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Original Research

c-Myb interferes with inflammatory IL1α-NF-κB pathway in breast cancer cells

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Abstract

The transcription factor c-Myb can be involved in the activation of many genes with protumorigenic function; however, its role in breast cancer (BC) development is still under discussion. c-Myb is considered as a tumor-promoting factor in the early phases of BC, on the other hand, its expression in BC patients relates to a good prognosis. Previously, we have shown that c-Myb controls the capacity of BC cells to form spontaneous lung metastasis. Reduced seeding of BC cells to the lungs is linked to high expression of c-Myb and a decline in expression of a specific set of inflammatory genes. Here, we unraveled a c-Myb-IL1α-NF-κB signaling axis that takes place in tumor cells. We report that an overexpression of c-Myb interfered with the activity of NF-κB in several BC cell lines. We identified IL1α to be essential for this interference since it was abrogated in the IL1α-deficient cells. Overexpression of IL1α, as well as addition of recombinant IL1α protein, activated NF-κB signaling and restored expression of the inflammatory signature genes suppressed by c-Myb. The endogenous levels of c-Myb negatively correlated with IL1α on both transcriptional and protein levels across BC cell lines. We concluded that inhibition of IL1α expression by c-Myb reduces NF-κB activity and disconnects the inflammatory circuit, a potentially targetable mechanism to mimic the antimetastatic effect of c-Myb with therapeutic perspective.

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Introduction

Breast cancer (BC) is represented by a group of malignancies heterogeneous in their biological and clinical behavior [1]. Whereas overall survival rates improved over the years, the prognosis of patients diagnosed with metastatic disease remains poor, with an estimated 5-year survival rate lower than 30% [2,3]. Therefore, considerable attention is being paid to the investigation of molecular pathways governing the establishment of metastatic lesions.

The transcription factor c-Myb is known as a mammal homologue of the v-Myb oncoprotein causing leukemia in birds, and its expression has been connected with several human malignancies [4]. Studies of leukemia linked c-Myb to worse prognosis [5,6]; however, its role in colon and breast cancer remains controversial [7–9]. Although c-Myb is a prerequisite for mammary carcinogenesis in murine models *in vivo* [10], elevated c-Myb expression in BC is linked to excellent prognosis [11,12]. Expression of c-Myb in BC correlates with estrogen receptor (ER) positivity [13] as *MYB* is identified as a direct target of ER signaling [14]. Survival of patients diagnosed with the ER-positive BC is longer since they may benefit from the adjuvant ER-targeted endocrine therapy [15,16]. In addition, antiestrogens may induce *MYB* expression [17,18], thus the contribution of c-Myb to patient survival must be assessed with care and may vary in subgroups of BCs [12,19]. There are several studies suggesting that ER may not always be essential for increased c-Myb expression in BC [10,20], it may be induced or suppressed by various microenvironmental cues [21–23]. Initially, c-Myb was shown to maintain proliferation and impede differentiation of mammary

Abbreviations: BC, breast cancer; CBA, cytometric bead array; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; TEM, trans-endothelial migration.

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cells [17,24]. Emerging data extended function of the c-Myb protein in adjusting plasticity of BC cells, as high proliferative state endowed by c-Myb was coupled with the acquisition of epithelial traits in some tumor cells [21]. Epithelial-to-mesenchymal transition (EMT) occurs in carcinoma cells exiting the primary tumor site, furthermore, the reverse transition (MET) in disseminated cells appears to be required for outgrowth of secondary tumors. The direct transcriptional repression of c-Myb by EMT regulator ZEB1 is required for stabilization of a mesenchymal phenotype, proliferation arrest and possibly precedes seeding of tumor cells in distant locations [21]. However, the role of c-Myb in EMT appears to be more complex, presumably context-dependent, varying with different stimuli and stage of the transition [21,22,25,26]. In our previous report, we have shown that high levels of c-Myb in ER-negative BC cells reduced their lung seeding capacity *in vivo* accompanied by decreased expression of a specific set of inflammatory genes (*Ccl2, Cxcl1, Cxcl2, Cxcl6, Cxcl16, Icam1, Il1a, Tnfrsf9, Lcn2, Ikbke* - denoted as the c-Myb-inflammatory signature). Inhibition of *Ccl2* expression by c-Myb was detrimental for migration of tumor cells through the lung endothelium, linking the c-Myb-governed transcriptional program with the control of transendothelial migration (TEM) [19]. Whether c-Myb may inhibit the inflammatory circuit by direct binding to the regulatory elements of the remaining signature genes and/or by interfering with relevant signaling pathways in BC cells remains to be elucidated. Here, we show that high c-Myb expression suppressed activity of NF-κB, a key inflammatory mediator, in BC.

The NF-κB protein family comprises pleiotropic transcription factors implicated in the control of expression of genes related to proliferation, survival, angiogenesis, metastasis, and immune response [27]. High NF-κB activity has been linked to worse prognosis in BC patients [28–30]. The NF-κB activation in cancer cells may be caused by mutations that affect signaling components or by the exposure to inflammatory cytokines in the tumor microenvironment [31]. IL1α is a cytokine that is expressed by epithelial, endothelial, and stromal cells under homeostatic conditions and its expression can be stimulated by a broad spectrum of inflammatory stimuli [32]. IL1α binds to the interleukin 1 receptor type 1 (IL-1R1) which can subsequently lead to NF-κB, c-Jun N-terminal kinase (JNK), and p38 MAPK pathways activation [33]. Autocrine IL1α signaling in malignant BC cells, driven besides other stimuli by HER2, supports cancer stem-like cell maintenance and tumorigenesis by activating NF-κB and STAT3 pathways [34]. Similarly, in ER-positive MCF7 cells overexpression of IL1α lead to NF-κB activation and promoted tumor growth [35], while other studies connect this cytokine with the metastatic spread of BC cells [36,37]. Inhibition of IL-1R1 signaling by anakinra, clinically licensed IL-1R1 antagonist, reduced tumor burden and bone metastases in ER-negative and positive BC cells [38].

Here, we report that c-Myb inhibited cytokine IL1α expression in BC cells, which in turn led to a decline in autocrine signaling affecting the NF-κB pathway and the ability of BC cells to migrate and cross the endothelial barrier.

Material and methods

Cell culture, plasmids and reagents

E0771.LMB cells were kindly provided by Dr. Robin Anderson [39], all other cell lines were purchased from American Type Culture Collection. 4T1, MDA-MB-231, MCF7, T47D, BT-474 and BT-549 were cultured in RPMI-1640 (Sigma-Aldrich), SKBR3 were cultured in McCoy's 5A Modified Medium (Sigma-Aldrich), MDA-MB-468 and E0771.LMB cells were cultured in DMEM (Sigma-Aldrich). The cells of all lines were cultured at 37°C in a humidified incubator with 5% CO_2, media were supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Lonza). The medium of 4T1 cells was further supplemented with 1 mM sodium pyruvate and 4500 μg/mL D-Glucose (Sigma-Aldrich). HUVECs were cultured in Endothelial Basal Medium supplemented with EGM SingleQuots (Lonza). All cell lines were routinely tested for mycoplasma contamination by PCR. Plasmids are listed in Table S1. All prepared constructs were verified by Sanger sequencing. *Il1a* and *IL1A* marked murine and human genes respectively, and uniform IL1α is used for the protein. Similarly, we used *Myb* (mouse gene), *MYB* (human gene), and c-Myb for protein.

