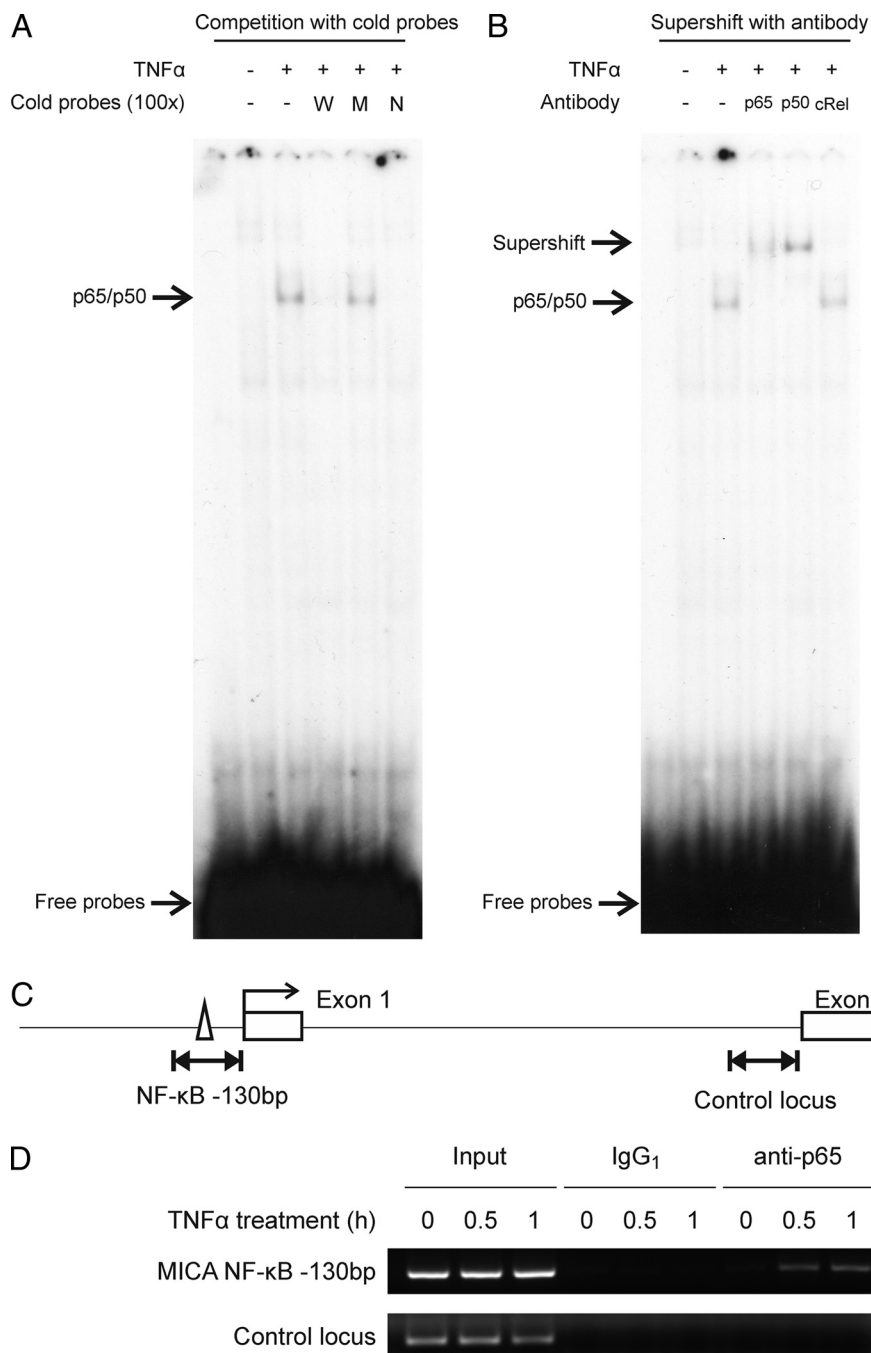
***MICA* Is Up-regulated on Endothelial Cells in Atherosclerotic and Inflamed Vessels**—Atherosclerotic lesions are characterized by the presence of pro-inflammatory lipid material and inflammatory cells, including macrophages that can produce TNFα (28). In this environment, our data suggest that local TNFα production could potentially influence endothelial cell *MICA* expression. Therefore, *MICA* expression was analyzed in normal and diseased human vessels using immunohistochemistry. In nondiseased vessels very occasional endothelial cells showed low levels of *MICA* expression, but the vast majority were negative on *MICA* immunostaining (Fig. 4, *A* and *B*). In contrast, in atheromatous vessels, endothelial cells showed strong immunostaining for *MICA*, indicating high levels of expression (Fig. 4, *C* and *D*). In giant cell arteritis, a form of large vessel vasculitis, the majority of endothelial cells showed *MICA* expression (Fig. 4, *E* and *F*), but at a lower level than that in the endothelial cells in atheromatous vessels. Significant *MICA*

expression was also seen on foamy macrophages associated with atherosclerotic lesions.

