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Abstract

The IFN-inducible human IFI16 gene is highly expressed in endothelial cells as well as epithelial and hematopoietic tissues. Previous gene array analysis of human umbilical vein endothelial cells overexpressing IFI16 has revealed an increased expression of genes involved in inflammation and apoptosis. In this study, protein array analysis of the IFI16 secretome showed an increased production of chemokines, cytokines and adhesion molecules responsible for leukocyte chemotaxis. Functional analysis of the promoter for CCL20, the chemokine responsible for leukocyte recruitment in the early steps of inflammation, by site-specific mutation demonstrated that NF-κB is the main mediator of CCL20 induction at the transcriptional level. Finally, both Langerhans DC and B-lymphocyte migration triggered by supernatants from IFI16-overexpressing endothelial cells was partially inhibited by Ab inactivating CCL4, CCL5 and CCL20 chemokines. Altogether, these results demonstrate that the IFI16 gene, through its secretome, regulates proinflammatory activity of endothelial cells, thus corroborating its role in the early steps of inflammation.

Introduction

The IFI16 gene, a member of the HIN200 family, encodes a nuclear phosphoprotein 1–3 believed to belong to the DNA repair system and is triggered by various stimuli including IFN (IFN-α/β and -γ), oxidative stress, cell density and some proinflammatory cytokines, such as TNF-α 4, 5. In addition to partially conserved repeat motifs of 200 amino acids, designated A, B and C, the IFI16 protein contains a DAPIN/PYRIN domain within its N-terminus 6, 7. This domain was identified as a putative protein–protein interaction domain at the N-terminus of several other proteins believed to function in inflammatory signalling pathways. Consistent with these observations, prominent in vivo IFI16 expression has been demonstrated in lymphocytes, monocytes, stratified squamous epithelia and endothelial cells (EC) isolated from both blood and lymph vessels 8, 9, suggesting a role for IFI16 in the modulation of inflammation and the immune response. We have previously shown that IFI16 overexpression in EC triggered at the transcriptional level the expression of both adhesion molecules (such as ICAM-1) and chemokines (such as CCL2 and CCL20) 9. The treatment of cells with short hairpin RNA, targeting IFI16 significantly inhibited ICAM-1 induction by IFN-γ demonstrating that IFI16 is required for proinflammatory gene stimulation by this cytokine. Moreover, functional analysis of the ICAM-1 promoter demonstrated that NF-κB, one of the main transcription factors activated during inflammation, is the main mediator of IFI16-driven ICAM-1 induction by IFN-γ. Additionally, anti-IFI16 autoAb are present at much greater levels in patients with autoimmune diseases, such as cutaneous systemic sclerosis, systemic lupus erythematosus and Sjogren's syndrome, compared with normal controls 10, 11.

A common complication in autoimmune connective tissue diseases is vascular involvement 12. A reduction in the number of capillaries has been observed associated with endothelial swelling, basement membrane thickening and hyperplasia of the intima with infiltration of inflammatory cells into the skin 12. Considering this scenario in mind, one can hypothesize that IFI16 is involved in the early steps of inflammation resulting in EC activation – a necessary condition for the development of autoimmune diseases.

The aim of this study was to verify whether inflammatory molecule induction by IFI16 is confined to adhesion molecules, such as ICAM-1, or if it can also be extended to proinflammatory chemokines that are responsible for inflammatory cell recruitment, such as CCL4, CCL5 and CCL20, thereby reinforcing the physiological relevance of IFI16 in the early steps of inflammation.