Recombinant IL1α (211-11A, PeproTech) was used in listed concentrations. JSH-23 (J4455, Sigma-Aldrich) was applied in concentration 20μM, IRAK1/4 inhibitor (I5409, Sigma-Aldrich) in concentration 10μM, and recombinant mouse IL-1ra/IL-1F3 protein (480-RM-010, R&D Systems) in concentration 300 ng/mL.

Transfections

For all transfections, Lipofectamine LTX (Life Technologies) was used. 4T1, E0771.LMB cells with constitutive c-Myb overexpression (Myb^{high}) and MDA-MB-231 c-Myb knock-outs were described previously [19]. 4T1 and E0771.LMB cells overexpressing IL1α were prepared by transfection with pcDNA3.mIl1a (Table S1) and selected with G418 (800 μg/mL and 500 μg/mL, respectively) and cloned by limiting dilution. Similarly, MDA-MB-231 cells overexpressing human *IL1A* were transfected using pCMV14-3xflag_hIL1a (Table S1). Pool of transfected cells was selected with 500 μg/mL G418. To derive 4T1 IL1α knock-outs, 4T1 cells were transfected with one of the lentiCRISPRv2 plasmids (Table S1), selected with puromycin (1 μg/mL) for 2 wk and cloned by limiting dilution. PCR primers spanning potential sites of mutation were designed (Table S2), mutations were confirmed by the Sanger sequencing. IL1α overexpression/knock-out was verified by Cytometric Bead Array (CBA).

Transactivation assay

4T1 and E0771.LMB cells were transfected with the luciferase reporter plasmid pNF-κB-luc/mIl1a-luc, and CMV-βgal concomitant with wt *Myb*/M303V *Myb*/mock control vector and processed for luciferase and β-galactosidase assays 18 h after transfection as described elsewhere [40]. The luciferase activity was expressed in relative light units and normalized according to protein content measured by Bradford assay or for transfection efficiency according to the β-galactosidase activity.

RNA isolation and quantitative PCR (qPCR)

Total RNA was isolated using the GenElute Total RNA Purification Kit (Sigma-Aldrich) and cDNA using the QuantiTect RT Kit (Qiagen). qPCR was performed with KAPA SYBR Fast Master mix (KAPA Biosystems) with primers spanning exon-exon junctions (Table S3) using the LightCycler 480 (Roche).

Immunoblotting

Cell lysis and western blot analysis were performed as described elsewhere [41]. Following antibodies were used: c-Myb (05-175, Millipore), α-tubulin (T9026, Sigma), and horse antimouse (7076, CS) secondary antibody conjugated to peroxidase. Densitometry analysis was done using ImageJ (NIH).

Cytometric bead array (CBA)

Supernatants of cells cultured for 48 h were collected and the CBA kits (all from BD Biosciences) were used for determination of the amount of murine IL1α (560157), murine Cxcl1 (558340), and human IL1α (560153) according to manufacturer's instructions.

Flow cytometry analysis

The following antibodies were used for staining: PE antimouse CD54 (116107, Biolegend), PE antimouse CD137 (106106, Biolegend), and PE Syrian Hamster IgG Isotype Control (402008, Biolegend). For IκBα-miRFP703 reporter detection, cells were transfected with pIκBα-miRFP703 and pEGFP-C1 (GFP). The percentage of IκBα-miRFP703-positive cells was expressed from 5000 GFP-positive cells. Data were collected using a BD FACSVerse and analyzed by FlowJo v10.6.2.

Trans-endothelial migration (TEM) assay

TEM assay was performed as described previously [41].

Data-mining and correlation analysis

Derivation and RNA sequencing of 4T1 *Myb*^{high} were described previously [19,42]. The expression levels of the NF-κB targets/regulators were clustered using FGCZ Heatmap tool (http://fgcz-shiny.uzh.ch). The RNAseq data are available in Gene Expression Omnibus (GEO, NCBI) under the accession number GSE104264 [19]. *MYB* and *IL1A* transcript levels were looked up in microarray expression datasets GSE14027 [43] and GSE14405 [44].

The Broad Institute CCLE (https://portals.broadinstitute.org/ccle) was used to correlate *MYB* and *IL1A* expression in human BC cell lines. ER expression was evaluated according to Jiang *et al.* [45] and Dai *et al.* [46]. Correlations between *MYB* expression and NF-κB activity were calculated in a panel of 33 BC cell lines from dataset GSE44552, subtyping were done according to the Yamaguchi *et al.* [47]. Pearson correlations were calculated with the GraphPad Prism v6.07.

To detect the over-representation of transcription binding sites (TFBS) for a set of coexpressed genes we used cREMaG database interface with default settings and 47 input genes deregulated in *Myb*^{high} as identified previously [19].

Statistics

Statistical analysis was performed with the GraphPad Prism v6.07. All data are presented as mean ±SD and were analyzed with unpaired T-test unless stated otherwise.

Results

c-Myb reduces activity of the NF-κB pathway

Analysis of expression dataset (GSE44552) showed that *MYB* expression and constitutive NF-κB activity inversely correlated in human BC cell lines (Fig. 1a). Noting that c-Myb is downstream of the ER signaling in BC [24], we split the cell lines into the ER-positive (specifically luminal) and ER-negative (basal) subtypes. The inverse correlation between *MYB* expression and NF-κB activity was significant within the cell lines of luminal subtype, and the same trend was apparent for basal subtype, suggesting that c-Myb may provide an ER-independent effect (Fig. S1a). We have previously identified the c-Myb-inflammatory signature consisting of immune response genes that are repressed by c-Myb in ER-negative BCs [19]. *In silico* analysis using cREMaG database showed that the most over-represented transcription factor-binding sites within the promoters of the c-Myb-inflammatory signature genes are the NF-κB family motifs (Table S4). Therefore, we hypothesized that c-Myb may attenuate the NF-κB signaling.

To test the effect of c-Myb on activity of NF-κB in a cellular model, we performed luciferase assays using the NF-κB reporter plasmid (NF-κB-luc) containing the firefly luciferase gene under control of the NF-κB response elements. The NF-κB-luc vector was used with the *MYB*-coding or control plasmids for transient transfection of human MDA-MB-231 and MDA-MB-436 cells. In both cell lines, the NF-κB transactivation activity significantly decreased in the presence of c-Myb (Fig. 1b). We repeated this experiment with a vector coding for a mutant c-Myb harboring an amino acid substitution in its transactivation domain (M303V) that abrogates transcriptional activation/repression of the c-Myb target genes [48,49]. The NF-κB-luc plasmid was used together with vectors coding for wild-type (wt) c-Myb, M303V c-Myb, or mock control for transfection of mouse 4T1 and E0771.LMB cell lines. The NF-κB transactivation activity significantly decreased in the presence of wild-type c-Myb, but not M303V c-Myb (Fig. 1c, S1b), suggesting that suppression of NF-κB by c-Myb required trans-activation/repression of either directly NF-κB family member(s) or NF-κB pathway regulator(s).

