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RelB/p50 complexes regulate cytokine-induced YKL-40 expression¹

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Abstract

The secreted protein, YKL-40, has been proposed as a biomarker of a variety of human diseases characterized by ongoing inflammation, including chronic neurological pathologies such as multiple sclerosis (MS)² and Alzheimer's disease. However, inflammatory mediators and the molecular mechanism responsible for enhanced expression of YKL-40 remained elusive. Using several mouse models of inflammation, we now show that YKL-40 expression correlated with increased expression of both IL-1 and IL-6. Furthermore, IL-1 together with IL-6 or the IL-6

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²Abbreviations used in this article

ChIP chromatin immunoprecipitation

DOX doxycycline

EAE experimental autoimmune encephalomyelitis

GBM glioblastoma multiforme

IKK I κ B kinase

MS multiple sclerosis

NIK NF- κ B-inducing kinase

OSM oncostatin M

sIL-6R soluble IL-6 receptor

TAT transactivator of transcription

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family cytokine, oncostatin M (OSM), synergistically upregulated YKL-40 expression in both primary human and mouse astrocytes *in vitro*. The robust cytokine-driven expression of YKL-40 in astrocytes required both STAT3 and NF- κ B binding elements of the YKL-40 promoter. Additionally, YKL-40 expression was enhanced by constitutively active STAT3 and inhibited by dominant-negative I κ B α . Surprisingly, cytokine-driven expression of YKL-40 in astrocytes was independent of the p65 subunit of NF- κ B and instead required subunits RelB and p50. Mechanistically, we show that IL-1-induced RelB/p50 complex formation was further promoted by OSM and that these complexes directly bound to the YKL-40 promoter. Moreover, we found that expression of RelB was strongly upregulated during inflammation *in vivo* and by IL-1 in astrocytes *in vitro*. We propose that IL-1 and the IL-6 family of cytokines regulate YKL-40 expression during sterile inflammation via both STAT3 and RelB/p50 complexes. These results suggest that IL-1 may regulate the expression of specific anti-inflammatory genes in non-lymphoid tissues via the canonical activation of the RelB/p50 complexes.

Introduction

In the past several years YKL-40 (also known as chitinase 3-like protein 1, human cartilage glycoprotein 39, and breast regression protein 39) has attracted growing attention as a marker of ongoing inflammation and oncogenic transformation (1). This secreted glycoprotein, expressed in both invertebrates and vertebrates, belongs to the 18-glycosylhydrolase family of proteins but lacks glycolytic activity (2, 3). Although its biological functions are not fully understood, it is expressed by many cell types, including macrophages, neutrophils, synoviocytes, chondrocytes, smooth muscle cells, endothelial cells, microglia, and astrocytes, suggesting that its biological effects are not restricted to a particular cell type. Indeed, YKL-40 stimulates proliferation of synoviocytes and chondrocytes (4), promotes adhesion and migration of vascular smooth muscle and endothelial cells (5-7), and is a migration factor for astrocytes (8). Surprisingly, unchallenged YKL-40 knockout mice appear normal; however, when challenged with antigen they display impaired Th2-dependent immune responses, show diminished fibrosis and tissue inflammation, decreased accumulation of M2 macrophages, and increased apoptosis of CD4⁺ T cells and macrophages (9). In contrast, these mice display an exacerbated response to experimental autoimmune encephalomyelitis (EAE) (10) and enhanced inflammatory responses to hyperoxia (11), suggesting that YKL-40 differentially affects inflammatory responses depending on the type of immune activation and tissue involved. In agreement with the role of YKL-40 during inflammatory responses in mice, YKL-40 levels are elevated in patients with a wide array of human diseases characterized by ongoing inflammation, including rheumatoid arthritis, atherosclerosis, type 2 diabetes, pelvic inflammatory disease, chronic pancreatitis and secondary diabetes, severe pediatric asthma, cirrhosis, Crohn's disease, and others (12-19). Elevated levels of YKL-40 are also present in the cerebrospinal fluids of patients with a variety of acute and chronic neurological pathologies, such as MS, Alzheimer disease, viral encephalitis, HIV-associated dementia, brain infarction, and traumatic brain injury (20-23), with activated microglia and reactive astrocytes both producing YKL-40 in the central nervous system. Thus, over the last decade it has become evident that elevated YKL-40 expression correlates with both infection-induced inflammation and sterile inflammation, a paradigm triggered by physical,

chemical or metabolic noxia. Accordingly, YKL-40 is also expressed by several solid tumors, such as osteosarcoma, ovarian carcinoma and glioblastoma multiforme (GBM) (24, 25), and promotes angiogenesis and radioresistance of GBM tumors and angiogenesis of breast and colon cancer cells (26, 27). Since elevated expression of YKL-40 correlates with ongoing inflammation that is a component of many diseases, YKL-40 has been proposed as a biomarker for many pathologies, including cardiovascular disease, asthma, arthritis, MS, Alzheimer disease, and many cancers, including GBM, melanoma, hepatocellular carcinoma, breast and pancreatic cancers (20, 24).

Despite the numerous reports documenting elevated expression of YKL-40, relatively little is known about the inflammatory mediators and specific molecular mechanisms that control its expression. Proinflammatory cytokines, including TNF and IL-1, induce expression of YKL-40 in chondrocytes (28) and astrocytes (8); however, to a much lower extent than the conditioned media of macrophages (29). Both IL-1 and TNF are known to trigger a classical I κ B kinase (IKK) γ -dependent activation of NF- κ B, which involves IKK β -mediated phosphorylation and subsequent degradation of I κ Bs, followed by the release and subsequent nuclear translocation of the p65/p50 NF- κ B complexes (30). In contrast, these cytokines do not efficiently activate a noncanonical IKK γ -independent NF- κ B pathway, which involves NF- κ B-inducing kinase (NIK), IKK α -dependent processing of NF- κ B2 p100, and generation of RelB/p52 complexes (31-33). Concordantly, the p65/p50 complex has been proposed to mediate TNF- and IL-1-induced YKL-40 expression in chondrocytes (28). In contrast however, p65/p50 has also been shown to recruit histone deacetylases-1 and -2 to the YKL-40 promoter and repress its expression in response to TNF in GBM cells (34). In addition, IL-6 and OSM moderately upregulate YKL-40 expression in human astrocytes, which requires STAT3 and formation of a complex between STAT3 and nuclear factor I-X3 on the YKL-40 promoter (8). However, profound activation of YKL-40 expression observed during ongoing inflammatory processes *in vivo* has not been adequately recapitulated in the *in vitro* experiments. Therefore in this study, we set up experiments to identify molecular mechanisms that govern YKL-40 expression during inflammation.

