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Article

Keywords: Tumor-specific MHC-II, KIF17, Kinesin, Antigen presentation, Cancer immunotherapy

Posted Date: November 11th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11443622/v1

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Additional Declarations: (Not answered)

KIF17 governs a trafficking checkpoint for tumor-specific MHC-II presentation and breast cancer immunity

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Abstract

Background: Tumor-specific major histocompatibility complex class II (tsMHC-II) expression is associated with favorable prognosis and improved responsiveness to immune checkpoint blockade. However, the intracellular mechanisms controlling tsMHC-II trafficking and surface presentation remain poorly understood. This study investigated the molecular machinery regulating tsMHC-II surface presentation in breast cancer.

Methods: The tsMHC-II interactome was mapped across breast cancer subtypes using immunoprecipitation-coupled quantitative proteomics. Bioinformatic analyses, subcellular fractionation, flow cytometry, co-immunoprecipitation, proximity ligation, recombinant protein pull-down and structural modeling were used to characterize KIF17-mediated trafficking. Orthotopic 4T1 models, multiplex immunofluorescence, RNA sequencing and spectral flow cytometry were used to assess tumor growth and tumor microenvironment (TME) remodeling. Splenic T-cell coculture assays with MHC-II blockade were performed to evaluate $CD4^+$ and $CD8^+$ T-cell activation and tumor-cell killing. Patient specimens and public immunotherapy datasets were analyzed to assess clinical associations. Statistical analyses included two-tailed Student's t-tests, analysis of variance, log-rank tests, correlation analysis, receiver operating characteristic analysis and random-effects meta-analysis.

Results: KIF17 was identified as a tsMHC-II-interacting protein that was downregulated in breast tumors and associated with immune activation and favorable survival. KIF17 interacted directly with HLA-DQB2 and increased its tumor-cell surface localization without altering total MHC-II abundance. In orthotopic models, Kif17 overexpression remodeled the TME and increased activated $CD4^+$ and $CD8^+$ T-cell and antigen-presenting-cell populations. In coculture assays, Kif17-overexpressing tumor cells promoted $CD4^+$ T-cell activation, $CD4^+$-supported $CD8^+$ T-cell activation and tumor-cell killing. These responses were attenuated by MHC-II blockade or omission of $CD4^+$ T cells. Kif17-overexpressing tumors also displayed improved tumor control and prolonged survival following programmed cell death protein 1 (PD-1) blockade. Elevated KIF17 expression was associated with favorable treatment outcomes in patient specimens and independent immunotherapy datasets.

Conclusions: KIF17 regulates an intracellular trafficking checkpoint controlling tsMHC-II surface presentation. These findings support a model in which KIF17 promotes coordinated $CD4^+$ and $CD8^+$ T-cell responses and establishes an immune context associated with improved responsiveness to PD-1 blockade.

Keywords: Tumor-specific MHC-II, KIF17, Kinesin, Antigen presentation, Cancer immunotherapy

Introduction

Major histocompatibility complex class II (MHC-II) molecules are traditionally regarded as professional antigen-presenting cell (APC)-restricted proteins that mediate the presentation of extracellular antigens to $CD4^+$ T lymphocytes and coordinate adaptive immune responses[1]. In addition to their established role in APCs, MHC-II expression has been increasingly reported in a variety of solid tumors, including melanoma, lung cancer, ovarian cancer, and breast cancer, a phenomenon commonly referred to as tumor-specific MHC-II (tsMHC-II) expression[2]. Importantly, tsMHC-II-positive tumors are consistently associated with favorable clinical outcomes, increased immune infiltration, and improved responses to immune checkpoint blockade[3]. These observations have established tsMHC-II as an important determinant of tumor immunogenicity and a promising biomarker for cancer immunotherapy.

The biological mechanisms governing tsMHC-II expression have therefore become an area of increasing interest. Current understanding primarily centers on transcriptional regulation. Interferon-γ (IFNγ)-mediated activation of the JAK–STAT pathway induces expression of class II transactivator (CIITA), the master regulator of MHC-II transcription, thereby promoting the expression of MHC-II subunits and associated antigen-presentation machinery[4]. In parallel, multiple studies have demonstrated that epigenetic mechanisms, including promoter methylation and chromatin remodeling, contribute to the suppression of MHC-II expression in cancer cells[5,6]. Together, these findings have generated a prevailing model in which tsMHC-II abundance is largely determined by transcriptional activation or repression of MHC-II genes. However, the presence of MHC-II transcripts and proteins alone does not necessarily guarantee successful antigen presentation. Following synthesis in the endoplasmic reticulum, MHC-II complexes must undergo a series of tightly regulated intracellular trafficking events involving transport through the Golgi apparatus, endosomal sorting, peptide loading within MHC class II compartments (MIICs), and eventual delivery to the plasma membrane[7]. While these trafficking processes have been extensively studied in professional APCs, the molecular machinery governing intracellular transport and surface localization of tsMHC-II in tumor cells remains poorly defined.

This knowledge gap may be particularly relevant in breast cancer. Although immune checkpoint inhibitors have transformed the treatment landscape of several malignancies, their efficacy in breast cancer remains limited to selected triple negative subtype. Breast cancer is generally considered an immunologically cold tumor type characterized by relatively low immune infiltration and modest responsiveness to immunotherapy. Multiple mechanisms have been proposed to explain this phenotype, including low neoantigen burden, impaired antigen presentation, defective interferon signaling, stromal-mediated immune exclusion, and the accumulation of immunosuppressive immune populations[8]. Nevertheless, considerable heterogeneity

exists among individual tumors, and the biological factors underlying these differences remain incompletely understood. Recent studies have highlighted tsMHC-II as a favorable prognostic and predictive biomarker in breast cancer[9,10], suggesting that tumor-cell antigen presentation may contribute to the development of more immunologically active tumor microenvironments. However, despite the growing clinical significance of tsMHC-II, the intracellular mechanisms regulating its surface presentation in breast cancer cells remain largely unknown.

Intracellular transport of membrane proteins and vesicular cargoes is tightly regulated by microtubule-associated motor proteins[11]. Members of the kinesin superfamily mediate ATP-dependent transport along microtubules and play essential roles in membrane trafficking, organelle positioning, and signal transduction[12]. Notably, previous studies have implicated the heterotrimeric kinesin-2 complex KIF3A/KIF3B/KAP in the anterograde transport of MHC-II-containing vesicles in professional APCs, supporting the concept that kinesin-dependent mechanisms may participate in antigen-presentation pathways[13]. Whether analogous transport systems regulate tsMHC-II trafficking in tumor cells remains unknown. KIF17 is a distinct member of the kinesin-2 family that functions as a plus-end-directed motor protein involved in cargo transport in multiple cellular contexts[14]. Although KIF17 has been implicated in epithelial homeostasis and cancer progression[15], its potential role in antigen presentation and tumor immunity has not been explored.

To address this question, we systematically mapped the tsMHC-II molecular interactome across multiple breast cancer subtypes using immunoprecipitation (IP)-coupled quantitative proteomics. By integrating proteomic, transcriptomic, mechanistic, and functional analyses, we sought to identify molecular regulators governing the intracellular trafficking and surface presentation of tsMHC-II in breast cancer. Our findings reveal a previously unrecognized trafficking network that regulates functional tsMHC-II presentation and links tumor antigen presentation to immune remodeling and responsiveness to immune checkpoint blockade.

Materials and Methods

Cell culture and compound treatment

Human breast cancer cell lines ZR-75-1, SK-BR-3, and MDA-MB-231, as well as the murine breast cancer cell line 4T1, were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (P/S) under standard culture conditions (37°C, 5% CO_2). All cell lines were routinely tested and confirmed negative for mycoplasma contamination. Stable KIF17-overexpressing and CIITA-overexpressing cell lines were generated using lentiviral vectors and expression constructs synthesized by Fitgene Biotech as previously described. For trafficking inhibition experiments, cells were treated with brefeldin A (BFA; HY-16592, MedChemExpress) or monensin (HY-N4302, MedChemExpress) at the indicated concentrations and durations.