To confirm our hypothesis that c-Myb suppresses activity of NF-κB, we analyzed expression levels of the NF-κB family members and targets in unstimulated 4T1 and E0771.LMB cells exhibiting high ectopic c-Myb expression (*Myb*^{high} cells) [19]. A significant decrease of several of these genes in *Myb*^{high} cells (Fig 1d, S1c) support the hypothesis that c-Myb has a negative impact on the activity of the NF-κB pathway.

IL1α expression is reduced by c-Myb but not by the NF-κB inhibitor JSH-23

The link between NF-κB signaling and c-Myb expression has been described for colorectal cancer, where the NF-κB proteins trigger transcription of *Myb* [50]. To test whether expression of *Myb* itself could be stimulated by NF-κB in our experimental settings, we treated 4T1 cells with the NF-κB-specific inhibitor JSH-23 [51]. Inhibition of NF-κB did not affect the *Myb* transcription but resulted in attenuation of the inflammatory signature gene expression (Fig. 2a), thereby resembling an effect of c-Myb overexpression [19]. Since expression of some genes from the signature (*Il1a, Ikbke,* and *Cxcl16*) were not altered by the NF-kB inhibition, these genes may act upstream of NF-κB.

IL1α is a known activator of the NF-κB pathway [52] and has been described as one of the most abundant molecules in triple-negative BC cells upon NF-κB stimulation [53]. Given that transcription of *Il1a* is significantly reduced in 4T1 *Myb*^{high} cells [19], we investigated the relationship between c-Myb and IL1α. Lower levels of IL1α in the conditioned medium of 4T1 *Myb*^{high} cells were detected using flow-cytometric bead array (CBA) (Fig. 2b). Vice versa, MDA-MB-231 c-Myb knock-out cells showed an increase in IL1α production (Fig. 2c). This reverse relationship between *Myb* and *Il1a* expression was also detected in E0771.LMB cells using qPCR (Fig. 2d).

Overexpression of IL1α stimulates the NF-κB pathway and the inflammatory signature

Next, we prepared several independent clones of 4T1 and E0771.LMB stably overexpressing IL1α (IL1α up, Fig. 2e-f). We detected significant increases in the transcription of the NF-κB genes *Nfkb2, Nfkbia, Birc3* in E0771.LMB IL1α up clones compared to the mock control (Fig. 2g). To verify IL1α as an NF-κB activator, we used a reporter vector coding for the NF-κB inhibitor (IκBα) fused with miRFP703 fluorescent tag for cotransfection of 4T1 cells with pcDNA3.1 (mock) and pcDNA3.Il1a (IL1α). The increase of NF-κB activity was accompanied by a rapid degradation of IκBα [54]. Thus, the IκBα-miRFP703^{+} cells were considered as NF-κB inactive, while the IκBα-miRFP703^{-} cells represented the NF-κB-activated population. To exclude nontransfected cells from the analysis, we cotransfected a GFP-coding vector and analyzed only the GFP^{+} cells. The significant decrease of GFP^{+} IκBα-miRFP703^{+} cells in the presence of IL1α showed that IL1α activated the NF-κB pathway, as hypothesized (Fig. 2h). To test, whether exogenous IL1α can also stimulate NF-κB, we determined transactivation of the NF-κB-luc reporter in E0771.LMB cells treated with recombinant IL1α. The NF-κB-luc activity was induced upon