Materials and Methods

Mice

The doxycycline (DOX)-inducible, brain-specific HIV-Tat₁₋₈₆ transgenic mice have been described previously (35). Tat expression was induced with chow containing 6 mg/g DOX (Harlan, Indianapolis, IN) for 1 week. C57BL/6 mice were obtained from Jackson Laboratory. Mice were housed at Virginia Commonwealth University according to guidelines of the Institutional Animal Care and Use Committee of Virginia Commonwealth University. The mouse protocols were approved by the Institutional Animal Care and Use Committee. Male and female mice 6–16 weeks of age were used.

Turpentine- and LPS-induced inflammation

Turpentine abscesses were initiated by subcutaneous injection of pure gum spirits of turpentine (50 μ l) into anesthetized age-matched male and female mice (6–8 weeks of age). For LPS-induced inflammation, mice were injected subcutaneously with 50 μ g LPS (in 50 μ l

PBS). Mice were killed after 8h and the skin and underlying muscles at the site of injection was collected for mRNA analysis.

Experimental autoimmune encephalomyelitis (EAE)

Each mouse received intradermally 300 µg of MOG₃₅₋₅₅ peptide (AnaSpec, Fremont, CA) emulsified in CFA containing 500 µg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) and intraperitoneally 200 ng of Pertussis toxin (Enzo Life Sciences, Farmingdale, NY). An additional dose of 200 ng of Pertussis toxin was administered two days post immunization. A booster dose MOG₃₅₋₅₅/CFA/Mycobacterium tuberculosis H37Ra was given seven days post immunization. Mice were examined daily (the first day following booster injection was assigned as day 1), weighed, and the severity of the disease was quantitated using a five point scale: 0 – no symptoms, 1 – limp tail, 2 – limp tail with loss of righting, 3 – paralysis of single hind limb, 4 – paralysis of both hind limbs, and 5 – death. Tissues were collected at day 28 for RNA analysis.

Irradiation

Irradiations were carried out using a MDS Nordion Gamma-cell 40 research irradiator with a ¹³⁷Cs source delivering a dose of 1.05 Gy/min to the head only. Mice received a dose of 20 Gy.

Clinical samples

Biopsy samples of oligodendroglioma tumors were obtained from VCU's brain tumor bank (Department of Neurosurgery, Richmond, VA). Normal cortical tissue samples were obtained from healthy brain regions of patients with oligodendroglioma or epileptic patients. The biopsy samples represent primary grade II and III, and recurrent tumors.

Cell culture

Human glioblastoma U373-MG cells were obtained from American Type Culture Collection (Manassas, VA). Mouse astrocytes were prepared as described (36). Human cortical astrocytes were prepared from fetal tissue provided by Advanced Bioscience Resources and were cultured as previously described (37, 38). Primary human chondrocytes were purchased from PromoCell, Heidelberg, Germany. Cells were stimulated with 25 ng/ml IL-1, 25 ng/ml OSM, 25 ng/ml IL-6, 10 ng/ml TNF, or 25 ng/ml sIL-6R (all from R&D Systems, Minneapolis, MN).

Knock-down of target genes

Expression of RelB, cRel, p50, STAT3, p65 and p52 mRNAs was down-regulated using SmartPool siRNAs transfected into astrocytes using Dharmafect 1 (all from Dharmacon, Lafayette, CO).

Quantitative PCR

Total RNA was prepared utilizing TRIzol (Life Technologies, Carlsbad, CA). 1 µg of RNA was reverse-transcribed using the high capacity cDNA Archive kit (Life Technologies). Premixed primer-probe sets and TaqMan Universal PCR Master Mix (Life Technologies)

were employed to examine mRNA levels. cDNAs were diluted 10-fold (for the target genes) or 100-fold (for GAPDH) and amplified using the ABI7900HT cyclor. Gene expression levels were normalized to GAPDH and presented as a fold induction with the mean values \pm SEM. A Power SYBRGreen PCR kit (Life Technologies) was used for chromatin immunoprecipitation qPCR assays as previously described (8).

Western Blotting

The cells were lysed in 10 mM Tris, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM PMSF, and protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). Samples were separated using SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The anti-YKL-40, anti-RelB, anti-p65, anti-p50, anti- β -tubulin, and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-Stat3 antibody was obtained from Cell Signaling Technology (Danvers, MA). Antigen-antibody complexes were visualized by enhanced chemiluminescence using Immobilon Western blotting kit (Millipore, Temecula, CA).

Coimmunoprecipitation

200–300 μ g of protein lysates, prepared as described above, were pre-cleared with 10 μ l of the protein G-Sepharose beads (GE Healthcare, Pittsburgh, PA) for 1 h. The lysates were then incubated with 2 μ g of anti-RelB or anti-p50 antibodies overnight at 4°C, 25 μ l of protein G-Sepharose beads were added, and incubated for 1 h at 4°C. The beads were washed extensively with the lysis buffer, and immunoprecipitated proteins were eluted in sample buffer at 95 °C for 5 min.

Synthetic Oligonucleotides

The following oligonucleotides were synthesized to introduce mutations in the YKL-40 promoter: –669 NF-KB, 5'-CTGAATTCGATAGCTGTCTTTCCCTCTAA-3' and 5'-ACAGCTATCGAA TTCAGAATGCTTTAAGC-3'; –717 NF-KB, 5'-ATCTCGAGAATAAACAGAAGCAAAT AG-3' and 5'-TTATTCTCGAGATAAAGAGAGAGGATCTT-3'. Following oligonucleotides were used in EMSA: –669 NF-KB probe, 5'-GATCTTCTTGGAATTTCCC TGTC A-3' and 5'-GATCTGACAGGGAAATTTCCCAAGAAA-3'; –717 NF-KB probe, 5'-GATCTCTTTATGG GAATTTCAAACAA-3' and 5'-GATCTTGTTTTGAAATTTCCCATAAAGA-3'.