Co-immunoprecipitation (co-IP)

Cells were lysed in 1% digitonin-based lysis buffer supplemented with protease inhibitors and incubated on ice for 20–30 min. After centrifugation, protein concentrations were determined using a BCA assay. For each immunoprecipitation reaction, 10 mg total protein was incubated overnight at 4°C with 10 μg antibody. Mouse anti-human MHC-II antibody (MCA477, Bio-Rad) was used for MHC-II immunoprecipitation, whereas rabbit anti-KIF17 antibody (14615-1-AP, Proteintech) was used for KIF17 immunoprecipitation. Immune complexes were captured using Protein A/G agarose beads provided in the Co-Immunoprecipitation Kit (Bes3011, BersinBio) according to the manufacturer's instructions. Following extensive washing, bound proteins were eluted and subjected to western blotting or mass spectrometry analysis.

Label-free quantitative mass spectrometry

Immunoprecipitated protein samples were analyzed by Fitgene Biotech using an Ultimate 3000 RSLCnano liquid chromatography system coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific). Raw data were processed using MaxQuant software with protein, peptide, and peptide-spectrum match false discovery rates (FDRs) set at 1%. Label-free quantification (LFQ) was performed using a minimum ratio count of two. Differential enrichment analyses were subsequently performed using the limma package in R.

Bioinformatic analysis

Proteomic datasets were analyzed using Perseus (v1.6.15.0). Gene set enrichment analyses were performed using clusterProfiler (v4.18.4)[16]. Anti-tumor immune activity across the Cancer-Immunity Cycle was evaluated using the Tracking Tumor

Immunophenotype (TIP) platform[17]. Immune-cell abundance and transcriptional signature analyses were performed using IOBR (v2.2.0)[18].

Protein-protein interaction analysis

Protein-protein interaction (PPI) networks were constructed using STRING database v12.0[19]. Interaction networks were imported into Cytoscape (v3.9.4) for visualization and clustering. Functional modules were identified using the clusterMaker2 plugin with the Markov Cluster Algorithm (MCL) using STRING combined scores as edge weights and an inflation parameter of 4. Gene Ontology enrichment analysis was used to annotate individual clusters.

Subcellular fractionation

Subcellular fractionation was performed to isolate cytoplasmic, membrane, and nuclear protein fractions. Cells were harvested and processed using a Cell Fractionation Kit (Beyotime Biotechnology) according to the manufacturer's instructions. Briefly, cells were sequentially lysed to obtain cytoplasmic, membrane, and nuclear fractions. Protein concentrations were determined using a BCA assay and equal amounts of protein from each fraction were subjected to western blot analysis. GAPDH, ATP1A1, and PCNA were used as markers for cytoplasmic, membrane, and nuclear fractions, respectively, to verify fraction purity.

Western blot

Cells were lysed in 1% digitonin-based lysis buffer supplemented with protease inhibitor cocktail (78420, Thermo Fisher Scientific). Protein concentrations were determined by BCA assay, and equal amounts of protein (20 μg) were separated by SDS-PAGE and transferred onto PVDF membranes. For analysis of SDS-stable MHC-II dimers, samples were prepared in non-reducing loading buffer without boiling as previously described[20]. Parallel samples prepared under reducing and denaturing conditions were used to detect monomeric proteins. Membranes were incubated overnight at 4°C with primary antibodies against KIF17 (14615-1-AP, Proteintech), MHC-II (ab55152, Abcam), HLA-DQB2 (17315-1-AP, Proteintech), GAPDH (10494-1-AP, Proteintech), PCNA (10205-2-AP, Proteintech), and ATP1A1 (14418-1-AP, Proteintech). HRP-conjugated secondary antibodies and enhanced chemiluminescence substrate (Pierce) were used for signal detection.

RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using a Reverse Transcription Master Kit (Takara) with 2 μg RNA per reaction. Quantitative PCR was performed using SYBR Premix Ex Taq II (Takara) on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Relative gene

expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method with GAPDH as the internal reference control. All reactions were performed in triplicate.

Multiplex immunofluorescence

Multiplex immunofluorescence (mIF) staining was performed on 4 μm formalin-fixed paraffin-embedded (FFPE) tissue sections using the PANO 7-plex IHC Kit (Panovue, 0004100100) according to the manufacturer's instructions. Following deparaffinization and rehydration, antigen retrieval was performed using either citrate buffer (Solarbio, C1032) or EDTA buffer (Solarbio, C1034). Slides were blocked with 10% goat serum and sequentially incubated with primary antibodies against KIF17 (14615-1-AP, Proteintech), MHC-II (ab55152, Abcam), E-cadherin (60902-1-Ig, Proteintech), CD8 (ab237709, Abcam), CD4 (11056-2-AP, Proteintech), CD68 (25747-1-AP, Proteintech), CD11c (ab52632, Abcam), F4/80 (ab300421, Abcam), CD31 (ab28364, Abcam), pan-cytokeratin (26411-1-AP, Proteintech), PD-1 (66220-1-Ig, Proteintech), PD-L1 (28076-1-AP, Proteintech), and α-SMA (ab124964, Abcam). Signal amplification was achieved using TSA reagents supplied with the kit. Slides were counterstained with DAPI and scanned using the TissueFAXS multispectral imaging platform (TissueGnostics) at 20× magnification.

Digital pathology analysis

Digital pathology analysis was performed using QuPath v0.4.3. Whole-tumor regions of interest (ROIs) were manually annotated while excluding necrotic regions and imaging artifacts. Cell segmentation was performed based on DAPI nuclear staining, followed by marker-specific classification using fixed positivity thresholds established from negative controls. Positive-cell percentages and densities were quantified within tumor ROIs.

For spatial analyses, tumor boundaries were manually delineated and serial inward bands of 200 μm width were generated. Each detected cell was assigned to a corresponding spatial band and positive-cell abundance was quantified within each band. In parallel, the distance of individual cells from the tumor boundary was normalized to the maximal tumor radius to generate a continuous rim-to-core coordinate ranging from 0 (tumor edge) to 1 (tumor center).

Proximity ligation assay

Protein-protein interactions were evaluated using the Duolink® Proximity Ligation Assay (PLA) kit according to the manufacturer's instructions. Cells were incubated with mouse anti-human MHC-II antibody (MCA477, Bio-Rad) and rabbit anti-KIF17 antibody (14615-1-AP, Proteintech), followed by PLA probe incubation, ligation, and rolling-circle amplification. Fluorescence images were acquired using a Zeiss LSM800 confocal microscope. Mean fluorescence intensity (MFI) per cell was quantified and compared between experimental groups.

Protein expression and purification

Full-length human KIF17 and truncation mutants corresponding to amino acids 1–346 (Mut1), 347–846 (Mut2), and 847–1029 (Mut3) were cloned into pGEX-4T-1 vectors to generate GST-tagged fusion proteins. Constructs were transformed into Escherichia coli BL21 competent cells and verified by Sanger sequencing. Protein expression was induced with IPTG, and bacterial pellets were harvested and lysed by sonication. GST fusion proteins were purified using glutathione-Sepharose beads according to the manufacturer's instructions. Full-length HLA-DQB2 was cloned into the pET-28a vector to generate His-tagged recombinant protein and purified using the same strategy.

GST pull-down assay

Purified GST, GST-KIF17, GST-KIF17-Mut1, GST-KIF17-Mut2, and GST-KIF17-Mut3 fusion proteins immobilized on glutathione-Sepharose beads were incubated with recombinant His-tagged HLA-DQB2 protein at 4°C overnight with gentle rotation. Beads were extensively washed to remove unbound proteins. Bound proteins were eluted by boiling in SDS loading buffer and analyzed by western blotting using anti-His (84814-1-RR, Proteintech) and anti-GST (66001-2-Ig, Proteintech) antibodies. GST alone served as a negative control.

Protein docking

Protein structures of KIF17 and HLA-DQB2 were predicted using AlphaFold[21]. Protein-protein docking analyses were subsequently performed using the HDOCK server with default parameters. The top-ranked docking models were further analyzed to identify shared interaction sites and candidate binding domains.

Orthotopic tumor model

Female BALB/c mice (6 weeks old) were used for all studies. $4T1^{EV}$ and $4T1^{Kif17\text{-}OE}$ cells (1×10^6 cells per mouse) in logarithmic growth phase were resuspended in PBS and injected into the right inguinal mammary fat pad in a total volume of 100 μL. Tumor growth was monitored every 3 days by caliper measurement.

