Developmental endothelial locus-1 inhibits MIF production through suppression of NF-κB in macrophages

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Abstract. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that regulates leukocyte recruitment, thereby playing a pivotal role in the regulation of innate and adaptive immunity and tumor progression. Elevated levels of MIF are associated with numerous inflammatory disorders and cancers. To determine whether developmental endothelial locus-1 (Del-1) regulated MIF, RAW264.7 macrophages were treated with Del-1 and assessed using ELISA. The results showed that MIF was downregulated in macrophages by Del-1, an endogenous anti-inflammatory protein that was previously shown to limit leukocyte adhesion and migration. Treatment of RAW264.7 macrophages with Del-1 inhibited constitutive and lipopolysaccharide (LPS)-induced MIF secretion. Recombinant Del-1 protein attenuated the phosphorylation of IκBα induced by a relatively low concentration of LPS in THP-1 monocytes, but did not inhibit IκBα phosphorylation in response to a relatively high concentration of LPS. Concomitantly, translocation of NF-κB to the nucleus was inhibited by Del-1 in LPS-activated macrophages. In addition, conditioned medium harvested from cells transfected with a Del-1 expression plasmid suppressed NF-κB activation in response to relatively low concentrations of TNF-α, albeit not the activation that was induced by a relatively high concentration of TNF-α. On the other hand, although Del-1 enhanced the macrophage expression of p53, a known negative regulator of MIF production, MIF production was not significantly affected by the level of p53 in mouse bone marrow-derived macrophages. These findings suggested that Del-1 controls NF-κB-activated MIF production in macrophages, and the potential application of Del-1 to therapeutic modalities for chronic inflammation-associated cancers.

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Introduction

Macrophage migration inhibitory factor (MIF) was initially named for its ability to inhibit the migration of macrophages (1,2). However, it has since been found to stimulate the recruitment of macrophages and other leukocytes (3-6). MIF plays a key role in regulating innate and adaptive immunity, autoimmunity and inflammatory responses (7). MIF is produced by immune and non-immune cells that are stimulated by microbial products, complements and T cell-derived cytokines that stimulate NF-κB signaling (7-9). Once secreted, MIF binds to chemokine receptors, such as CXCR2 and CXCR4, activating integrin signaling and driving the chemotaxis of monocytes and T cells (4). It also binds to the receptor CD74. This interaction initiates a signaling cascade that activates immune cells (10). MIF then promotes the secretion of a broad array of proinflammatory mediators including TNF-α, IL-1β, IL-2, IL-6, IL-8, nitric oxide, COX-2, PGE2, chemokines and adhesion molecules via NF-κB activation, leading to the amplification of inflammatory and immune responses (3,7,11).

NF-κB is a key mediator in immune and inflammatory responses. In resting cells, the NF-κB is bound by IκB, which inhibits NF-κB signaling. A variety of stimuli, such as lipopolysaccharide (LPS), stress and cytokines, activate IKK, the IκB kinase, leading to its phosphorylation. This targets the phosphorylated IκB for proteasomal degradation, and NF-κB is released followed by translocation to the nucleus, and initiation of the transcription of its target genes (12).

High levels of MIF are indicative of autoimmune diseases and severe inflammatory states, such as asthma and sepsis (13,14). Mice lacking MIF exhibit less pulmonary inflammation and chronic colitis (13,15). Genetic alteration of human MIF expression correlates with the severity of asthma, cystic fibrosis, rheumatoid arthritis, inflammatory bowel disease, and ischemic injury (13,16-19). Furthermore, MIF inhibits p53 activity, resulting in the suppression of p53-mediated apoptosis, rendering proinflammatory responses and tumorigenesis (7,20,21). As such, overexpression of MIF has been

observed in many types of cancer (22). Accordingly, unsuccessful attempts have been made to develop reagents that inhibit MIF such as neutralizing antibodies and small molecule inhibitors (23,24). There is, therefore, a great need for the development of molecules that effectively inhibit MIF, which may be used to treat inflammatory and immune diseases as well as cancer.