Nuclear Extracts and EMSA

Nuclear extracts were prepared as previously described (39). Briefly, double-stranded DNA fragments were labeled by filling in the 5'-protruding ends with Klenow enzyme using [α -³²P]dCTP (3000 Ci/mmol). 5 μ g of nuclear extracts and ~10 fmol (10,000 cpm) of probe were utilized. Anti-RelB, anti-p50, anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used for supershift studies.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described previously (8) with the following exceptions. The cells were cross-linked with 1% formaldehyde for 10 min at 37 °C and then washed with ice-cold PBS containing 125 mM glycine. RelB and p50 antibodies or non-immune rabbit serum were used to precipitate cross-linked proteins. The following primers to the YKL-40 promoter were used in the qPCR: 5'-GTGCAGCCGCCCGTAG-3' and 5'-GCCTGAAACTGAGCGCTCC-3'.

Plasmids and transfections

The pYKL(-1300)Luc reporter was provided by Dr. Michael Rehli (University of Regensburg, Regensburg, Germany) (40). The site-directed mutagenesis was performed using QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the oligonucleotides described above and published previously (8). Plasmids expressing dominant negative I κ B α and constitutively active STAT3 were described before (8). Human astrocytes were transiently transfected in 12-well clusters using the FuGENE6 transfection reagent (Roche Applied Science). Twenty four hours post-transfection, the cells were stimulated with IL-1 or OSM for 20h and cell extracts were prepared. Luciferase assays were performed using a dual luciferase reporter assay kit (Promega Corporation, Madison, WI). Luciferase activities were normalized to Renilla luciferase activities.

Statistical analysis

All experiments were repeated at least three times with consistent results. Data are presented as mean \pm SEM. For mouse studies, four to six randomly chosen mice were used per experimental group. SPSS Statistics 21 software was used for statistical analyses. A Bonferroni post-hoc test was used for one-way ANOVA comparisons, with a P value of <0.05 being considered statistically significant. Independent sample Student's t-test was used for unpaired observations.

Results

Inflammation induces YKL-40, IL-1 and IL-6 expression

To model the induction of YKL-40 expression during inflammation, we first employed a turpentine mouse model of irritant-induced acute inflammation, which is IL-1R-dependent (41). Turpentine causes tissue destruction at the site of injection, local cytokine production, and infiltration of proinflammatory cells (38). The local reaction is subsequently followed by a systemic response, including the production of acute phase proteins in the liver (42). Indeed, YKL-40 mRNA expression was robustly activated in tissues surrounding the site of turpentine injection and its induction was accompanied by a very strong induction of both IL-1 and IL-6 mRNAs (Fig. 1A). To corroborate these findings in a central nervous system model of sterile inflammation, expression of YKL-40, IL-1, and IL-6 was subsequently analyzed using a mouse EAE model of MS. Immunization of mice with MOG₃₅₋₅₀ peptide resulted in the induction of EAE, and modest upregulation of YKL-40 mRNA expression in the spinal cords of animals (Fig. 1B). The magnitude of YKL-40 induction was comparable to the results previously reported by others (10). In addition, expression of both IL-1 and

IL-6 mRNAs was also enhanced. We also found an increase in the expression of glial fibrillary acidic protein mRNA, which indicated activation of astrocytes during EAE induction as reported previously (43). Subsequently, we analyzed expression of YKL-40, IL-1 and IL-6 in the brains of mice exposed to gamma irradiation, which is known to induce inflammation (44). In agreement with the turpentine and EAE data, levels of YKL-40, IL-1, and IL-6 mRNAs were significantly higher in the irradiated brains (Fig. 1C). We also used the HIV transactivator of transcription (TAT) transgenic mouse model in which induction of TAT protein expression in the brain induces local sterile inflammation and enhances expression of IL-1 and IL-6 (45). Using the TAT model, we found that YKL-40 mRNA expression was also enhanced upon activation of TAT expression (Fig. 1D). Lastly, we analyzed samples of human oligodendroglioma tumors and found that the expression of YKL-40, IL-1, and IL-6 mRNAs was strongly induced in comparison to normal brain (Fig. 1E). Similarly to sterile inflammation, YKL-40 mRNA expression also coincides with IL-1 and IL-6 mRNA expression during inflammation induced by bacterial LPS (Supplementary Fig. 1). We conclude that YKL-40 expression is enhanced during inflammation, which coincides with the increased expression of IL-1 and IL-6.

IL-1 and OSM synergistically induce YKL-40 expression in astrocytes

In the nervous system, both astrocytes and microglia express YKL-40 (8, 21) and these cell types also respond to a broad range of inflammatory stimuli (46). We used astrocytes as model cells to study activation of YKL-40 expression during sterile inflammation. Primary human astrocytes were stimulated with IL-1 and IL-6 together with soluble IL-6 receptor (sIL-6R) (due to the fact that human fetal astrocytes express limited amounts of IL-6R (37)). We also included OSM, a member of IL-6 cytokine family that signals via OSM receptors abundantly expressed by astrocytes. Although IL-1, IL-6/sIL-6R, or OSM alone only moderately activated YKL-40 mRNA expression, stimulation of astrocytes with IL-1 together with OSM resulted in synergistic activation (Fig. 2A). Since IL-1-treated astrocytes express IL-6, their costimulation with IL-1 and sIL-6R also caused synergistic induction of YKL-40 mRNA expression; however, to less extent than OSM alone. Similar effects of IL-1 and OSM on YKL-40 mRNA expression were also observed at the protein level (Fig. 2A, insert) and in mouse primary astrocytes (Fig. 2B). These data together with our *in vivo* studies suggest that YKL-40 expression is coordinately regulated by IL-1 and cytokines of the IL-6 cytokine family during sterile inflammation.