For immunotherapy experiments, mice were randomized on day 7 after tumor implantation and treated with either anti-mouse PD-1 (LALA-PG) antibody (HY-P991078, MedChemExpress) or control IgG. Anti-PD-1 antibody was administered intravenously at 10 mg/kg twice weekly. Tumor volume and body weight were monitored before each treatment. At experimental or ethical endpoints, tumors were excised, weighed, and processed for downstream analyses.

Spectral flow cytometry

Tumor tissues were dissociated into single-cell suspensions using a digestion cocktail containing collagenase I, collagenase II, neutral protease, and hyaluronidase. Cells were filtered through a 70 μm cell strainer. For immune profiling, single-cell suspensions were stained with a 18-marker spectral flow cytometry panel consisting of CD45 (APC/Cy7, 157204, BioLegend), CD3ε (BV510, 100353, BioLegend), CD25 (PE, 101904, BioLegend), CD19 (Pacific Blue, 115523, BioLegend), CD11c (PE/Cy7, 117318, BioLegend), F4/80 (BV605, 123133, BioLegend), Ly6C (PE/Dazzle 594, 128044, BioLegend), I-A/I-E (AF647, 107618, BioLegend), TCRγδ (BV421, 118120, BioLegend), Ly6G (BV650, 127641, BioLegend), CD4 (BV570, 100542, BioLegend), CD8a (PerCP-eFluor 710, 46-0081-82, eBioscience), FOXP3 (APC, 17-5773-82, eBioscience), CD69 (BV480, 746813, BD Biosciences), CD49b (BV711, 740704, BD Biosciences), CD11b (BB515, 564454, BD Biosciences), and Fixable Viability Stain 620 (564996, BD Biosciences). Intracellular FOXP3 staining was performed using the Transcription Factor Buffer Set (562574, BD Biosciences). Fc receptors were blocked using purified anti-mouse CD16/CD32 antibody (553141, BD Biosciences) prior to staining. Spectral unmixing was performed using single-stained reference controls according to the manufacturer's recommendations. Samples were acquired on a Cytek Northern Light 3000 spectral flow cytometer. Spectral unmixing was performed using instrument-generated reference controls, and data were analyzed using FlowJo software.

Splenocyte isolation and functional assay

Spleens from mice bearing EV or Kif17-OE 4T1 tumors were individually dissociated through a 70-μm cell strainer. Each splenocyte suspension was divided into two fractions, and untouched $CD4^+$ and $CD8^+$ T cells were isolated by negative selection using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (STEMCELL Technologies, cat. no. 19852) and Mouse $CD8^+$ T Cell Isolation Kit (cat. no. 19853), respectively. Isolation purity was confirmed by flow cytometry.

For $CD4^+$ T-cell activation assays, $CD4^+$ T cells were added to tumor cells at a T-cell-to-tumor-cell ratio of 10:1. For $CD8^+$ T-cell activation and killing assays, $CD8^+$ T cells were cultured alone or together with $CD4^+$ T cells, corresponding to CD8-to-CD4-to-tumor-cell ratios of 10:0:1 or 10:10:1. T cells from different mice were not pooled. For MHC-II blockade, tumor cells were pre-incubated for 30 min with anti-mouse I-A/I-E antibody (InVivoMAb anti-mouse MHC Class II, BE0108, clone M5/114, 10 μg/mL) or rat IgG2b isotype control. Antibodies were maintained throughout the 24-h coculture. Exogenous IL-2, anti-CD3/CD28 stimulation and IFN-γ pretreatment were not applied to experimental wells.

Following coculture, adherent and non-adherent cells were collected together and stained for CD3, CD4, CD8, CD25 and CD69. DAPI was added immediately before

flow-cytometric acquisition. After lymphocyte, singlet and viable-cell gating, $CD4^+$ and $CD8^+$ T cells were identified as $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ cells, respectively. $CD25^-CD69^-$ cells were classified as resting, $CD25^-CD69^+$ cells as early activated, and $CD25^+CD69^+$ cells as fully activated. $CD25^+CD69^-$ cells were quantified separately. Killing efficacy was calculated as the percentage of $DAPI^+$ cells among total gated tumor cells. Each mouse was considered an independent biological replicate, and technical replicates were averaged before statistical analysis.

RNA sequencing

Total RNA was extracted from tumor tissues and subjected to library preparation using the RNA Library Prep Kit for Illumina. Libraries were sequenced on the Illumina NovaSeq 6000 platform using 150 bp paired end reads. Sequencing reads were aligned to the reference genome using HISAT2. Differential gene expression analysis was performed using the limma package in R.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.6.0 and R software unless otherwise specified. Data are presented as mean ± standard deviation (SD). Comparisons between two groups were performed using unpaired two-tailed Student's t-tests. Comparisons among multiple groups were performed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, as indicated in the figure legends. Kaplan–Meier survival curves were compared using the log-rank (Mantel–Cox) test. Correlation analyses were performed using Pearson correlation coefficients. For transcriptomic and proteomic analyses, differential expression was determined using the limma package in R. A two-sided P value < 0.05 was considered statistically significant.

Results

Mapping the tsMHC-II molecular interactome in breast cancer.

To systematically characterize the molecular machinery associated with tsMHC-II in breast cancer cells, we established an IP-LFQ-MS workflow targeting MHC-II using three breast cancer cell lines representing distinct molecular subtypes, including ZR-75-1 ($HR^+/HER2^-$), SK-BR-3 ($HR^-/HER2^+$), and MDA-MB-231 ($HR^-/HER2^-$) (Fig. 1A). Comparison with IgG controls identified 384, 280, and 361 significantly enriched tsMHC-II-binding proteins (tsMHC-II-BPs) in each cell line, respectively (Fig. S1A). Reactome pathway enrichment revealed highly concordant biological signatures across all three cell lines, with MHC-II antigen presentation consistently emerging among the top enriched pathways (Fig. S1B). To further place the identified proteins within established MHC-II biology, we manually annotated tsMHC-II-BPs according to the Reactome MHC class II antigen presentation pathway (R-HSA-2132295) and Gene Ontology term MHC class II protein complex binding (GO:0023026). Given the complexity of the MHC-II trafficking route, the pathway was divided into three functional stages: transport of invariant chain-associated MHC-II complexes (Ii-MHC-II), endocytosis of Ii-MHC-II or peptide-loaded MHC-II (pMHC-II), and membrane trafficking of mature pMHC-II complexes (Fig. 1B). Proteins identified in each cell line could be mapped to multiple stages of this process, demonstrating that the tsMHC-II-BPs are functionally integrated within established MHC-II trafficking pathways and implicating diverse regulators in tsMHC-II transport.

Quantitative comparison of pathway enrichments using Jaccard similarity analysis demonstrated substantial overlap between cell lines, indicating that tsMHC-II is associated with a conserved molecular network despite marked differences in breast cancer subtype (Fig. S1C). Consistently, intersection analysis identified 118 proteins shared among all three datasets (Fig. S1D). Among these, 34 proteins were significantly enriched in every cell line and were defined as the core tsMHC-II interactome (Fig. 1C). To further characterize the functional organization of the conserved tsMHC-II interactome, protein-protein interaction analysis was performed using the 34 shared tsMHC-II-binding proteins. This analysis identified ten interconnected functional modules associated with diverse biological processes (Fig. 1D). Collectively, these modules encompassed key processes required for MHC-II biosynthesis, intracellular trafficking, peptide processing, complex maturation, and surface presentation. Together, these findings demonstrate that the conserved tsMHC-II interactome is functionally integrated with multiple stages of the MHC-II antigen presentation pathway, supporting the biological relevance of the identified tsMHC-II-binding proteins.