Developmental endothelial locus-1 (Del-1) is expressed in endothelial cells and a subset of macrophages, and regulates angiogenesis, apoptosis and cell adhesion and migration (25-28). Del-1 binds to the leukocyte integrin LFA-1, inhibiting the adhesion of leukocytes to vascular endothelial cells, thereby suppressing leukocyte migration (29). Therefore, Del-1 acts as an endogenous anti-inflammatory molecule. Although binding of Del-1 to LFA-1 is well established, subsequent downstream signaling events remain unclear.

Considering that MIF and Del-1 are expressed in macrophages and are key players in the regulation of inflammation, we aimed to investigate the involvement of Del-1 in the regulation of MIF-1.

Materials and methods

Cell culture. RAW264.7 and HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and streptomycin/penicillin. THP-1 cells were cultivated in RPMI-1640 supplemented with 10% FBS and antibiotics. Isolation of mouse bone marrow-derived monocytes/macrophages (BMDM) was performed as previously described (30,31). In brief, bone marrow cells were flushed with medium from the bones of wild-type (WT) and Del-1-deficient (Del-1$^{-/-}$) mice (provided by Professor T. Chavakis at Dresden University, Dresden, Germany), and WT, heterozygous (p53$^{+/-}$) and homozygous (p53$^{-/-}$) mice (obtained by crossing p53 heterozygous mice, purchased from the Jackson Laboratory, Bar Harbor, ME, USA), and then filtered on a cell strainer. The red blood cells were lysed and the remaining bone marrow cells were cultured in complete RPMI-1640 medium containing murine GM-CSF (10 ng/ml) (Peprotech, Rocky Hill, NJ, USA). After 4 days, the floating cells were removed and adherent cells were used. The cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless otherwise specified. All the animal studies were approved by the Institutional Animal Care and Use Committees of Asan Institute and Ewha Women's University (Seoul, Korea).

Production of Del-1 conditioned media using the Flp-In T-Rex system. An ~1.5 kb full-length mouse Del-1 cDNA containing the signal sequence and an HA epitope tag was cloned into the pcDNA5/FRT/TO plasmid (Invitrogen Life Technologies). The mDel-1 expression plasmid and pOG44 (Invitrogen Life Technologies) were used to transfect Flp-In T-Rex293 cells, and then the cells were cultured with 1 μg/ml doxycycline for 24 h. The conditioned media were analyzed by immunoblotting using an anti-HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Enzyme-linked immuno-sorbent assay (ELISA). RAW264.7 cells were seeded at 5x10^5 per well in a PLL-coated 24-well plate, incubated at 37˚C, 5% CO_2 for 12 h, and then further incubated for 24 h in the presence of recombinant Del-1 (R&D Systems, Minneapolis, MN, USA), LPS (*E. coli* 0111:B4; Sigma-Aldrich, St. Louis, MO, USA) or both, at the concentrations of 0, 0.005, 0.05, 0.5 and 5 μg/ml. The supernatants were collected, added to a MaxiSorp 96-well plate (Nunc A/S, Roskilde, Denmark) and incubated at 4˚C for 12 h. Serial dilutions of recombinant MIF protein were used as the standard. After being washed with 0.1% PBS-Tween-20 (PBST) three times, the plate was blocked with PBST containing 0.3% skim milk for 2 h at room temperature. The plate was then washed, incubated with a rabbit anti-MIF antibody (ab7207, Abcam, Cambridge, MA, USA) at room temperature for 4 h, washed again, and incubated with an HRP-conjugated anti-rabbit antibody (Cell Signaling Technology, Boston, MA, USA) at room temperature for 1 h. After four washes, the plate was incubated with the TMB Plus 2 (Kem-En-Tec Diagnostics, Taastrup, Denmark) substrate and the reaction was stopped with 1 N HCl. Absorbance was read at 450 nm on a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Immunoblot analysis. Cells were washed with ice-cold PBS and lysed in PRO-PREP protein extraction solution (Intron, Seongnam, Korea). Lysates were separated on a 12% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked and probed with antibodies against phospho-IκB-α (pS32/pS36; BD Pharmingen, San Diego, CA, USA), IκB-α (C-21), p53 (DO-1) (both from Santa Cruz Biotechnology), α/β-tubulin, or actin (both from Cell Signaling Technology). After washing, the blots were incubated with the appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology) and developed with the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific, Waltham, MA, USA).