STAT3 but not p65 (NF- κ B) regulate cytokine-induced YKL-40 expression

NF- κ B and STAT3 are the major transcription factors activated by IL-1 and OSM, respectively (30, 47). Since p65 subunit of NF- κ B and STAT3 were previously implicated in cytokine-induced expression of YKL-40 (8, 34), we knocked down their expression in human astrocytes. Downregulation of STAT3 (Fig. 3C) dramatically diminished IL-1/OSM-induced YKL-40 mRNA expression (Fig. 3A). Surprisingly, knockdown of p65 had no effect on YKL-40 mRNA expression (Fig. 3A), but did drastically diminish the expression of IL-8 mRNA, which is p65-dependent (Fig. 3B). To identify the mechanism of cytokine-induced YKL-40 expression, putative NF- κ B and STAT3 binding sites of the YKL-40 promoter were mutated and the generated reporters were analyzed in astrocytes (Fig. 3D). Although cytokine-driven expression of YKL-40 is p65-independent (Fig. 3A), mutation of

the proximal NF- κ B site (-669 to -660 bp) alone substantially diminished activation of the reporter, whereas mutation of the distal NF- κ B site (-717 to -708 bp) alone had no effect (Fig. 3D). In addition, mutation of the STAT3 site also drastically diminished reporter activity. In another approach, we overexpressed dominant-negative I κ B α and constitutively active STAT3 and analyzed expression of the YKL-40 reporter (Fig. 3E). Constitutively active STAT3 enhanced, whereas dominant-negative I κ B α diminished cytokine-responsiveness. Collectively, these results suggest that the proximal NF- κ B and STAT3 elements of the YKL-40 promoter are required for full responsiveness to IL-1 and OSM; however, and in contrast to previous reports (34), p65 is dispensable for this response.

RelB and p50 regulate cytokine-induced YKL-40 expression

To determine which component of the NF- κ B complex regulates cytokine-driven YKL-40 expression, expression of p65, cRel, RelB, p50 and p52 was effectively knocked-down in astrocytes (Fig. 4B). Knockdown of either RelB or p50 significantly diminished cytokine-induced YKL-40 mRNA expression, whereas knockdown of p65, cRel and p52 had no effect (Fig. 4A). This finding implicates both RelB and p50 in YKL-40 regulation. Interestingly, although human astrocytes constitutively express low levels of RelB, IL-1 induced dramatic RelB protein accumulation in these cells (Fig. 4C). Similarly, RelB mRNA expression was also up-regulated by IL-1 in mouse astrocytes (Fig. 4D). RelB mRNA expression was also strongly activated *in vivo* by turpentine in an IL-1-dependent model of irritant-induced sterile inflammation (Fig. 4E). Since TNF also efficiently activates NF- κ B, we tested whether, similarly to IL-1, TNF regulates YKL-40 expression by a RelB-dependent mechanism. Although TNF and OSM could not induce YKL-40 mRNA expression in astrocytes, YKL40 mRNA expression was RelB-dependent in response to these cytokines in U373 glioma cells (Supplementary Fig. 2). These data suggests that RelB regulates expression of YKL-40 in response to proinflammatory cytokines, such as IL-1 and TNF, which induce canonical activation of RelB/p50 complexes. In contrast to astrocytes and U373 cells, we found that basal expression of YKL-40 and RelB mRNA was very high in primary human chondrocytes and not stimulated by IL-1 or OSM (Supplementary Fig 3). In these cells, basal expression of YKL-40 is RelB-, p65-, and STAT3-independent, implicating other unknown factors. Cumulatively, these data suggest that in cells expressing relatively low levels of YKL-40, such as astrocytes, cytokine-driven RelB promotes YKL-40 induction *in vitro* and *in vivo*.

RelB/p50 complexes bind to the YKL-40 promoter

To determine whether RelB regulates YKL-40 expression directly or indirectly, binding of RelB and p50 to the YKL-40 promoter was analyzed by ChIP (Fig. 5A). These experiments were performed in U373 glioma cells, which similarly to human and mouse astrocytes, upregulate expression of both YKL-40 and RelB in response to IL-1 and OSM, and this cytokine-induced expression is diminished by the knockdown of p50 and RelB (Suppl. Fig. 4). Although RelB and p50 did not bind to the YKL-40 promoter in unstimulated U373 cells, binding of RelB and p50 was apparent in cytokine-treated cells (Fig. 5B), suggesting that RelB/p50 complexes directly regulate YKL-40 expression. To determine if IL-1/OSM-treatment induces RelB/p50 binding to the previously identified NF- κ B sites, EMSAs were performed using oligonucleotides containing distal and proximal NF- κ B sites. In agreement

with the mutational analysis that showed the importance of the proximal NF- κ B site (Fig. 3D), strong protein binding to the proximal, but not distal, NF- κ B site of the YKL-40 promoter was induced by IL-1 alone or together with OSM (Fig. 5C). Cytokine stimulation facilitated the binding of protein complexes to the proximal NF- κ B element *in vitro*, which super-shifted with anti-p65, anti-RelB and anti-p50 antibodies (Fig. 5D), indicating that the proximal NF- κ B site can bind both p65/p50 and RelB/p50 complexes. We conclude that RelB/p50 complexes can directly bind to the proximal NF- κ B site of the YKL-40 promoter and are essential for the cytokine-induced YKL-40 expression.

OSM enhances IL-1-induced RelB/p50 heterodimer formation

OSM and IL-1 efficiently regulated YKL-40 expression via STAT3 (Fig. 3A) and OSM promoted the recruitment of IL-1-induced p50 to the YKL-40 promoter (Fig. 5B). Since YKL-40 expression depends on RelB/p50 (Fig. 4A), we subsequently asked whether OSM promotes formation of IL-1-induced RelB/p50 heterodimers. RelB and p50 were immunoprecipitated from cytokine-treated cells and analyzed for the presence of coimmunoprecipitated RelB and p50 (Fig. 6). Indeed, p50/RelB complexes were formed in response to IL-1. More importantly, OSM significantly enhanced IL-1-induced formation of the p50/RelB complexes. These data suggest that in addition to STAT3 activation, OSM enhances YKL-40 expression by promoting formation of the RelB/p50 complexes, which bind to the YKL-40 promoter.