KIF17 governs a trafficking checkpoint for tumor-specific MHC-II surface presentation

To determine whether the identified tsMHC-II-BPs are associated with breast cancer immunity, consensus clustering was performed using the 34 core tsMHC-II-BPs in the TCGA-BRCA cohort (Fig. S2A). Two distinct patient populations (C1 and C2) were identified with clear separation in principal component analysis (Fig. 2A). Patients in C2 exhibited significantly prolonged overall survival compared with C1 (Fig. 2B). As a functional comparison, clustering based on the Gene Ontology term MHC class II protein complex binding (GO:0023026) produced highly similar patient stratification and survival outcomes (Fig. S2B-C). Despite sharing only two genes, the two approaches classified 99% of patients identically, supporting a strong functional association between the tsMHC-II interactome and MHC-II biology (Fig. S2D-E).

Given the favorable prognosis of C2 patients, we next examined anti-tumor immune activity using TIP analysis. Both tumor clusters exhibited higher activity than normal tissues (C3) during antigen release and antigen presentation. However, while C2 maintained elevated activity throughout the downstream steps of the Cancer-Immunity Cycle, immune activity in C1 declined markedly and fell below the normal reference level (Fig. 2C), suggesting impaired propagation of anti-tumor immunity despite comparable antigen availability.

To identify tsMHC-II-BPs associated with the favorable immune phenotype, differential expression analysis was performed between C1 and C2 tumors. Seven genes were significantly enriched in each subgroup (Fig. 2D). Comparison between tumor and normal tissues revealed that most tsMHC-II-BPs showed relatively stable expression, whereas KIF17 was significantly downregulated in tumors despite being enriched in C2 (Fig. 2E). Correlation analysis with immune cell infiltration further grouped tsMHC-II-BPs into two major clusters, one of which displayed strong similarity to HLA-DQB2, a canonical MHC-II subunit (Fig. 2F). Ranking genes according to their similarity with the HLA-DQB2 immune correlation profile identified KIF17 among the top candidates (Fig. 2G). Notably, KIF17 and HLA-DQB2 were also among the lowest expressed tsMHC-II-BPs across breast cancer cell lines (Fig. S2F).

Although our previous work identified KIF17 as a metastasis-suppressive protein that is broadly silenced in breast cancer[15], its potential role in tumor immunity remains unknown. We therefore further explored the association between KIF17 and the tumor microenvironment. KIF17 expression negatively correlated with tumor purity and positively correlated with microenvironment scores (Fig. 2H). Consistently, GSEA demonstrated enrichment of immune-related and MHC-II-associated pathways in KIF17-high tumors (Fig. 2I). Collectively, these findings identify KIF17 as a prominent tsMHC-II-associated candidate whose exceptionally low expression in breast cancer

suggests that KIF17 ablation may represent the rate-limiting regulator of a trafficking checkpoint restricting effective tsMHC-II-mediated anti-tumor immunity.

KIF17 facilitates the membrane trafficking of tsMHC-II by directly binding with HLA-DQB2

Given that KIF17 is a plus-end-directed kinesin-2 motor protein that transports specific cargos along microtubules through ATP-dependent movement, we hypothesized that KIF17 may participate in the intracellular trafficking of tsMHC-II. This hypothesis was further supported by previous studies showing that another kinesin-2 family member, the heterotrimeric KIF3A/KIF3B/KAP complex, mediates anterograde transport of MIICs to the plasma membrane in professional APCs[13]. We therefore investigated whether KIF17 performs a similar trafficking function for tsMHC-II in breast cancer cells. Stable KIF17-overexpressing cell lines were generated in ZR-75-1, SK-BR-3, and MDA-MB-231 cells. Consistent with our previous finding that KIF17 functions primarily through non-transcriptional mechanisms, we first examined its impact on MHC-II expression. Unlike the positive control CIITA, KIF17 overexpression did not alter the mRNA or total protein levels of MHC-II subunits (Fig. S3A-B), indicating that KIF17 is unlikely to regulate tsMHC-II expression.

Subcellular fractionation revealed that KIF17 overexpression induced a marked enrichment of tsMHC-II within the membrane fraction without affecting total cellular abundance (Fig. 3A). This effect was abolished by treatment with either brefeldin A or monensin, suggesting that KIF17 functions during post-Golgi trafficking of tsMHC-II-containing vesicles (Fig. 3B). Consistently, flow cytometric analysis of non-permeabilized cells demonstrated significantly increased surface tsMHC-II following KIF17 overexpression, whereas CIITA overexpression produced only a modest increase despite inducing MHC-II transcription (Fig. 3C and Fig. S3C).

To validate these findings in clinical specimens, we established an automated QuPath-based multiplex immunofluorescence analysis pipeline using a breast cancer tissue microarray comprising 148 primary patient samples. While no correlation was observed between total cellular KIF17 and tsMHC-II levels, membrane-localized tsMHC-II showed a significant positive correlation with KIF17 expression (Fig. 3D), supporting a specific role for KIF17 in regulating membrane localization of tsMHC-II.

To identify the molecular basis of this trafficking process, reverse anti-KIF17 IP-MS was performed. Among the proteins consistently identified across all three breast cancer cell lines, HLA-DQB2 emerged as a prominent candidate cargo protein (Fig. 3E). As HLA-DQB2 was also identified in the tsMHC-II interactome, we hypothesized that KIF17 may directly interact with tsMHC-II through this subunit. Co-IP confirmed the association

between KIF17 and HLA-DQB2 in all three cell lines (Fig. 3F), while PLA further demonstrated enhanced KIF17–HLA-DQB2 interactions following KIF17 overexpression (Fig. 3G). Importantly, GST pull-down assays showed direct binding between recombinant KIF17 and HLA-DQB2 but not HLA-DPB1, confirming the specificity of this interaction (Fig. 3H).

To further characterize the interaction interface, protein-protein docking analyses were performed. The top five predicted models displayed highly similar overall conformations, in which HLA-DQB2 occupied a binding pocket within KIF17 while adopting different rotational orientations (Fig. 3I and Fig. S3D). Although no universal residue-level interaction sites were shared among all models (Fig. S3E), consensus analysis identified recurrent interaction regions in both proteins (Fig. S3F). Integration of structural predictions revealed that the shared HLA-DQB2 interaction sites localized predominantly within its intramembrane region, whereas KIF17 interaction sites clustered within the tail domain and a disordered region of the stalk domain (Fig. S3G-H). Guided by these predictions, a series of KIF17 truncation mutants were generated and evaluated by GST pull-down assays. Deletion of either region weakened HLA-DQB2 binding relative to full-length KIF17, indicating that both domains contribute to stable cargo recognition (Fig. 3J).

Based on these findings, we propose that KIF17 directly recognizes HLA-DQB2 through coordinated interactions involving the tail domain and a disordered region within the stalk domain (Fig. 3K). We speculate that this interaction becomes accessible following endocytic entry of MHC-II-containing membranes into the MIIC pathway, where membrane topology is preserved and the intramembrane region of HLA-DQB2 remains exposed on the cytoplasmic surface of the transport vesicle. This configuration enables direct engagement by KIF17 and facilitates anterograde transport of peptide-loaded tsMHC-II vesicles toward the plasma membrane.

Kif17 overexpression reshapes the tumor microenvironment and induces spatial stromal–immune reorganization

To investigate the impact of Kif17 on the tumor microenvironment in vivo, orthotopic syngeneic mammary tumors were established by implanting EV or Kif17-overexpressing 4T1 cells into the mammary fat pads of mice (Fig. 4A). Kif17 overexpression significantly, but not dramatically, suppressed tumor growth throughout the experimental period, resulting in slightly reduced tumor volumes at the endpoint (Fig. 4B-C).

To characterize the underlying biological changes, bulk RNA sequencing was performed on excised tumors. Principal component analysis demonstrated clear separation between EV and Kif17-OE tumors (Fig. S4A), while differential expression analysis

revealed extensive transcriptional remodeling following Kif17 overexpression (Fig. 4D). Pathway-level analysis identified broad alterations in immune, stromal, and metabolic programs (Fig. S4B-C). Notably, immune-related signatures, including antigen presentation and immune cell infiltration pathways, were consistently enriched in Kif17-OE tumors (Fig. S4E). Immune deconvolution analyses further revealed increased abundance of dendritic cells, $CD4^+$ T cells, and a higher M1/M2 macrophage ratio, consistent across both CIBERSORT and IOBR-based analyses (Fig. S4D-F).