Immunocytochemistry. THP-1 cells were plated at 2x10^5 per well in a PLL-coated 24-well plate. The cells were pretreated with recombinant Del-1 for 1 h, and then stimulated with LPS for 10 min. The plate was placed on ice for 10 min to stop reactions. The cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde for 20 min, washed, and stained with antibody against p65 (Cell Signaling Technology) for 12 h at 4˚C. The cells were washed again, incubated with Alexa 488-conjugated secondary antibody (Invitrogen Life Technologies) for 1 h and DAPI for 10 min at room temperature, mounted with Fluoromount G, and observed under a confocal microscope (LSM 710; Zeiss, Oberkochen, Germany) at a magnification of x200.

Luciferase assay. One day prior to transfection, 293 T cells were plated at a density of 3x10^5 cells per well in a 12-well plate. The cells were transfected with an NF-κB luciferase construct using Effectene transfection reagent (Qiagen, Hilden, Germany) in Opti-MEM (Invitrogen Life Technologies), according to the manufacturer's instructions. Twelve hours later, the cells were treated with TNF-α at the indicated concentrations in the control or Del-1-conditioned media. Luciferase activity was measured 24 h post-treatment using a luciferase reporter assay system (Promega, Madison, WI, USA) and a Luminometer (Victor X3, Perkin-Elmer,

Waltham, MA, USA), according to the manufacturer's instructions.

Real-time RT-PCR. Total RNA was isolated using TRIzol (Invitrogen Life Technologies) and cDNA was synthesized using the ImProm-II reverse transcriptase kit (Promega). The cDNA was amplified using LightCycler 480 SYBR-Green I Master and a LightCycler 480 machine (Roche, Mannheim, Germany). Primer sequences used were: Del-1, forward: 5'-CTTGGTAGCAGCCTGGCTTT-3' and reverse: 5'-GCC TTCTGGACACTCACAGG-3'; MIF, forward: 5'-CGCACA GTACATCGCAGTG-3' and reverse: 5'-GGCAGCGTTCATG TCGTAAT-3'; 18S forward: 5'-CGCGGTTCTATTTTGT TGGT-3' and 18S reverse: 5'-AGTCGGCATCGTTTA TGGTC-3'. The following PCR cycle was used: 95˚C for 15 min; 50 cycles of 30 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C; and 95˚C for 15 min. Melting curve analyses were performed on all PCR products to ensure that specific PCR products were generated. Data were analyzed using the comparative C_t method (32), and the levels of mRNA were expressed as the relative fold change.

Statistical analysis. Data are presented as the means ± standard deviation (SD). Data were compared using the Student's t-test. $P<0.05$ was considered to indicate statistical significance.

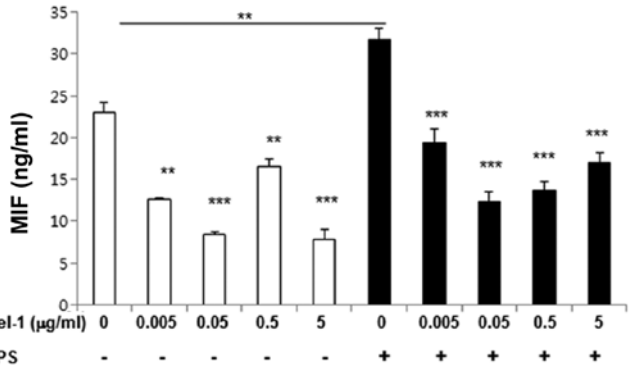

Figure 1. Developmental endothelial locus-1 (Del-1) decreases macrophage migration inhibitory factor (MIF) secretion by macrophages in a dose-dependent manner. RAW264.7 cells were incubated for 24 h with the increasing concentrations of recombinant Del-1 (0, 0.005, 0.05, 0.5 and 5 μg/ml) in the absence (open bars) or presence (closed bars) of lipopolysaccharide (LPS) (0.1 μg/ml). The supernatants were collected and MIF production was analyzed by enzyme-linked immuno-sorbent assay (ELISA). Values are the means ± standard deviation (SD) from three independent experiments, $^{**}P<0.01$, $^{***}P<0.001$.

