The Mincle/Syk/NF-κB Signaling Circuit Is Essential for Maintaining the Protumoral Activities of Tumor-Associated Macrophages

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ABSTRACT

Tumor-associated macrophages (TAM) have important roles in cancer progression, but the signaling behind the formation of protumoral TAM remains understudied. Here, by single-cell RNA sequencing, we revealed that the pattern recognition receptor Mincle was highly expressed in TAM and significantly associated with mortality in patients with non–small cell lung cancer. Cancer cells markedly induced Mincle expression in bone marrow–derived macrophages (BMDM), thus promoting cancer progression in invasive lung carcinoma LLC and melanoma B16F10 in vivo and in vitro. Mincle was predominately expressed in the M2-like TAM in non–small cell lung carcinoma and LLC tumors, and silencing of Mincle unexpectedly promoted M1-like phenotypes in vitro. Mechanistically, we discovered a novel Mincle/Syk/NF-κB signaling pathway in TAM needed for executing their TLR4-independent protumoral activities. Adoptive transfer of Mincle-silenced BMDM significantly suppressed TAM-driven cancer progression in the LLC-bearing NOD/SCID mice. By modifying our well-established ultrasound microbubble-mediated gene transfer protocol, we demonstrated that tumor-specific silencing of Mincle effectively blocked Mincle/Syk/NF-κB signaling, therefore inhibiting the TAM-driven cancer progression in the syngeneic mouse cancer models. Thus, our findings highlight the function of Mincle as a novel immunotherapeutic target for cancer via blocking the Mincle/Syk/NF-κB circuit in TAM.

Introduction

Cancer invasion and metastasis depend on cancer cells and the tumor microenvironment (TME; refs. 1–4). Cancer cell–derived cytokines trigger the establishment of a protumoral microenvironment, thus preventing immune surveillance (5, 6). Increasing evidence suggests that tumor-associated macrophages (TAM) are one of the predominant stromal cell types in the TME that promote cancer progression (7). TAMs are able to facilitate the survival, proliferation, extracellular matrix integrity, invasion, angiogenesis, and metastasis of tumors via indirect cross-talk with cancer cells (8–10). A better understanding of the underlying mechanisms of TAM-driven tumor promotion may reveal new cancer treatment strategies (11–13).

C-Type Lectin Domain Family 4, Member E (Clec4e, also called Mincle) is expressed by inflammatory macrophages and plays a key role in acute kidney injury (14). Adoptive transfer of Mincle-silencing macrophage significantly reduces renal inflammation in two mouse kidney injury models induced by cisplatin and unilateral ureteral obstruction (14). Mincle also involves in the development of subarachnoid hemorrhage and autoimmune disease of the eye (15, 16). Its pathogenic roles are demonstrated in a number of cancers including pancreatic cancer and urethelial cancer (17, 18); however, there is no specific inhibitor available for targeting Mincle, limiting its further development as a potential anticancer therapy.

By single-cell RNA sequencing (scRNA-seq), we uncovered a Mincle+ TAM subset in two syngeneic mouse cancer models that significantly associated with the mortality of patients with non–small cell lung carcinoma (NSCLC). Mincle was highly expressed in M2 TAM, rather than in M1 seen in kidney disease models (14), and contributed to the suppression of M1 phenotypes. We discovered a novel Mincle/Syk/NF-κB signaling circuit in TAM that promoted cancer invasion and metastasis. Adoptive transfer of Mincle-silenced bone marrow–derived macrophage (BMDM) abrogated the TAM-driven cancer growth in the macrophage-malfunctional NOD/SCID mice. Using ultrasound microbubble (USMB)–mediated gene transfer technology (19–21), we demonstrated that tumor-specific silencing of Mincle effectively blocked the progression of both LLC and B16F10 tumors in mice. Our results identified a role of Mincle in TAM regulation, and the novel Mincle/Syk/NF-κB signaling circuit may represent a promising target for cancer immunotherapy.

Materials and Methods

Patient samples

Frozen sections of eight liver and eight lung cancer biopsies were collected from Prince of Wales Hospital, the Chinese University of Hong Kong.
Hong Kong, Hong Kong SAR, and ten oral squamous cell carcinoma biopsies were collected from West China Hospital of Stomatology, Sichuan University, China. Archival formalin-fixed paraffin-embedded (FFPE) tissue specimens were retrieved from 90 male patients with lung carcinoma aged between 50 and 70 who underwent colectomy at the Prince of Wales Hospital, Hong Kong SAR. The study was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all the patients. This study was approved by the Ethics Committee of the corresponding hospitals and the Clinical Research Ethics Committee of Joint Chinese University of Hong Kong-New Territories East Cluster (CREC Ref. No.: 2019.368 and 2018.054).