***Regulatory Control Site Integrates Regulatory Input from the NF-κB and Heat Shock Pathways***—Given the potential importance of TNFα-mediated up-regulation of *MICA* in endothelial cells in vascular inflammation and in the pathogenesis of atherosclerotic vascular disease, we sought to identify ways to target this up-regulating activity on the *MICA* gene. The NF-κB site at –130 bp of the *MICA* promoter overlaps with a heat shock element (HSE)<sup>2</sup> previously shown to mediate the induction of *MICA* by heat shock (8). We hypothesized that this overlap creates a master regulatory control site that integrates input from these two important pathways of transcriptional regulation. To test this, we designed and constructed detailed sets of mutations to this site to differentially disrupt either the NF-κB-binding elements or the heat shock factor-binding elements alone. The design was based on the different sequence specificities for NF-κB binding and heat shock factor binding (Fig. 5*A*) (29, 30). Mutation of the NF-κB elements alone abolished induction through this site by NF-κB but not by heat

<sup>2</sup> The abbreviation used is: HSE, heat shock response element.

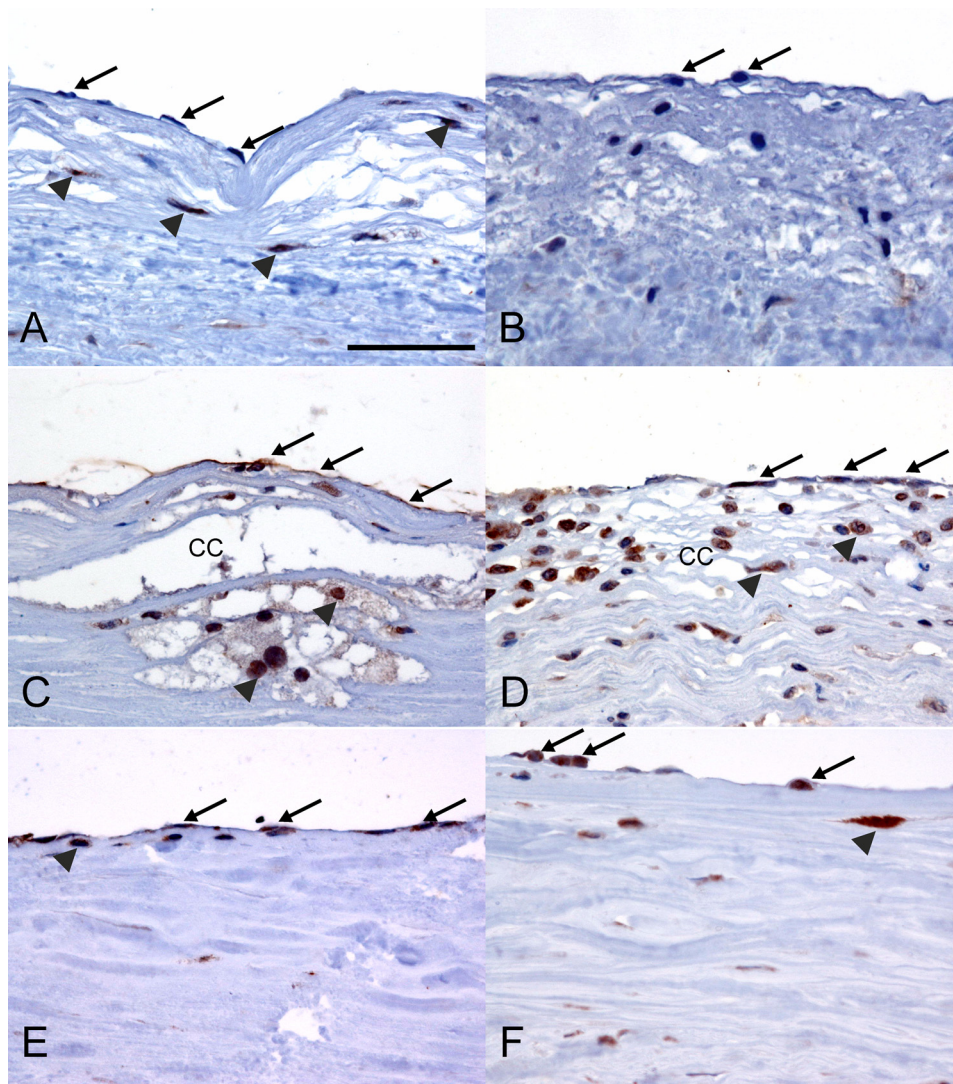
## NF- $\kappa$ B Regulates MICA Transcription in Endothelial Cells