Results

Extracellular Del-1 protein inhibits constitutive and LPS-induced MIF production in macrophages. To determine whether Del-1 regulated the production of MIF in macrophages, RAW264.7 macrophages were treated with Del-1 in the absence or presence of LPS, and secreted MIF was assessed by ELISA. Levels of secreted MIF were reduced in the media harvested from the cells treated with Del-1 compared to control cells, in the LPS-treated and -untreated groups (Fig. 1). These results indicated that Del-1 may be involved in the downregulation of MIF.

Del-1 suppresses NF-κB activation in macrophages in a stimulation intensity-dependent manner. As MIF expression is promoted by the activation of NF-κB (9,33), the regulation of LPS-activated NF-κB activity by Del-1 was investigated. Levels of phospho-IκBα and IκBα, both of which are key players in the modulation of NF-κB activity, were examined by immunoblot analysis in RAW264.7 mouse macrophages or THP-1 human monocytes. The cells were pretreated with human recombinant Del-1 prior to LPS stimulation. Subsequently, the cells were collected and lysates were immunoblotted using anti-phospho-IκBα and anti-IκBα antibodies. Notably, Del-1 decreased phospho-IκBα protein levels in monocytes stimulated with a low concentration of LPS (0.1 μg/ml), but not in monocytes stimulated with a high concentration of LPS (1 μg/ml) (Fig. 2A). The total IκBα levels were comparable between samples treated with LPS alone and LPS plus Del-1 (data not shown). Comparable results were obtained when conditioned media from mouse Del-1-transfected cells (known as Del-1-conditioned media hereafter) were used to treat macrophages, instead of recombinant Del-1 (data not shown). In addition, recombinant Del-1 attenuated the translocation of NF-κB to the nucleus induced by LPS (0.1 μg/ml) (Fig. 2B). To confirm these findings, human embryonic kidney HEK293T cells were transfected with an NF-κB reporter plasmid and stimulated with TNF-α at varying concentrations in Del-1-conditioned media, and luciferase activity was measured. As expected, the cells treated with the Del-1-conditioned media showed lower luciferase activity than the control cells, but only when a low concentration of TNF-α was present (Fig. 2C and D). Taken together, these data suggest that Del-1 regulates NF-κB activity by suppressing the phosphorylation of IκBα in the presence of low concentrations of inflammatory stimuli.

Del-1-induced p53 does not affect the macrophage production of MIF. Previous studies have shown that NF-κB and p53 pathways often interact in the regulation of gene transcription (33,34). The potential contribution of p53 to the Del-1-mediated suppression of MIF production in macrophages was therefore examined. Del-1 enhanced the level of p53 protein in RAW264.7 cells (Fig. 3A). MIF protein levels were also assessed by ELISA in BMDM isolated from $p53^{+/+}$ or $p53^{-/-}$ or $p53^{+/-}$ null for p53. The levels of MIF protein in these BMDMs were not significantly different from one another (Fig. 3B), indicating that p53 does not regulate MIF production in macrophages.

Intracellular Del-1 fails to inhibit MIF production within macrophages. Del-1 has been reported to be expressed in a subset of macrophage cell lines and some primary macrophages (25). To determine whether intracellular Del-1 regulates the expression of MIF within macrophages, we first assessed the levels of Del-1 by RT-PCR in BMDMs isolated from WT ($Del\text{-}1^{+/+}$) and $Del\text{-}1^{-/-}$ mice. Del-1 was present in the WT BMDM, but not in the $Del\text{-}1^{-/-}$ BMDM (Fig. 4A), and its expression decreased following LPS stimulation in the WT BMDM (Fig. 4B), suggesting that Del-1 is expressed in macrophages and that it could respond to inflammatory stimuli. MIF expression in the BMDM was then