**Animals**

C57BL/6j and NOD/SCID (8- to 10-week-old) mice were purchased from The Chinese University of Hong Kong Laboratory Animal Services Centre. All experimental procedures were approved by the Animal Ethics Experimental Committee of The Chinese University of Hong Kong (AEEC Ref No.: 18-241-ITF).

**ScRNA-seq data analysis**

The human NSCLC dataset in cloupe format was downloaded from the 10X Genomic website (https://support.10xgenomics.com/single-cell-gene-expression/datasets). Mincle CD68+ and Mincle CD68− cells were clustered with the expression of Mincle and CD68 by cutoff > 2.0 using Loupe Cell Browser 3.0.1 version. The gene expression matrix was generated by Cell Ranger 3.0.2 for coexpression analysis. The public available dataset of 31 human melanoma tumors (GSE115978) was downloaded from Gene Expression Omnibus database for visualizing Mincle+ and CD68+ cells with R 3.6.0 and Seurat 3.1.1. The unbiased gene regulatory networks were constructed based on differentially expressed genes of Mincle+ TAM from the 10X scRNA-seq by using MetaCore analytical suite (version 4.2 build 8168; GeneGo), a Web-based suite for functional analysis of experimental data in the context of manually collected protein interactions, canonical pathways, and knowledge base ontologies of biological functions and diseases (22).

**Cancer cell culture**

The murine lung adenocarcinoma LLC (CRL-1642, ATCC) and melanoma B16F10 (CRL-6475, ATCC) were obtained from the ATCC in 2016. The luciferase overexpression stable cell lines of LLC and melanoma B16F10 (CRL-6475, ATCC) were obtained from the ATCC previously described (28). In brief, after blocking nonspecific binding, primary antibodies against GAPDH (Millipore, MAB374), Mincle (Abcam, ab3993), phospho-Syk (Abcam, ab58575), p65 (Cell Signaling Technology, #6956), and phospho-p65 (Abcam, ab47395) and IRDye800-conjugated secondary antibodies (Rockland Immunocchemicals). Signals were detected by Odyssey imaging system (LI-COR Biosciences), and results were further quantified by Image J. The ratio of protein expression was normalized to GAPDH expression.

**Primary culture of BMDM**

For primary culture of BMDM, bone marrow cells were harvested from 8- to 10-week-old C57BL/6j mice as previously done (23–25). In brief, bone marrow cells were harvested from femur and tibia via fine dissection. Red blood cells were lysed, and the remaining bone marrow cells were cultured in DMEM/F12 medium with 10% heat-inactivated FBS, 1% penicillin and streptomycin, and 50 ng/mL M-CSF (Invitrogen) in 5% CO2 at 37°C. BMDM were harvested after 7 days of M-CSF-mediated macrophage differentiation. In TAM stimulation experiments, BMDM were treated with DMEM/F12 (negative control), 10% LLC-CM in DMEM/F12 (LLC-CM group), or 10% B16F10-CM in DMEM/F12 (B16F10-CM group). For signaling experiments, DMSO (negative control), 1 ng/mL Cli-095, 0.5 μmol/L Bay11–7082, and 25 μmol/L piceatannol (PIC) were used for 1 hour prior to experiments (the inhibitor was optimal for 100% cell viability of the treated BMDM). For in vitro knockdown assays, 50 nmol/L of nonsense control (NC) or Mincle-specific siRNA (siMincle; sense 5′-CCUUAGAACUGGAAACAUUTT-3′, antisense 5′-AUGUAAAGGUAUGCCUUGT-3′; GenePharma) were transfected into BMDM with lipofectamine RNAiMAX (Invitrogen) 24 hours prior to experiments (14).

**Cell invasion and metastasis assays**

B16F10 melanoma cells were treated with 50% conditioned media derived from B16F10-CM–treated BMDM (TAM-CM) and received NC or siMincle treatment for additional 24 hours. For studying the role of IL6, IL6 (2 ng/mL), IL6-neutralizing antibody (0.25 μg/mL), or control IgG (0.25 μg/mL) were added prior to the TAM-CM treatment. Cell migration was detected via wound healing assay (26), and cell invasion was detected by transwell invasion assay (27).

