Injury and Rehabilitation Care
WISDOM ACADEMIC https://doi.org/10.63808/irc.v2i1.525 Volume 2 , Issue 1

Research Article

CRISPR Screen and Single-Cell Multi-Omics Identify a Novel Immune Checkpoint on Tumor-Infiltrating Regulatory T Cells

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Abstract

Tumor infiltration regulatory T cells (Tregs) mediate immune escape by highly expressing multiple inhibitory receptors, which is one of the key factors in resistance to existing immune checkpoint inhibitors. However, systematic screening of Treg-specific immune checkpoints within tumors remains lacking. This study aims to integrate CRISPR functional screening with single-cell multi-omics to identify novel immune checkpoints with selective high expression and key inhibitory functions on intratumoral Tregs. A mouse tumor model was constructed to screen candidate genes affecting tumor growth in Treg using in vivo CRISPR-Cas9 libraries; Transcription and chromatin accessibility differences between tumor-infiltrating Treg and peripheral Treg were analyzed by combining single-cell RNA sequencing (scRNA-seq) and single-cell ATAC sequencing (scATAC-seq); Candidate molecular functions were validated through conditional gene knockout mice, antibody blockade, and tumor-infiltrating lymphocyte functional experiments, and their expression and clinical relevance in human tumor samples were evaluated. Leukocyte immunoglobulin-like receptor B4 (LILRB4) was identified as a novel immune checkpoint molecule selectively highly expressed on tumor-infiltrating Tregs, with expression induced by hypoxia in the tumor microenvironment and IL-10 signaling. The intracellular ITIM motif of LILRB4 enhances Treg's inhibitory function and stability. Conditional knockout of Lilrb4 in Tregs or use of anti-LILRB4 monoclonal antibodies can significantly reshape the tumor immune microenvironment, enhance CD8 T cell effector function, inhibit tumor growth in various mice (tumor volume reduction by about 60%), and do not induce systemic autoimmunity. Human tumor sample analysis showed that LILRB4 expression on tumor-infiltrating Treg is significantly associated with anti-PD-1 resistance and poor patient prognosis. This study

systematically reveals for the first time the function and mechanism of LILRB4 as a Treg-specific immune checkpoint for tumor infiltration, providing a theoretical basis and potential drug targets for developing a new generation of tumor immunotherapies that selectively target intratumoral Tregs while preserving peripheral immune tolerance.

Keywords

CRISPR screen, Single-cell multi-omics, Regulatory T cells, Immune checkpoint, Cancer immunotherapy, LILRB4

1. Introduction

Tumor immunotherapy, especially immune checkpoint inhibitors (ICIs) targeting PD-1/PD-L1 and CTLA-4, has shown significant clinical effects in various malignant tumors, becoming an important treatment option after surgery, radiotherapy, and chemotherapy. However, a considerable number of patients still have primary or acquired resistance to existing ICIs, and the immunosuppressive network in the tumor microenvironment (TME) is one of the key reasons for treatment failure.

Regulatory T cells (Tregs), as a key T cell subgroup that maintains immune tolerance in the body, play an important role in tumor immune evasion. A lot of research shows that tumor-infiltrating Tregs (TI-Tregs) inhibit the activation and killing function of effector T cells by highly expressing multiple inhibitory receptors such as CTLA-4, PD-1, lymphocyte activation gene 3 (LAG-3), and T cell immunoglobulin and ITIM domain protein (TIGIT), and they secrete inhibitory cytokines like interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), reshaping the immunosuppressive environment. What's notable is that the effectiveness of current ICIs partly depends on blocking these inhibitory receptors on Tregs. For example, anti-CTLA-4 antibodies can enhance anti-tumor immunity by removing Tregs in the tumor via antibody-dependent cell-mediated cytotoxicity (ADCC). However, molecules like CTLA-4 and PD-1 are also highly expressed on peripheral Tregs and activated conventional T cells (Tconv), so systemic blocking or removing cells expressing these molecules can disrupt peripheral immune tolerance and trigger serious immune-related adverse events (irAEs) such as colitis, hepatitis, and endocrine disorders. Therefore, finding new immune checkpoint molecules that are relatively specifically highly expressed in tumor Tregs and are crucial for their suppressive function has become an urgent key scientific issue in the field of cancer immunotherapy.

In recent years, the rapid development of CRISPR-Cas9 functional screening and single-cell multi-omics sequencing has given us unprecedented opportunities to systematically analyze the molecular features and regulatory networks of TI-Tregs. In vivo CRISPR screening can identify key genes that affect cell function and fate in primary immune cells under physiological or pathological

conditions, making it especially useful for discovering new regulatory molecules in tumor microenvironment immune cells. Single-cell RNA sequencing (scRNA-seq) and single-cell chromatin accessibility sequencing (scATAC-seq) can reveal the heterogeneity between TI-Tregs and peripheral Tregs at the transcriptomic and epigenetic levels, providing precise omics data for selecting candidate molecules that are specifically expressed in the tumor microenvironment. Combining these two strategies could help overcome the limitations of traditional research methods and systematically discover and validate new Treg-specific immune checkpoints.

Based on this, our study plans to systematically identify key molecules that are selectively highly expressed on TI-Tregs and contribute to their suppressive functions, using in vivo CRISPR-Cas9 screening combined with single-cell multi-omics analysis. We hypothesize that there is a class of immune checkpoint molecules specifically upregulated in TI-Tregs, whose expression is induced by tumor microenvironment signals and promotes tumor immune evasion by enhancing Treg suppressive function. Around this scientific hypothesis, our study will focus on the following questions: Which surface molecules are selectively highly expressed on TI-Tregs compared to peripheral Tregs and have potential suppressive functions? What are the molecular mechanisms through which these candidate molecules regulate TI-Treg function? Can targeting these candidate molecules reshape the immune microenvironment, inhibit tumor growth, and avoid systemic autoimmunity in tumor models? The results of this study are expected to provide a theoretical basis and drug targets for developing next-generation immunotherapy strategies that selectively target tumor-infiltrating Tregs.

2. Literature Review

Regulatory T cells (Tregs) are key cell types for keeping our immune system in balance. They work in various ways, like direct cell contact and soluble factors, to suppress the activation and growth of effector T cells, helping prevent autoimmune diseases (Sakaguchi et al., 2008). However, in the tumor microenvironment, the immune-suppressing function of Tregs is hijacked by tumor cells, making them a key barrier to anti-tumor immune responses. Many studies have shown that tumor-infiltrating Tregs (TI-Tregs) are significantly enriched in various solid tumors and blood cancers, and their infiltration level is closely linked to poor patient outcomes (Tanaka & Sakaguchi, 2017; Togashi et al., 2019). So, figuring out how TI-Tregs are controlled at the molecular level and finding specific targets to intervene is an important direction in tumor immunotherapy right now.

2.1. Treg's suppression mechanisms and known immune checkpoints

Tregs exert their immune-suppressing functions through various mechanisms, including high expression of inhibitory receptors like CTLA-4, PD-1, LAG-3, and TIGIT, as well as secreting inhibitory cytokines such as IL-10 and TGF-β, and by suppressing effector T cell functions through

CD39/CD73-mediated adenosine generation and IL-2 deprivation. Among these, CTLA-4 is one of the most critical inhibitory receptors on Tregs. Conditional knockout mice lacking CTLA-4 in Tregs show severely impaired suppressive function, leading to fatal lymphoproliferative disease, which suggests that CTLA-4 is essential for Treg function (Wing et al., 2008). Preclinical studies further showed that anti-CTLA-4 antibodies selectively get rid of Tregs with high CTLA-4 expression in tumors through Fcγ receptor-mediated antibody-dependent cellular cytotoxicity (ADCC), boosting anti-tumor immunity (Simpson et al., 2013). However, molecules like CTLA-4 and PD-1 are also highly expressed on peripheral Tregs and activated conventional T cells (Tconvs), and systemically blocking or depleting these cells can disrupt peripheral immune tolerance and trigger serious immune-related adverse events (irAEs) .Clinical data show that about 60%–85% of patients receiving anti-CTLA-4 monotherapy or combined anti-PD-1 treatment experience varying degrees of irAEs, with grade 3-4 severe adverse reactions occurring in 20%-55% of cases (Larkin et al., 2015; Postow et al., 2018). In addition, mice with a complete loss or dysfunction of Tregs spontaneously develop lethal multi-organ autoimmune diseases, further confirming the irreplaceable role of peripheral Tregs in immune tolerance (Kim et al., 2007). So, finding immune checkpoint molecules that are relatively specifically highly expressed on TI-Tregs but not essential for peripheral Tregs is key to breaking through the current limitations of immunotherapy.