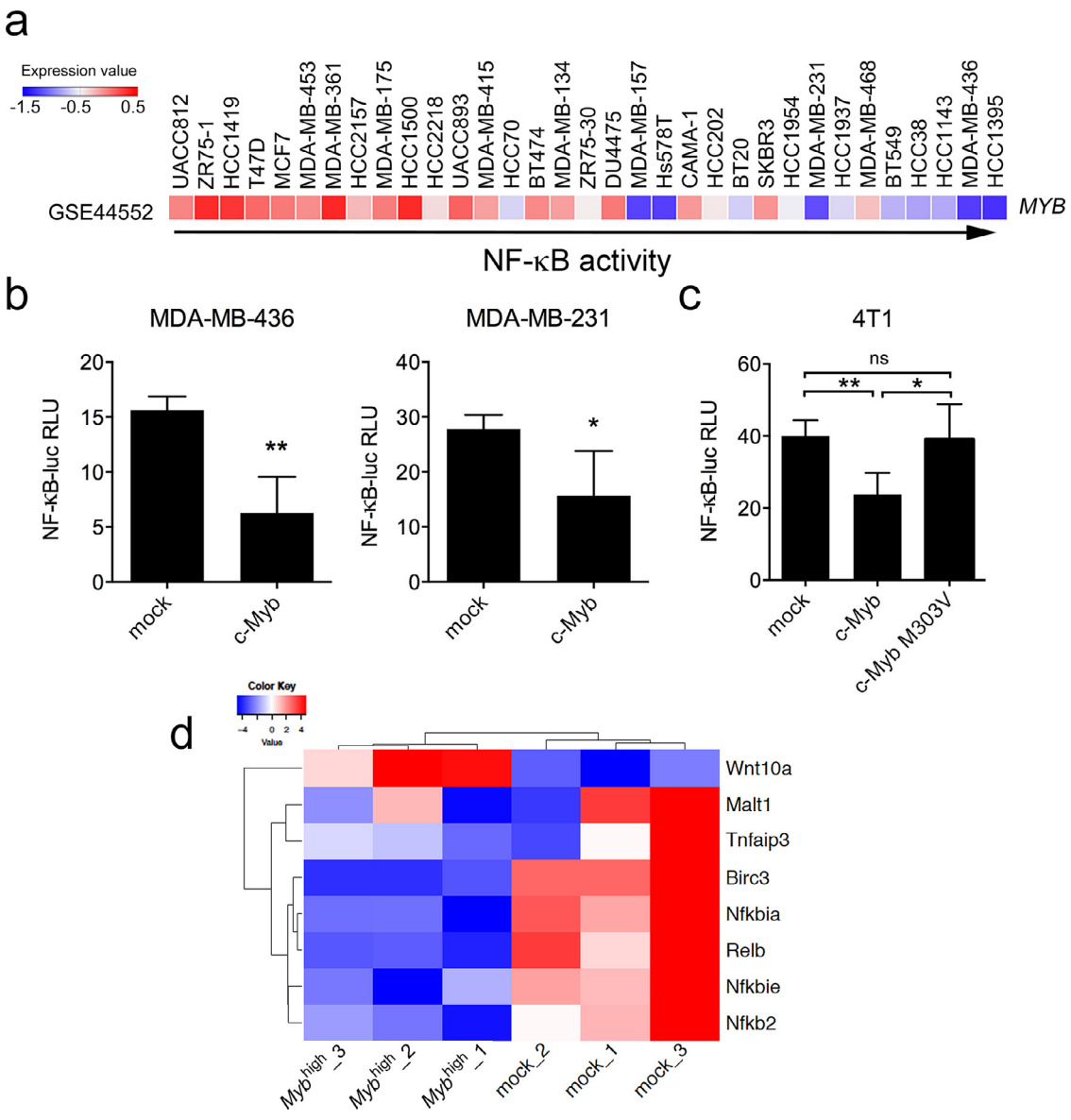

Fig. 1. c-Myb overexpression interferes with NF-κB signaling in breast cancer cells lines. (A) Heatmap of *MYB* mRNA expression in human breast cancer cell lines aligned according to their constitutive NF-κB activity as measured in [47]. (B) Transactivation of NF-κB-luc reporter in human MDA-MB-436 and MDA-MB-231 cells transiently overexpressing *MYB*, luciferase activity was normalized to β-galactosidase activity and expressed as relative light units. Average normalized relative light units (RLU) from 3 independent experiments are shown, comparison to mock transfected cells. (C) Transactivation of NF-κB-luc reporter in murine 4T1 cells transiently overexpressing wt *Myb* and *Myb* harboring M303V amino acid substitution. Luciferase activity was normalized to the protein concentration. Average normalized relative light units (RLU) from 3 independent experiments are shown in comparison to mock-transfected cells. (D) NF-κB target genes [64] that are differentially expressed in 4T1 Myb^{high} cells compared to mock control cells, heatmap of RNA sequencing data. Significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$) are indicated.

IL1α treatment in a dose-dependent manner from 0.1 ng/mL and peaked at 1 ng/mL (Fig. S2a).

Analysis of the effect of IL1α overexpression on transcription of the c-Myb-inflammatory signature revealed a significant increase in the amount of all the transcripts in E0771.LMB IL1α up cells, except for *Cxcl16* (Fig. 2i). Flow cytometric analysis confirmed the significant increase of Cxcl1 (Fig. 2j) as well as Icam1 and Tnfrsf9 protein production by E0771.LMB IL1α up cells (Fig. 2k, S2b). Similarly, elevated expression of the signature genes *Cxcl1, Cxcl5, Icam1,* and *Lcn2* was found in 4T1 IL1α up cells by qPCR (Fig. S2c). Elevated secretion of Cxcl1 was confirmed in conditioned media of 4T1 IL1α up cells (Fig. S2d). In contrast, expression of nearly all signature genes declined in 4T1 IL1α KO cells (Fig. S2e-f). To further confirm the effect of human IL1α on expression of the signature genes, we prepared MDA-MB-231 cells overexpressing human IL1α (Fig. S2g). We found *CCL2, CXCL1, CXCL2, ICAM1, IKBKE, TNFRSF9* transcripts to be elevated (Fig. S2h). Altogether these results indicate that human and murine IL1α produced by BC cells can be involved in autocrine signaling, leading to elevated expression of a subset of the genes from the NF-κB pathway and its targets.

Suppression of the NF-κB inflammatory circuit by c-Myb depends on IL1α

To verify that IL1α stimulates expression of the c-Myb inflammatory genes through activation of NF-κB signaling, we blocked IL1α and NF-κB using specific inhibitors. We used either IRAK1/4 (interleukin-1 receptor-