**Flow cytometry analysis**

Total tumor tissues were digested by Trypsin-EDTA (Roche Inc.) into cell suspension and fixed with 1% Fixation Buffer (eBioscience) for 30 minutes as previously described (13). Cells then were stained with Mincle antibody (Santa Cruz Biotechnology; sc-161489) that was FITC-conjugated by using the Lighting-Link fluorescein kit (Innova Biosciences 707-0030) and PE-conjugated anti-CD68 (Biolegend 137014). Isotype control antibodies were used as negative controls. After being extensively washed, single cells were analyzed by FACSCalibur flow cytometer (BD Biosciences) as described previously (14).

**Western blot analysis**

Protein from cultured cells and tumor tissues was extracted using the RIPA lysis buffer (Santa Cruz Biotechnology) according to the manufacturer’s instructions. Western blot analysis was performed as previously described (28). In brief, after blocking nonspecific binding with 5% BSA, membranes were incubated overnight at 4°C with primary antibodies against GAPDH (Millipore, MAB374), Mincle (MBL, D292-3), iNOS (Abcam, ab3523), Syk (Abcam, ab3993), phospho-Syk (Abcam, ab58575), p65 (Cell Signaling Technology, #6956), and phospho-p65 (Abcam, ab47395) and IRDye800-conjugated secondary antibodies (Rockland Immunocchemicals). Signals were detected by Odyssey imaging system (LI-COR Biosciences), and results were further quantified by Image J. The ratio of protein expression was normalized to GAPDH expression.

**Real-time PCR analysis**

Total RNA was extracted using Trizol reagent (Molecular Research Center) according to the instructions of the manufacturer, and RNA concentration was measured by Nanodrop. For each reaction, 20 ng of total RNA was used as the template, and RNA expression was quantified by real-time PCR using SYBR Green Supermix (Life Technologies) with three replicates (14, 29, 30). Primers are listed in Supplementary Table S1. The relative expression of detected genes was normalized against internal control GAPDH and calculated by the 2−ΔΔCt method.
Histology, opal multiplex IHC, and immunofluorescence

Tissue microarray (TMA) from Department of Anatomical and Cellular Pathology in The Chinese University of Hong Kong was performed on 5 μm FFPE tissue sections from human lung carcinoma samples and stained with the antibody against Mincle (Santa Cruz Biotechnology, sc-390806; 1:100). The protein expression on the TMA slides was calculated based on the histoscore (H-score) method. Samples were imaged on the Ny-u Light Microscope (Nikon) and analyzed by Aperio ImageScope (Leica Biosystems).

For Opal multiplex IHC, TMA sections were treated with endogenous horseradish peroxidase blocking and heat-induced epitope retrieval in citrate buffer. Primary antibodies were incubated overnight at 4°C, and the fluorescence was developed using OPAL 4-color IHC kit (NEL820001KT, Perkin Elmer) according to the manufacturer's protocol. Samples were imaged on the Mantra quantitative pathology workstation (Perkin Elmer) and analyzed by inForm image analysis software (Perkin Elmer).

Immunofluorescence was performed on 5 μm fresh tissue sections from human tumors, mouse tumors, and spleen tissues and stained with antibodies against Mincle (Santa Cruz Biotechnology, sc-390806 Alexa 546/Alexa 488) and CD68 (Biolegend 137012/137014). Antibodies were diluted to be 1:100 in staining buffer (eBioscience 00-4222-57) and applied on the samples at 4°C for overnight. The unbound antibodies were washed out with PBST 3 times and then the samples were sealed with DAPI mounting buffer (Invitrogen S36938). All stained samples were imaged under a fluorescence microscope (Axio Observer.Z1; Carl Zeiss; ref. 19).

Enzyme-linked immunosorbent assay

Medium from stimulated BMDM or serum from tumor-bearing mice was collected to detect the cytokine production using the ELISA kit as previously described (4, 28). IL6 (R&D Systems M6000B) was measured according to the instructions of the manufacturer.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed with the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, #9003S) as previously described (24). In brief, immunoprecipitation was performed with the antibody against NF-κB subunit p65 (Cell Signaling Technology, #6956) or a normal IgG as the negative control. Precipitated DNA fragments were detected by PCR using a specific primer of the promoter region of Mincle that was predicted by CellMiner (Table S1) and applied on the samples at 2 W/cm² for a total of 5 minutes). To maintain the transgene expression, mice were transfected with EV or shMincle plasmid on days 4, 7, and 10 and sacrificed on day 13 for the B16F10 melanoma model and on days 7, 12, and 17 and sacrificed on day 22 for the LLC lung carcinoma model. The schedule difference was due to the much faster tumor progression of B16F10.

Adoptive transfer of Mincle-silencing BMDM

The adoptive transfer model was induced by subcutaneous inoculation of 1 × 10⁶ of LLC or B16F10 cells into syngeneic male NOD/SCID mice. Eight mice for each group were measured, and tumor-bearing mice were sacrificed on day 18.