Histological analysis revealed profound architectural remodeling of the tumor microenvironment following Kif17 overexpression. While EV tumors displayed a relatively homogeneous architecture with tumor and stromal components broadly intermixed, Kif17-OE tumors exhibited pronounced spatial compartmentalization characterized by distinct fibroblast-rich stromal regions and tumor-cell-rich regions (Fig. 4E). Notably, within the remaining tumor-cell-rich areas, fibroblasts were substantially reduced and direct tumor–fibroblast interactions appeared less frequent. This reorganization was accompanied by an increase in vascular structures and the formation of stromal corridors separating tumor cell clusters, suggesting extensive remodeling of the physical tumor landscape. Multiplex immunofluorescence further demonstrated significantly increased infiltration of $CD4^+$ T cells, $CD8^+$ T cells, $CD11c^+$ dendritic cells, $CD68^+$ macrophages, $F4/80^+$ macrophages, and $CD68^+F4/80^+$ double-positive macrophages in Kif17-OE tumors (Fig. 4F and Fig. S4G). Spatial analysis revealed that EV tumors exhibited a classical immune-excluded phenotype, with most immune cells confined to the tumor margin and showing limited penetration into the tumor core (Fig. 4G). In contrast, Kif17-OE tumors displayed extensive immune infiltration throughout the tumor parenchyma. Importantly, different immune populations exhibited distinct spatial distribution patterns. $CD4^+$ T cells and macrophage populations showed prominent accumulation within tumor-cell-rich regions, indicating increased opportunities for direct interaction with tumor cells, whereas $CD11c^+$ dendritic cells were more broadly distributed throughout the tumor mass. These observations suggest that Kif17 overexpression not only increases immune cell recruitment but also alters the spatial organization of immune populations within tumors.

Together, these findings demonstrate that Kif17 overexpression drives large-scale stromal and immune remodeling of the tumor microenvironment, promoting a transition from an immune-excluded phenotype toward a more immune-inflamed state. Given the role of tsMHC-II in local antigen presentation, the enhanced immune penetration and redistribution observed in Kif17-OE tumors are consistent with improved intratumoral immune activation and immune–tumor cell engagement.

KIF17-mediated surface tsMHC-II promotes T-cell activation and enhances anti-PD-1 responsiveness

Given the extensive immune remodeling induced by Kif17 overexpression, we next investigated its potential impact on immune checkpoint signaling. Analysis of bulk RNA-seq data revealed significant enrichment of immune checkpoint-related transcriptional signatures in Kif17-OE tumors (Fig. 5A). Consistently, multiplex immunofluorescence demonstrated an increased frequency of PD-1$^+$ cells and a trend toward increased PD-L1$^+$ cells in Kif17-OE tumors compared with EV controls (Fig. 5B–C). These findings indicate increased engagement of immune checkpoint pathways within the tumor immune microenvironment remodeled by Kif17 overexpression.

To determine whether these changes influence immunotherapy responsiveness, we established an orthotopic 4T1 model combining Kif17 overexpression with anti-PD-1 treatment (Fig. 5D). Anti-PD-1 treatment exerted only limited effects on the growth of EV tumors, whereas Kif17-OE tumors displayed improved tumor control following PD-1 blockade (Fig. 5E). The combination treatment did not induce complete tumor regression but resulted in a sustained reduction in tumor progression. Consistent with this effect, mice bearing Kif17-OE tumors and receiving anti-PD-1 treatment showed the most favorable survival outcome among the four experimental groups (Fig. 5F). Thus, Kif17 overexpression enhanced tumor responsiveness to PD-1 blockade and promoted sustained immune control of tumor progression.

To characterize the immune features associated with the improved response to PD-1 blockade, dissociated tumors were subjected to 18-marker multiplex spectral flow cytometry followed by comprehensive immune profiling (Fig. S5). Kif17 overexpression induced extensive remodeling of the tumor immune landscape, which was further enhanced by anti-PD-1 treatment (Fig. 5G). Although total immune-cell infiltration increased only modestly, pronounced changes were observed in immune-cell composition and functional state. In particular, Kif17-OE tumors contained increased frequencies of activated CD4$^+$ and CD8$^+$ T-cell subsets, accompanied by reductions in their resting counterparts. These changes were most evident in the Kif17-OE plus anti-PD-1 group. Antigen-presenting-cell-associated populations, including dendritic cells, macrophages and B cells, were also enriched following Kif17 overexpression and were further increased after anti-PD-1 treatment.

Principal component analysis demonstrated progressive separation of the immune states across the four experimental groups (Fig. 5H). EV tumors treated with control IgG or anti-PD-1 clustered closely, consistent with the limited immune response to PD-1 blockade in the absence of Kif17 overexpression. In contrast, Kif17-OE tumors occupied a distinct immune state, whereas the Kif17-OE plus anti-PD-1 group showed the greatest separation from EV controls. The Kif17-OE plus control IgG group occupied an

intermediate position, indicating that Kif17 overexpression alone partially remodeled the tumor immune microenvironment and established an immune state that was further modified by PD-1 blockade. Representative flow-cytometry plots further illustrated the increased frequencies of $CD4^+$ T cells and dendritic cells in Kif17-OE tumors, particularly following anti-PD-1 treatment (Fig. 5I).

The spectral flow cytometry results identified increased activation of both $CD4^+$ and $CD8^+$ T cells as a prominent feature of Kif17-mediated immune remodeling. The concordance between increased T-cell activation and improved tumor control suggested that Kif17-induced changes in T-cell function might contribute to the enhanced response to PD-1 blockade. We therefore examined whether T cells isolated from tumor-bearing mice could functionally respond to Kif17-OE tumor cells and whether this response required MHC-II.

$CD4^+$ T cells isolated from Kif17-OE tumor-bearing mice displayed the highest frequency of $CD25^+/CD69^+$ cells following re-exposure to Kif17-OE 4T1 cells (Fig. 5J). Comparatively weaker activation was observed when $CD4^+$ T cells were isolated from EV tumor-bearing mice or when EV 4T1 cells were used as target cells. Blocking I-A/I-E markedly reduced $CD4^+$ T-cell activation in cocultures containing Kif17-OE tumor cells. These results indicate that both previous exposure to Kif17-OE tumors in vivo and MHC-II expression on the target tumor cells contributed to the enhanced $CD4^+$ T-cell response.

We next examined whether MHC-II-dependent $CD4^+$ T-cell activation was associated with $CD8^+$ T-cell function. Coculture of $CD4^+$ and $CD8^+$ T cells isolated from Kif17-OE tumor-bearing mice with Kif17-OE 4T1 cells produced the highest frequency of activated $CD25^+/CD69^+$ $CD8^+$ T cells (Fig. 5K). This increase was attenuated by I-A/I-E blockade and by the omission of $CD4^+$ T cells from the coculture. These findings indicate that the enhanced $CD8^+$ T-cell response associated with Kif17 overexpression requires MHC-II engagement and is supported by $CD4^+$ T cells.

Consistent with the T-cell activation profiles, Kif17-OE tumor cells showed the greatest killing when cocultured with both $CD4^+$ and $CD8^+$ T cells isolated from Kif17-OE tumor-bearing mice (Fig. 5L). Tumor-cell killing was significantly reduced by I-A/I-E blockade and by the omission of $CD4^+$ T cells. Thus, the increased activation of $CD4^+$ and $CD8^+$ T cells observed in Kif17-OE tumors was accompanied by enhanced cytotoxic activity against Kif17-OE tumor cells in vitro.

Together, the spectral flow cytometry and functional coculture results support a model in which KIF17-mediated surface tsMHC-II enhances tumor recognition by $CD4^+$ T cells, which subsequently supports $CD8^+$ T-cell activation and tumor-cell killing. This coordinated T-cell response provides a potential mechanistic explanation for the

immune-inflamed microenvironment and improved response to PD-1 blockade observed in Kif17-OE tumors. Although the causal contribution of MHC-II and $CD4^+$ T cells to anti-PD-1 efficacy was not directly tested in vivo, these findings establish a functional connection between the KIF17–tsMHC-II axis, coordinated $CD4^+$ and $CD8^+$ T-cell responses, and enhanced immunotherapy responsiveness.