Results

We have previously analyzed transcriptomes from EC overexpressing IFI16 and found that IFI16 upregulates a complex array of cellular genes encoding inflammatory molecules responsible for leukocyte recruitment 9. Moreover, we showed that IFI16 triggers the expression of EC ICAM-1 9 – an adhesion molecule involved in the enrolment of cells at the site of inflammation during the first steps of inflammation 13. In this study, in order to determine whether IFI16 also induces the secretion of chemokines and cytokines, we first analyzed the IFI16 secretome for 174 common chemokines, cytokines and growth factors using RayBio human cytokine array G Series 2000 Ab arrays. A comparison of the supernatants from cultured human umbilical vein EC (HUVEC)-overexpressing IFI16 with those from control HUVEC cultures infected with the LacZ transgene indicated 12 significantly induced molecules (Table 1). The most abundant inflammatory factors in the IFI16 secretome included the chemokines/cytokines CCL3, CCL4, CCL5, CCL20 and IL-1β, along with the growth regulatory factor amphiregulin (AREG). Consistent with the previous results showing induction of ICAM-1 at the transcriptional level, IFI16 overexpression also induced the expression of the soluble form of ICAM-1. Validation of the protein array analysis for some of the proteins identified from the secretome analysis was performed using real-time PCR (RT-PCR). Primer sequences were designed using the program qPrimerDepot (http://primerdepot.nci.nih.gov.offcampus.dam.unito.it/) directed at both the 3′ and 5′ ends of the gene sequence. The gene-specific primers used in this study are listed in Table 2. RT-PCR analysis largely confirmed secretome analysis. As shown in Fig. 1, IFI16 modulates the expression of endothelial genes, such as ICAM-1, implicated in the early steps of inflammation. Indeed, CCL3, CCL4, CCL5, CCL20 and IL-1β were significantly upregulated (33.5-fold, 21-fold, 9.5-fold, 18.5-fold and 28.5-fold, respectively), indicating that the RT-PCR results were generally consistent with the expression patterns observed in the secretome analysis (Table 1).

Table 1. Analysis of IFI16 secretome in EC

	AdVLacZ secretome average intensity ± SD	AdVIFI16 secretome average intensity ± SD	Fold change	Student's t-test *p*-value
AgRP	1651.450 ± 657.550	4954.350 ± 271.654	3.0	0.001
AREG	381.273 ± 67.032	1448.839 ± 128.596	3.8	< 0.001
Cardiotrophin-1	973.540 ± 639.844	878.183 ± 368.983	0.9	0.834
CCL3	1030.750 ± 306.773	3461.770 ± 826.773	3.4	0.008
CCL4	602.257 ± 209.482	3854.443 ± 1878.550	6.4	0.041
CCL5	1545.243 ± 236.382	9116.934 ± 1520.283	5.9	0.001
CCL7	659.540 ± 146.767	1239.935 ± 279.654	1.9	0.034
CCL20	8233.340 ± 1568.556	41 990.034 ± 2547.992	5.1	< 0.001
CCL26	621.643 ± 102.163	590.270 ± 236.028	0.9	0.843
CXCL1	35 311.203 ± 13 752.043	25 553.327 ± 5800.789	0.7	0.321
CXCL6	5995.737 ± 1244.631	18 586.785 ± 10 706.380	3.1	0.113
CXCL11	759.550 ± 253.434	3266.065 ± 146.767	4.3	< 0.001
GCSF	347.883 ± 104.481	450.161 ± 141.627	1.3	0.371
ICAM-1	192.340 ± 75.805	506.993 ± 46.562	2.6	0.004
IL-1β	52.150 ± 24.581	250.320 ± 48.248	4.8	0.003
Leptin	18 583.860 ± 3952.464	16 097.310 ± 6391.782	0.9	0.597
MIF	737.776 ± 315.361	1991.995 ± 330.532	2.7	0.009
PDGF-BB	7108.980 ± 1863.687	13 507.062 ± 918.387	1.9	0.006
TNF-α	1475.370 ± 212.806	1895.350 ± 679.587	1.3	0.365
uPAR	872.607 ± 360.851	1221.649 ± 132.546	1.4	0.191
VE-cadherin	189.780 ± 130.023	169.650 ± 42.131	0.9	0.811

Factor values (in arbitrary units) were determined by RayBiotech protein array analysis. Averages7SD are shown for AdVLacZ- and AdVIFI16-infected intensity values.