Discussion

Although YKL-40 is upregulated in a broad range of human diseases associated with ongoing sterile inflammation, its biological functions still remain elusive (48-50). Nevertheless, exacerbated EAE (10) and enhanced responses to hyperoxia (11) in YKL-40 knock-out mice suggest that cytokine-induced YKL-40 is needed for the proper resolution of inflammation. Once inflammation is resolved, YKL-40 expression ceases. In contrast, chronic inflammation leads to continuous aberrant upregulation of YKL-40 expression, which is continuously driven by proinflammatory cytokines. Our data from the turpentine, TAT-overexpression, and EAE mouse models together with human oligodendroglioma data, demonstrate that YKL-40 expression correlates with the expression of both IL-1 β and IL-6. Importantly, IL-1 α and IL-1 β are both critical initiators of sterile inflammation released in response to broad range of danger-associated molecular patterns. IL-1 β is processed from its inactive precursor in response to inflammasome activation and subsequently secreted from activated cells, while active intracellular IL-1 α , which does not require processing, is liberated from damaged cells (51). Since IL-1 efficiently activates expression of IL-6 in many cell types (52, 53), it is not surprising that these two cytokines are found at the sites of local inflammation. The synergistic upregulation of YKL-40 expression by IL-1 and IL-6 (or OSM) in *in vitro* experiments therefore likely recapitulates the regulation of YKL-40 *in vivo*. Similarly to the regulation of YKL-40, IL-1 and IL-6 (or OSM) are known to control expression of acute phase proteins in the liver, which are released to the blood to limit inflammation-associated damage and allow for the return to homeostasis (54). Thus, acute cytokine-driven expression of YKL-40 is likely highly beneficial and allows for proper resolution of inflammation. Nevertheless the mechanism by which YKL-40 limits

inflammation remains elusive due to limited understanding of YKL-40 receptors and their signaling. In fact YKL-40 induces the interaction of $\alpha_v\beta_3$ integrins with syndecan-1 in endothelial cells (27), it activates ERK, AKT, and Wnt/ β -catenin signaling in macrophages via IL-13 receptor alpha 2-dependent mechanism (55), and activates ERK, AKT, and p38 via protease activated receptor 2 in bronchial smooth muscle cells (56). It remains to be established whether these proposed receptors/mechanisms are important for the biological functions of YKL-40, which are associated with inflammation. However, in agreement with the proposed role of YKL-40 in limiting inflammation, it has recently been shown that YKL-40 also inhibits NF- κ B activation and expression of IL-6, IL-8 and MCP-1 by skeletal muscle cells by a protease activated receptor 2-dependent mechanism (57).

It is generally accepted that IL-1 triggers a classical NF- κ B pathway in many cell types, which leads to the induction of p65/p50 target genes, including those encoding proinflammatory cytokines such as IL-8 and IL-6 (58). It is also accepted that RelB is activated in lymphoid cells, such as dendritic cells, by a noncanonical NF- κ B pathway and generation of RelB/p52 complexes that are important for proper dendritic cell functions (59). This non-canonical NF- κ B activation pathway is activated by ligands such as CD40 and lymphotoxin- β (60), but cannot be activated by IL-1. However, it has recently been recognized that in dendritic cells, RelB can also be activated by a canonical pathway in response to TNF, LPS and CpG, and this activation results in RelB/p50 complex formation (60). This canonical RelB activation depends on TRAF6 (61), which is further supported by findings that the RelB/p50 complexes are bound to I κ B α and I κ B ϵ in the cytoplasm (60). It has been also suggested that canonical activation of RelB in dendritic cells is possible because of a higher level of RelB expression in these cells and the constitutive activation of noncanonical pathway (60). The physiological importance of the RelB/p50 complexes is strongly supported by a more deleterious phenotype of RelB-deficient than the NIK-deficient mice (62), suggesting that RelB has NIK-independent functions that likely can be attributed to RelB/p50 complexes. Nevertheless, the mechanism of specific RelB/p50-dependent gene regulation is not clear since RelB/p50 and p65/p50 complexes bind the same regulatory sequences.

Although existence of RelB/p50 complexes in non-lymphoid tissues has been shown more than a decade ago (33), the function of these complexes remained elusive. Our data clearly demonstrate that RelB expression is strongly upregulated during sterile and LPS-induced inflammation *in vivo*, which is likely mediated by IL-1. Furthermore, IL-1 strongly activates expression of RelB in astrocytes *in vitro*. RelB and YKL-40 expression can also be upregulated strongly in cells responding to TNF. RelB subsequently forms complexes with p50 and activation of these complexes is controlled by I κ B α . Once the RelB/p50 complexes are activated, they regulate expression of YKL-40 and likely other RelB/p50-responsive genes. Thus, it appears that RelB/p50 complexes may play critical role(s) during acute inflammation that normally leads to a return to homeostasis. However, chronic inflammation may also lead to pathological RelB/p50-dependent gene expression. Indeed, YKL-40 has recently been proposed as a marker of a particularly aggressive mesenchymal subtype of GBM (63). In agreement with our current findings indicating that RelB regulates YKL-40 expression, RelB is strongly expressed in mesenchymal subtype of GBM and promotes

expression of mesenchymal genes, including YKL-40 (63). Furthermore, loss of RelB expression attenuates GBM cell survival, motility and invasion (64).

Our previous (8) and current data suggest that activation of YKL-40 expression requires both STAT3 and RelB. Although mechanistic details of YKL-40 promoter activation are not clear, robust cytokine-induced YKL-40 expression involves induction of RelB expression, binding of both STAT3 and RelB/p50 complexes to the YKL-40 promoter, and acetylation of histones. It remains to be established whether increased STAT3-dependent acetylation of histones (and opening of chromatin) allows for efficient binding of newly formed RelB/p50 complexes. Surprisingly, OSM also promotes formation of the RelB/p50 complexes by a mechanism, which currently remains unclear, but these complexes do not contain STAT3 (data not shown).