**FIGURE 3. TNF $\alpha$  induces NF- $\kappa$ B transcription factor binding to the -130-bp control site.** *A* and *B*, *in vitro* binding of NF- $\kappa$ B to the -130-bp MICA NF- $\kappa$ B site demonstrated by EMSA in primary human arterial endothelial cells.  $^{32}$ P-Labeled DNA probes containing the -130-bp site were incubated with nuclear extract from primary endothelial cells treated with TNF $\alpha$  for 1 h or from untreated cells. In the competition assay (*A*), nuclear extracts were preincubated with 100-fold excess of unlabeled probe containing the intact wild-type -130-bp site (*W*), a mutated site (*M*), or a consensus NF- $\kappa$ B site (*N*); in the supershift assay (*B*), nuclear extracts were preincubated with antibodies against NF- $\kappa$ B p65, p50 or c-Rel before the addition of  $^{32}$ P-labeled probe. Protein-probe complexes were resolved by native PAGE. Results are representative of four experiments using different batches of independently purified nuclear extracts. *C*, locations of the PCR amplicons used in the ChIP assay at the -130-bp site or at a control locus in the first intron of the MICA gene. *D*, *in vivo* binding of p65 to the MICA locus demonstrated by ChIP assay. Primary endothelial cells were treated with TNF $\alpha$  for the times shown or untreated. Chromatin was immunoprecipitated with anti-NF- $\kappa$ B p65 antibody (*anti-p65*) or IgG<sub>1</sub> isotype control (*IgG<sub>1</sub>*) and analyzed by PCR in parallel with 50-fold diluted pre-immunoprecipitated chromatin (*input*).

shock (Fig. 5*B*). In contrast, mutation of the heat shock element alone abolished the induction through the site by heat shock but not by NF- $\kappa$ B. This demonstrates that NF- $\kappa$ B and heat shock regulate MICA transcription through overlapping, but independent, elements at the -130-bp NF- $\kappa$ B/HSE regulatory control site.

*A Dominant Negative Truncated HSF1 Blocks NF- $\kappa$ B Binding to the -130-bp Regulatory Control Site*—The existence of a single overlapping regulatory control site suggests a potentially powerful approach to inhibit the TNF $\alpha$ -mediated up-regulation of MICA on endothelial cells. We hypothesized that a dominant negative HSF1, which lacks a transactivation domain,



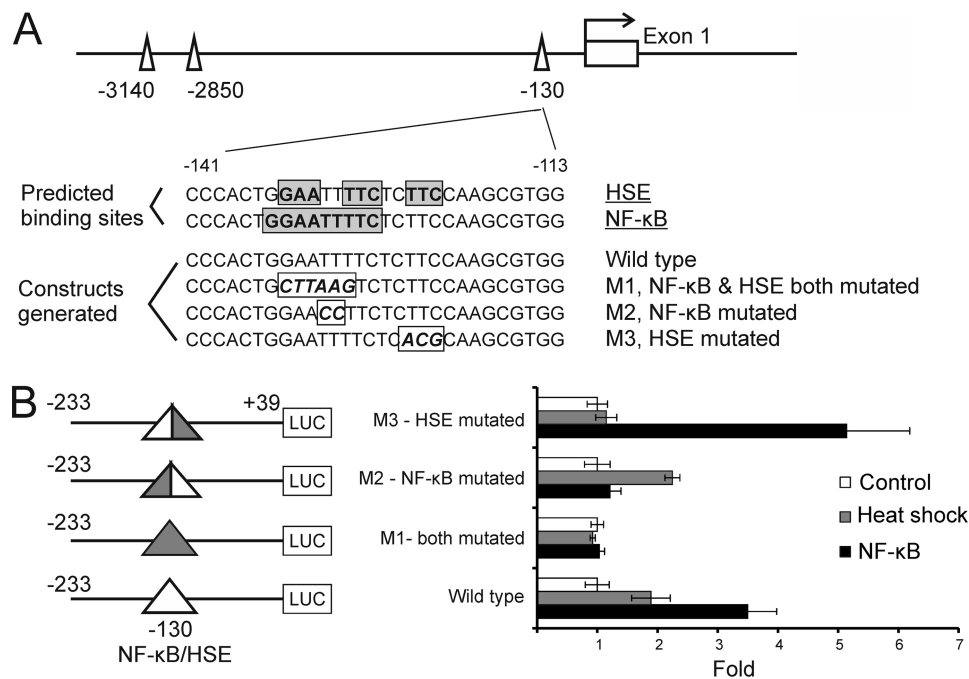
**FIGURE 4. MICA is up-regulated in endothelial cells in atherosclerotic and inflamed human arteries.** Sections of human aorta were immunostained for MICA (brown) using the peroxidase method. *A* and *B*, normal aorta showing minimal staining of endothelial cells (indicated with arrows). Some positive immunostaining of medial smooth muscle cells (arrowheads) is also seen in *A*. The scale bar in *A* (50  $\mu$ m) refers to all panels. *C* and *D*, atheromatous aorta showing strong staining of endothelial cells for MICA (arrows). Staining is also seen in foamy macrophages (arrowheads) associated with cholesterol clefts (CC) in plaques. *E* and *F*, in giant cell arteritis, there is less intense staining of endothelial cells (arrows) with some scattered MICA-positive macrophages seen (arrowheads).