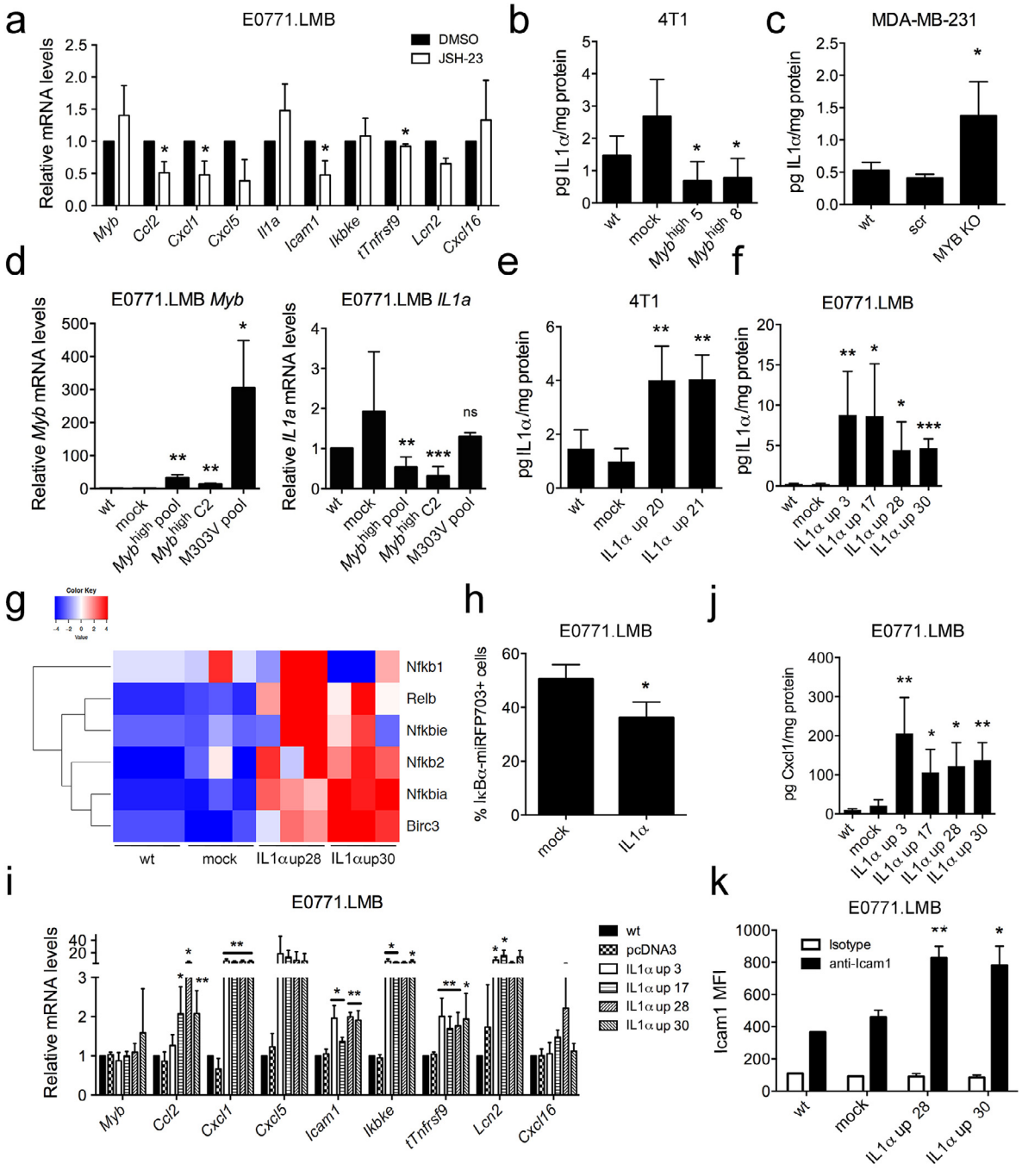

Fig. 2. IL1α is downregulated by c-Myb and its overexpression inversely impacts the inflammatory signature and NF-κB pathway. (A) mRNA levels of indicated genes in JSH-23-treated (20 μM, 24 h) E0771.LMB cells were normalized to *Gapdh*, relative fold change to DMSO-treated cells is shown for each gene. An average of 3 independent experiments is shown. Secreted IL1α protein levels in 4T1 cells overexpressing wt c-Myb (Myb^{high} 5 and 8) (B) and MDA-MB-231c-Myb knock-out cells (*MYB* KO) (C) as measured by Cytometric Bead Array (CBA). An average of 3 independent experiments is shown, comparison to mock-transfected and scrambled-transfected cells, respectively (D) *Myb* (left) and *Il1a* (right) mRNA expression in E0771.LMB cells overexpressing wt c-Myb (Myb^{high} pool, Myb^{high} C2) and M303V mutant c-Myb (M303V pool). Data were normalized to *Gapdh*, fold change values relative to wt cells from 3 independent experiments are shown, comparison to mock-transfected cells. Clones overexpressing mouse *Il1a* (IL1α up) were derived from 4T1 (E) and E0771.LMB (F) cells. Secreted IL1α protein levels were measured in 3 biological replicates by CBA, higher IL1α concentrations are compared to mock-transfected cells. (G) Expression of selected NF-κB family members/targets in E0771.LMB IL1α up cells as determined by qPCR, normalized to *Gapdh* and shown in triplicates as a heatmap. (H) IκBα levels in E0771.LMB cells cotransfected with pcDNA3.1 (mock), resp. pcDNA3.IL1a (IL1α), and pIκBα-miRFP703 vectors. Graph shows average frequency of miRFP703+ cells from 3 independent experiments. (I) mRNA levels of indicated genes in IL1α up E0771.LMB cells as determined by qPCR were normalized to *Gapdh* and expressed as fold change to wt cells. Significance calculated from 3 independent experiment is shown, comparison to mock-transfected cells. Proteins levels of Cxcl1 and Icam1 were determined in E0771.LMB IL1α up cells by CBA (J) and flow cytometry, respectively (K). Significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) are indicated.

associated kinase 1 and 4) inhibitor or IL-1ra (interleukin 1 receptor antagonist) to inhibit IL1α activity and JSH-23 to inhibit NF-κB. All inhibitors significantly reduced the secretion of Cxcl1 (Fig. 3a), and surface levels of Icam1 and Tnfrsf9 in E0771.LMB IL1α up cells (Fig. 3b-c). In addition, inhibition of NF-κB resulted in a significant decline in the expression of the majority of the c-Myb-inflammatory signature genes in E0771.LMB IL1α up cells as determined by qPCR (Fig. 3d). Altogether, these data demonstrate that IL1α-controlled expression of the c-Myb-inflammatory signature genes is sensitive to NF-κB inhibition.

To test whether c-Myb inhibits the inflammatory signature by limiting the IL1α expression, we treated *Myb*^{high} cells with recombinant murine IL1α and analyzed the expression of the signature genes. IL1α derepressed the expression of the signature genes in both E0771.LMB (Fig. 3e) and 4T1 (Fig. 3f) *Myb*^{high} cell lines. Of note, *Tnfrsf9* expression was induced only in E0771.LMB and *Cxcl16* only in 4T1 cells. On protein level, treatment of E0771.LMB *Myb*^{high} cells with IL1α increased the production of Icam1 (Fig. 3g, S3a) and Cxcl1 (Fig. S3b) in a dose-dependent manner. To verify that the effect of IL1α on gene expression is associated with activation of NF-κB signaling, we took advantage of the fluorescent IκBα-miRFP703 reporter and simultaneous staining of the Icam1 protein. After IL1α stimulation, we detected a decrease of GFP$^+$ IκBα-miRFP703$^+$ cells, proving the NF-κB activation in 4T1 *Myb*^{high} cells (Fig. 3h, S3c). IL1α stimulation led to a higher Icam1 expression, importantly, Icam1$^+$ cells were predominantly IκBα-miRFP703 negative (Fig. S3d), confirming that Icam1 expression induced by IL1α was dependent on NF-κB signaling.

To validate the proposed c-Myb-IL1α-NF-κB axis, we determined the effect of c-Myb on the transactivation of the NF-κB-luc reporter in 4T1 cells deficient in IL1α expression (IL1α KO cells). Transactivation by NF-κB was suppressed upon cotransfection with the NF-κB-luc reporter and a vector coding for c-Myb in control (scrambled) cells, but not in IL1α KO cells (Fig. 3i). We analyzed the murine *Il1a* promoter sequence and found 5 potential Myb-binding sites (Fig. 3j). To test, whether c-Myb directly modulates *Il1a* expression, we constructed a luciferase reporter vector containing the murine *Il1a* promoter sequence (Il1a-luc). In 4T1 cells, cotransfected with this Il1a-luc reporter and the c-Myb-expressing plasmid, we detected a significant decrease of *Il1a* promoter activity (Fig. 3k). We concluded that transactivation of the *Il1a* promoter is negatively regulated by c-Myb.

IL1α facilitates TEM and inversely correlates with c-Myb in BC cell lines

To prove the functional significance of c-Myb-IL1α-NF-κB signaling, we inspected the TEM ability of IL1α up cells. We have shown previously that c-Myb has a negative impact on TEM [19]. Here, we found that ectopic expression of IL1α in 4T1 cells enhanced transmigration capacity through primary endothelial cells (Fig. 4a, S4a). Similar results were obtained with MDA-MD-231 IL1α up cells transmigrating through a layer of HUVEC cells (Fig. 4b), underlining the opposite role of IL1α to c-Myb in sustaining transmigration capability in BC cells.

In order to investigate the expression pattern of c-Myb and IL1α, we analyzed the level of these proteins in several human BC cell lines. We determined the amount of secreted IL1α in the conditioned medium using CBA (Fig. 4c) and the amount of c-Myb in the cell lysates by immunoblotting (Fig. 4d). The highest level of secreted IL1α correlated with almost no detectable expression of c-Myb in MDA-MB-436 and MDA-MB-231 cells. In contrast, cells with strong c-Myb expression (MCF7, BT-474, MDA-MD-468, and T47D) secreted barely detectable IL1α. This corresponds with levels of *MYB* and *IL1A* in RNAseq data of the Cancer Cell Line Encyclopedia (CCLE), showing a strong negative correlation between these 2 genes (Fig. 4e, S4b). After splitting the lines into ER-negative and ER-positive subgroups (Fig. S4c), the inverse correlation remained only in the ER-negative subgroup. Although the ER-positive subgroup comprises lower number of samples, further variables, such as low *IL1A* expression in the ER-positive cell lines, may contribute to this outcome.