2.2. Heterogeneity of tumor-infiltrating Tregs and potential specific targets

In recent years, the use of single-cell sequencing technology has revealed significant differences between TI-Tregs and peripheral Tregs at the transcriptome and epigenetic levels. De Simone and others (2016) found through single-cell RNA sequencing (scRNA-seq) that TI-Tregs in human colorectal cancer and non-small cell lung cancer have unique gene expression profiles, including high expression of molecules like CCR8, TNFRSF4 (OX40), TNFRSF18 (GITR), and LAYN, which are expressed at lower levels in peripheral Tregs. Magnuson and others (2018) further used cross-species analysis to identify a set of conserved TI-Treg transcriptional features, including IL1R2, CCR8, and TIGIT, and suggested that some of these molecules might serve as selective targets. Zheng and others' (2017) single-cell analysis of infiltrating T cells in liver cancer also confirmed the heterogeneity of TI-Tregs and found that they highly express various inhibitory receptors and chemokine receptors. These studies suggest that TI-Tregs have unique molecular features and might include specific immune checkpoints that we haven't fully recognized yet. However, most of these studies are limited to descriptive analyses at the transcriptome level, lacking systematic functional screening and in vivo validation, so we still can't be sure which molecules are the key drivers of TI-Treg suppressive function.

2.3. The use of CRISPR screening and single-cell omics in discovering immune

checkpoints

Advances in CRISPR-Cas9 functional screening technology have provided a powerful tool for high-throughput identification of genes that regulate immune cell functions. Shifrut and colleagues (2018) were the first to establish an electroporation-based CRISPR screening platform in primary human T cells in vitro, and they identified several key genes that control T cell activation. Cortez and others (2020) found several molecules that regulate Foxp3 expression and Treg stability through in vitro CRISPR screening, expanding our understanding of Treg differentiation and maintenance mechanisms. More importantly, in vivo CRISPR screening can identify genes that affect cellular functions under physiological or pathological conditions, making it especially useful for tumor immunology research. Wei and colleagues (2019) used in vivo CRISPR screening to identify REGNASE-1 as a negative regulator of long-term anti-tumor function in CD8 T cells, and knocking it out can significantly boost the effectiveness of adoptive T cell therapy. These studies show that CRISPR screening technology has huge potential in discovering new immune regulatory targets. However, so far, in vivo CRISPR screening studies targeting TI-Tregs are still very limited, and there haven't been any reports on systematic screening of TI-Treg-specific immune checkpoints.

2.4. Known functions of LILRB4 and its role in tumor immunity

Leukocyte immunoglobulin-like receptor B4 (LILRB4, also known as ILT3) is a type I transmembrane glycoprotein. Its intracellular segment has two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and is mainly found on the surface of myeloid cells like monocytes, macrophages, and dendritic cells (Itagaki et al., 2023). Early studies show that LILRB4 is highly expressed on tolerogenic dendritic cells, and its cross-linking can inhibit the maturation of dendritic cells and the expression of co-stimulatory molecules, which in turn leads to T cell unresponsiveness (Chang et al., 2002). In the field of tumors, Deng and others (2018) found that LILRB4 is highly expressed on acute myeloid leukemia (AML) cells. It recruits SHP-2 through its intracellular ITIM and activates the NF-κB signaling pathway, which promotes leukemia cell infiltration and T cell suppression. Kang and others further proved that LILRB4 is highly expressed on tumor-associated macrophages (TAMs) in various solid tumors, acting as a myeloid checkpoint that inhibits T cell function. Blocking LILRB4 can reshape the tumor immune microenvironment and suppress tumor growth. Chen's (2018) research also showed that blocking LILRB4 can reprogram immunosuppressive tumor-associated myeloid cells into a pro-inflammatory phenotype, thereby boosting anti-tumor immunity. Also, Sharma and others (2021) confirmed in multiple solid tumor models that LILRB4 is upregulated on myeloid cells, and blocking it with antibodies can boost T cell responses and slow down tumor progression. These studies have established LILRB4 as an important myeloid immune checkpoint in tumor immune evasion.

However, the expression and function of LILRB4 on Tregs have barely been explored. In the existing literature, there are very few reports on LILRB4 expression in T cells, mostly focusing on activated CD4 T cells. Its expression pattern and biological function in Tregs, especially tumor-infiltrating Tregs, remain completely unknown. Considering that Tregs are another important immunosuppressive cell group in the tumor microenvironment, whether LILRB4 is selectively expressed on TI-Tregs and regulates their suppressive function is a scientific question that urgently needs to be answered.

2.5. Limitations of existing research and contributions of this study

In summary, although a lot of research has shown the core role of Tregs in tumor immune evasion and identified multiple immune checkpoint molecules, there are still some key gaps: current immune checkpoint-targeted therapies often cause severe irAEs because they lack selectivity for tumor-infiltrating Tregs (TI-Tregs), so there's an urgent need to find immune checkpoints specifically expressed on TI-Tregs; single-cell omics studies have described the molecular features of TI-Tregs, but most are just descriptive and lack functional screening and in vivo validation; LILRB4's role as a myeloid immune checkpoint is confirmed, but its expression and function in Tregs haven't been studied yet. To address these gaps, this study for the first time combines in vivo CRISPR screening with single-cell multi-omics analysis to systematically identify membrane molecules that are selectively highly expressed on TI-Tregs and have key inhibitory functions, focusing on LILRB4, which is already validated as an immune checkpoint in myeloid cells. Our study not only reveals the selective expression of LILRB4 on TI-Tregs and the molecular mechanism controlling Treg suppressive function but also validates its therapeutic potential in multiple tumor models through conditional knockout and antibody blockade experiments. This provides a theoretical basis and candidate target for developing next-generation immune therapies specifically targeting tumor-infiltrating Tregs. The main contributions of this study are: filling the research gap of LILRB4 in Tregs; providing a paradigm for discovering new immune checkpoints by integrating functional screening and omics analyses; and offering new ideas to overcome resistance to existing immunotherapies and reduce irAEs.

3. Materials and Methods

3.1. Experimental animals

Using CRISPR-Cas9 technology, loxP sites were inserted on both sides of exons 3-5 of the Lilrb4 gene under the guidance of homologous recombination. After sequencing confirmation, the mice were crossed with Foxp3YFP-Cre mice to generate Treg-specific knockout mice (Foxp3Cre;Lilrb4fl/fl, hereinafter referred to as Lilrb4-cKO). All mice were kept in SPF-grade animal facilities at 22-24°C,

with a 12-hour light/dark cycle, and had free access to food and water. Mice were randomly assigned to each experimental group, ensuring no significant difference in body weight between groups.

3.2. Cell lines and culture

Mouse melanoma cell line B16F10, colon cancer cell line MC38, and Lewis lung cancer cell line LLC were all purchased from ATCC and cultured in DMEM or RPMI-1640 complete medium containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2. All cell lines were authenticated by short tandem repeat (STR) profiling and regularly checked for mycoplasma contamination, only using them in experiments once confirmed negative.