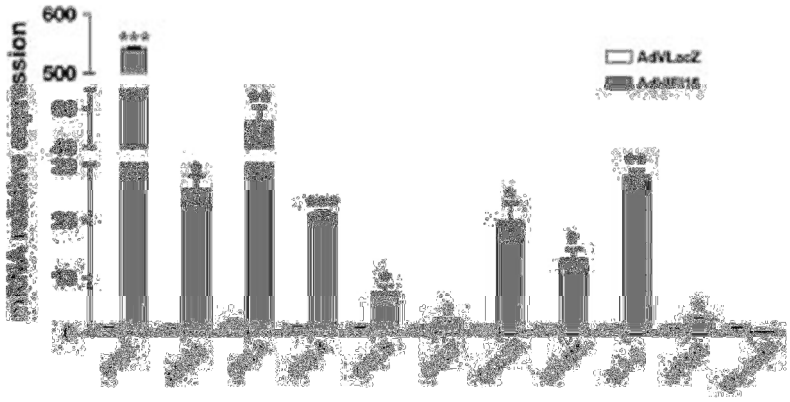

Figure 1. Validation of IFI16 secretome analysis in EC at the mRNA level. Total cellular RNA was extracted from HUVEC infected with AdVIFI16 or AdVLacZ at an MOI of 300 for 24 h and reverse transcribed into cDNA; the expression levels of the indicated genes were assessed by RT-PCR. Data show mean±SEM from three independent experiments. *p<0.05, **p<0.01, ***p<0.001; unpaired Student's t-test.

Table 2. List of primers for secretome validation

Gene name	Forward primer	Reverse primer
AREG	5′-TGGAAGCAGTAACATGCAAATGTC-3′	5′-GGCTGCTAATGCAATTTTTTGATAA-3′
β-actin	5′-GTTGCTATCCAGGCTGTG-3′	5′-TGTCCACGTCACACTTCA-3′
CCL3	5′-TGCAACCAGTTCCTCTGCATC-3′	5′-TGGCTGCTCGTCTCAAAGTA-3′
CCL4	5′-CTTCCTCGCAACTTTGTGGT-3′	5′-GGATTCACTGGGATCAGCAC-3′
CCL5	5′-GCACTTCTGTGTTCTGCTGCT-3′	5′-GATGTACTCCCGAACCCA-3′
CCL7	5′-GCACTTCTGTGTTCTGCTGCT-3′	5′-CAGCCTCTGCTTAGGGATTTT-3′
CCL20	5′-CCAAGAGTTTGCTCCTGGCT-3′	5′-TGCTTGCTGCTTCTGATTCG-3′
ICAM-1	5′-CAACCGGAAGGTGTATGAAC-3′	5′-CAGCGTAGGGTAAGGTTC-3′
IFI16	5-ACTGAGTACAACAAAGCCATTTGA-3′	5′-TTGTGACATTGTCCTGTCCCCAC-3′
IL-1β	5′-TCCCCAGCCCTTTTGTTGA-3′	5′-TTAGAACCAAATGTGGCCGTG-3′
uPAR	5′-CTGGAGCTGGTGGAGAAAAG-3′	5′-TAACGGCTTCGGGAATAGG-3′
Ve-cadh	5′-CCTACCAGCCCAAAGTGTGT-3′	5′-GACTTGGCATCCCATTGTCT-3′

As IFI16 increases the expression of genes encoding inflammatory chemokines, to confirm these inductions at the protein level, representative chemokines were also quantified by ELISA in supernatants from both LacZ and IFI16 HUVEC supernatants 60 h postinfection. As shown in Fig. 2, the CCL4 protein levels are 28-fold higher in supernatants from IFI16 HUVEC-infected cells compared with those in the supernatants from LacZ-infected cells (86±24 versus 3±4 pg/mL, mean±SEM), the CCL5 protein levels are fourfold higher (273±39 versus 74±32 pg/mL) and the CCL20 protein levels are about threefold higher in supernatants from IFI16 HUVEC-infected cells (312±30 versus 102±8 pg/mL). This analysis provides the first glimpse into the complexity of the IFI16 secretome and confirms its ability to trigger proinflammatory activity in EC.