USMB-mediated gene transfer

USMB-mediated gene transfer was performed as previously described (4, 19). The syngeneic mouse models were induced by subcutaneous inoculation with 1 × 10⁶ LLC or B16F10 cells into the back of 8-week-old male or female C57BL/6j mice.

For tumor-specific Mincle knockdown in vivo, 8 mice for each group were treated with pSuper.puro empty vector (EV) or shMincle recombinant plasmid via USMB-mediated gene transfer according to a modified protocol from our previous studies (19–21). In brief, each mouse was intravenously injected 250 μL mixed microbubble (SonoVue, Bracco Suisse SA) solution containing 100 μg of EV or shMincle and immediately treated using an ultrasound transducer (Therasonic, Electro Medical Supplies) by directly placing the transducer on the skin of the back against the tumor (with the output of 1 MHz at 2 W/cm² for a total of 5 minutes). To maintain the transgene expression, mice were transfected with EV or shMincle plasmid on days 4, 7, and 10 and sacrificed on day 13 for the B16F10 melanoma model and on days 7, 12, and 17 and sacrificed on day 22 for the LLC lung carcinoma model. The schedule difference was due to the much faster tumor progression of B16F10.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 5 (GraphPad Software). All data were presented as mean ± SEM. Statistical significance was determined by P < 0.05 in the Student t test or one-way or two-way ANOVA.

Results

Mincle was highly expressed in TAM and associated with NSCLC mortality

Mincle plays an important role in macrophage modulation (31, 32), but its function in TAM is largely unexplored. By analyzing a publicly available 10X scRNA-seq dataset of human NSCLC biopsy from 10X Genomics, we found a Mincle+- TAM (red) subset that has a distinguishable transcriptome profile compared with the Mincle-CD68+ cells (Fig. 1A). Our internal cohort showed that both stromal and tumoral expression of Mincle significantly associated with poor disease-specific survival in patients with NSCLC (n = 90, log-rank test P = 0.0151, Fig. 1B). Mincle+- TAMs were positively correlated with the expression level of Mincle in cancer cells by Opal multiplex IHC system (Fig. 1C). This Mincle+- TAM subset was also found in patients with melanoma, NSCLC, hepatoma, and oral squamous cell carcinoma by immunofluorescence and scRNA-seq (Fig. 1D and E; Supplementary Fig. S1).

Cancer condition induced Mincle expression in TAM

We next investigated the role of Mincle in TAM-mediated cancer progression by using two syngeneic mouse models bearing lung carcinoma LLC or melanoma B16F10. Mincle dramatically increased TAM but not splenic macrophages in tumor-bearing mice (Fig. 2A). Mincle expression was largely increased in the TAM of LLC and B16F10 tumors compared with the splenic macrophages of the B16F10-bearing mice analysis (Fig. 2B), implying a TME-specific mechanism for inducing Mincle expression in TAM.

To investigate how the TME promoted Mincle expression in TAM, CD68+ macrophages were sorted from the B16F10 tumors. mRNA expression of Mincle was dramatically increased in TAM but low in the splenic macrophages and CD68+ stromal cells from B16F10 tumors (Fig. 2C). Lipopolysaccharide (LPS) did not further increase Mincle expression in the flow-sorted splenic macrophages (Supplementary Fig. S2), implying that expression of Mincle maybe specifically induced by the TME. To prove this hypothesis, we stimulated BMDM in vitro with 10% of cancer conditioned medium derived from either lung carcinoma LLC (LLC-CM) or melanoma B16F10 (B16F10-CM). LLC- and B16F10-CM markedly increased both mRNA and protein expression of Mincle in the BMDM (Fig. 2D and E). More than 95% of BMDMs expressed Mincle compared with the untreated controls.
Figure 1.
Figure 2.
TME-driven Mincle expression in macrophages. Immunofluorescence (A) and flow cytometry (B) of Mincle+/macrophages in spleen and tumor of B16F10-bearing mice on day 10 and LLC tumor on day 15, respectively, in vivo. C, Mincle expression in TAM, splenic macrophages, and CD68-negative cells of B16F10-bearing mice on day 10 in vivo detected by real-time PCR. D–F, Mincle expression in BMDM under stimulation with cancer conditional medium (10% of B16F10- or LLC-CM) in vitro found via real-time PCR (D), Western blotting (WB; E), and flow cytometry (F) analysis. B and C, Data represent mean ± SEM of 5 mice/group, one-way ANOVA, ***, P < 0.001 versus all other groups and ###, P < 0.001 versus splenic macrophages. D and E, Data represent mean ± SD for three independent experiments, two-way ANOVA. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus control. Scale bars, 50 μm.
Mincle promoted TAM-mediated cancer progression in vivo and in vitro. A, Tumor volume and growth curve of LLC-bearing NOD/SCID mice that received BMDM pretreated with siMincle or NC. B, Mincle expression in TAM of the LLC tumors by immunofluorescence. Real-time PCR (C) and Western blot (D) analysis of Mincle expression in siMincle-or NC-pretreated BMDM after 24-hour B16F10-CM stimulation. Wound healing (E) and transwell (F) assays of B16F10 melanoma cells under stimulation with conditioned medium obtained from B16F10-CM–stimulated BMDM pretreated with siMincle or NC in vitro.