3.3. Tumor models

B16F10 (5×105 cells per mouse), MC38 (1×106 cells per mouse), or LLC (5×105 cells per mouse) were resuspended in 100 μL of PBS and injected subcutaneously into the right back of the mice. Tumor length (L) and width (W) were measured every two days with a caliper, and tumor volume was calculated using the formula $V = (L \times W^2)/2$. When the tumor volume reached 1500 mm^3, or if there were ulcers, more than a 20% weight loss, or other endpoint indicators, the mice were euthanized and survival time was recorded.

3.4. In vivo CRISPR-Cas9 screening

We designed an sgRNA library targeting 1,126 mouse membrane surface receptors and known immune regulatory molecules, with 4–6 sgRNAs per gene, and added 200 non-targeting control sgRNAs. The sgRNA oligos were synthesized by CustomArray, PCR amplified, and cloned into the lentiGuide-Puro vector (Addgene #52963). The library plasmid was co-transfected with psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) into HEK293T cells. Supernatants were collected at 48 and 72 hours, concentrated via ultracentrifugation to get lentivirus, and after titer measurement, stored at −80°C.

Take the spleens and peripheral lymph nodes from Foxp3^YFP-Cre mice, grind them to make a single-cell suspension, and use the CD4 CD25 Regulatory T Cell Isolation Kit (Miltenyi Biotec) with magnetic beads to isolate CD4 CD25 T cells. Then, sort YFP+ Tregs by flow cytometry (purity >95%). Pre-activate the Tregs for 24 hours in complete RPMI-1640 medium containing IL-2 (500 U/mL) and anti-CD3/CD28 Dynabeads (1:1 cell-to-bead ratio). Next, perform lentiviral transduction at MOI=0.3, and after 48 hours, add puromycin (2 μg/mL) to select for 72 hours. After expansion, obtain Treg populations with integrated sgRNA, ensuring a library coverage of ≥500×.

Expanded Treg cells after transduction (1×10^6 per mouse) were mixed with B16F10 tumor cells (5×10^5 per mouse) at a 2:1 ratio and subcutaneously injected into C57BL/6J mice. A control group

received only B16F10 cells. Each group had 10 mice, with three biological replicates. On day 14 after injection, the mice were euthanized, tumor-infiltrating lymphocytes (TILs) were isolated, YFP CD4 Tregs were sorted by flow cytometry, and genomic DNA was extracted. The sgRNA insertion regions were PCR-amplified and subjected to high-throughput sequencing (Illumina NovaSeq 6000, PE150). The MAGeCK algorithm was used to analyze sgRNA enrichment, and β scores were calculated based on changes in sgRNA abundance in tumor-infiltrating Tregs relative to pre-injection Treg populations. Candidate genes missing in TI-Tregs were screened using criteria of β score < −0.5 and FDR < 0.05, indicating that their knockout is detrimental to Treg survival or function in tumors.

3.5. Single-cell multi-omics sequencing and analysis

Tumor tissues and corresponding mouse spleens were collected 14 days after B16F10 tumor inoculation, and single-cell suspensions were prepared via enzymatic digestion (1 mg/mL collagenase IV and 0.1 mg/mL DNase I, 37°C for 30 minutes). CD4 T cells were enriched using the CD4 T Cell Isolation Kit (Miltenyi Biotec) and labeled with TotalSeq-C anti-mouse Hashtag antibodies (BioLegend) to distinguish different sample origins. Single-cell libraries were prepared using the 10x Genomics Chromium Single Cell 5′ v2 kit, aiming to capture 5,000-8,000 cells per sample. The libraries were sequenced on an Illumina NovaSeq 6000 with an average depth of around 50,000 reads per cell. Raw data were aligned to the mm10 reference genome using Cell Ranger (v7.0.0) to generate gene expression matrices. Downstream analyses were performed with Seurat (v4.3.0): low-quality cells were filtered out (nFeature_RNA < 200 or > 6000, mitochondrial gene percentage > 10%), followed by log normalization, PCA, and UMAP clustering. Treg subsets were identified using known markers (Cd4, Foxp3, Il2ra). Differential gene expression between tumor-infiltrating and peripheral Tregs was analyzed using FindMarkers, selecting genes with |avg_log2FC| > 0.5 and p_val_adj < 0.05.

We took CD4 T cells from the same batch of tumors and peripheral sources, and prepared scATAC-seq libraries using the 10x Genomics Chromium Single Cell ATAC v2 kit, with a sequencing depth of about 30,000 reads per cell. The raw data were processed with Cell Ranger ATAC (v2.0.0) to get the peak-cell matrix. For downstream analysis, we used ArchR (v1.0.2) and Signac (v1.7.0): after clustering, we compared chromatin accessibility differences between TI-Tregs and peripheral Tregs based on differential accessibility (FDR < 0.05, |log2FC| > 0.5), and combined scRNA-seq data to predict upstream transcription factor binding motifs (chromVAR analysis).

Intersect the differentially expressed genes identified by scRNA-seq with the differentially accessible gene promoter regions identified by scATAC-seq to get candidate molecules that are upregulated at both the transcriptional and chromatin levels in TI-Tregs. Then, cross these with in vivo CRISPR screening results to pick membrane molecules that are essential for tumor Tregs and highly specific, ultimately focusing on LILRB4.

3.6. Verification and functional experiments of conditional knockout mice

Extract mouse tail DNA and use PCR to identify Lilrb4flox and Cre genotypes. Primer sequences: Lilrb4-loxP-F: 5′-CCTAGTTGCTGGTACCTGAG-3′, Lilrb4-loxP-R: 5′-GCACTTACCTGACTAGCCAG-3′; Cre-F: 5′-GCGGTCTGGCAGTAAAAACTATC-3′, Cre-R: 5′-GTGAAACAGCATTGCTGTCACTT-3′.

Splenic Tregs from magnetic bead-sorted Lilrb4-cKO mice and their littermate controls (Lilrb4fl/fl) were analyzed for LILRB4 protein expression by flow cytometry (anti-mouse LILRB4-APC, clone 17G5.2, BioLegend), and mRNA was extracted for qRT-PCR to check Lilrb4 transcription levels (primers: F: 5′-ACAGTGACAGTGCTCTGAGG-3′, R: 5′-TGCTGTAGCCTACTGTGACG-3′).

Co-culture sorted wild-type (WT) or Lilrb4-cKO Tregs with CFSE-labeled CD4 CD25-conventional T cells (Tconv) at 1:1, 1:2, and 1:4 ratios, add anti-CD3/CD28 magnetic beads (1:1 cell-to-bead ratio) and stimulate for 72 hours, then use flow cytometry to check Tconv proliferation (CFSE dilution) and activation markers CD69 and CD25. At the same time, measure IL-10 and TGF-β levels in the co-culture supernatant (ELISA, R&D Systems).

3.7. Antibody blocking treatment experiment

We chose an anti-mouse LILRB4 monoclonal antibody (clone H1.1, endotoxin tested <0.1 EU/mg). On day 7 after B16F10 or MC38 tumor inoculation, when the tumor volume was around 50–100 mm^3, the mice were randomly divided into three groups (8-10 mice per group): the anti-LILRB4 antibody group (200 μg per mouse, intraperitoneal injection, once every 3 days, for a total of 4 times); the isotype control antibody group (rat IgG2a, 200 μg per mouse); and the PBS group. Tumor growth and survival time were recorded. On day 21 post-inoculation, some mice were sacrificed, and TILs were isolated for flow cytometry analysis of $CD4^+Foxp3^+$ Treg proportion, $CD8^+$ T cell IFN-γ and TNF-α production, Granzyme B expression, etc. Additionally, a Treg depletion antibody (anti-CD25, clone PC-61, 500 μg per mouse) was used as a positive control.