KIF17 serves as a predictive biomarker for breast cancer immunotherapy

Having established that KIF17-mediated surface tsMHC-II expression enhances responsiveness to PD-1 blockade, we next investigated whether KIF17 could serve as a predictive biomarker for breast cancer immunotherapy. Multiplex immunofluorescence analysis of pre-treatment biopsy samples from patients receiving neoadjuvant anti-PD-1 therapy revealed significantly higher KIF17 expression in patients achieving pCR compared with non-pCR patients, whereas tsMHC-II levels showed no significant difference between the two groups (Fig. 6A). Similarly, in an independent cohort of recurrent or metastatic breast cancer patients receiving anti-PD-1 therapy, responders (PR+CR) exhibited significantly higher KIF17 expression than non-responders (PD+SD), while tsMHC-II expression remained comparable between groups (Fig. 6B).

To further evaluate the predictive value of KIF17, five independent cancer immunotherapy cohorts were analyzed. Although individual cohort comparisons did not reach statistical significance, responders consistently displayed higher KIF17 expression than non-responders across all datasets (Fig. 6C). Consistent with the limited sample size of individual cohorts, ROC analyses demonstrated only modest predictive performance when each cohort was analyzed separately (Fig. 6D). Nevertheless, meta-analysis revealed a uniform positive effect across all cohorts, with a pooled standardized mean difference favoring responders (Fig. 6E). This trend became significant when cohort-level mean expression values were compared directly, demonstrating consistently elevated KIF17 expression in responder groups across independent studies (Fig. 6F). Finally, pooling all cohorts substantially improved predictive performance, yielding an AUC of 0.714 (95% CI: 0.63–0.798), exceeding that observed in any individual cohort (Fig. 6G).

Collectively, these findings identify KIF17 as a clinically relevant biomarker associated with favorable response to PD-1-based immunotherapy and support its potential utility for cancer patient stratification.

Discussion

Here, we systematically mapped the tsMHC-II interactome in breast cancer and identified KIF17 as a previously unrecognized regulator of tsMHC-II trafficking. KIF17 directly interacted with HLA-DQB2 and promoted surface localization of tsMHC-II without affecting MHC-II transcription or total protein abundance. Restoration of KIF17 remodeled the tumor microenvironment, enhanced responsiveness to PD-1 blockade, and was associated with favorable immunotherapy outcomes. Together, these findings identify intracellular trafficking as an important regulatory layer of tsMHC-II biology.

Tumor-specific MHC-II expression has emerged as an important biomarker associated with favorable prognosis and responsiveness to immune checkpoint blockade across multiple cancer types[3,9,10,22]. Current models of tsMHC-II regulation primarily focus on IFNγ signaling, CIITA activation, and transcriptional control of MHC-II genes[2]. However, despite the requirement for MHC-II complexes to undergo extensive intracellular trafficking before reaching the cell surface, the molecular machinery governing this process in tumor cells remains poorly understood. Our findings suggest that intracellular trafficking represents an additional layer controlling tsMHC-II function. Unlike canonical regulators such as CIITA, KIF17 increased membrane-localized tsMHC-II without altering transcript or total protein abundance, indicating that MHC-II expression and MHC-II functionality are not necessarily equivalent. By identifying KIF17-dependent trafficking as a determinant of surface tsMHC-II expression, our study expands the current paradigm of tsMHC-II regulation beyond transcriptional control.

The identification of KIF17 as a tsMHC-II trafficking regulator also extends current understanding of kinesin-dependent antigen presentation. Previous studies have implicated vesicular trafficking machinery, including Rab GTPases and kinesin-2 complexes such as KIF3A/KIF3B/KAP, in MHC-II transport within professional antigen-presenting cells[23,24]. In contrast, we identify KIF17 as a cargo-selective regulator of tsMHC-II transport in breast cancer cells[14]. KIF17 directly interacted with HLA-DQB2 and promoted membrane localization of tsMHC-II, supporting a model in which KIF17 couples microtubule-based transport to surface delivery of tumor-derived MHC-II complexes. The predicted interaction between KIF17 and the intramembrane region of HLA-DQB2 further provides a plausible topological explanation for how a cytoplasmic motor protein engages MHC-II-containing vesicles following endocytic entry into the MIIC pathway.

Breast cancer is generally considered an immunologically cold malignancy, and multiple mechanisms have been proposed to explain its limited responsiveness to immune checkpoint blockade, including low neoantigen burden, impaired antigen presentation, defective IFNγ signaling, stromal-mediated T-cell exclusion, and immunosuppressive myeloid populations[8,25]. Most of these mechanisms focus either on upstream regulation of antigen presentation or downstream suppression of anti-tumor immunity. Our findings suggest that disruption of tsMHC-II trafficking may represent an additional layer of

immune evasion. KIF17 was markedly reduced in breast cancer cells and tumors, while restoration of KIF17 enhanced surface tsMHC-II expression, promoted immune infiltration, and converted tumors from an immune-excluded to a more immune-inflamed phenotype. Together with our previous work identifying KIF17 as a metastasis-suppressive protein frequently silenced during breast cancer progression, these findings suggest that KIF17 loss may provide dual selective advantages by simultaneously promoting metastatic potential and limiting anti-tumor immune surveillance.

Recent studies have further suggested that tsMHC-II-positive tumors represent a distinct immune ecosystem rather than merely expressing an additional antigen-presentation molecule. A recent single-cell spatial atlas demonstrated that MHC-II-positive tumor cells reside within highly inflamed immune niches characterized by enhanced immune activation, increased checkpoint signaling, and elevated PD-1 expression in neighboring T cells[26]. Consistent with this model, restoration of KIF17 increased surface tsMHC-II abundance, promoted infiltration of $CD4^+$ T cells and antigen-presenting cells, enhanced PD-1 and PD-L1 expression, and improved responsiveness to PD-1 blockade. These findings support a model in which KIF17 functions upstream of the immune ecosystem associated with tsMHC-II-positive tumors by facilitating membrane delivery of functional tsMHC-II complexes. Across independent cohorts, elevated KIF17 expression was consistently associated with favorable responses to PD-1-based immunotherapy, supporting its potential utility as a predictive biomarker.

Several limitations of the present study should be acknowledged. First, our mechanistic investigations primarily relied on gain-of-function models. Although loss-of-function approaches are commonly employed to validate protein function, endogenous KIF17 expression is nearly undetectable in most breast cancer cell lines and patient tumors, consistent with our previous findings and public transcriptomic datasets. We therefore focused on restoration of KIF17 expression as a biologically relevant model to investigate its contribution to tsMHC-II trafficking and tumor immunity. Second, although multiple orthogonal approaches, including co-immunoprecipitation, proximity ligation assays, GST pull-down assays, structural modeling, and mutational analyses, consistently supported a direct interaction between KIF17 and HLA-DQB2, the dynamic trafficking process itself was not directly visualized. Future studies employing live-cell imaging, endogenous protein tagging, and super-resolution microscopy will be valuable for defining the spatiotemporal dynamics of KIF17-mediated tsMHC-II transport. Finally, while KIF17 demonstrated promising predictive performance across multiple immunotherapy cohorts, these analyses were retrospective and involved relatively limited sample sizes. Prospective validation in larger clinical cohorts will be required to establish the utility of KIF17 as a predictive biomarker and to determine whether

therapeutic restoration of KIF17-mediated trafficking can be exploited to enhance responses to immune checkpoint blockade.

Conclusion

Collectively, our findings identify KIF17 as a previously unrecognized regulator of a trafficking checkpoint governing tumor-specific MHC-II surface presentation and establish intracellular trafficking as an additional regulatory layer controlling tumor immunogenicity. By linking KIF17-dependent tsMHC-II surface presentation to immune ecosystem remodeling, improved responsiveness to PD-1 blockade, and favorable clinical outcomes, this study extends the current paradigm of tsMHC-II regulation beyond transcriptional control and identifies intracellular antigen-presentation trafficking as a potential therapeutic vulnerability in immune-cold breast cancers.