Recently, ectopic c-Myb expression was induced in a genetically engineered mouse model (GEMM) of spontaneous basal-like BC. In WB1P_Myb (*WapCre;Brca1*$^{F/F}$*; Trp53*$^{F/F}$*;Col1a1*$^{invCAG\text{-}Myb2\text{-}IRES\text{-}Luc/+}$) mice, the mammary-specific expression of Cre is driven by whey acidic protein (*Wap*) and induces mammary-specific inactivation of *Brca1* and *Trp53*, concomitant with the overexpression of *Myb* [55,56]. Spontaneous mammary tumors were analyzed by RNA-sequencing [56] and showed a significant downregulation of *Il1a* expression in the WB1P_Myb mice compared to WB1P control mice (*WapCre;Brca1*$^{F/F}$*; Trp53*$^{F/F}$) (Fig. 4f). This further supports our results of c-Myb directed inhibition of *Il1a*. Together, we showed an inverse pattern of c-Myb and IL1α expression in BC cells, which may reflect their opposing roles in the metastatic capability of BC cells (Fig. 4g).

Discussion

Inspection of the *MYB* expression across BC cell lines revealed that high *MYB* level correlated with diminished constitutive NF-κB activity. Since the decrease of NF-κB activity is linked to ER signaling [57] and c-Myb expression can be under the control of ER in BC [14], we selected several ER-negative BC cell lines for delineating the proposed role of c-Myb in the NF-κB pathway. To verify the potential inhibitory effect of c-Myb, we used human MDA-MB-231 and MDA-MB-436 cells with high constitutive NF-κB activity [47]. c-Myb decreased the activity of NF-κB in both cell lines, in addition, similar results were obtained from murine 4T1 and E0771.LMB cells. The M303V variant of c-Myb, defective in the control of target genes transcription [48], had no effect on transactivation by NF-κB, indicating an indirect suppression of the NF-κB pathway. M303V c-Myb is unable to interact with the CBP/p300 coactivator, resulting in the abrogation of c-Myb-mediated transactivation, and likewise repression of the c-Myb target genes [49,58]. Although the requirement of p300 for the c-Myb-directed transcriptional repression is not fully understood, the outcome of c-Myb/p300 interaction at regulatory elements can be gene-specific [59]. Depending on the MBS position, it can activate transcription of noncoding RNA molecules, thus leading to post-transcriptional repression [48]. Additional regulatory molecules may be essential to facilitate access of the p300 active site [60] or other interacting partners may be involved [61]. Overall, the effect of p300 recruitment to the gene regulatory elements appears to be context-dependent [62,63]. About half of its target genes are estimated to be repressed by c-Myb [48]. We described earlier that c-Myb acts as a potent inhibitor of a specific subset of genes in BC sharing the NF-κB-responsive elements in their regulatory regions [19]. Here, the crosstalk between c-Myb and NF-κB was further supported by decreased transcription of several genes of the previously published NF-κB signature [64] in cells with elevated c-Myb expression.

We assume that NF-κB signaling in ER-negative BC cells can be attenuated by c-Myb-governed inhibition of IL1α expression. While c-Myb significantly decreased transactivation activity of NF-κB in IL1α producing cells, it had no impact on transactivation activity of NF-κB in IL1α-knock-out cells. IL1α is known as an NF-κB signaling activator as well as a direct target, capable of inducing a positive feedback loop [52]. We confirmed that IL1α efficiently activates the NF-κB pathway and overrules the c-Myb inhibitory effect on the expression of the inflammatory signature genes. Previous studies provide evidence of elevated IL1α autocrine and paracrine production by cancer cells of various origins, including breast cancer cells [65,66]. IL1α expression is predominantly found in ER-negative BC [67,68]. There are studies showing an estradiol-mediated decrease of IL1α production by macrophages [69,70]. Therefore, we can speculate that c-Myb works in cooperation with ER in ER-positive BC, importantly, we showed that c-Myb suppression of IL1α occurs in the absence of ER signaling. In a panel of human BC cell lines, we demonstrated an inverse correlation of c-Myb