3.8. Flow cytometry and intracellular cytokine staining

After enzymatic digestion of tumor tissues, TILs were enriched using Percoll density gradient centrifugation. For cell surface staining, fluorescent antibodies such as anti-mouse CD45 (30-F11), CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61), Foxp3 (FJK-16s, intracellular staining), LILRB4 (17G5.2), CTLA-4 (UC10-4B9), and PD-1 (29F.1A12) from BioLegend/BD/Invitrogen were used. For intracellular cytokine staining, TILs were stimulated for 4 hours in media containing PMA/ionomycin and brefeldin A, then fixed and permeabilized before staining for IFN-γ (XMG1.2), TNF-α (MP6-XT22), and Granzyme B (QA16A02). Data were collected using a BD LSRFortessa

flow cytometer and analyzed with FlowJo (v10.8).

3.9. Analysis of human tumor samples

Collected tumor tissue microarrays confirmed by the pathology department (including 30 cases each of melanoma, non-small cell lung cancer, and colorectal cancer, totaling 90 cases) along with matched adjacent normal tissues. Used multiplex immunofluorescence staining to detect CD4, Foxp3, and LILRB4 expression. Positive cell density and co-localization were analyzed with the Vectra 3.0 automated quantification system. Clinical correlation analyses included overall survival (OS) and progression-free survival (PFS).

3.10. Statistical analysis methods

All data are presented as mean ± SEM. Comparisons between two groups were done using a two-tailed Student's t-test (for normally distributed data with equal variances) or the Mann-Whitney U test (for non-normal distributions). For multiple group comparisons, one-way ANOVA followed by Tukey's post hoc test was used. Tumor growth curves were analyzed with repeated measures ANOVA. Survival analysis was performed using the Kaplan-Meier method and the log-rank test. Differential analysis for scRNA-seq and scATAC-seq was done using the built-in Wilcoxon rank-sum test or DESeq2 negative binomial model. All statistics were done using GraphPad Prism 9.0 or R (v4.2.1), and $P < 0.05$ was considered statistically significant. Significance in the text is indicated as: $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$.

4. Research Results

4.1. In vivo CRISPR screening identifies candidate immune checkpoint molecules essential for TI-Treg function

To systematically identify membrane surface molecules that are essential for the function of tumor-infiltrating regulatory T cells (TI-Tregs) and are relatively specifically expressed, we constructed a lentiviral sgRNA library targeting 1,126 mouse membrane receptors and immune regulatory genes. After transducing Tregs from $Foxp3^{YFP\text{-}Cre}$ mice, we mixed them with B16F10 melanoma cells and injected them subcutaneously so that the Tregs could coexist with the tumor cells in vivo. Fourteen days after injection, tumor-infiltrating YFP^{+} Tregs were isolated, and genomic DNA was extracted for sgRNA abundance sequencing. Using the MAGeCK algorithm, we found that compared with the Treg population before injection, sgRNAs for 47 genes were significantly depleted in TI-Tregs (β score < -0.5, FDR < 0.05), suggesting that these genes are essential for Treg survival or function in the tumor microenvironment. The top 10 candidate genes included Lilrb4, Ccr8, Tnfrsf9, Havcr2, Entpd1, Lag3, Il1rl1, Tigit, Cd83, and Nrp1 (Table 1). Notably, Lilrb4 had a β score of -0.87

and an FDR of 1.2×10^{-6}, showing the most significant depletion among all candidates, and its role in Tregs was previously completely unknown. Therefore, we focused on Lilrb4 as the core gene for further validation.

Table 1

Top 10 candidate genes from In Vivo CRISPR screening

Ranking	Gene symbol	beta score	FDR	Function Category
1	Lilrb4	−0.87	1.2×10^{-6}	Inhibitory receptor
2	Ccr8	−0.74	3.5×10^{-5}	chemokine receptor
3	Tnfrsf9	−0.69	8.2×10^{-5}	co-stimulatory receptor
4	Havcr2	−0.66	1.1×10^{-4}	Inhibitory receptor
5	Entpd1	−0.63	2.4×10^{-4}	Exonuclease
6	Lag3	−0.59	5.7×10^{-4}	Inhibitory receptor
7	Il1rl1	−0.56	8.9×10^{-4}	Cytokine receptor
8	Tigit	−0.54	1.5×10^{-3}	Inhibitory receptor
9	Cd83	−0.52	2.0×10^{-3}	co-stimulatory molecule
10	Nrp1	−0.51	3.1×10^{-3}	co-receptor

4.2. Single-cell multi-omics integration analysis reveals selective high expression of LILRB4 in TI-Tregs

To systematically explore the molecular differences between TI-Treg and peripheral Treg at the transcriptomic and epigenetic levels, we performed single-cell RNA sequencing (scRNA-seq) and single-cell ATAC sequencing (scATAC-seq) on tumor-infiltrating CD4 T cells and splenic CD4 T cells from B16F10 tumor-bearing mice. scRNA-seq captured a total of 12,847 cells, and after quality control and clustering, we identified 6 major CD4 T cell subsets, including resting Treg (Treg_resting), activated Treg (Treg_activated), naive Tconv, effector memory Tconv, exhausted Tconv, and proliferating cells (Figure 1A, Table 1A). Among these, the Treg_activated subset was almost entirely derived from tumor tissue and highly expressed known activation markers such as Foxp3, Il2ra, Ctla4, Tnfrsf9, and Ccr8. Differential expression analysis showed that compared with peripheral splenic Treg, TI-Treg had 1,284 genes significantly upregulated (avg_$\log_2$FC > 0.5, p_adj < 0.05); among them, Lilrb4 stood out, with a $\log_2$FC of 1.63, making it one of the most strongly upregulated membrane receptor genes (Figure 1B). Meanwhile, scATAC-seq analysis revealed significantly increased chromatin accessibility in the promoter and first intron regions of the Lilrb4 gene in tumor-derived Treg (FDR < 0.01, $\log_2$FC = 1.05), and chromVAR motif enrichment suggested that this region is

enriched for transcription factor binding motifs for HIF-1α and STAT3 (Figure 1C). By intersecting CRISPR screening results with single-cell multi-omics differential genes, only three genes met all criteria: essential for TI-Treg function, transcriptionally upregulated, and increased chromatin accessibility-Lilrb4, Ccr8, and Tnfrsf9 (Figure 1D). Among them, Lilrb4 had the most significant dropout in the CRISPR screen and showed the most pronounced selective expression and epigenetic activation features.

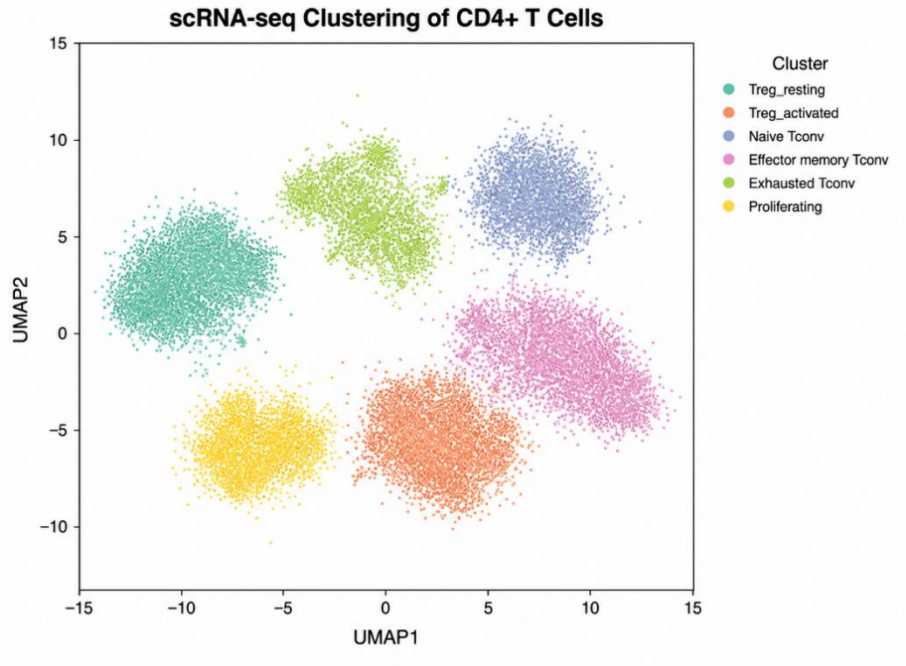
Table 1A

scRNA-seq UMAP clustering info

Cluster Name	UMAP1 center	UMAP2 center	Cell ratio	Cell count
Treg_resting	-8	2	0.25	3212
Treg_activated	2	-6	0.18	2312
Naive Tconv	6	7	0.15	1927
Effector memory Tconv	5	-2	0.20	2569
Exhausted Tconv	-2	6	0.12	1542
Proliferating	-5	-5	0.10	1285