could compete with NF- $\kappa$ B for binding to this master regulatory control site and so block the NF- $\kappa$ B-mediated induction of MICA by TNF $\alpha$ . To establish whether there was direct competition between truncated HSF1 and NF- $\kappa$ B for binding to the -130-bp MICA promoter site, EMSAs were undertaken using nuclear extracts from TNF $\alpha$ -treated endothelial cells transfected with lentivirus expressing truncated HSF1 (HSF1T). The TNF $\alpha$ -induced binding of NF- $\kappa$ B to the -130-bp regulatory element was inhibited by truncated HSF1 overexpression, as seen when the probe concentration was limiting (Fig. 6A). When abundant probe is available in the binding reaction, TNF $\alpha$ -induced binding of NF- $\kappa$ B to the probe is similar in the presence or absence of truncated HSF1 (Fig. 6B), confirming that NF- $\kappa$ B activation by TNF $\alpha$  is not affected by truncated HSF1 expression. These observations demonstrate that truncated HSF1 competes directly with NF- $\kappa$ B for binding to the -130-bp regulatory site of the MICA promoter.

In addition to the NF- $\kappa$ B p65/p50 heterodimer induced by TNF $\alpha$ , there is also constitutive basal level HSF1 binding to the -130-bp site (Fig. 6A), as confirmed by supershift assay (supplemental Fig. 4). With abundant probe, the TNF $\alpha$ -induced band seen for NF- $\kappa$ B bound to the -130-bp site is much stronger than that for HSF1, which is not seen with a short exposure time (Fig. 6B), although with limiting probe the bands are comparable (Fig. 6A). This demonstrates that the level of NF- $\kappa$ B-probe complex falls more than that of the HSF1-probe complex when the probe concentrations are reduced. Therefore, the affinity of NF- $\kappa$ B for the -130-bp regulatory site is weaker than that of HSF1 for the site. For this reason, HSF1 can compete successfully with NF- $\kappa$ B for binding to the -130-bp overlapping site.

The ability of truncated HSF1 to block NF- $\kappa$ B-mediated transactivation of the MICA promoter was tested by reporter assay. To explore this, cells were co-transfected with MICA

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**FIGURE 5. MICA regulatory control site integrates input from both heat shock and NF- $\kappa$ B pathways.** *A*, locations and sequences of the mutations introduced to the reporter constructs. Bases corresponding to the predicted binding sites for NF- $\kappa$ B or heat shock factor (HSE) sites are boxed and highlighted. Mutations designed to specifically disrupt either the NF- $\kappa$ B or HSE site in the corresponding reporter constructs are shown in boxed italics. *B*, different sets of mutations at the  $-130$ -bp NF- $\kappa$ B/HSE site selectively abolish the induction of the MICA promoter by NF- $\kappa$ B or heat shock. HeLa cells were transfected with the indicated reporter constructs bearing site specific mutations to the  $-130$ -bp site together with either an NF- $\kappa$ B p65 expression vector (NF- $\kappa$ B) or empty control vector (Control and Heat Shock), and cells were harvested 48 h post-transfection for reporter assay (NF- $\kappa$ B and Control) or subjected to heat shock (Heat Shock) for 1 h at 42 °C followed by 5-h recovery at 37 °C before cells were harvested. The error bars represent standard deviations of three replicates. LUC, luciferase.

promoter reporter constructs and expression plasmids for NF- $\kappa$ B and for a truncated HSF1 lacking a transactivation domain. As shown in Fig. 6C, co-transfection of the truncated HSF1 completely blocks the induction of the MICA promoter by NF- $\kappa$ B, confirming that a functional interaction occurs at this site and that transcriptional regulation by NF- $\kappa$ B at this site can be inhibited by the truncated HSF1.