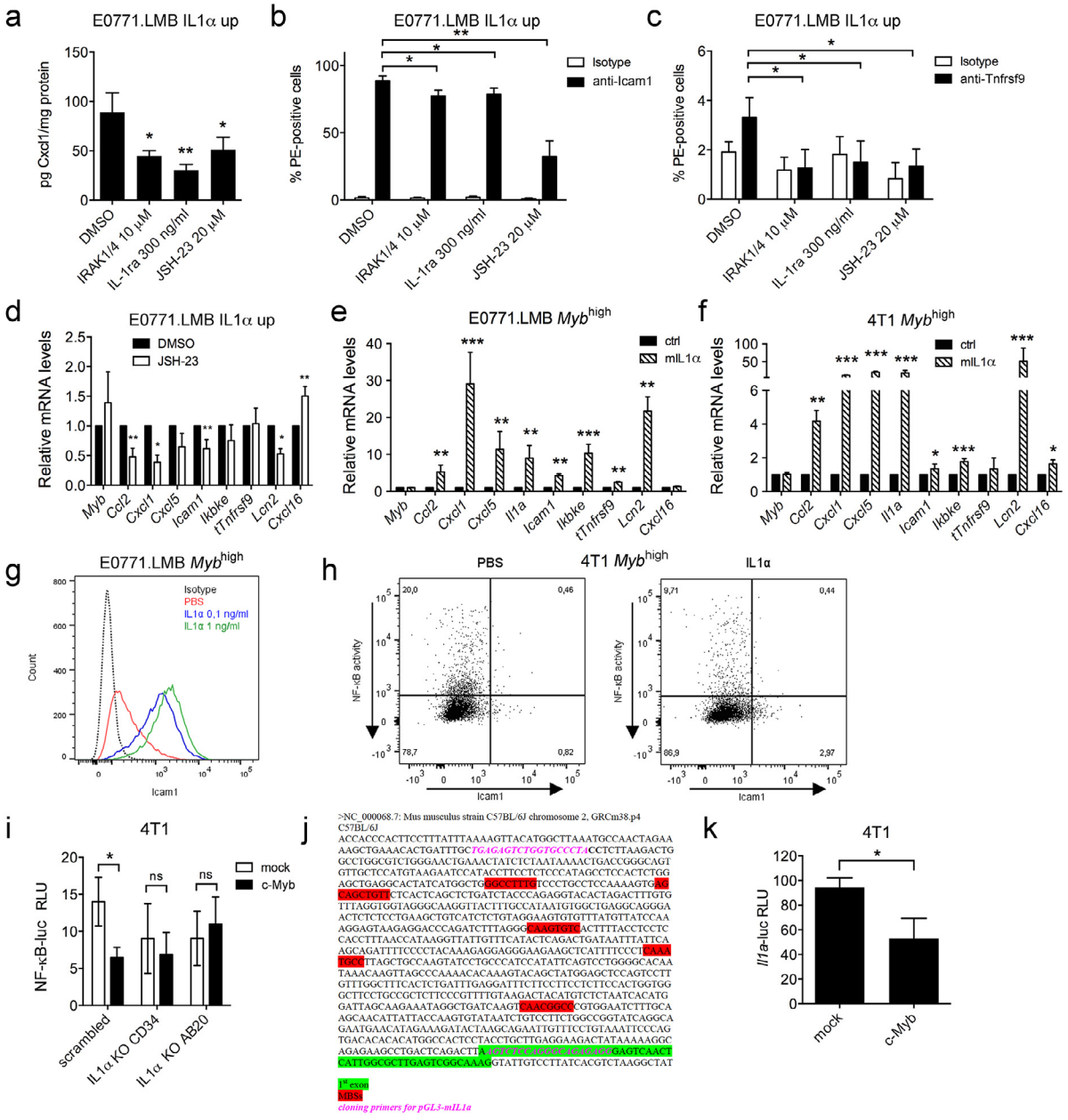

Fig. 3. Suppression of NF-κB and the inflammatory circuit by c-Myb depends on IL1α. IL1α and NF-κB inhibition in IL1α up cells reversed the up-regulation of inflammatory genes: Cxcl1 (A), Icam1 (B), Tnfrsf9 (C) upon treatment with IRAK1/4, IL1-Ra and JSH-23. An average from 3 independent experiments is shown. Indicated concentration of inhibitors were added to E0771.LMB IL1α up 28 cells (IL1α up 28), after 24 h Cxcl1 concentrations were determined by CBA and Icam1 and Tnfrsf9 surface expression was measured by flow cytometry and expressed as frequency of positive cells, comparison to vehicle (DMSO)-treated cells. (D) qPCR detection of signature genes in JSH-23-treated (10 μM, 24 h) E0771.LMB IL1α up cells (IL1α up 28). Values were normalized to *Gapdh* and expressed as a fold change of DMSO-treated cells. An average of 3 independent experiments is shown. E0771.LMB (E) and 4T1 (F) cells overexpressing wt c-Myb (E0071.LMB Myb^{high} C2, 4T1 Myb^{high} 7) supplemented with recombinant mIL1α (1 ng/mL, 24 h) induces the expression of the signature genes. mRNA levels of indicated genes are determined by qPCR relative to vehicle-treated cells (ctrl) and normalized to *Gapdh*. An average of 3 independent experiments is shown. (G) Dose-dependent increase in surface Icam1 upon IL1α stimulation (24 h) of E0771.LMB cells overexpressing wt c-Myb (Myb^{high} C2) as determined by flow cytometry. (H) Surface Icam1 expression is restored by recombinant IL1α in 4T1 cells overexpressing wt c-Myb (Myb^{high} 7) concomitant with loss of IκBα-miRFP703. Myb^{high} 7 cells cotransfected with pEGFP-C1 (GFP) and pIκBα−miRFP703 were treated with 1 ng/mL IL1α for 24h, Icam1 was stained and determined in GFP+ cells by flow-cytometry in parallel with IκBα levels. (I) Two 4T1 IL1α KO clones (IL1α KO CD34 and AB20), and scrambled control cells, were cotransfected with pcDNA3.c-Myb (c-Myb)/pcDNA3 (mock) and the NF-κB reporter. Luciferase activity (RLU) was measured 18 h later and normalized to β-galactosidase activity. An average of 3 independent experiments is shown. (J) Putative MYB-binding sites (MBSs, red) in the murine *Il1a* promoter sequence (980 bp upstream TSS, green). Primer pair used for cloning Il1a-luc reporter construct is indicated in pink. (K) Transactivation assay using 4T1 cells cotransfected with c-Myb or empty vector and Il1a-luc reporter. *Il1a* promoter activity is expressed as luciferase relative light units normalized to total protein levels. An average of 3 independent experiments is shown. Significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) are indicated.