In summary, we propose that inflammation leads to the expression of YKL-40, which is induced by IL-1 and cytokines of the IL-6 family. Mechanistically, both STAT3 and the RelB/p50 complexes drive the expression of YKL-40 via the elements within the YKL-40 promoter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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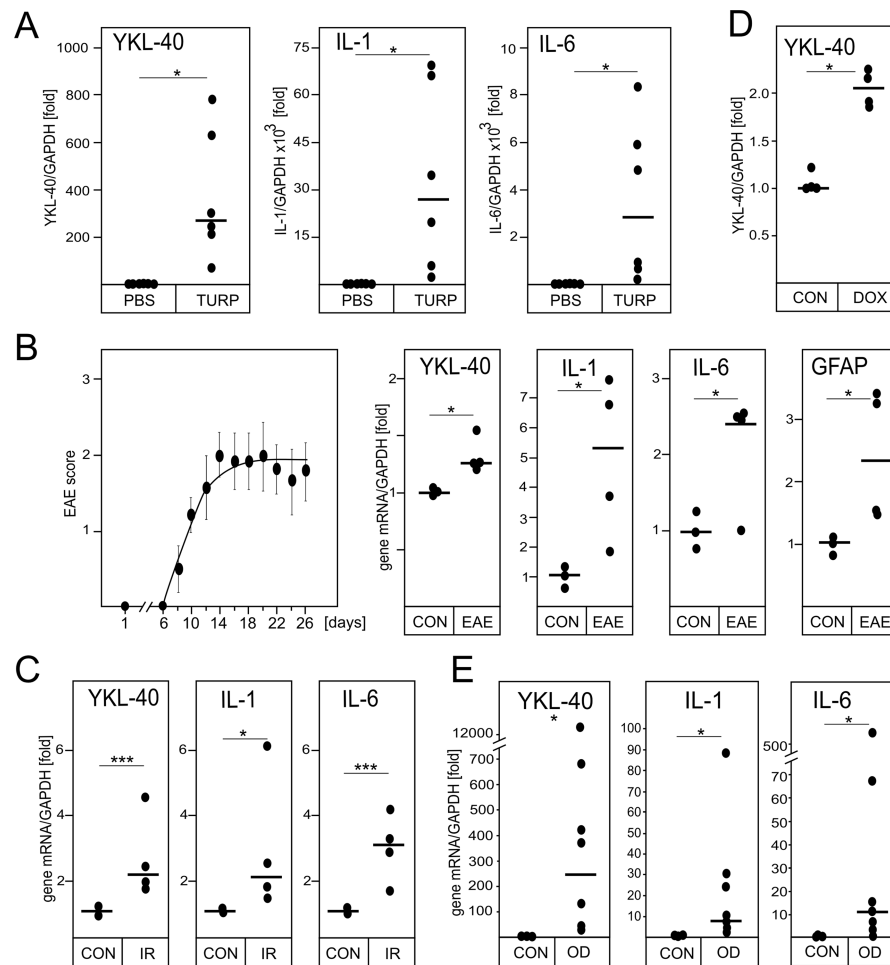


Fig. 1. Sterile inflammation induces YKL-40, IL-1 and IL-6 expression

(A) Mice were injected s.c. with 50 μ l turpentine or PBS (n=6). Tissue at the site of injection was collected after 8h and the expression of YKL-40, IL-1 and IL-6 mRNA was analyzed by qPCR. (B) EAE was induced in mice (n=6) and scored as described in methods section (left panel). Expression of YKL-40, IL-1, IL-6 and GFAP mRNA was analyzed in spinal cords at day 28 by qPCR (right panels). (C) Expression of YKL-40, IL-1 and IL-6 was analyzed in the hippocampus of control (CON) or irradiated (IR) mice after 24 h. (D) Tat expression was induced by DOX in Tat transgenic mice and the expression of YKL-40 mRNA was analyzed by qPCR. (E) Expression of YKL-40, IL-1 and IL-6 mRNA was analyzed by qPCR in biopsy samples of oligodendroglioma tumors (OD) and healthy brain tissue. Lines indicate median values. * $P < 0.05$ and *** $P < 0.001$ (t-test).

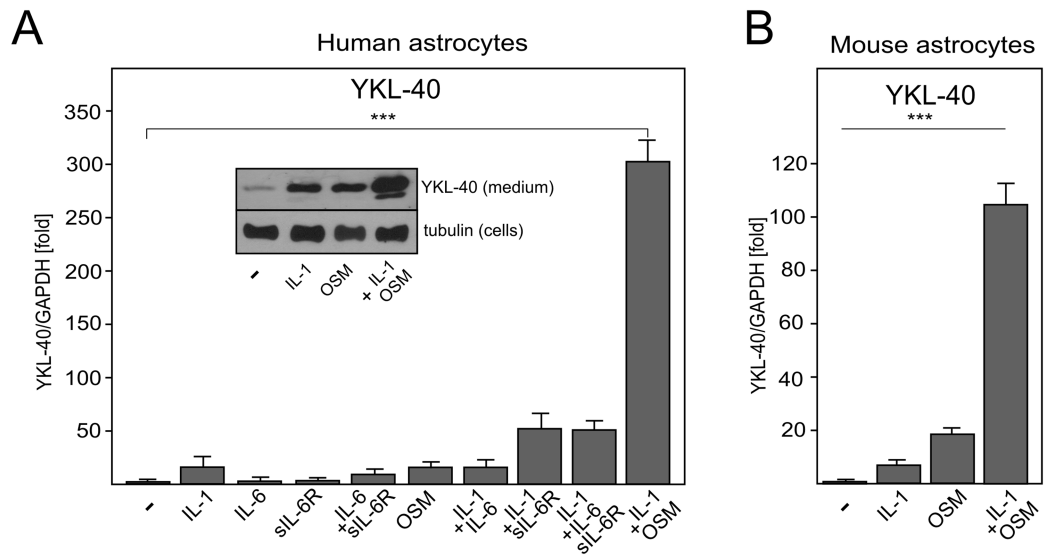


Fig. 2. Proinflammatory cytokines induce YKL-40 expression in astrocytes

Primary human (A) and mouse (B) astrocytes were stimulated with IL-1, IL-6, OSM and siIL-6R for 18h and expression of YKL-40 mRNA was analyzed by qPCR. Secreted YKL-40 was analyzed by Western blotting in the culture medium (insert). *** $P < 0.001$ (one-way ANOVA).