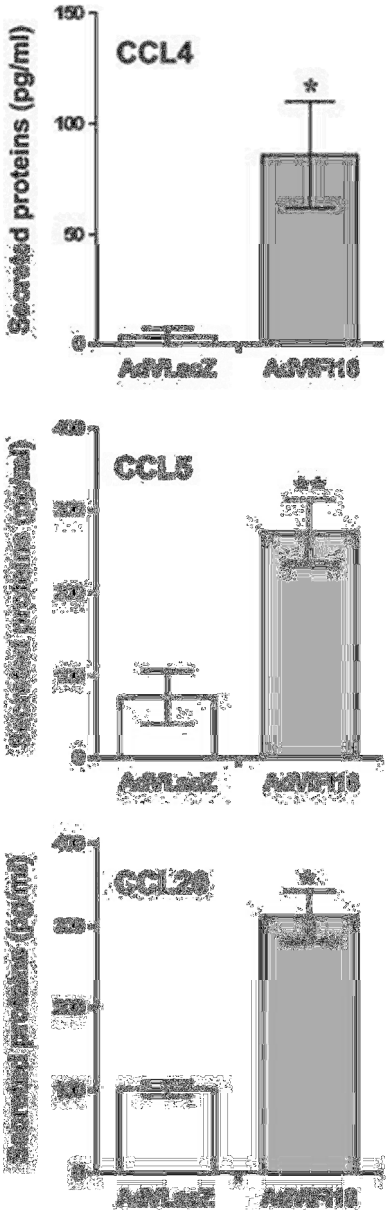

Figure 2. ELISA analysis of IFI16-induced chemokines. Supernatants from AdVLacZ- and AdVIFI16-infected HUVEC (MOI 300) were collected 60 h postinfection and analyzed by ELISA (R&D Systems). Data show mean±SEM from three independent experiments. *p<0.05, **p<0.01; unpaired Student's t-test.

The IFI16 gene is known to be induced by IFN, however, to confirm the role of IFI16 as the mediator of IFN pro-inflammatory activity, we investigated whether the array of inflammatory molecules stimulated in HUVEC by treatment with IFN-β overlapped with that observed in IFI16-infected cells. To do so, EC were treated with IFN-β or left untreated. After 24 h, total RNA were extracted, retrotranscribed into cDNA and analyzed by RT-PCR and the arrays of expressed

proinflammatory genes compared. As shown in Fig. 3, treating HUVEC with IFN-β resulted in the upregulation of a series of proinflammatory genes, including ICAM-1, CCL3, CCL4, CCL5, CCL20 and IL-1β (6.35-fold, 10.4-fold, 6.1-fold, 58.7-fold, 26.8-fold and 8.71-fold, respectively) that were also observed to be upregulated in HUVEC overexpressing IFI16.

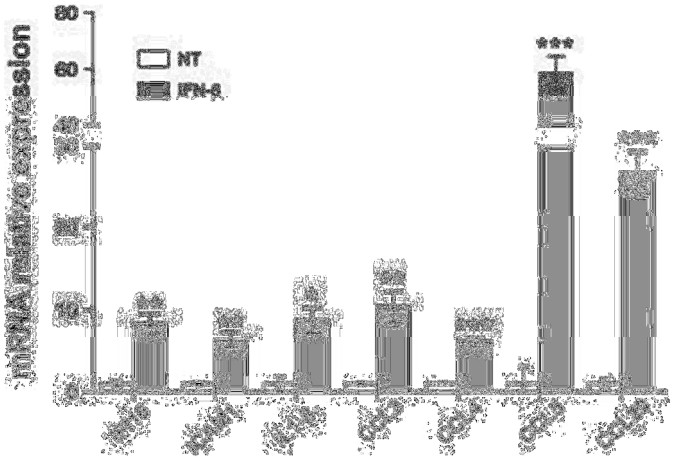

Figure 3. IFI16 and secretome induction following IFN-β treatment in EC. Total cellular RNA was extracted from HUVEC treated with 1000 U/mL of IFN-β for 24 h or left untreated (NT) and reverse transcribed into cDNA. Then the expression levels of the indicated genes were assessed by RT-PCR. Data show mean±SEM from three independent experiments. *p<0.05, **p<0.01, ***p<0.001; unpaired Student's t-test.

To determine whether the increase in expression of inflammatory molecules was a consequence of stimulating the encoding genes at the transcriptional level, we analyzed the effects of IFI16 on the expression of the transiently transfected luciferase reporter gene driven by the promoters of either CCL20 or ICAM-1. HUVEC were transiently transfected with the indicated plasmids and then infected with either adenovirus containing the IFI16 gene (AdVIFI16) or AdVLacZ, or otherwise left uninfected. Thirty-six hours postinfection, cell extracts were prepared and assayed for luciferase activity. As shown in Fig. 4, IFI16 overexpression led to an increase in the expression of the luciferase reporter gene driven by either the CCL20 promoter (3.8-fold) or the ICAM-1 promoter (11.5-fold) (used as positive control) compared with extracts from AdVLacZ-infected HUVEC.