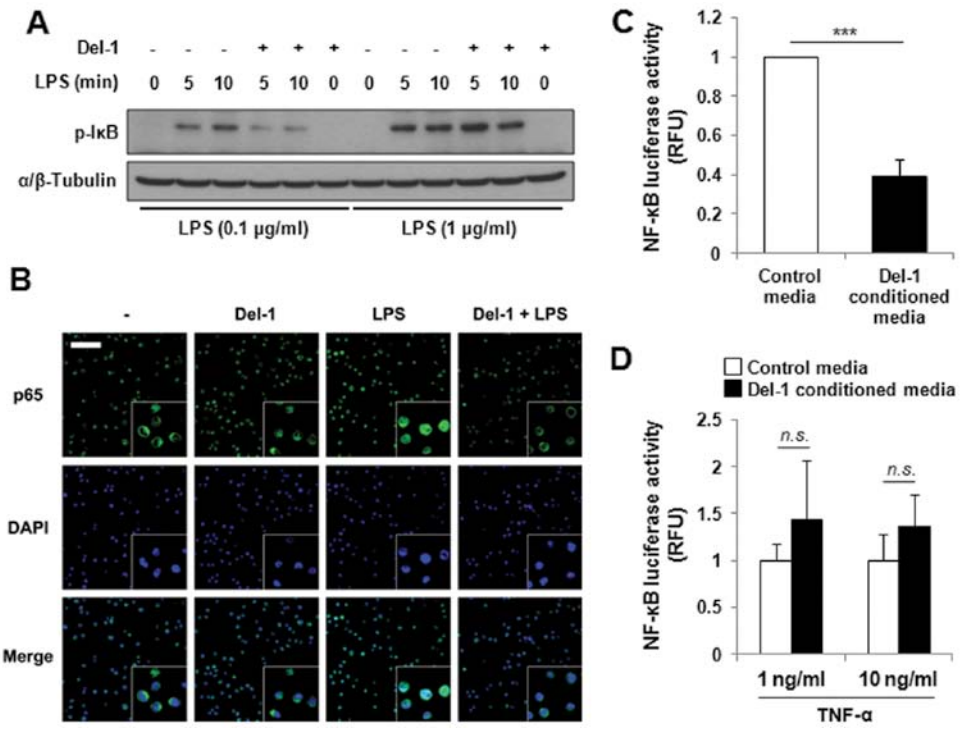

Figure 2. Developmental endothelial locus-1 (Del-1) inhibits NF-κB activated in response to low-grade stimuli. (A) THP-1 cells were pretreated with 0.5 μg/ml Del-1 for 1 h, and then stimulated with 0.1 μg/ml (low-grade stimulus) or 1 μg/ml lipopolysaccharide (LPS) (high-grade stimulus) for the indicated time periods. The cells were lysed and protein levels of p-IκB-α were assessed by immunoblot analysis. α/β-tubulin was used as a loading control. Data show three independent experiments. (B) THP-1 cells were pretreated with 0.5 μg/ml Del-1 for 1 h, and then stimulated with 0.1 μg/ml LPS for 10 min. The translocation of p65 was assessed by immunocytochemistry. p65 is shown in green and the nucleus was stained blue with DAPI. Scale bar, 100 μm. (C) 293 T cells were transfected with a NF-κB luciferase construct and then incubated in either control media or Del-1 conditioned media, together with TNF-α at 0.05 ng/ml. Values are the means ± standard deviation (SD) from four independent treatments. ***P<0.001. (D) 293 T cells were transfected with a NF-κB luciferase construct and then incubated in control or Del-1 conditioned media, together with TNF-α at the indicated concentrations. Twenty four hours later, the cells were lysed and the luciferase activity was measured. Values are the means ± SD from three independent treatments. n.s., not significant.