A and B, Data represent mean ± SEM of 5 mice/group, one-way ANOVA: *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001 versus control; #, *P < 0.05 and ###, *P < 0.001 versus NC-BMDM. C–F, Data represent mean ± SD for three independent experiments, two-way ANOVA: *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001 versus control; #, *P < 0.01 and ###, *P < 0.001 versus NC group. Scale bars, 50 μm.
Figure 4.

Mincle facilitated M2 polarization of TAM by suppressing M1 phenotypes. A and B, Mincle expression of human CD163⁺ and mouse CD206⁺ M2-like TAM in NSCLC and LLC tumor detected by flow cytometry analysis. C and D, Level of M1 marker (iNOS) and effectors (MCP-1 and TNFα) in Mincle-knockdown BMDM (siMincle) after 24 hours of LLC-CM stimulation in vitro found by Western blot and real-time PCR. E, Expression of the M1 marker iNOS and M2 marker CD206 in TAM of LLC-bearing NOD/SCID mice that received Mincle-silenced or NC-treated BMDM revealed by immunofluorescence. A and B, Data represent mean ± SEM of 5 mice/group or 5 NSCLC biopsies, t test. ***, P < 0.001 versus M0/M1 or M1 TAM. C and D, Data represent mean ± SD for three independent experiments, two-way ANOVA. **, P < 0.01 and ###, P < 0.001 versus control; ##, P < 0.01 and ###, P < 0.001 versus siMincle group under LLC-CM stimulation. E, Data represent mean ± SEM of 5 mice/group, one-way ANOVA. **, P < 0.01 and ***, P < 0.001 versus control; #, P < 0.05 and ##, P < 0.01 versus NC-BMDM. Scale bars, 50 μm.
Mincle promoted TAM-mediated cancer progression in vivo and in vitro

Gene ontology analysis showed that the differentially expressed genes (DEG) of Mincle− TAM significantly contributed to carcinogenesis (Supplementary Fig. S3). To examine the functional role of TAM-specific Mincle in cancer progression, we employed the macrophage adoptive transfer approach used in our previous studies (4, 28) in a model of lung carcinoma induced in macrophage-deficient NOD/SCID mice. Adoptive transfer of BMDMs (NC-BMDM) dramatically promoted the growth of LLC tumors in NOD/SCID mice compared to the controls that only received LLC cells; cancer progression was largely suppressed in mice receiving siMincle-BMDMs (Fig. 3A and B). Silencing of Mincle showed no significant effects on the proliferation of both LLC and B16F10 cancer cells in vitro (Supplementary Fig. S4), suggesting that Mincle expression in TAM drove cancer progression.

We next investigated the role of Mincle in TAM-mediated cancer promotion in vitro. As shown in Fig. 3C and D, we successfully blocked the B16F10-CM–induced Mincle expression in BMDM by siRNA-mediated gene silencing (siMincle) in vitro. Conditioned media derived from the B16F10-CM–stimulated BMDM (TAM-CM, NC) significantly enhanced the migration and invasion of B16F10 cancer cells compared with the conditional medium derived from BMDM (BMDM-CM, NC; Fig. 3E and F). The cancer-promoting effects of TAM-CM were significantly reduced when Mincle was knocked down in the B16F10-CM–stimulated BMDM (TAM-CM, siMincle). Our findings demonstrated the importance of Mincle in promoting TAM-mediated cancer invasion and metastasis.