List of abbreviations

ANOVA, analysis of variance
APC, antigen-presenting cell
AUC, area under the receiver operating characteristic curve
BCA, bicinchoninic acid
BFA, brefeldin A
CIITA, class II major histocompatibility complex transactivator
CR, complete response
DAPI, 4′,6-diamidino-2-phenylindole
DC, dendritic cell
DMEM, Dulbecco's modified Eagle medium
EDTA, ethylenediaminetetraacetic acid
EV, empty vector
FCS, fetal calf serum
FDR, false discovery rate
FFPE, formalin-fixed paraffin-embedded
GSA, Genome Sequence Archive
GSEA, gene set enrichment analysis
GST, glutathione S-transferase
H&E, hematoxylin and eosin
HLA-DQB2, human leukocyte antigen DQ beta 2
HR, hormone receptor
IACUC, Institutional Animal Care and Use Committee
IFNγ, interferon gamma
IgG, immunoglobulin G
IHC, immunohistochemistry
IOBR, Immuno-Oncology Biological Research
IP, immunoprecipitation
IPTG, isopropyl β-D-1-thiogalactopyranoside

JAK, Janus kinase
KIF17, kinesin family member 17
LFQ, label-free quantification
MCL, Markov clustering
MFI, mean fluorescence intensity
MHC-II, major histocompatibility complex class II
mIF, multiplex immunofluorescence
MIIC, MHC class II compartment
NR, non-responder
OE, overexpression
panCK, pan-cytokeratin
pCR, pathological complete response
PD, progressive disease
PD-1, programmed cell death protein 1
PD-L1, programmed death-ligand 1
PLA, proximity ligation assay
PPI, protein–protein interaction
PR, partial response
PVDF, polyvinylidene difluoride
R, responder
RNA-seq, RNA sequencing
ROC, receiver operating characteristic
ROI, region of interest
RT-qPCR, reverse-transcription quantitative polymerase chain reaction
SD, standard deviation
SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis
STAT, signal transducer and activator of transcription
TCGA-BRCA, The Cancer Genome Atlas Breast Invasive Carcinoma cohort
TCR, T-cell receptor
TIP, Tracking Tumor Immunophenotype
TME, tumor microenvironment
Treg, regulatory T cell
TSA, tyramide signal amplification
tsMHC-II, tumor-specific major histocompatibility complex class II
WT, wild type
αSMA, alpha-smooth muscle actin

Declarations

Ethics approval and consent to participate

The use of human tissue specimens was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (approval no. [2023]723). The study was conducted in accordance with the Declaration of Helsinki and relevant institutional guidelines. Written informed consent was obtained from all participants before sample collection and use for research purposes. All animal experiments were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Sun Yat-

sen University (approval no. [2023]243) and were conducted in accordance with institutional animal-welfare guidelines.

Consent for publication

Not applicable.

Availability of data and materials

TCGA-BRCA transcriptomic data were obtained from the UCSC Xena platform. Publicly available immunotherapy datasets analyzed in this study included GSE67501, GSE111636, GSE115821, GSE126044 and GSE194040. The mouse RNA-sequencing data generated in this study have been deposited in the Genome Sequence Archive under accession number CRA044319 (https://ngdc.cncb.ac.cn/gsa). Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Natural Science Foundation of China (grant no. 82372781) and the Science and Technology Development Fund of the Macao Special Administrative Region (grant no. FDCT-0009/2022/AKP).

Authors' contributions

TL contributed to conceptualization, formal analysis, investigation, methodology, software, validation, visualization, project administration and preparation of the original draft. DZ contributed to formal analysis, investigation, methodology, software and preparation of the original draft. ZW contributed to investigation, methodology, validation and preparation of the original draft. YS, MZ and SC contributed to resources and data curation. NS contributed to conceptualization, resources, data curation, supervision, project administration and preparation, review and editing of the manuscript. YL contributed to conceptualization, resources, data curation, supervision, funding acquisition, validation, project administration and preparation, review and editing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figure Legends

Figure 1. Mapping the tumor-specific MHC-II (tsMHC-II) interactome in breast cancer.
A. Workflow of the immunoprecipitation (IP) and label-free mass spectrometry (LFQ) based tsMHC-II interactome analysis pipeline.
B. Step-wise annotation of the tsMHC-II trafficking process and related molecular participants. Known hits were summarized from each cell type.
C. Volcano plot of shared tsMHC-II binding proteins identified across subtypes in all three cell lines. Top10 proteins were annotated with gene names. $P < 0.05$ was considered as significant.
D. Protein-protein interaction clusters of the shared tsMHC-II binding proteins. Gene Ontology-biological process enrichments were used to define the function of each clusters.

Figure 2. KIF17 ablation is the rate-limiting step in the intracellular trafficking of tsMHC-II.
A. Consensus clustering of TCGA-BRCA tumor patients based on the expressional profile of tsMHC-II-BP revealed two distinct clusters.
B. Overall survival analysis of tsMHC-II-BP based clustering of TCGA-BRCA patients, comparison was made by log-rank test.
C. Comparisons of anti-cancer immunity status across the seven-step Cancer-Immunity Cycle in different TCGA-BRCA patient clusters. C1 and C2 were tumor samples; C3 contained normal samples.
D. Differential expression of tsMHC-II-BPs between C1 and C2 cancer patient clusters.
E. Differential expression of tsMHC-II-BPs between tumor and normal samples from TCGA-BRCA cohort.
F. Correlation between gene expression of tsMHC-II-BPs and immune cell infiltration across TCGA-BRCA tumors.
G. Genes ranked by functional similarity to the MHC-II immune correlation profile (HLA-DQB2) in TCGA-BRCA cohort.
H. Correlation analyses between the expression of KIF17 and tumor purity/microenvironment score.
I. GSEA functional enrichments of KIF17 in TCGA-BRCA cohort.

Figure 3. KIF17 facilitates the membrane trafficking of tsMHC-II by directly binding with HLA-DQB2.
A. Western blot of whole cell lysate and different cell component lysates comparing KIF17 OE and Wild Type (WT) groups of all three cell lines.
B. Western blot of whole cell lysate and membrane specific lysates comparing KIF17 OE and Wild Type (WT) groups. All three cell lines were treated with 5μM of brefeldin A or monensin for 24 hours.

C. Flow cytometry measurement of membrane tsMHC-II staining between WT and KIF17/CIITA OE. Isotype control was included for unspecific binding control.
D. Automated pipeline of QuPath based multiplex immunofluoresence microarray analysis. Correlation between KIF17 protein level and cytoplasmic/membrane tsMHC-II protein level were quantified using dot plot and linear regression. (N = 148).
E. Heatmap of shared KIF17 binding proteins identified across subtypes in all three cell lines. Top10 proteins were annotated with gene names. $P < 0.05$ was considered as significant.
F. Western blot of anti-KIF17 immunoprecipitation elution lysates in all three cell lines.
G. Proximity ligation assay (PLA) of KIF17 and HLA-DQB2 in SK-BR-3 cell line. Mean intensity per cell was calculated to compare between WT and KIF17 OE groups. Scale bar: 50 μm.
H. GST-pull down assay of KIF17 and HLA-DQB1/2. GST-KIF17 and 6*his-HLA-DQB1/2 were constructed and purified.
I. Protein-protein interaction docking of KIF17 and HLA-DQB2. The top5 models predicted were illustrated here with the top 2 interaction sites from model 1 highlighted.
J. GST-pull down assay of different KIF17 mutations and HLA-DQB2. Different mutations of KIF17 were illustrated here.
K. Proposed model of KIF17-mediated tsMHC-II trafficking. Following peptide loading within MIIC compartments, the intramembrane region of HLA-DQB2 remains exposed on the cytoplasmic surface of the transport vesicle due to preservation of membrane topology during endocytic trafficking. KIF17 directly recognizes this region through coordinated interactions involving its tail domain and disordered stalk region, thereby facilitating microtubule-dependent transport of peptide-loaded tsMHC-II vesicles toward the plasma membrane.