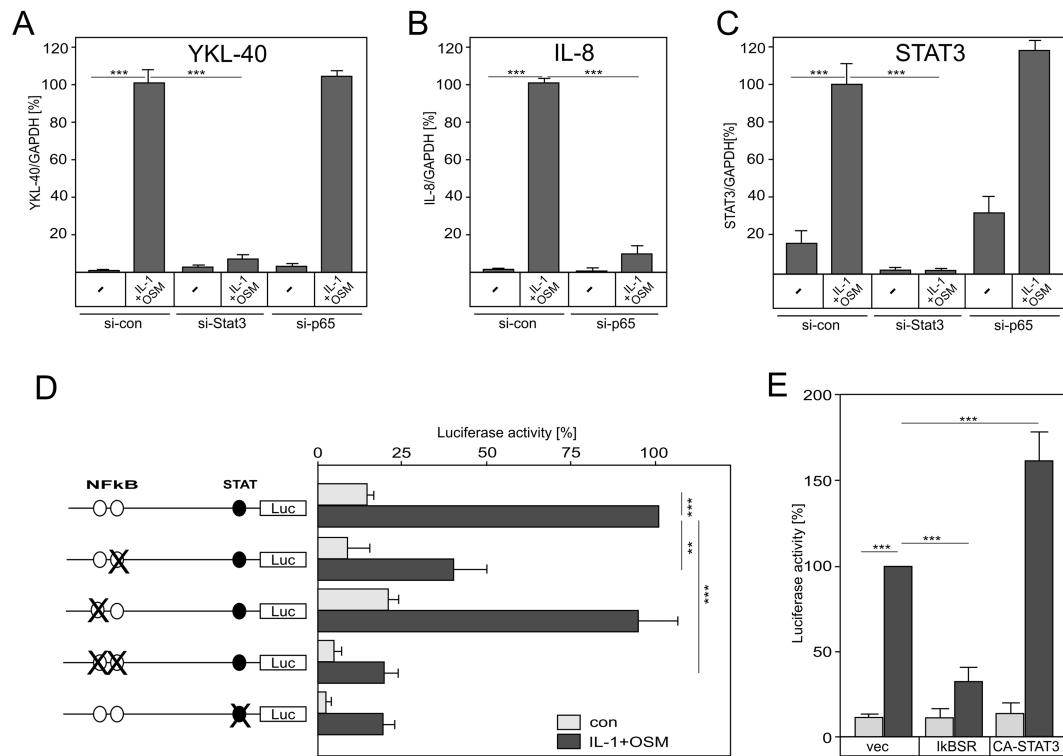


Fig. 3. Induction of YKL-40 expression by IL-1 and OSM is STAT3-dependent, but p65-independent

Human astrocytes were transfected with the indicated si-RNAs. Forty hours post transfection, cells were stimulated with IL-1 and OSM for 18 hours and expression of YKL-40 (A), IL-8 (B), and STAT3 (C) was analyzed by qPCR. (D) Astrocytes were transfected with the indicated reporters, stimulated with IL-1 and OSM for 18 hours, and luciferase and renilla activities were determined. Data are presented in comparison to the induced wild-type reporter (set as 100%). (E) Astrocytes were transfected with pYKL(-1300)Luc and plasmids expressing either vector, dominant-negative I κ B (IkBSR), or constitutively-active (CA-STAT3). Cells were stimulated with IL-1 and OSM for 18 hours and processed as described in D. $**P < 0.01$ and $***P < 0.001$ (one-way ANOVA).

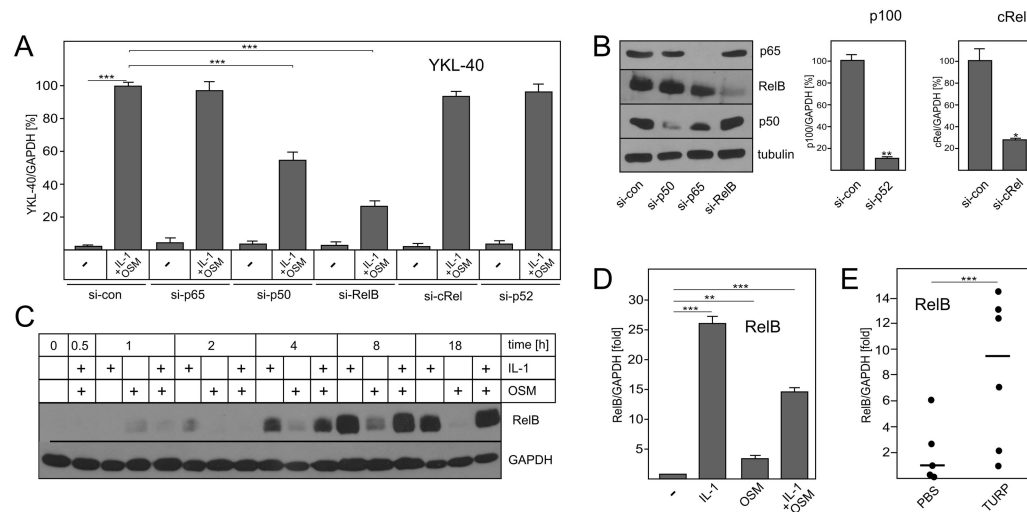


Fig. 4. Cytokine-induced RelB/p50 complexes regulate YKL-40 expression

Human astrocytes were transfected with the indicated si-RNAs. Forty hours post-transfection, cells were stimulated with IL-1 and OSM for 18 hours. Expression of YKL-40 (A), p100 and cRel (B, right panel) was analyzed by qPCR. Expression of p65, RelB, p50 and β -tubulin was analyzed by Western blotting (B, left panel). (C) Human astrocytes were stimulated with IL-1 and OSM for the indicated times and expression of RelB and GAPDH was analyzed by Western blotting. (D) Mouse astrocytes were stimulated with IL-1 and OSM for 18 hours and expression of RelB was analyzed by qPCR. (E) Mice were injected s.c. with 50 μ l turpentine or PBS (n=6). Tissue at the site of injection was collected after 24h and the expression of RelB mRNA was analyzed by qPCR. Lines indicate median values. ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA).