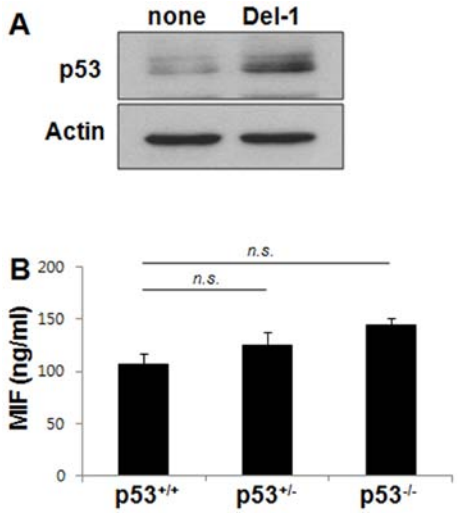

Figure 3. Macrophage migration inhibitory factor (MIF) production by monocytes/macrophages is not affected by p53. (A) Developmental endothelial locus-1 (Del-1) elevated p53 protein in macrophages. RAW264.7 cells were incubated in the absence or presence of Del-1 (0.5 μg/ml) for 12 h. The protein level of p53 was assessed by immunoblot analysis. (B) MIF production was not altered by the level of p53. Bone marrow-derived monocytes/macrophages (BMDM)s were isolated from wild-type (WT) ($p53^{+/+}$), homozygous ($p53^{-/-}$) and heterozygous ($p53^{+/-}$). MIF secretion from BMDM after 24 h was measured by enzyme-linked immuno-sorbent assay (ELISA). Values are the means ± standard deviation (SD) (n=5, 5 and 2; $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mice, respectively). Data from one of three independent experiments are shown. n.s., not significant.

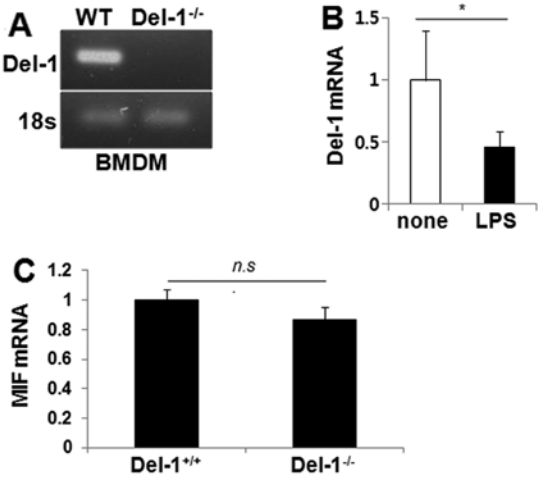

Figure 4. Macrophage-derived developmental endothelial locus-1 (Del-1) does not affect macrophage migration inhibitory factor (MIF) production by monocytes/macrophages. (A) Del-1 is expressed in monocytes/macrophages. Del-1 mRNA in bone marrow-derived monocytes/macrophages (BMDM) from wild-type (WT) and Del-1-deficient ($Del\text{-}1^{-/-}$) mice was analyzed by semi-quantitative reverse transcription (RT)-PCR. (B) Del-1 is downregulated by lipopolysaccharide (LPS). WT BMDM was treated with 100 ng/ml LPS for 6 h, and the Del-1 mRNA level was assessed by real-time RT-PCR. (C) Macrophage-derived Del-1 does not alter MIF levels in monocytes/macrophages. MIF mRNA isolated from BMDM of WT and $Del\text{-}1^{-/-}$ mice was analyzed by quantitative RT-PCR. Values are the means ± standard deviation (SD) (n=5 mice per group). Data from one of three independent experiments are shown. *P<0.05. n.s., not significant.