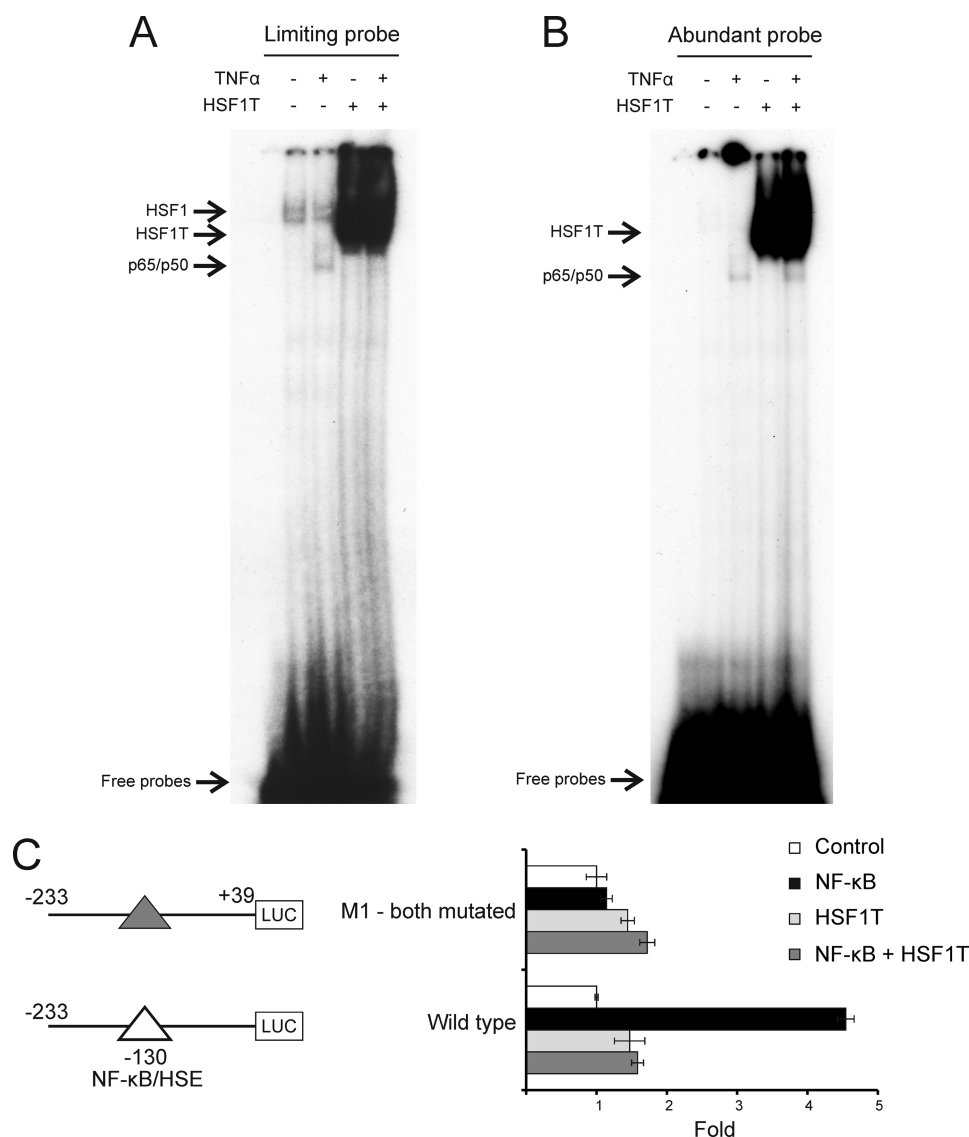
**Gene-based Functional Antagonism of MICA Up-regulation at the Master Regulatory Control Site**—To formally test the hypothesis that truncated HSF1 can block NF- $\kappa$ B-mediated MICA up-regulation by TNF $\alpha$  in endothelial cells, truncated HSF1 was overexpressed in primary human arterial endothelial cells using lentiviral gene delivery, and the cells were treated with TNF $\alpha$  and analyzed for MICA expression. Expression of truncated HSF1 in endothelial cells had an inhibitory effect on TNF $\alpha$ -induced up-regulation of cell surface MICA (Fig. 7A). Quantitative real time RT-PCR demonstrated that lentiviral infection of endothelial cells with the truncated HSF1 significantly inhibited the transcriptional up-regulation of MICA by TNF $\alpha$ , as did a dominant negative I $\kappa$ B $\alpha$  (Fig. 7B). This result confirms the importance of the  $-130$ -bp control site in the up-regulation of MICA by TNF $\alpha$  in endothelial cells and suggests a potential gene-based therapeutic approach to influence immune activation and inflammation in vascular disease utilizing overlapping transcription factor-binding sites.