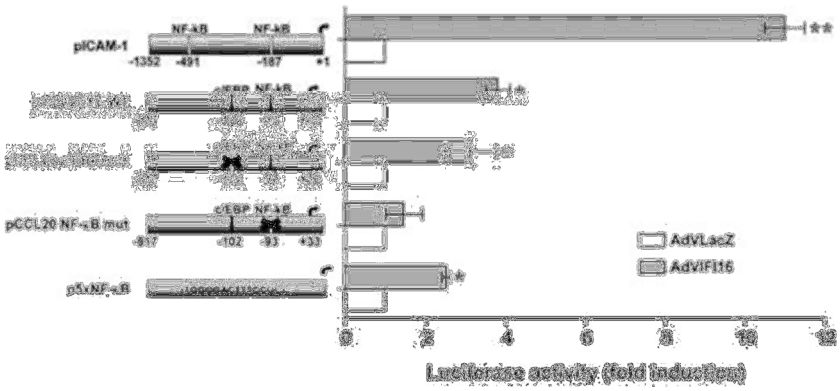

Figure 4. Mutational analysis of putative IFI16-responsive elements in the CCL20 promoter. HUVEC were transfected with the indicated constructs and 24 h later infected with either AdVIFI16 or AdVLacZ at an MOI of 300. After a further 36 h of incubation, protein extracts were prepared and assessed for luciferase activity. The p5×NF-κB-luc vector was used to test IFI16 activity on NF-κB binding sites. Data show mean±SEM from three independent experiments are shown; data are expressed as fold changes in the induction of promoter activity compared with the AdVLacZ-infected cells. *p<0.05, **p<0.01; unpaired Student's t-test.

Previous results have demonstrated that NF-κB is the main mediator of IFI16-driven ICAM-1 induction responsible for leukocyte adhesion to the endothelium 9. To determine whether the interaction of IFI16 with NF-κB may also be responsible for the induction of other genes within the IFI16 secretome, functional analysis of the CCL20 gene promoter was performed. To do this, transient transfection assays were performed using either one of the two human CCL20 promoter-luciferase constructs: pCCL20 c/EBPmut, containing the full-length human CCL20 promoter bearing the mutated c/EBP site; and the pCCL20 NF-κBmut, containing the full-length CCL20 promoter bearing the mutated NF-κB site 14. As shown in Fig. 4, site-specific mutation of the single NF-κB responsive motif almost completely blocked the ability of IFI16 to trigger luciferase activity. In contrast, mutation of the single C/EBP site only slightly decreased luciferase activity compared with the wild-type CCL20 promoter.

In order to provide definitive evidence supporting the role of NF-κB as the mediator of CCL20 promoter activation by IFI16, HUVEC were transfected with the indicator plasmid 5× NF-κB luc 15, infected thereafter with AdVIFI16 or AdVLacZ and reporter gene activity subsequently measured 24 h later. As shown in Fig. 4, overexpression of IFI16 significantly increased NF-κB transactivation of the reporter gene although at levels lower than those observed with the endogenous CCL20 promoter. Altogether, these results demonstrate that IFI16 interacts with NF-κB in order to trigger CCL20 promoter activity, in line with the results obtained from the ICAM-1

promoter analysis. However, NF-κB does not appear to be the only transactivator stimulated by IFI16 in order to trigger CCL20 promoter.

The ligand–receptor pair CCL20-CCR6 is believed to be responsible for the chemoattraction of CD34-derived immature DC, Langerhans DC (L-DC), effector/memory T cells and B cells, and it plays a role at skin and mucosal surfaces under homeostatic and inflammatory conditions 16, 17. If this is the case, it is important to verify a functional link between the ability of IFI16 to trigger CCL4, CCL5 and CCL20 release by HUVEC and DC and B-lymphocyte chemoattraction. Using a transwell migration assay, we demonstrate that both L-DC and B cells migrate to a significantly greater degree in response to the supernatants from IFI16-infected HUVEC compared with the supernatants from LacZ-infected HUVEC (Fig. 5). This migration was significantly reduced by pre-incubation with the anti-CCL4, anti-CCL5 and anti-CCL20 mAb, but only when added to the supernatants from IFI16-infected HUVEC. In contrast, addition of an unrelated mAb of the same isotype, used as an internal control, did not influence cell migration (data not shown). These results confirm that the secretion of CCL4, CCL5 and CCL20 by IFI16-infected HUVEC is functional and important for inducing L-DC and B-cell migration into the mucosa and skin where these cells are particularly abundant. Moreover, the only partial anti-CCL4, anti-CCL5 and anti-CCL20 mAb-induced inhibition of cell migration can be explained by the finding that the IFI16 secretome also contains other chemoattractant chemokines (Table 1) that contribute to the modulation of L-DC and B-cell migration.