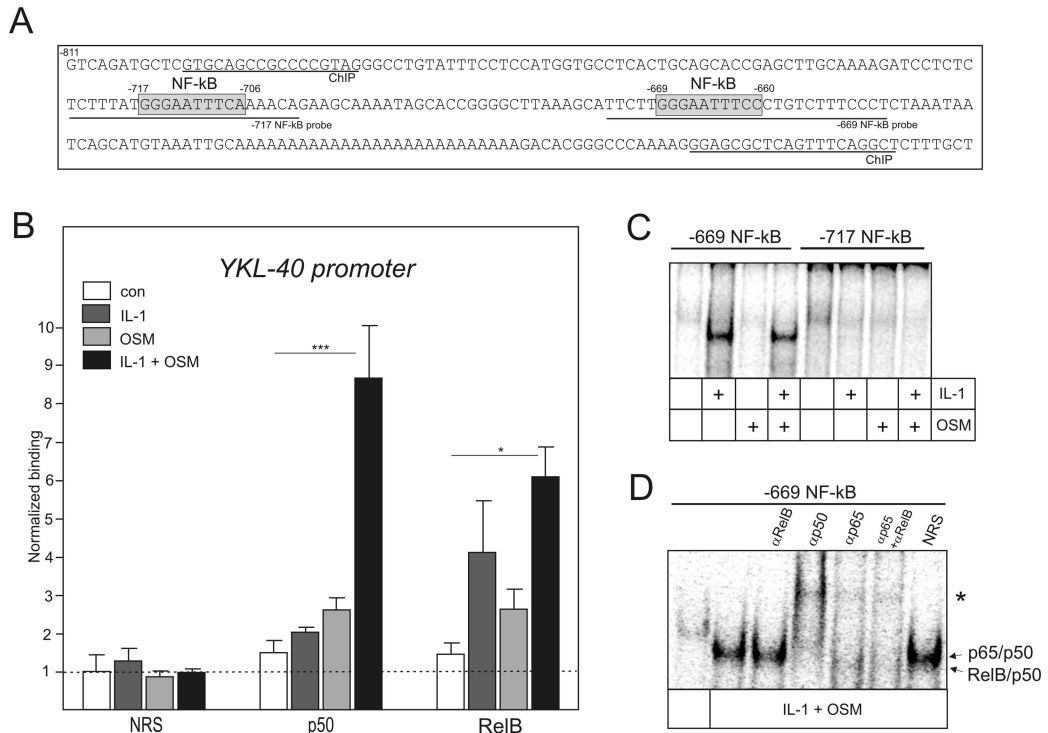


Fig. 5. RelB/p50 complexes bind at the distal NF-κB site of YKL-40 promoter
 (A) YKL-40 promoter. NF-κB sites are indicated by grey boxes. Positions of ChIP primers and probes used in EMSA (–717 and –669 probe) are indicated. (B) ChIP was performed using chromatin prepared from U373 cells treated with IL-1 and OSM for 2h. Binding of p50 and RelB to the YKL-40 promoter was analyzed using the antibodies described in the experimental procedures. NRS indicates normal rabbit serum used for immunoprecipitation. Results are shown as normalized binding (binding of NRS-immunoprecipitated untreated samples were set as 1). Experiments were performed three times. * $P < 0.05$ and *** $P < 0.001$ (two-way ANOVA). (C-D) Nuclear extracts were prepared from U373 and glioma cells stimulated with IL-1 and OSM for 8 hours. The binding was then analyzed by EMSA using the ^{32}P -labeled oligonucleotide probes derived from the 5' flanking region of the YKL-40 (–717 NF-κB and –669 NF-κB, as indicated). (C) Binding to the –717 NF-κB and –669 NF-κB elements in U373 glioma cells. (D) Binding to the –669 NF-κB probe in U373 cells. Specific antibodies or NRS were added to the binding reaction. Asterisk indicates super-shifted complexes.

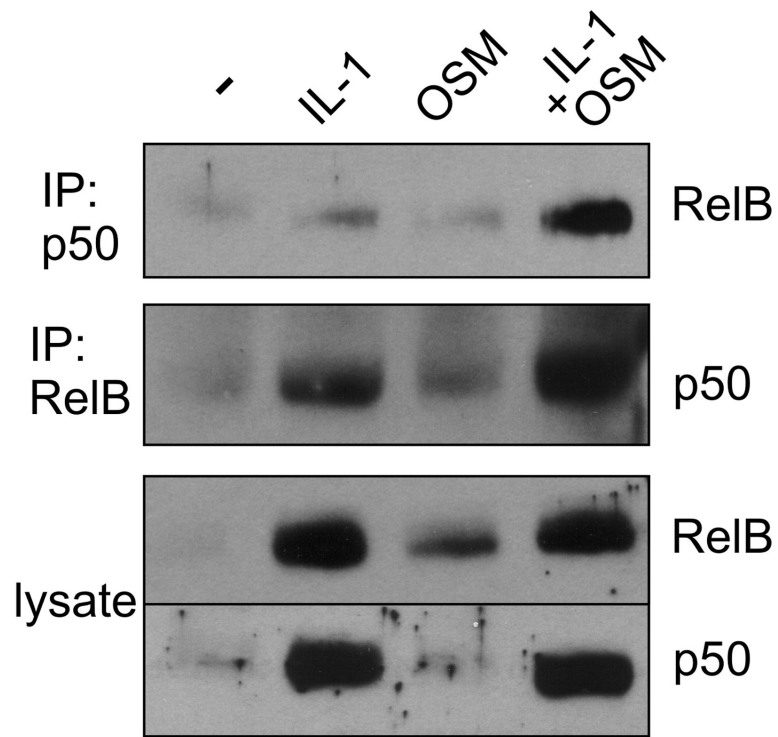


Fig. 6. OSM enhances IL-1-induced RelB/p50 heterodimer formation

Human astrocytes were stimulated with IL-1 or OSM for 8 hours, p50 or RelB were immunoprecipitated, and co-immunoprecipitated p50 and RelB were detected by Western blotting, as indicated. Expression of RelB and p50 in the lysates is shown the lower panels (lysate).