## DISCUSSION

The role of innate immunity in human disease is increasingly recognized, as is the role of inflammation in vascular disease. We have demonstrated that the activating immune ligand

MICA is up-regulated on human endothelial cells by the pro-inflammatory cytokine TNF $\alpha$ . This up-regulation is controlled at the transcriptional level by a master regulatory control element positioned  $-130$ -bp upstream of the MICA transcription start site. This element integrates input from the NF- $\kappa$ B and heat shock pathways and in the presence of NF- $\kappa$ B activation exerts a strong influence on MICA expression at the cell surface. The overlapping nature of the control element for these two pathways allows targeted transcriptional blockade that inhibits TNF $\alpha$ -induced up-regulation of MICA. These results define a precise molecular mechanism of MICA up-regulation, of particular relevance in the pro-inflammatory state. The persistent binding of NF- $\kappa$ B to the regulatory site and the long half-life of the MICA transcript (supplemental Fig. 5) indicate that TNF $\alpha$  stimulation will cause prolonged activation at the  $-130$ -bp site, and transcript levels will continue to rise for a considerable time while the stimulus persists. Slow prolonged NF- $\kappa$ B-mediated transcriptional up-regulation has been described for genes with long transcript half-lives (31). In arterial endothelial cells, the main NF- $\kappa$ B species bound is p65/p50 heterodimer, whereas in venous endothelial cells there is significant p65/p65 homodimer binding. This may be a factor in the slightly stronger induction of MICA in venous cells as p65/p65 homodimers contain two transactivating domains, whereas the p65/p50 heterodimers contain only one. We found MICA expression on the endothelium overlying atherosclerotic lesions and in autoimmune vascular disease. The display of MICA on the surface of endothelial cells will promote immune attack of the endothelial cells (3, 4), resulting in damage to the





**FIGURE 6. Truncated HSF1 blocks transactivation of MICA promoter by NF- $\kappa$ B through direct competition with NF- $\kappa$ B for binding to the -130-bp site.** A and B, direct competition between NF- $\kappa$ B and truncated HSF1 for the -130-bp NF- $\kappa$ B site is demonstrated by EMSA under limiting probe condition.  $^{32}$ P-Labeled DNA probes containing the -130-bp site were incubated with nuclear extract from primary arterial endothelial cells transduced with lentivirus expressing a dominant negative truncated HSF1 that lacks a transactivation domain (*HSF1T*) or with empty vector control and treated with TNF $\alpha$  for 1 h. The binding reaction was carried out under limiting probe conditions (A) or standard abundant probe conditions (B) as described under "Experimental Procedures." The protein-probe complex was resolved by native PAGE, and the gel was subjected to autoradiography for 72 h (A) or 9 h (B). The identities of the complexes indicated by the arrows were confirmed by supershift assay (supplemental Fig. 4). A, it can be seen that TNF $\alpha$  induces an NF- $\kappa$ B band, but the truncated HSF1 successfully competes for probe binding resulting in loss of this band. C, dominant negative HSF1 blocks the induction of MICA promoter by NF- $\kappa$ B through the -130-bp NF- $\kappa$ B site. HeLa cells were transfected with the indicated luciferase (LUC) reporters (as illustrated in Fig. 5A) together with either an NF- $\kappa$ B p65 expression vector (NF- $\kappa$ B) or a truncated HSF1 expression vector (HSF1T) or an NF- $\kappa$ B p65 and a truncated HSF1T expression vector together (NF- $\kappa$ B + HSF1T) or an empty control vector (Control). Cells were harvested 48 h post-transfection for reporter assay. The error bars represent standard deviations of three replicates.

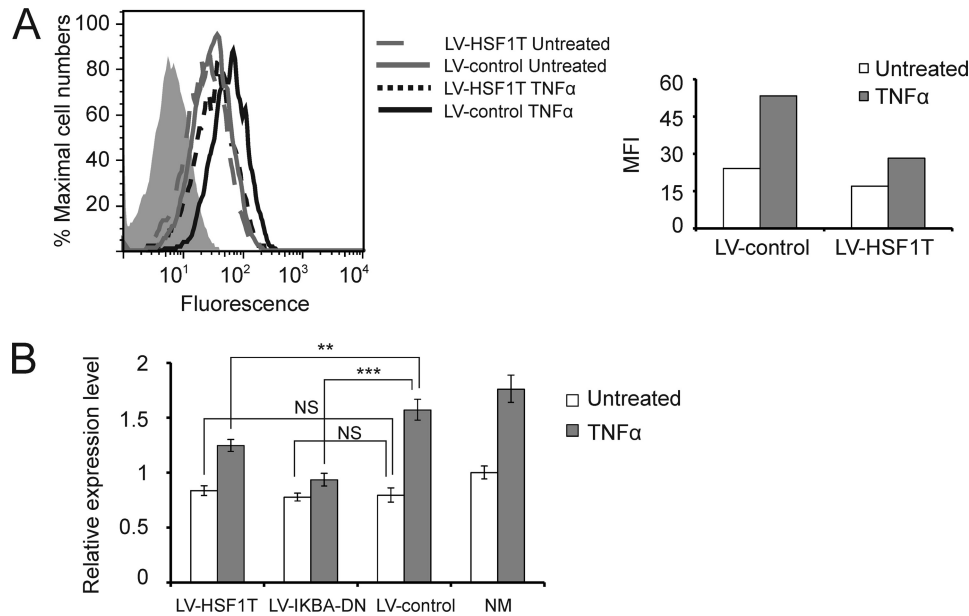
endothelium and contributing to ongoing vessel inflammation. Our data define a new mechanism whereby MICA expression could play a role in the development of vascular damage.