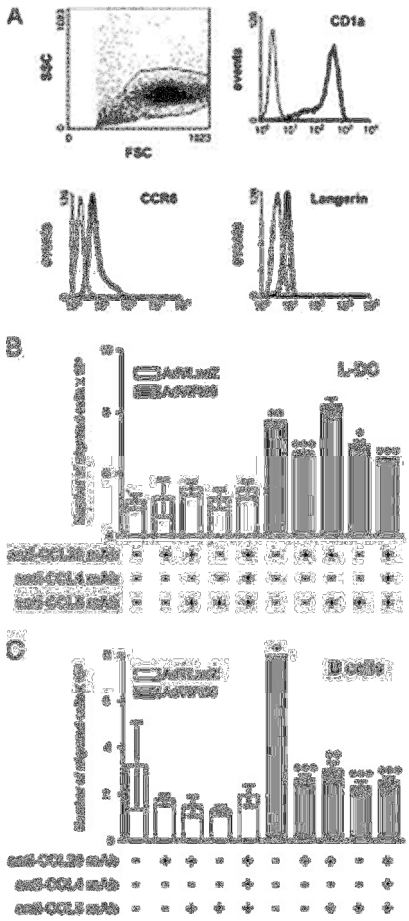

Figure 5. The supernatants from IFI16-infected HUVEC induce L-DC and B-cell migration. (A) Monocyte-derived L-DC express CD1a, CCR6 and langerin. The thin line shows the negative control, the thick line L-DC stained with the indicated mAb. Data are representative of six independent experiments with different donors. (B) In vitro-generated L-DC and (C) purified B cells were cultured in the presence of AdVLacZ-infected (white columns) and AdVIFI16-infected (grey columns) HUVEC supernatants for 2 h in the upper chamber of transwell plates in the presence of specific anti-CCL4, anti-CCL5 and anti-CCL20 mAb. Data show mean±SEM of three independent experiments. *$p<0.05$, **$p<0.01$: for AdVIFI16 versus AdVLacZ; °$p<0.05$, °°$p<0.01$, °°°$p<0.001$: for AdVIFI16-treated cells versus AdVIFI16-untreated cells; unpaired Student's t-test.

Discussion

Chemokines, basic proteins that strongly bind to heparin, can induce leukocyte chemotaxis and activation and are intimately involved in various biological processes, including inflammatory responses, hematopoietic regulation and neoangiogenesis 18–20. The chemokines CCL4, CCL5 and

CCL20 have been reported as being capable of attracting memory/activated T cells, whereas immature DC and B cells express CCR6 – its specific CC chemokine receptor 20, 21.

Previous DNA microarray analysis has revealed that IFI16 overexpression in EC triggers the expression of proinflammatory adhesion molecules, and functional analysis of the ICAM-1 promoter by site-specific mutagenesis has demonstrated that NF-κB is the main mediator of IFI16-driven gene induction 9. However, definitive prove that IFI16 regulates the proinflammatory activity of EC at the functional level has been missing. In this study, protein array analysis of the IFI16 secretome reveals that IFI16 triggers the expression of both intercellular adhesion molecules and chemokines responsible for leukocyte recruitment in vivo. Consistent with these observations, significant increases in the protein levels of CCL4, CCL5 and CCL20 were identified by ELISA in the supernatants of HUVEC overexpressing IFI16. Moreover, studying CCL20 as a representative chemokine, we demonstrate that NF-κB is the relevant mediator of CCL20 gene transcriptional activation following IFI16 overexpression. The relevance of this interaction is highlighted by the finding that the supernatants of IFI16-overexpressing HUVEC trigger the migration of both CCR6-positive L-DC and B cells and that this migration is significantly downregulated by the addition of Ab that neutralize CCL4, CCL5 and CCL20.