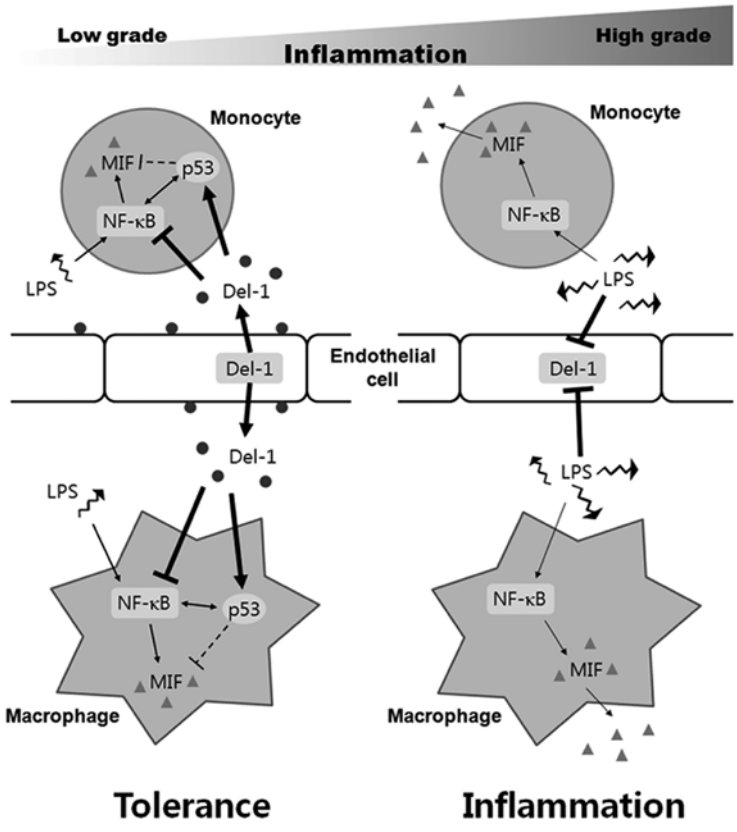

Figure 5. Schematic diagram on the proposed mechanism by which developmental endothelial locus-1 (Del-1) acts as an inflammation gatekeeper.

assessed by quantitative RT-PCR. No difference in the level of MIF was found between WT and Del-1$^{-/-}$ BMDM (Fig. 4C). These findings suggest that macrophage-derived Del-1 does not regulate MIF within macrophages.

Discussion

We previously demonstrated that Del-1 has an anti-inflammatory function through its inhibition of leukocyte adhesion (29). The present study provides an additional mechanism by which Del-1 inhibits inflammation by downregulating the potent proinflammatory cytokine, MIF.

Treatment of monocytes/macrophages with recombinant Del-1 protein attenuated IκB phosphorylation, indicating that the decrease in MIF secretion is due to the suppression of NF-κB signaling. However, this suppression occurred only when monocytes/macrophages were stimulated with a low dose of LPS, but not with a high dose of LPS. Similarly, Del-1-conditioned media suppressed NF-κB activity only when a low dose of TNF-α was present, suggesting that the activation of cells that act as a first line of defense should depend on the intensity of the stimulus. To limit unnecessary inflammatory responses caused by the initiation of inflammation by negligible stimuli, Del-1 may be a key player in this tolerance, by suppressing the initiation of inflammation by trivial stimuli.

On the other hand, previous studies have suggested that the activity of p53 and MIF is reciprocally regulated (20,21,33). Inflammation, cell stress, and hypoxic conditions activate hypoxia-inducible factor-1α (HIF-1α). p53 represses HIF-1α activity and inhibits HIF-1α-dependent transcription of multiple cytokines, including MIF (35-37). Del-1 elevated the level of p53, which may counteract NF-κB activity (34).However, in our studies, WT BMDM did not express decreased levels of MIF compared to BMDM with reduced p53, suggesting that p53 activated in response to the stimulation of macrophages with extracellular Del-1 may not play a role in regulating MIF production in macrophages. Alternatively, the basal level of p53 in macrophages may not be sufficiently high to modulate MIF production.

Under inflammatory conditions, we found that Del-1 is decreased in macrophages, which is in agreement with our previous result showing that Del-1 is decreased in vascular endothelial cells (29). However, the molecular mechanism underlying the inflammation-mediated decrease in Del-1 remains to be determined. NF-κB activated by inflammation may suppress p53 activity, which in turn decreases Del-1 transcription (31). Because Del-1 activates p53 and p53 decreases MIF, we initially expected that Del-1 deficiency would increase MIF production. However, this did not occur, suggesting that the basal level of Del-1 in macrophages is insufficient to decrease MIF. Although we found that Del-1 attenuates NF-κB activation, it is still premature to conclude that Del-1 is a global inhibitor of proinflamatory cytokine production. Further studies may clarify this issue.

In summary, as shown in Fig. 5, once inflammation is initiated, NF-κB suppresses p53 activity in monocytes, macrophages, and vascular endothelial cells, all of which are first-line defense cells, thereby repressing the anti-inflammatory molecule Del-1 and increasing proinflammatory molecules including MIF. As a result, beneficial inflammation

ensues. High-grade inflammatory stimuli, but not low-grade inflammatory stimuli, may inhibit Del-1 sufficiently for inflammation to be initiated. For this reason, Del-1 may control MIF only under low-grade inflammatory conditions or in resting cells. Taken together, these data indicate that Del-1 inhibits NF-κB-dependent proinflammatory cytokine production by monocytes/macrophages, thereby acting as an inflammation gatekeeper.

Acknowledgements

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