Mincle facilitated M2 polarization of TAM by suppressing M1 phenotypes

We characterized the Mincle− TAM subset in NSCLC and found that Mincle+ TAMs were predominately M2-like, accounting for >95% of CD163+ cells in NSCLC and >85% murine CD206+ cells in LLC tumor, respectively (Fig. 4A and B). As Mincle+ macrophages are M1-like in kidney disease models via a toll-like receptor–dependent mechanism (14, 34), we investigated the specific role of Mincle in TAM. Unexpectedly, silencing of Mincle dramatically enhanced expression of M1 phenotypes (iNOS, MCP-1, and TNFα) in the LLC-CM–stimulated BMDM at 24 hours in vitro and in the LLC tumor of NOD/SCID mice that received siMincle-BMDM in vivo (Fig. 4C–E). In contrast, there were relatively mild changes detected in M2 phenotypes in vitro (Supplementary Fig. S5), suggesting that Mincle may be responsible for promoting M1/M2 polarization of TAM by suppressing M1 phenotypes in the TME.

Mincle enhanced the protumoral activity of TAM via the Syk/NF-κB/B/IL6 axis

To identify the mechanism of Mincle in TAM-mediated cancer promotion, we elucidated the 10X scRNA-seq data of NSCLC in Fig. 1A with unbiased network and enrichment analysis (24). By using the DEG of Mincle− TAM in NSCLC, a regulatory gene network enriched with NF-κB signaling pathway was reconstructed by MetaCore bioinformatic platform (Fig. 5A; ref. 24). We detected that both the NF-κB p65 subunit and Syk were substantially expressed in the Mincle− TAM (Fig. 5B), in line with other studies suggesting that Mincle expression is associated with the activation of Syk and NF-κB during inflammation (35, 36). The Mincle ligand SAP130 activated Syk and synergistically enhanced the phosphorylation of NF-κB p65 subunit in the LLC-CM–stimulated BMDM in vitro (Fig. 5B and Supplementary Fig. S6), uncovering a role of Mincle/Syk signaling in NF-κB activation of TAM.

To investigate the function of this Mincle/Syk/NF-κB pathway in TAM, we examined the potential regulatory role of Mincle in the direct NF-κB–targeted genes by PCR array in vitro. We found Mincle knockdown altered the expression of NF-κB–dependent genes in the B16F10-CM–stimulated BMDM, where a protumoral inflammatory cytokine IL6 was the most suppressed (Fig. 5C). IL6 is a cancer promoter (21, 22) mainly produced by TAM (23) and recognized to be a prognostic factor for various cancers (24, 25). We confirmed that the silencing of Mincle dramatically blocked the IL6 production from B16F10-CM–stimulated BMDM (Fig. 5D). The addition of IL6 rescued the loss of protumoral activities in the TAM-CM derived from Mincle-silenced BMDM in vitro (Fig. 5E and F). The inhibition of Syk and NF-κB with specific inhibitors PIC and Bay11-7082 substantially reduced the Mincle-dependent IL6 production in BMDM under B16F10-CM stimulation (Fig. 5G–I). Thus, Mincle facilitated TAM-mediated cancer promotion through the Syk/NF-κB/IL6 inflammatory pathway, and Mincle may represent a new therapeutic target for invasive cancers.

A TLR4-independent Mincle/Syk/NF-κB circuit in TAM

We found that blockade of Syk and NF-κB, downstream of Mincle signaling, altered the B16F10-CM–induced Mincle expression in BMDM in vitro (Fig. 6A and B). Both TLR4 activator HMBG1 and inhibitor CL1-095 (23, 37) did not affect Mincle expression in B16F10-CM–stimulated BMDM (Fig. 6C and Supplementary Fig. S7), although Mincle is reported as the downstream of TLR4 signaling under LPS stimulation (14, 38). To dissect this underlying mechanism, we first demonstrated that the silencing of Mincle effectively inhibited B16F10-CM–induced Syk and NF-κB activation in BMDM and confirmed Syk and NF-κB p65 were the downstream targets of Mincle.

Figure 5.