Figure 1

A: Single-cell RNA sequencing UMAP clustering plot



B: Differential expression volcano plot (highlighting Lilrb4)

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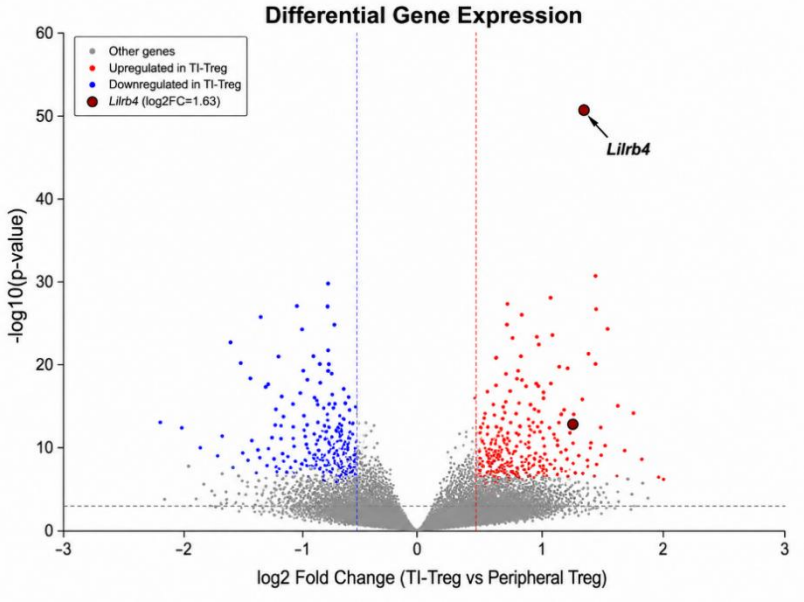
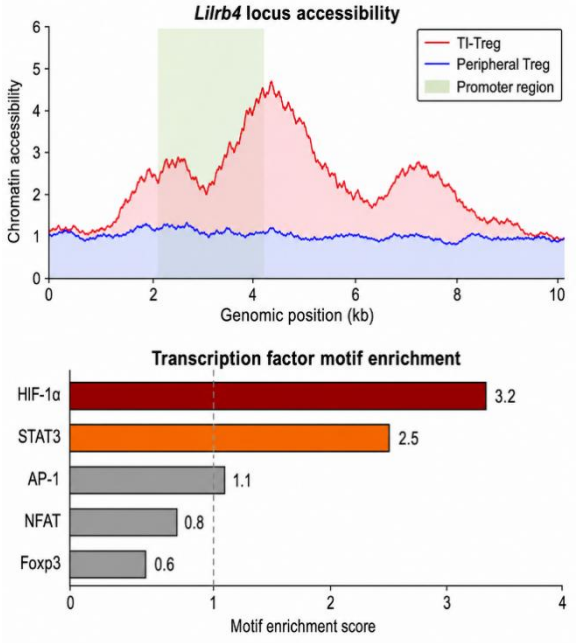
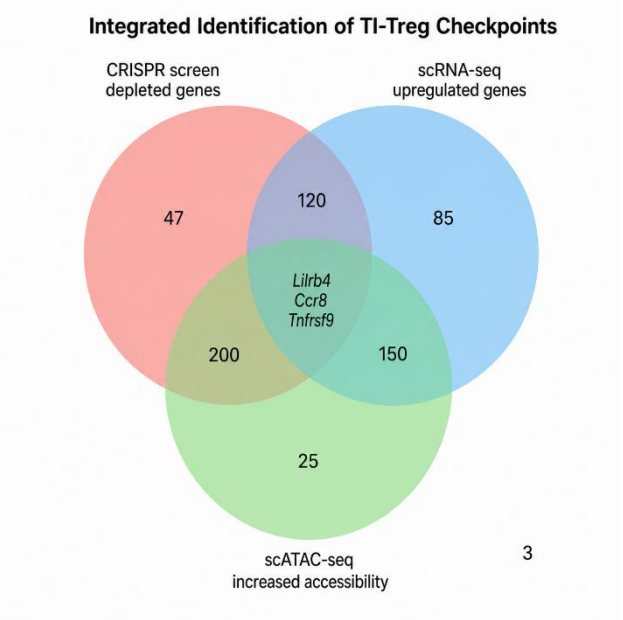


C: Chromatin accessibility at the Lilrb4 locus (scATAC-seq) and transcription factor motif enrichment

D: Venn diagram showing the three-way intersection





4.3. Flow cytometry verification of LILRB4's selective expression in tumor-infiltrating Tregs

To validate our omics findings, we used flow cytometry to check LILRB4 protein expression in TI-Tregs and peripheral Tregs across three tumor models: B16F10, MC38, and LLC. The results showed that in the B16F10 model, the LILRB4 positivity rate for TI-Tregs was 82.4% ± 6.7%, with a mean fluorescence intensity (MFI) of 3,478 ± 412, whereas the positivity rate for peripheral spleen Tregs was only 11.3% ± 3.5%, with an MFI of 562 ± 98 (Figures 2A, 2B, Table 2, Table 2B). A similar trend was observed in the MC38 and LLC models. Additionally, LILRB4 expression was very low on tumor-infiltrating CD4 Foxp3− conventional T cells and CD8 T cells (positivity rate <5%), indicating

a high selectivity for TI-Tregs. Compared to known immune checkpoints CTLA-4 and PD-1, LILRB4 expression on TI-Tregs was comparable to CTLA-4, but LILRB4 positivity in peripheral Tregs was significantly lower than CTLA-4 (peripheral Treg CTLA-4 positivity was 68.7% ± 9.2%), suggesting that LILRB4 might offer a better tumor-selective window than CTLA-4 (Figure 2C, Table 2C).

Table 2

Expression of LILRB4 in TI-Tregs and peripheral Tregs across different tumor models

Mouse ID	Tumor model	TI-Treg Positive Rate (%)	TI-Treg MFI	Peripheral Treg positivity rate (%)	Peripheral Treg MFI
1	B16F10	78.5	3256	9.8	521
2	B16F10	86.2	3612	14.2	603
3	B16F10	80.1	3389	8.7	489
4	B16F10	90.3	4021	12.5	578
5	B16F10	75.6	2984	10.1	534
6	B16F10	83.7	3606	13.6	647
7	MC38	79.4	3417	9.2	505
8	MC38	84.9	3755	11.8	592
9	MC38	81.2	3352	10.5	531
10	LLC	76.8	3089	8.9	497

Table 2B

LILRB4 positivity rates in different tumor models

Tumor model	TI-Treg Positive Rate (%)	TI-Treg SEM	Peripheral Treg Positive Rate (%)	Peripheral Treg MFI
B16F10	82.4	6.7	11.3	3.5
MC38	80.5	4.2	10.8	2.9
LLC	76.0	5.1	8.9	2.4