Inflammation is a complex defence mechanism, which aims to contain and resolve harmful processes (such as infections, toxic stress and tissue damage) and protect the integrity of the human body. At sites of inflammation, infection or vascular injury, both local proinflammatory and pathogen-derived stimuli render the vessel endothelium surface attractive for incoming leukocytes 22. This innate immune response of the endothelium consists of a well-defined and regulated multi-step cascade involving consecutive steps of release of leukocyte-recruiting chemokines by EC and adhesive interactions between the leukocytes and the endothelium; thus the proinflammatory activation of EC is important for the tight regulation of the mechanisms underlying the chemoattraction of leukocytes to lesions – mechanisms that are known to involve components of the NF-κB complex; indeed, the NF-κB complex is considered to be the major transcription factor regulating the expression of EC adhesion molecules and chemokine release 23–25. Consistent with this, in this study we show that IFI16 triggers the expression of proinflammatory genes by activating the NF-κB complex. The existence of a positive interaction between IFI16 and NF-κB transcription factors may have far reaching implications because many genes that play a role in host-defense mechanisms contain NF-κB elements in their respective promoter regions. In conclusion, these findings reinforce the role of IFI16 as a mediator of the immunomodulatory and proinflammatory activities of IFN that regulate the early defence mechanisms against infections.

Materials and methods

Cell lines and reagents

HUVEC cultured in endothelial growth medium (EGM-2, Lonza, Milan) containing 2% fetal bovine serum, human recombinant vascular endothelial growth factor, basic fibroblast growth factor, human epidermal growth factor, IGF-1, hydrocortisone, ascorbic acid, heparin, gentamycin and amphotericin B (1 μg/mL each) were seeded into 60 mm culture dishes coated with 0.2% gelatin. Experiments were performed with cells between passages 2 and 6. Human embryo kidney 293 cells (Microbix Biosystems) were cultured in minimum Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma, Milan, Italy), 2 mM glutamine, 100 units of penicillin per milliliter and 100 μg/mL of streptomycin sulfate.

Recombinant adenovirus preparations and HUVEC infection

Adenovirus-derived vectors expressing either IFI16 or LacZ were generated as described previously 9. Briefly, the pAC-CMV IFI16 containing the human IFI16 cDNA linked to a FLAG tag at the N-terminus was cotransfected together with pJM17 into human embryonic kidney 293 cells. After several rounds of plaque purification, the AdVIFI16 was amplified on 293 cell monolayers and purified from cell lysates by banding twice on CsCl gradients. Recombinant AdVIFI16 was tested for IFI16 expression by Western blotting using an anti-FLAG Ab (Sigma). For cell transduction, preconfluent HUVEC were washed once with PBS and incubated with either AdVIFI16 or AdVLacZ (used as a control) at a MOI of 300 in EGM-2. After 60 min at 37°C, the virus was washed off and fresh medium added. Cells were cultured for 36 h before use in the experiments.

RT-PCR analysis

RT-PCR analysis was performed on an Mx 3000 PTM (Stratagene) using the SYBR Green I dye (Fermentas) as a nonspecific PCR product fluorescence label. Total cellular RNA was isolated using the Nucleospin Extract RNA II (Macherey Nagel). RNA (1 μg) was then retrotranscribed at 42°C for 60 min in PCR buffer (1.5 mM MgCl2) containing 5 μM random primers, 0.5 mM dNTP and 100 units of RevertAid H Minus M-MuLV Reverse Transcriptase in a final volume of 20 μL. cDNA (1 μL), or water as control, were amplified in duplicate by RT-PCR using the Brilliant SYBR Green QPCR master mix (Fermentas) in a final volume of 25 μL. Primer sequences are summarized in Table 2. The Ct values for each gene were normalized to the Ct values for β-actin using the Ct equation. The level of target RNA, normalized to the endogenous reference and relative to the mock

infected and untreated cells, was calculated by the comparative Ct method using the $2-\delta\delta Ct$ equation.

Plasmids and transfection assays

For transfection experiments, HUVEC grown to subconfluence were detached and transfected with 0.6 μg of the reporter vector of interest with a MicroPorator (Digital Bio) according to the manufacturer's instructions (1200 V, 30 ms pulse width, one impulse). Transfected cells were added to antibiotic-free EGM-2 in 12-well costar multiwell cell culture plates and incubated overnight at 37°C. The medium was then replaced with complete EGM-2 medium containing 2% fetal bovine serum. Two hours later, cells were either infected with AdVIFI16, AdVLacZ (MOI of 300) or mock-infected. After 36 h, protein extracts were prepared and chemiluminescence was measured using the Dual Luciferase Reporter Assay System kit (Promega) at the Lumino luminometer (Stratec Biomedical Systems, Birkenfeld, Germany).