Mincle promoted Syk/NF-κB pathway–mediated protumoral activity of BMDM in vitro. A, Regulatory gene network of Mincle− TAM in NSCLC predicted by MetaCore bioinformatics. B, Expression of Syk and p65 NF-κB subunit in Mincle− and Mincle+ CD68 cells determined via in-house bioinformatic analysis, and BMDM under LLC-CM stimulation in vitro. C, The mRNA expression profile of NF-κB-targeted genes in B16F10-CM–stimulated BMDM with or without Mincle knockdown at 24 hours revealed by PCR array. The columns represent the relative expression fold change, where only genes with mRNA fold change over 1.5 or below 0.67 are presented. D, Production of IL6 from B16F10-CM–stimulated BMDM detected by ELISA. Wound healing (E) and transwell (F) assays of B16F0 melanoma cells under stimulation with conditioned medium of B16F10-CM–stimulated BMDM (TAM-CM) in vitro. Real-time PCR (G and H) and ELISA (I) detected mRNA expression and secretion of IL6 in BMDM under B16F10-CM stimulation with inhibitor of Syk (PIC) or NF-κB (Bay11-7082) in vitro. Data represent mean ± SD for three independent experiments: one-way (B, E, F, and D) and two-way (G, H, and I) ANOVA, *P < 0.01 and **P < 0.001 versus control; ###P < 0.001 versus B16F10-CM–stimulated NC group. E and F, *P < 0.05 and **P < 0.001 versus NC group; #P < 0.05 and ##P < 0.01 versus siMincle group. G–I, ***P < 0.001 versus control; ###P < 0.001 versus B16F10-CM–stimulated DMSO control.
A novel Mincle/Syk/NF-κB signaling circuit in TAM. BMDM were pretreated with Syk (PIC), NF-κB (Bay11-7082), or TLR4 (Cli-095) inhibitors 1 hour prior to the B16F10-CM stimulation. A–C, Mincle expression in BMDM under B16F10-CM stimulation at 6 hours. D, Activation of Syk and NF-κB in B16F10-CM–stimulated BMDM pretreated with siMincle or NC at 24 hours detected by Western blot. E, Mincle level in B16F10-stimulated BMDM under inhibition of NF-κB, Syk, or TLR4 at 24 hours shown by Western blot. F, Direct binding of NF-κB p65 subunit on Mincle promoter region in BMDM under B16F10-CM stimulation shown by ChIP assay. Data represent mean ± SD for three independent experiments, two-way ANOVA. ***, P < 0.001 compared with control group; #, P < 0.05 and ###, P < 0.001 compared with NC or DMSO group under B16F10-CM stimulation.
USMB-shMincle effectively inhibited progression of B16F10 and LLC in mice. B16F10 tumor–bearing mice were treated with EV (USMB-EV) or a plasmid containing the Mincle shRNA sequence (USMB-shMincle) on days 4, 7, and 10. A, Bioluminescence imaging of B16F10–bearing mice, tumor volume, and weight on day 13. B, Western blot examined Mincle expression in B16F10 tumor on day 13. ELISA detected IL6 (C) and tumor growth (D) of mice bearing B16F10 tumor. LLC tumor–bearing mice were treated with USMB-EV or USMB-shMincle on days 7, 12, and 17. E, Bioluminescence imaging of LLC–bearing mice, tumor volume, and weight on day 22. Real-time PCR (F) and flow cytometry (G) analysis detected Mincle expression in the LLC tumors on day 22. ELISA detected serum IL6 (H) and tumor growth (I) of mice bearing LLC tumor. Data represent mean ± SEM of 5 mice/group, t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with USMB-EV control group.
USMB-shMincle effectively inhibited tumor progression in mice

As there is no synthetic molecule or antibody commercially available, we further developed a virus-free gene therapy, USMB-shMincle, for silencing Mincle in a tumor-specific manner in vivo. By modifying our well-established USMB-mediated platform (19–21), we effectively delivered our constructed plasmid specifically to tumor tissues in mice (Supplementary Fig. S9). USMB delivery of pSuper.puro plasmid expressing Mincle-specific shRNA (USMB-shMincle) dramatically blocked the growth of B16F10 tumors compared with the EV control group (USMB-EV; Fig. 7A). USMB-shMincle effectively silenced Mincle in B16F10 tumors compared with the USMB-EV group (Fig. 7B), resulting in a significant reduction of serum IL6 in the treated mice; this associated with a decelerated tumor growth (Fig. 7C and D). We next examined the anticancer efficiency of USMB-shMincle on the mice with syngeneic lung carcinoma LLC, and we found that USMB-shMincle significantly suppressed the progression of LLC tumor growth with the USMB-EV control group (Fig. 7E). USMB-shMincle effectively silenced Mincle in the LLC tumor, especially on TAM (Fig. 7F and G). In line with the B16F10 tumor data, USMB-shMincle significantly reduced IL6 production and suppressed the growth of LLC tumor in mice (Fig. 7H and I). Thus, we successfully developed USMB-shMincle for blocking the protumoral Mincle/Syk/NF-κB signaling circuit in TAM (Supplementary Fig. S10), which may represent a therapeutic strategy for invasive cancers.