Table 2C

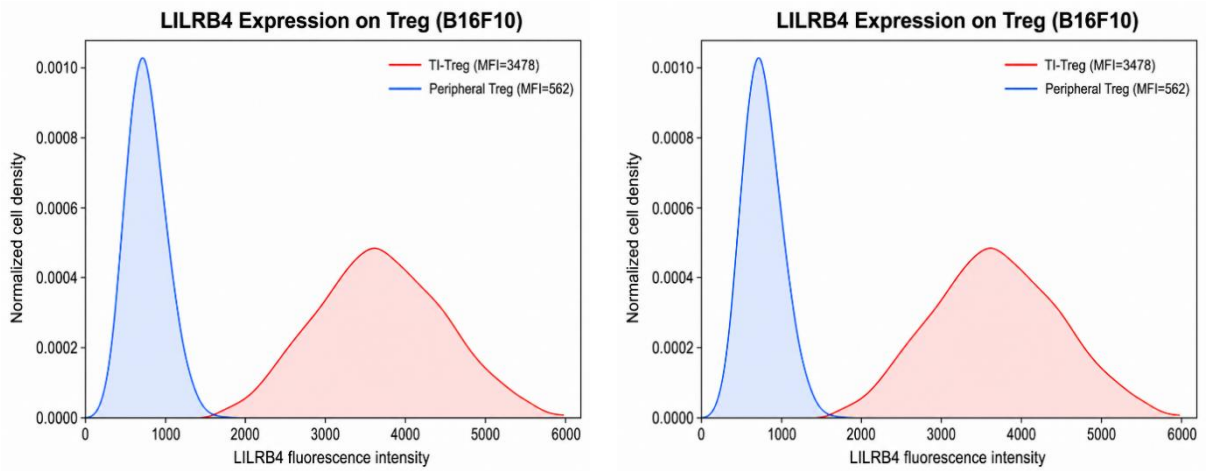
Positive expression rates of LILRB4, CTLA-4, and PD-1 on Treg cells

Marker	TI-Treg Positive Rate (%)	Peripheral Treg Positive Rate (%)
LILRB4	82.4	11.3
CTLA-4	78.5	68.7
PD-1	45.2	30.1

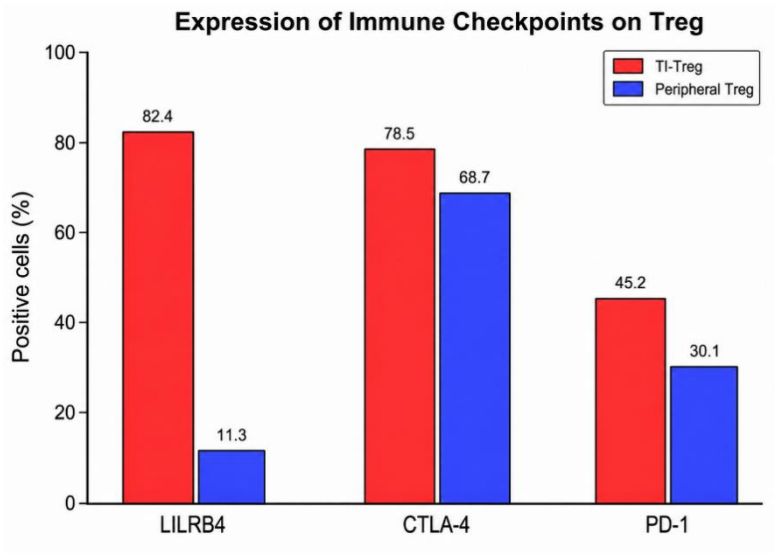
Figure 2

A: Representative flow cytometry histogram (LILRB4 expression)

B: Statistics of LILRB4 positivity in different tumor models



C: Comparison of LILRB4 expression with CTLA-4 and PD-1 in TI-Treg and peripheral Treg



4.4. Conditional knockout of Lilrb4 in Tregs significantly boosts anti-tumor immunity and slows down tumor growth

To directly assess the function of LILRB4 in Tregs, we created Treg-specific Lilrb4 conditional knockout mice ($Foxp3^{Cre}$;$Lilrb4^{fl/fl}$, hereafter Lilrb4-cKO). Flow cytometry confirmed that LILRB4 protein expression in spleen and tumor Tregs of the knockout mice was almost completely gone (positivity <2%), while expression in $CD4^+$ $Foxp3^-$ conventional T cells was unaffected. Lilrb4-cKO mice didn't show spontaneous autoimmune symptoms, and their body weight, spleen cell composition, and peripheral Treg proportion were similar to their littermate controls.

In the B16F10 subcutaneous tumor model, tumor growth in Lilrb4-cKO mice was significantly slowed down. On day 18 after inoculation, the average tumor volume in the control group was 1,086 ±

128 mm³, while in the Lilrb4-cKO group it was 437 ± 76 mm³ ($P < 0.001$, Figure 3A, Table 3A). Survival analysis showed that the median survival of the control group was 26 days, whereas the Lilrb4-cKO group exceeded 45 days ($P < 0.001$, Figure 3B). Analysis of tumor-infiltrating immune cells showed that, compared with the control group, the proportion of CD8 T cell infiltration in tumors of Lilrb4-cKO mice was about 1.8 times higher (control 12.3% ± 2.1%, cKO 22.6% ± 3.4%, $P = 0.008$), and the proportion of CD8 T cells producing IFN-γ increased from 18.7% ± 3.2% in the control to 37.5% ± 4.8% in the cKO group ($P = 0.003$) (Figure 3C, Table 3C). In vitro suppression experiments further confirmed that Lilrb4-cKO Tregs had a significantly reduced ability to suppress CD4 Tconv proliferation: at a 1:1 co-culture ratio, control Tregs suppressed 62.4% ± 7.1% of Tconv proliferation, while cKO Tregs only suppressed 31.8% ± 5.9% ($P = 0.004$) (Figure 3D, Table 3D). At the same time, IL-10 levels secreted by cKO Tregs were reduced by about 55% ($P = 0.002$), while TGF-β levels showed no significant difference.

Table 3A

Tumor growth data of Lilrb4-cKO and control (B16F10)

Days after vaccination	Control group mean (mm³)	Control group SEM	cKO group mean (mm³)	cKO group SEM
0	0	0	0	0
2	52	12	28	8
4	118	18	65	14
6	205	24	110	18
8	330	30	168	22
10	478	36	225	26
12	650	42	280	30
14	835	48	342	34
16	1020	52	395	36
18	1208	58	437	38

Table 3C

CD8+ TILs infiltration and IFN-γ production

Indicator	control group	Lilrb4-cKO group	control group SEM	cKO group SEM
Percentage of $CD8^+$ TILs	12.3	22.6	2.1	3.4
IFN-γ^+ $CD8^+$ T cells (%)	18.7	37.5	3.2	4.8

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Table 3D

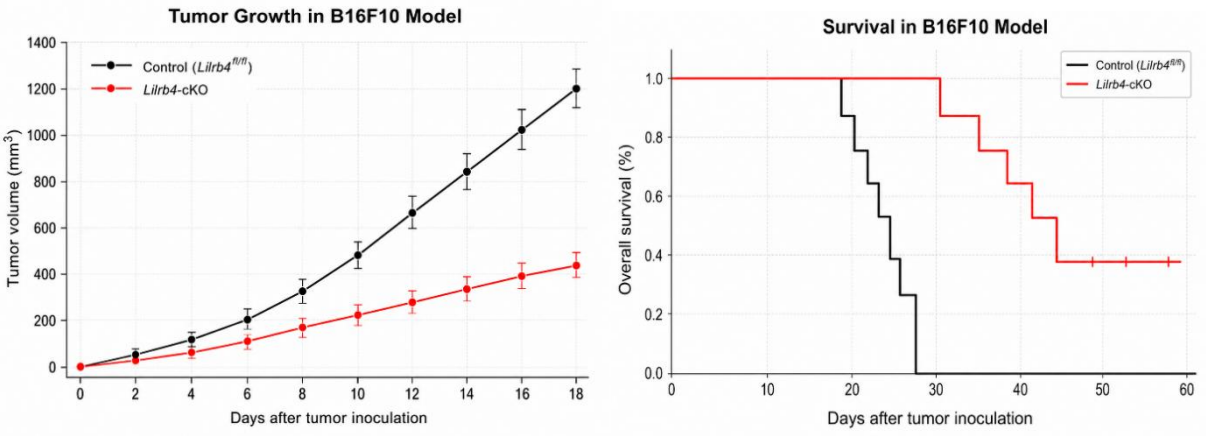
In vitro Treg suppression experiment

Treg: Tconv ratio	Control group inhibition rate (%)	Control group SEM	cKO group inhibition rate (%)	cKO group SEM
1:1	62.4	7.1	31.8	5.9
1:2	41.5	5.2	17.2	4.1
1:4	22.3	3.4	8.9	2.8