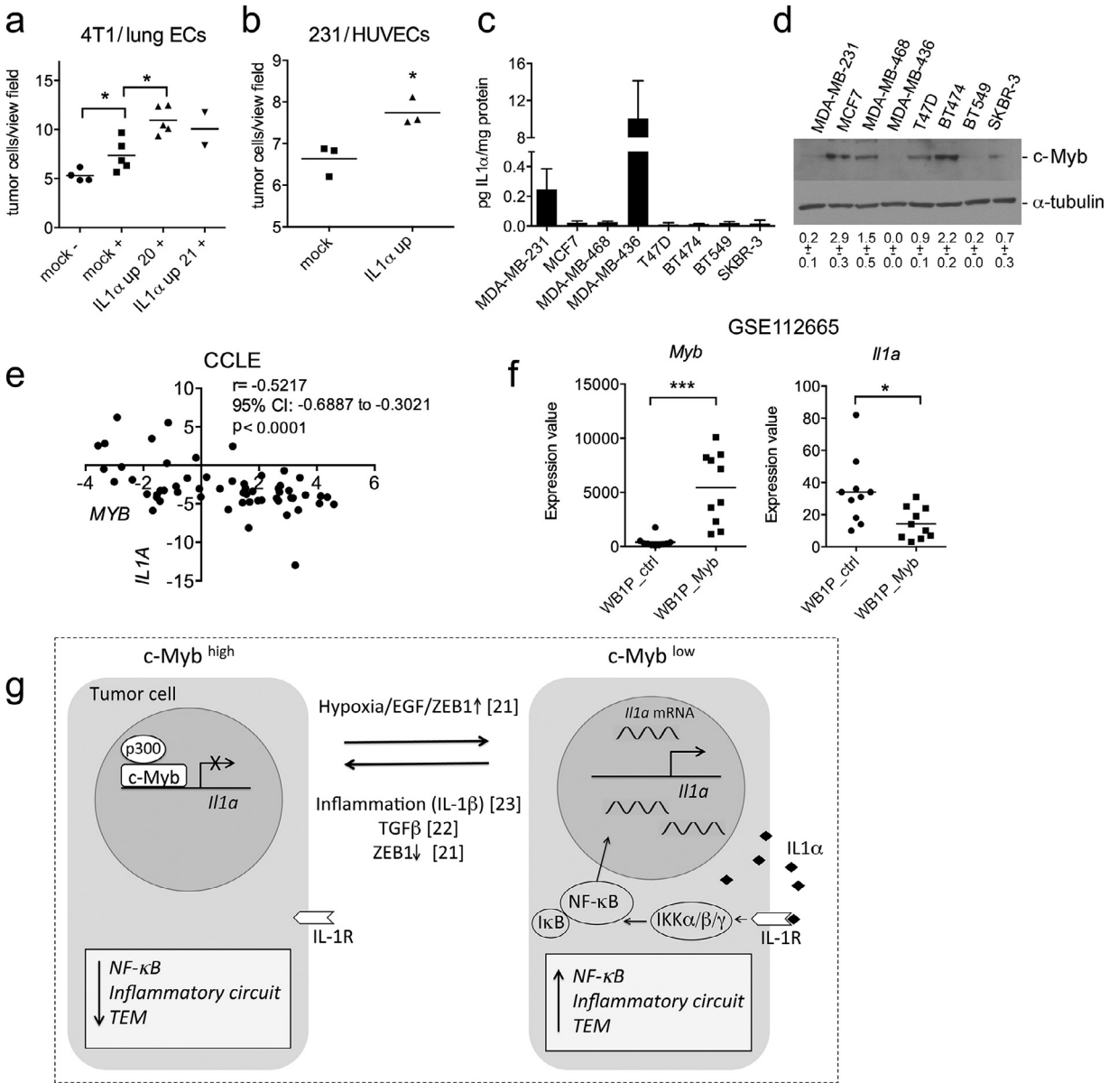

Fig. 4. *IL1A* facilitates transendothelial migration and inversely correlates with *MYB* in BC cells. (A) Monocyte-assisted TEM of 4T1 cells across primary lung ECs *in vitro* is enhanced by ectopic IL1α (IL1α up clones 20 and 21). –/+ bone marrow derived CD115+ monocytes. (B) Human *IL1A* overexpressed by MDA-MB-231 potentiates TEM across HUVECs *in vitro*. (C) Concentrations of IL1α protein in CM from a panel of human BC cell lines as measured by CBA. Average values of 3 independent experiments are shown. (D) Endogenous c-Myb levels in indicated BC cell lines as determined by immunoblotting, α-tubulin was used as a loading control. (E) Inverse correlation between *IL1A* and *MYB* mRNA levels as determined by RNAseq in BC cell lines in a Cancer Cell Line Encyclopedia (CCLE). r = Pearson correlation coefficient, CI = confidence interval. (F) *Myb* and *Il1a* transcript levels in tumors developed in genetically engineered mouse models of BC overexpressing *Myb* (*WapCre; Brca1$^{F/F}$; Trp53$^{F/F}$; Col1a1$^{invCAG\text{-}Myb2\text{-}IRES\text{-}Luc/+}$* mice, "WB1P_Myb") and controls (*WapCre; Brca1$^{F/F}$; Trp53$^{F/F}$* mice, "WB1P_ctrl") as determined by RNAseq involved in GSE112665 [56]. (G) Proposed role of c-Myb protein in the regulation of NF-κB activity in BC. High level of c-Myb repress IL1α expression which in turn leads to a decline in NF-κB activity and TEM ability of the BC cells. Significant differences ($^{*}P < 0.05$, $^{***}P < 0.001$) are indicated.

and IL1α basal protein levels. CCLE showed a strong inverse correlation between *MYB* and *IL1A* transcript levels only in a subgroup of the ER-negative cell lines, highlighting the significance of c-Myb as an inhibitor of IL1α in the absence of ER. We showed that upon overexpression of c-Myb, the ER-negative BC cells strongly decreased the production of IL1α and contrariwise, c-Myb knock-out cells showed increased production of IL1α. c-Myb significantly decreased transactivation of the luciferase gene reporter equipped with the *Il1a* promoter sequence. In line with our hypothesis, *IL1A* has been described as one of the genes repressed by c-Myb in the human monocytic cell line THP-1 [71]. In addition, affinity of c-Myb to the *IL1A* promoter region has been observed by ChIP in ERMYB myeloid progenitor cells [48] and BC cell line MCF7 [72]. Together, we propose a mechanism of NF-κB signaling inhibition in ER-negative BC cells determined by the inhibition of IL1α expression governed by c-Myb.

Numerous studies declare importance of the NF-κB pathway in promotion of the metastatic capability of BC cells [73,74]. Similarly, most

reports declare IL1α expression in highly metastatic BC cells [35,36]. IL1α belongs to the top upregulated secreted proteins in the metastatic triple negative BC cells compared with the nonmetastatic counterpart [37]. In line with this, metastatic MDA-MB-436 and MDA-MB-231 cells were the most potent producers of secreted IL1α. Moreover, overexpression of IL1α provided 4T1, E0771.LMB as well as MDA-MB-231 cells with increased transmigration capacity. Inflammatory cytokines are essential for endothelial activation and tumor cell extravasation [75,76]. c-Myb has a negative impact on the TEM by limiting CCL2 production in BC cells [19]. The release of *Ccl2* transcription in Myb^{high} cells by IL1α and increased *Ccl2* expression in IL1α up cells further underline the opposite effect of IL1α and c-Myb on TEM capability of BC cells. Furthermore, IL1α secreted by BC cells induces the production of thymic stromal lymphopoietin by myeloid cells that serve as a crucial survival factor for cancer cells in the primary lesion and on the site of metastasis, specifically in the lungs [36]. High IL1α levels are connected with shorter distant metastasis-free survival in BC patients. *Vice versa*, IL1α knock-out dramatically reduces tumorigenicity of human BC cells HCC1954 [34]. On the contrary, IL1α acts as a tumor suppressor in a PyMT-driven tumorigenesis model [77]. It is noteworthy that in this PyMT model PyMT/IL1$\alpha^{-/-}$ mice have been used, whereas, in the previously mentioned study, IL1α has been selectively knocked-out in HCC1954 cancer cells. This implies that the impact of the IL1α cytokine may depend on the source of its production or its local concentration in the tumor microenvironment.

Importance of the genetic makeup of breast tumors in dictating prometastatic systemic inflammation has been demonstrated by comparison of immune landscapes of series of GEMMs of BC [56]. Deeper understanding of the interactions between cancer cell-intrinsic genetic events and the immune landscape is required to design personalized immune interventions for cancer patients [78]. In this study, we unraveled a novel c-Myb-IL1α-NF-κB signaling axis that takes place in tumor cells and alters an outward inflammatory network that likely modulates heterotypic cell interactions within the tumor/metastatic microenvironment and dictates the outcome of tumor-stromal cell encounter during dissemination. Overall, we showed that high c-Myb restrains the tumor-driven inflammatory circuit and NF-kB pathway in ER-negative BC cells by transcriptional control over IL1α cytokine, thereby prevents efficient transmigration of tumor cells. While in primary tumor c-Myb may be responsible for diverse functions, its anti-inflammatory action may affect circulating disseminated tumor cells and forestall metastatic cascade. Preclinical studies are needed to validate these findings and set the principles for clinical usage.

Authors' contribution

Dúcka Monika – Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft.

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Červinka Jakub - Data curation.

Biglieri Elisabetta - Data curation.

Šmarda Jan - Writing – review and editing, Resources.

Borsig Lubor - Writing – review and editing, Resources.

Beneš Petr - Writing – review and editing, Resources.

Knopfová Lucia – Conceptualization, Supervision, Visualization, Data curation, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2021.01.002.

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