MICA may contribute to the pathogenesis of vascular disease at one or more stages of its development. Risk factors for atherosclerosis are well appreciated and include elevated lipid levels, diabetes, hypertension, age, and genetic risk. These factors apply systemically, but the disease is patchy and favors certain regions of the vascular tree. The cause for this distribution is unclear, but in some anatomical locations physical stressors, such as pressure or flow characteristics, may play a role. Flow-related stresses can have substantial effects on endothelial cells

function and could be significant factors in the development of vascular disease and its anatomical distribution (32, 33). MICA is known to respond to a wide range of cellular stresses (9, 34), and it is possible that it may be up-regulated by flow-related stresses at particular locations in the vascular tree.

Inflammatory cytokines such as TNF $\alpha$  are implicated in the pathogenesis of vascular disease. Patients with atherosclerosis have elevated levels of TNF $\alpha$  in their blood compared with matched controls (18, 35). Traditional risk factors correlate with markers of inflammation in patients with atherosclerosis (17, 35). This association may indicate that traditional risk factors, such as raised oxidized lipid levels, act by triggering

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**FIGURE 7. Gene-based functional antagonism of MICA up-regulation at the master control site.** *A*, truncated HSF1 inhibits induction of MICA surface expression by TNF $\alpha$  in primary arterial endothelial cells. Human primary arterial endothelial cells were infected with lentivirus expressing a dominant negative truncated HSF1 that lacks a transactivation domain (*LV-HSF1T*) or empty vector control (*LV-control*). 48 h later, cells were treated with TNF $\alpha$  for 24 h and analyzed for MICA surface expression by flow cytometry. The shaded histogram represents an isotype control. The right panel illustrates the median fluorescence intensity (MFI) of MICA staining after background isotype control subtraction. *B*, quantitative real time RT-PCR analysis demonstrates that a truncated HSF1 inhibits induction of *MICA* transcription by TNF $\alpha$  in primary arterial endothelial cells. Endothelial cells were infected with the lentivirus expressing either a truncated HSF1 lacking a transactivation domain (*LV-HSF1T*) or a dominant negative I $\kappa$ B $\alpha$  (*LV-IKBA-DN*) or an empty vector control (*LV-control*) and treated with TNF $\alpha$ . The transcript expression level relative to the uninfected untreated sample is shown. Statistical significance was assessed using Student's *t* test: NS, not significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

inflammation. TNF $\alpha$  levels increase with age in humans, as does atherosclerosis (36). Polymorphisms in TNF $\alpha$  receptors are associated with risk for age-related atherosclerosis in humans (37). Our data raise the possibility that TNF $\alpha$  acts via NF- $\kappa$ B on the *MICA* gene, by the mechanism that we have defined, to lower the threshold required for local anatomically variable stimuli, such as abnormal flow or pressure to up-regulate MICA and so promote endothelial cell attack by the immune system. There are precedents for this type of synergism between TNF $\alpha$  and a physical stressor, and the effect of TNF $\alpha$  on endothelial cell recruitment of monocytes has been shown to increase by an order of magnitude in the presence of nonuniform shear stress (38). In addition, there is evidence that TNF $\alpha$  can increase the susceptibility of endothelial cells to cytotoxic destruction by natural killer cells (39).

Up-regulation of MICA expression on endothelial cells by other stimuli could also contribute to vascular damage. Cytomegalovirus can infect endothelial cells and cause MICA expression (4), which will promote immune attack with the potential for endothelial damage and consequent influx of blood components, including immune cells and lipid species into the vessel wall. There is evidence that cytomegalovirus infection is associated with increased cardiovascular mortality long after primary infection (40–42).

The mechanism of MICA up-regulation that we have defined could clearly play a role in the propagation of an established atherosclerotic lesion. The lesions are rich in inflammatory cells, especially macrophages and lymphocytes (including T cells and natural killer cells) (2, 43). Macrophages release TNF $\alpha$  in response to lipid species, especially oxidized low density lipoprotein (44).