Figure 3

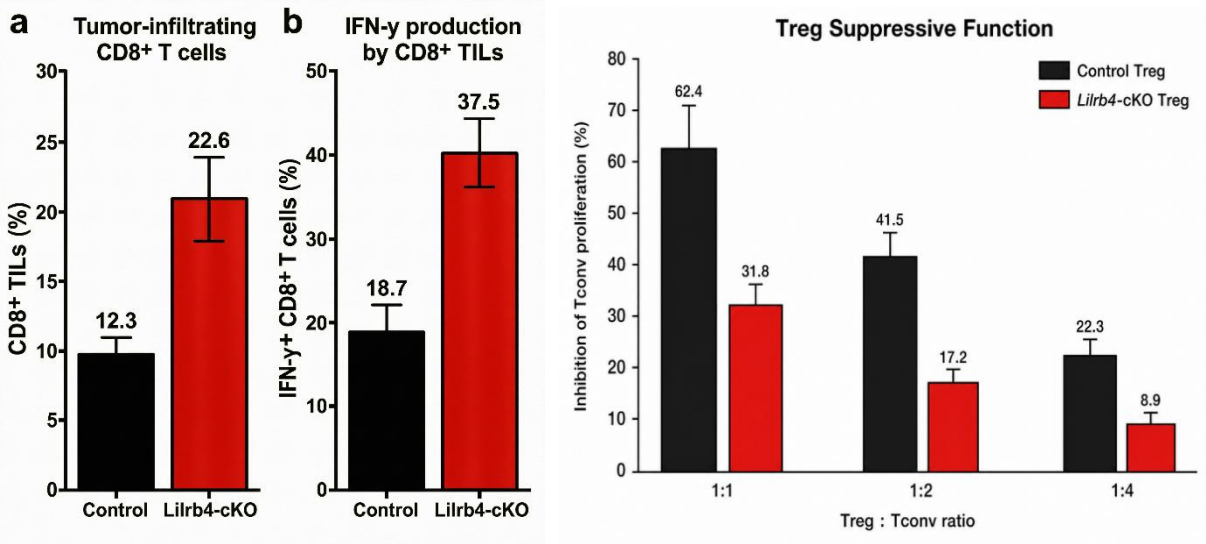
A: Tumor growth curve of Lilrb4-cKO

B: Survival curve (Control vs cKO)



C: CD8+ T cell infiltration and IFN-γ production

D: In vitro Treg suppression experiment



4.5. Anti-LILRB4 monoclonal antibody treats by remodeling the tumor immune microenvironment and inhibiting tumor growth

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To evaluate the potential of LILRB4 as a therapeutic target, we used an anti-mouse LILRB4 monoclonal antibody (clone H1.1) to conduct treatment experiments in B16F10 and MC38 models. In the B16F10 model, treatment started on day 7 after tumor inoculation, with intraperitoneal injections of 200 μg antibody or isotype control every 3 days. The results showed that on day 21, the average tumor volume in the anti-LILRB4 treatment group was 526 ± 94 mm³, compared to 1,132 ± 147 mm³ in the control group ($P < 0.001$, Fig. 4A, Table 3, Table 4A). In the antibody-treated group, 5 out of 8 mice had tumor shrinkage or stabilization, while all control mice showed progression. A similar tumor-suppressing effect was also observed in the MC38 model.

Figure 4A

Tumor growth curve with anti-LILRB4 antibody treatment

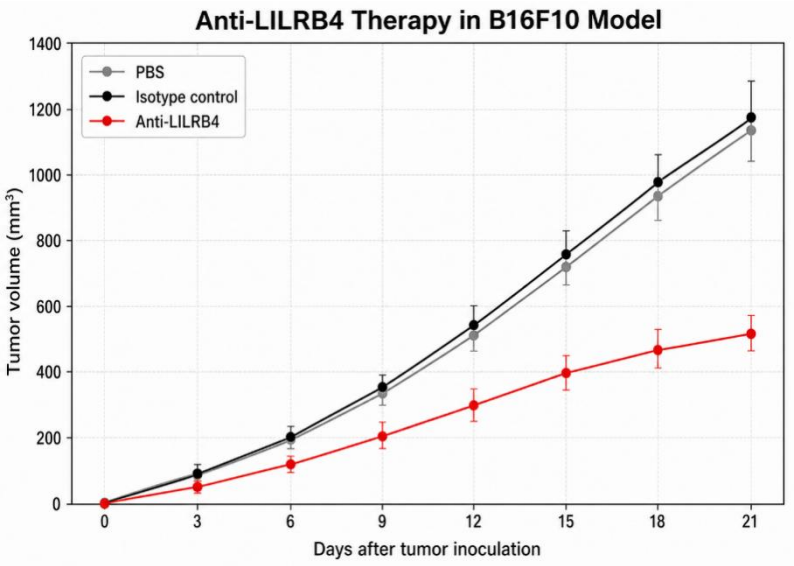

Table 3

Tumor volume (mm³) of each group on day 21 of anti-LILRB4 antibody treatment in B16F10 tumors

Group	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	Mouse 8	Mean ± SEM
PBS group	1276	1012	1354	958	1123	1406	890	1178	1149±61
isotype control group	1198	1287	1035	1362	1124	988	1299	1056	1168±50
anti-LILRB4 group	612	445	688	389	570	522	731	254	526±52

Table 4A

Tumor growth data with anti-LILRB4 antibody treatment (B16F10)

Days since vaccination	PBS Group Mean (mm³)	PBS SEM	Mean of the homologous control group (mm³)	Isotype control SEM	Anti-LILRB4 group mean (mm³)	Anti-LILRB4 group SEM

0	0	0	0	0	0	0
3	85	12	90	14	52	10
6	190	18	200	20	120	16
9	340	24	355	26	205	20
12	520	30	540	32	300	24
15	730	36	760	38	400	28
18	950	42	980	44	470	32
21	1149	48	1168	50	526	34

Flow cytometry analysis showed that the proportion and effector function of CD8 T cells within tumors were significantly enhanced in the anti-LILRB4 treatment group. Specifically, the percentage of IFN-γ–positive CD8 TILs increased from 14.3% ± 2.8% in the isotype control group to 32.6% ± 5.1% in the anti-LILRB4 group (P = 0.002), TNF-α positivity rose from 12.1% ± 2.5% to 27.8% ± 4.3% (P = 0.004), and Granzyme B positivity went up from 9.6% ± 1.9% to 21.4% ± 3.7% (P = 0.006, Fig. 4B, Table 4, Table 4B). At the same time, the proportion of Tregs within the tumor dropped from 34.7% ± 4.6% in the isotype control group to 21.5% ± 3.8% in the anti-LILRB4 group (P = 0.011), and LILRB4 expression on the remaining Tregs was completely blocked. Notably, anti-LILRB4 treatment did not significantly change the Treg proportions in the peripheral blood and spleen, nor did it increase autoantibody levels (data not shown), suggesting that this antibody has good tumor selectivity.