Figure 4. Kif17 overexpression reshapes the tumor immune microenvironment and induces spatial stromal–immune reorganization.
A. Schematic overview of the in vivo tumor model and downstream analyses. Orthotopic syngeneic 4T1 mammary tumors were established by injecting EV- or Kif17-OE 4T1 cells into the mammary fat pad of BALB/c mice.
B. In vivo tumor growth of empty vector (EV) and Kif17-overexpressing (Kif17-OE) 4T1 tumors (n = 5 mice per group)
C. Endpoint tumor volume of EV and Kif17-overexpressing 4T1 tumors at day 21. Each dot represents one mouse (n = 5 per group). Statistical significance was assessed by unpaired two-tailed Student's t-test.
D. Differential gene expression between EV and Kif17-OE 4T1 tumors. Volcano plot showing $\log_2$ fold change versus $-\log_{10}(P\text{value})$ from bulk RNA-seq analysis.
E. Kif17 overexpression induces spatial tumor–stroma compartmentalization. Representative H&E staining and multiplex immunofluorescence images of EV and Kif17-OE 4T1 tumors

stained for panCK (tumor cells), αSMA (cancer-associated fibroblasts), CD31 (endothelial cells), and DAPI. Scale bar: 200 μm.
F. Multiplex immunofluorescence validation of increased immune cell infiltration in Kif17-OE tumors. Representative multiplex IF images and quantification of $CD4^+$, $CD8^+$, $CD11c^+$, $CD68^+$, and $F4/80^+$ cells, as well as $CD68^+F4/80^+$ double-positive macrophages, in EV and Kif17-OE 4T1 tumors. Data are presented as the percentage of marker-positive cells. Statistical comparisons were performed between groups using unpaired two-tailed Student's t-test. Scale bar: 200 μm.
G. Kif17 overexpression promotes deeper immune cell infiltration and spatial redistribution within tumors. Tumors were segmented into 200 μm concentric bands from the tumor rim to the core, and the percentage of marker-positive cells was quantified in each band. Spatial distance was normalized from rim (0) to core (1). Scale bar: 200 μm.

Figure 5. KIF17-mediated surface tsMHC-II promotes T-cell activation and enhances anti-PD-1 responsiveness.
A. Up-regulation of immune checkpoint transcriptional signatures in Kif17-OE 4T1 tumors. Signatures inferred from bulk RNA-seq data using the IOBR package. Statistical comparisons were performed between groups using unpaired two-tailed Student's t-test.
B. Quantification of $PD\text{-}1^+$ and $PD\text{-}L1^+$ cells in EV and Kif17-OE 4T1 tumors. Data are presented as the percentage of marker-positive cells. Statistical comparisons were performed between groups using unpaired two-tailed Student's t-test.
C. Kif17 overexpression induces increased PD-1 and PD-L1 positive cells. Representative multiplex immunofluorescence images of EV and Kif17-OE 4T1 tumors stained for panCK (tumor cells), PD-1, PD-L1, and DAPI. Scale bar: 100 μm.
D. Schematic overview of the in vivo tumor model combining Kif17 overexpression and anti-PD-1 immunotherapy. Orthotopic syngeneic 4T1 mammary tumors were established by injecting EV- or Kif17-OE 4T1 cells into the mammary fat pad of BALB/c mice. Mice carrying different cell models were further randomized into two groups one week after injection receiving either anti-IgG or anti-PD-1 immunotherapy. Anti-PD-1 antibody was administered intravenously at 10 mg/kg twice weekly.
E. Tumor volume change of all groups separately and combined. Comparison of tumor volume between groups were done using Two-way ANOVA and Tukey's multiple comparison.
F. Kaplan–Meier survival analysis of tumor-bearing mice receiving anti-PD-1 immunotherapy. Mice bearing EV or Kif17-OE tumors were treated with anti-PD-1 antibody (10 mg/kg, i.v., twice weekly) or corresponding control IgG. Survival was monitored over time and plotted using Kaplan–Meier analysis. Survival differences between groups were analyzed using the log-rank (Mantel–Cox) test.
G. Multiplex spectral flow cytometry analysis of tumor immune microenvironment remodeling following KIF17 overexpression and anti-PD-1 immunotherapy. Bar plots

represent mean ± SD with individual mice shown as dots. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test.
H. Principal component analysis (PCA) of multiplex spectral flow cytometry profiling revealed distinct immune-state remodeling across treatment groups.
I. Multiplex spectral flow cytometry dotplot of $CD4^+$ T cells and dendritic cells (DC) in all four groups.
J. Quantification of activated $CD4^+$ T cells following co-culture with 4T1 tumor cells. $CD4^+$ T cells were isolated from the spleens of mice bearing EV or Kif17-OE 4T1 tumors and co-culture with EV or Kif17-OE 4T1 cells at an effector-to-target ratio of [10 : 1]. For MHC-II blockade, tumor cells were pre-incubated with anti-mouse I-A/I-E antibody (clone M5/114,10 μg/mL) or the corresponding rat IgG2b isotype control. The antibody was maintained throughout the 24-h co-culture. Activated $CD4^+$ T cells were identified as live $CD45^+CD3^+CD4^+CD8^-CD25^+/CD69^+$ cells and presented as the percentage of parent $CD4^+$ T cells. Bar plots represent mean ± SD with individual mice shown as dots. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test.
K. Quantification of activated $CD8^+$ T cells following co-culture with 4T1 tumor cells. $CD8^+$ T cells +/- $CD4^+$ T cells isolated from EV or Kif17-OE 4T1 tumor-bearing mice were co-cultured with EV or Kif17-OE 4T1 cells at an effector-to-target ratio of [10: 10: 1]. MHC-II blockade was performed as above. Activated $CD8^+$ T cells were identified as live $CD45^+CD3^+CD4^-CD8^+CD25^+/CD69^+$ cells and presented as the percentage of parent $CD8^+$ T cells. Bar plots represent mean ± SD with individual mice shown as dots. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test.
L. Quantification of tumor-cell killing following co-culture with splenic $CD4^+$ and $CD8^+$ T cells. EV or Kif17-OE 4T1 cells were co-cultured with $CD8^+$ T cells +/- $CD4^+$ T cells isolated from the indicated tumor-bearing mice for 24 hours as described above. Dead cells were stained with DAPI and measured with flow cytometry. Killing efficacy was calculated as the percentage of dead cells among total identified tumor cells. Bar plots represent mean ± SD with individual mice shown as dots. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test.

Figure 6. KIF17 serves as a predictive biomarker for cancer immunotherapy.
A. KIF17-tsMHC-II immunofluorescence staining of pre-neoadjuvant patient biopsy samples. Patients then received anti-PD-1 immunotherapy and pathological evaluation was done afterwards. 3 pathological complete response (pCR) and 3 non-pathological complete response patients were selected. Immunofluorescent images were quantified using QuPath. Mean fluorescent intensity (MFI) of KIF17 and tsMHC-II were quantified by normalizing to corresponding DAPI signal of the cell. Statistical comparisons were performed between groups using unpaired two-tailed Student's t-test. Scale bar: 100 μm.
B. KIF17-tsMHC-II immunofluorescence staining of late-stage recurrent or distal metastatic patient biopsy samples. Patients then received anti-PD-1 immunotherapy and pathological evaluation was done afterwards. 3 Responders (R) and 3 non-Responders (non-R/NR) were selected. Quantification was done as above.

C. Comparisons of KIF17 expression between R and NR patients receiving anti-PD-1immunotherapy from different cohorts. Comparisons were done using student T tests.
D. Receiver operating characteristic (ROC) curve analysis of KIF17 expression for prediction of immunotherapy response across independent breast cancer immunotherapy cohorts. The area under the curve (AUC) is indicated within each panel. Grey diagonal lines indicate random-classification performance. Cohort names and R/NR sample numbers are shown above each plot.
E. Forest plot meta-analysis of KIF17 expression differences between immunotherapy R and NR across independent breast cancer immunotherapy cohorts. Standardized mean differences (SMDs) and corresponding 95% confidence intervals were calculated for each cohort and visualized as individual effect sizes. The pooled random-effects model is shown at the bottom of the plot. Dashed vertical lines indicate no effect (SMD = 0).
F. Cohort-level paired comparison of mean KIF17 expression between immunotherapy R and NR groups across independent breast cancer immunotherapy cohorts. Statistical significance was evaluated using a paired two-tailed Student's t-test.
G. ROC analysis evaluating the predictive performance of KIF17 expression for immunotherapy response across pooled breast cancer immunotherapy cohorts. AUC and corresponding 95% confidence interval indicated within the plot.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.pdf
- WBraw.pdf