Protein microarray

Preconfluent HUVEC were washed once with PBS and incubated with either AdVIFI16 or AdVLacZ (used as a control) at an MOI of 300 in EGM. After 2 h at 37°C, the virus was washed off and fresh medium was added. After 60 h of incubation, supernatants were collected, centrifuged and transferred to new tubes for the chemokine/cytokine analysis according to the manufacturer's instructions. The RayBio human cytokine array (G Series 2000 Ab arrays; RayBiotech, Norcross, GA, USA) is a glass slide format. The signals from G series arrays are detected using a laser scanner for the detection of 174 human cytokines in single experiment. In brief, after blocking, the arrays were incubated with the indicated samples. Unspecific bound proteins were removed and the arrays were incubated with a cocktail of biotin-Ab and then fluorescent dye-conjugated streptavidin. Spots were visualized using detection buffer loaded to cover the entire surface and incubated for 5 min. Image fluorescence signals were scanned and a software used that allows the fluorescence from all samples to be detected simultaneously or each sample to be detected on an individual basis as required. Spots were digitized into pixel densities. The densities were exported into spreadsheet software (Excel; Microsoft, Redmond, WA, USA) and the background intensity subtracted. The data were normalized to the positive control values provided by the manufacturer as 100% 26. LacZ- and IFI16-infected samples were compared for significance using Student's t-tests. p-Values of <0.05 were considered statistically significant.

Detection of proteins

CCL4, CCL5 and CCL20 chemokines were quantified in LacZ- and IFI16-infected HUVEC supernatants by ELISA (R&D Systems, by SPACE, Milan, Italy) in accordance with the manufacturer's instructions.

Generation of monocyte-derived L-DC and purification of B cells

Human PBMC were isolated from venous blood of voluntary healthy donors using HistoPaque (Sigma) density gradient centrifugation. L-DC were generated as described previously 27 starting from monocytes purified with a monocyte isolation kit II (Miltenyi Biotech, Bologna, Italy) by negative selection. After 6 days of culture, cells were >95% CD1a+ and almost CCR6+ (from 65 to 85%) and langerin+ (from 50 to 70%) as determined by FACSCalibur (BD Bioscences, Milano, Italy). B cells were purified with an isolation Kit (Miltenyi Biotech) by negative selection and used immediately. The resulting preparations were consistently >90% CD19+CCR6+. After separation cells were resuspended in PBS (Sigma), supplemented with 0.2% BSA and 0.01% sodium azide, and incubated with fluorochrome-conjugated mAb and isotype-matched negative controls (DakoCytomation, Milan, Italy) after blocking nonspecific sites with rabbit IgG (Sigma) for 30 min at 4°C. The following PE-conjugated mAb were used: anti-CD1a, anti-CCR6 (both from R&D Systems), anti-langerin (BD Biosciences). FACS analysis was performed with an FACSCalibur and CELLQuest software (BD Biosciences). Cells were gated according to their light-scatter properties to exclude cell debris and contaminating lymphocytes. Migration measurements were made in duplicate using a transwell system (24-well plates; 5.0 μm pore sizes; Costar, Corning, NY, USA). A total of 600 μL of supernatant from LacZ and IFI16 infected HUVEC preincubated or not in the presence of anti-CCL4, anti-CCL5 and anti-CCL20 mAb for 30 min at room temperature were added to the lower chamber. A total of either 1.5×105 L-DC or B cells in 100 μL were added to the upper chamber and incubated at 37°C for 2 h. Cells that migrated into the lower chamber were harvested and counted by flow cytometry acquiring events for a fixed time of 30 s. The range of the control titration curves obtained by testing increasing concentrations of cells. The results are expressed as the mean number of migrated cells±SEM 28. Unpaired Student's t-tests were used to determine whether the differences in migration were statistically significant.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

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PBMC, B cells and DC were derived from the peripheral blood of healthy donors from the Blood Bank under an Institutional Review Board-approved protocol.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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