Discussion

Mincle is highly expressed in TAM, but its regulatory mechanism and functional role in cancer promotion are still largely unstudied (17, 18). Here, we demonstrated that Mincle is involved in promoting the protumoral activity of M2-like TAM by suppressing M1 phenotypes. As a Mincle-specific inhibitor is not available worldwide, we developed a tumor-specific Mincle-targeted therapy USMB-shMincle and demonstrated its anticancer efficiency on two syngeneic mouse cancer models. Mechanistically, we discovered a novel TLR4–independent Mincle/Syk/NF-κB circuit in TAM for maintaining protumoral activity. Targeting of Mincle effectively cut this signaling circuit, significantly suppressing the TAM-mediated cancer progression. Thus, USMB-shMincle may represent an effective immunotherapy for cancer.

Mincle is expressed on a number of immune cells during inflammation, including macrophages (34, 39), dendritic cells (40), neuronal, and endothelial cells (41). In tumors, Mincle is expressed on some types of cancer cells and leukocytes including TAM (17, 18). Here, Mincle was predominantly expressed in the TAM of murine melanoma and patients with lung, liver, or oral cancer. Half of the Mincle-positive cells were TAMs, with neutrophils as the second most abundant cell type to express Mincle. Neither B16F10 nor LLC murine cancer cells expressed Mincle in the animal models, which differed from observations in urothelial cancer cells (18); however, the B16F10 melanoma is an ideal platform for studying the role of Mincle in TAM-driven cancer progression. Although there might be differences in the function and ligands of Mincle between human and mice (42, 43), our data indicated that mouse is an appropriate model to explore function of Mincle in the TME (44).

The functional role of Mincle in cancer development is still largely unexplored. Mincle has an immunoregulatory role in pancreatic oncogenesis (17). SAP130 released from pancreatic ductal adenocarcinoma can bind on Mincle in the basal cells (45), where deletion of Mincle attenuates oncogenesis and immunogenic reprogramming of the TME. Here, Mincle was highly expressed in M2-like TAM and was essential for maintaining their protumoral activities via increasing the production of proinflammatory cytokines (e.g., IL1β, IL6, CSF-2, etc.). These cytokines are not only involved in immunomodulation (46), but also play important roles in the cancer progression (47, 48). TAM-derived IL6 supports tumor progression in several settings (49–51). Mincle was also responsible for suppressing the M1 phenotypes of BMDM, thus enhancing the M2 phenotypes of TAM. BMDM-specific knockdown of Mincle effectively inhibited the TAM-mediated tumor growth in NOD/SCID mice associated with reduction of M1/M2 transition in vivo, and Mincle was essential in maintaining the TAM-mediated cancer migration and invasion in vitro. Thus, Mincle is crucial in M2 TAM-mediated tumor progression.

Although a number of TLR4 activators are in the TME (52), we found no evidence supporting the link between TLR4 and TME-induced Mincle expression. Instead, Mincle was directly regulated in BMDM by Syk/NF-κB signaling in a TLR4-independent manner in the TME, which led to the Mincle/Syk/NF-κB circuit in TAM; this idea is supported by various studies showing that Mincle could be activated via Syk and NF-κB (53, 54). This novel circuit explains why targeting Mincle largely suppressed the protumoral activity of TAM, resulting in a significant anticancer effect in vivo.

We developed a virus-free gene therapy for targeting Mincle as no commercial specific inhibitor was available. In brief, the microbubble-capsule plasmid expressing Mincle shRNA was specifically and effectively released at the tumor site by ultrasound guidance, which was modified according to our well-established protocol on the renal disease models (19–21). There was a significant reduction of Mincle in the TME, especially in TAM, after USMB-shMincle, which associated with a significant reduction in tumor size. We found that tumor-specific knockdown of Mincle effectively reduced serum IL6 in mice, and this was associated with slowed tumor growth. Mincle was essential for maintaining the TAM-mediated cancer migration and invasion in an IL6-dependent manner in vitro. Mincle/Syk/NF-κB circuit was important for maintaining the protumoral activity of M2-like TAM, with USMB-shMincle effectively abrogating this signaling circuit and resulting in tumor regression. Whether Mincle/Syk/NF-κB circuit commonly occurs in NSCLC and if it is a viable treatment target remain to be seen. Regardless, USMB-shMincle may represent as a novel immunotherapeutic strategy for cancer.

Disclosure of Potential Conflicts of Interest

P.M.-K. Tang reports a U.S. patent 62/987,079 that is pending. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

C. Li: Conceptualization, data curation, investigation, methodology, writing–original draft. V.W. Xue: Data curation, formal analysis, validation, investigation, writing–review and editing. Q.-M. Wang: Validation. G.-Y. Lian: Validation.
K.-F. To: Resources, funding acquisition. P.M.-K. Tang: Conceptualization, resources, data curation, supervision, funding acquisition, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing. H.-Y. Lan: Conceptualization, resources, supervision, funding acquisition, project administration, writing—review and editing.

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