Atherosclerotic lesions contain a range of pro-inflammatory lipid species, and the macrophages within the lesions have been shown to produce TNF $\alpha$  (45, 46). Within lesions, endothelial cells are often physically close to macrophages or foam cells, and production of TNF $\alpha$  by these cells will expose overlying endothelial cells to high local levels of TNF $\alpha$ . Thus, in the development of inflammatory atherosclerotic lesions, macrophages that have migrated beneath the endothelium release TNF $\alpha$  that can activate NF- $\kappa$ B in the overlying endothelial cells. Binding of activated NF- $\kappa$ B components to the regulatory control site in the *MICA* gene would trigger expression of MICA on the surface of the endothelial cells; this would promote an immune attack resulting in further vascular damage with loss of endothelial integrity and influx of blood cells and components to the subendothelial space. The consequence of this is likely to be further macrophage influx, further local TNF $\alpha$  release and further MICA expression on neighboring endothelial cells, thus propagating and exacerbating an inflammatory immune attack on the vessel wall.

Even chronic low grade immune attack of endothelial cells may damage endothelial integrity and reduce the capacity of the vessel wall to generate NO. Such endothelial dysfunction has been implicated in the pathogenesis of vascular disease and has been shown to arise in the context of inflammation and improve with resolution of the inflammation (47). MICA-mediated immune damage to the endothelium could contribute to the development of endothelial dysfunction. Endothelial damage could also expose subendothelial structures to blood, potentially triggering thrombosis and vessel occlusion. MICA could play a role in the pathology of unstable plaques or plaque

rupture if MICA expression results in endothelial destruction and local immune activity sufficient to cause plaque herniation into the vessel lumen and consequent thrombosis.

Genome-wide analyses have not identified a strong association between MICA and atherosclerotic vascular disease. This indicates either that MICA does not play a role in the disease or that its effect does not differ between individuals with different MICA alleles. Although MICA is highly polymorphic, the regulatory control site is conserved unchanged in over 70 alleles that have been sequenced to date. This is consistent with an important function for this site and selection for an effective pathway for NF- $\kappa$ B and heat shock to drive MICA expression in endothelial cells and likely other cells too. Furthermore, the site is completely conserved across those species that have been sequenced (chimpanzee, rhesus, and orangutan). In the marmoset sequence, there is only a single base difference, and this is in a part of the site not involved in NF- $\kappa$ B or heat shock factor binding (29, 30). This pathway is likely to be particularly important in endothelial cells, which are a key interface between the circulating immune system and infected or diseased tissue. Our data indicate that the use of the regulatory site by one of NF- $\kappa$ B or heat shock factor is exclusive of the other. Therefore, there will be no additive effect of heat shock and NF- $\kappa$ B on MICA expression. This could prevent low level activation of the two pathways combining to trigger MICA expression but allow high level stimulation of just one pathway to do so.

Atherosclerotic vascular disease is the major cause of death globally (48). Despite improvements in both treatment and prevention of vascular disease, there is a pressing need for a detailed understanding of all aspects of the biology of atherosclerotic vascular disease. Statin drugs, which have had a major beneficial effect on cardiovascular disease, lower lipid levels by inhibiting HMG-CoA reductase, but they also have some inhibitory action on NF- $\kappa$ B (49). Different statins differ in their ability to block NF- $\kappa$ B in monocytes (50), so developing statins with a more pronounced NF- $\kappa$ B inhibitory effect in specific cellular contexts might improve their efficacy.

Future studies will be required to investigate the effects of modulating MICA expression on the development of atherosclerotic disease *in vivo*, but as rodents do not have a polymorphic NKG2D ligand like MICA, such studies will require careful design. Direct studies of activation of primary immune cells by MICA on endothelial cells are also limited by the need to avoid allo-responses, ideally by using autologous endothelial cells, which is not generally possible. Our findings establish a new mechanism whereby endothelial cells can flag themselves as potential targets for cytotoxic destruction by the immune system in atherosclerotic vascular disease. Other factors may contribute to the regulation of MICA on endothelial cells, and it will be important to define these factors. We have shown that targeted lentivirus-mediated genetic intervention can down-regulate the MICA response to TNF $\alpha$  in primary human endothelial cells. The identification of the overlapping NF- $\kappa$ B-HSE site in the MICA gene allows specific genetic targeting of this NF- $\kappa$ B site while leaving other NF- $\kappa$ B sites unaffected. In the longer term, it may prove possible and beneficial to engineer specific blood-borne therapeutics to control MICA expression in endothelial cells. These could be in the form of genetic ther-

apy, recombinant protein, or small molecules. Such approaches may be of benefit in a wide range of circumstances including atherosclerosis, other inflammatory vascular conditions, and organ transplantation. Furthermore, our results indicate that therapeutic regulation of other genes with overlapping transcription factor-binding sites may be possible using gene therapy with an altered form of one of the transcription factors lacking an activation domain. This approach will be relatively gene-specific in its blockade of the target transcription factor.

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