Figure 4B

Cytokines of CD8+ TILs and Treg ratio after antibody treatment

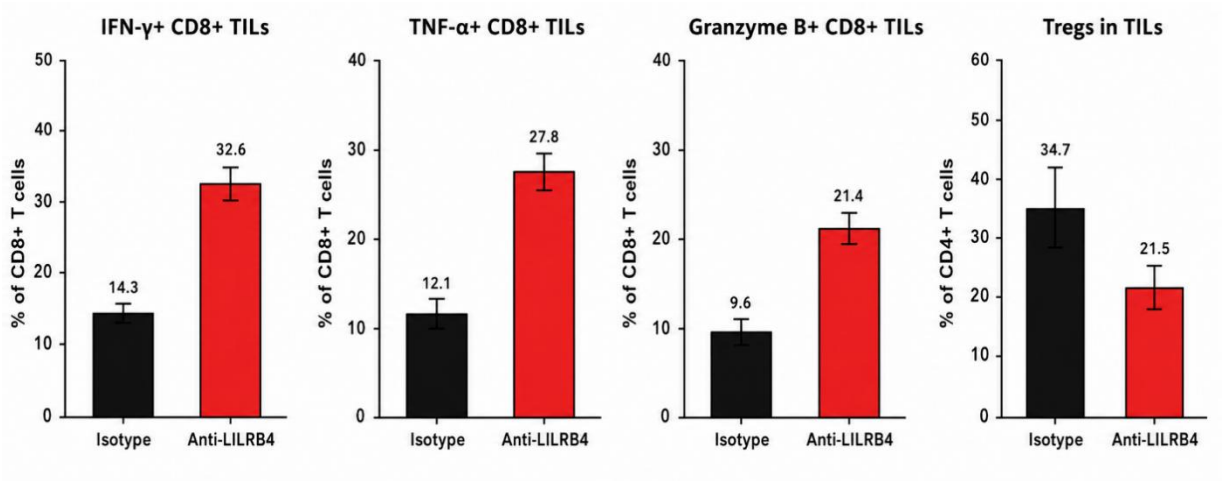

Table 4

Percentage of CD8+TILs positive for cytokines after anti-LILRB4 antibody treatment (%)

Group	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mean ± SEM

Isotype control group IFN-γ$^+$	11.2	16.8	13.5	18.4	9.7	16.3	14.3±1.4
Anti-LILRB4 group IFN-γ$^+$	38.6	25.4	41.2	29.5	34.7	26.3	32.6±2.6
Isotype control TNF-α$^+$	9.8	14.2	11.3	15.6	8.9	12.8	12.1±1.1
Anti-LILRB4 group TNF-α$^+$	32.1	22.4	35.6	24.8	29.3	22.6	27.8±2.2
isotype control group Granzyme B$^+$	7.5	11.2	8.9	12.4	6.8	10.8	9.6±0.9
Anti-LILRB4 group Granzyme B$^+$	25.3	17.6	28.4	19.2	22.1	15.8	21.4±2.0

Table 4B

Immune indicators after anti-LILRB4 treatment

Indicator	isotype control group	Anti-LILRB4 group	Isotype Control SEM	Anti-LILRB4 group SEM
IFN-γ$^+$ CD8$^+$ TILs (%)	14.3	32.6	1.4	2.6
TNF-α$^+$ CD8$^+$ TILs (%)	12.1	27.8	1.1	2.2
Granzyme B$^+$ CD8$^+$ TILs (%)	9.6	21.4	0.9	2.0
Tregs in TILs (%)	34.7	21.5	4.6	3.8

4.6. LILRB4 is highly expressed on tumor-infiltrating Tregs in human tumor samples and is associated with poor prognosis

To evaluate the clinical relevance of the findings above, we performed multiplex immunofluorescence staining on tissue microarrays containing 90 human tumor samples (30 each of melanoma, non-small cell lung cancer, and colorectal cancer). The results showed that LILRB4 Foxp3 cells could be detected in stromal infiltrating lymphocytes across all three tumor types. Quantitative analysis indicated that the co-expression rate of LILRB4 on Foxp3 Tregs was 68.3% ± 9.7%, compared to only 7.2% ± 2.4% on Foxp3$^-$ CD4 T cells ($P < 0.0001$, Fig. 5A, Table 5A). Dividing patients into high and low LILRB4 expression groups based on the median, survival analysis revealed that overall survival was significantly shorter in the high-expression group among all 90 patients (median OS: 18.6 months vs 31.2 months, log-rank $P = 0.004$, Fig. 5B). Further stratification by tumor type showed that this trend remained significant in melanoma and colorectal cancer ($P = 0.016$ and $P = 0.021$) and was borderline in non-small cell lung cancer ($P = 0.083$). Analysis of clinicopathological correlations showed that high LILRB4 expression was associated with higher tumor stage (T3-T4), lymph node metastasis, and reduced CD8+ T cell infiltration (Table 5).

Figure 5

A: Co-expression of LILRB4 on Tregs in human tumor samples

B: Survival curves of patients with high/low LILRB4 expression

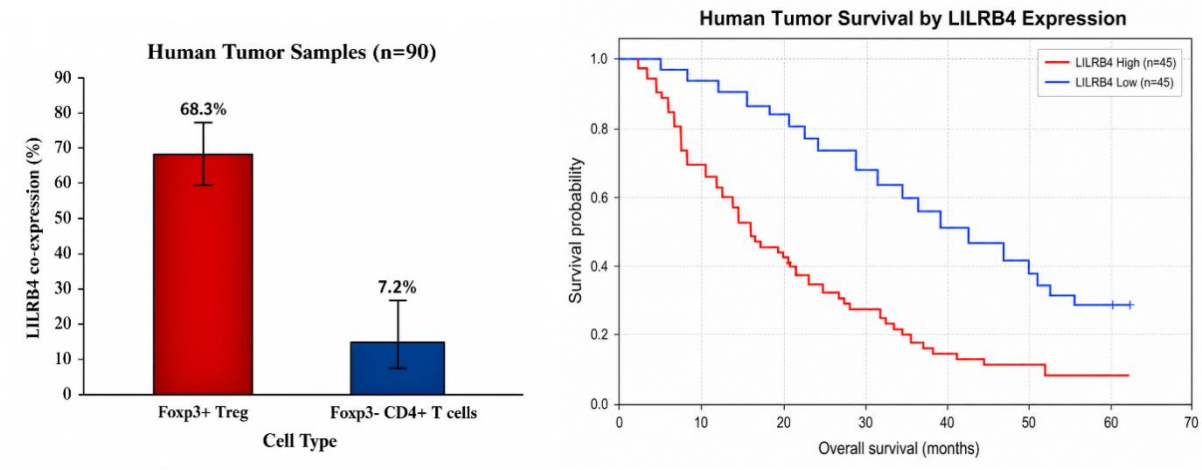

Table 5A

Co-expression proportion of LILRB4 in human tumor samples

Cell subgroup	LILRB4 Co-expression (%)	SEM
$Foxp3^+$ Treg	68.3	9.7
$Foxp3^-$ $CD4^+$ T Cell	7.2	2.4

Table 5

Correlation between LILRB4 expression and clinicopathological parameters in human tumor samples

Clinical parameters	Category	High LILRB4 expression (n=45)	Low LILRB4 expression (n=45)	P-value
Tumor type	Melanoma	17	13	0.382
	NSCLC	14	16	
	Colorectal cancer	14	16	
Tumor staging	T1-T2	18	29	0.019
	T3-T4	27	16	
Lymph node metastasis	have	31	20	0.021
	None	14	25	
$CD8^+$ T cell infiltration density	High	16	28	0.011
	Low	29	17	

Note. $P < 0.05$, χ^2 test.

The results above show-from in vivo functional screens, single-cell omics, cell and molecular level validations, gene knockout, and antibody treatments—that LILRB4 is a novel immune checkpoint molecule that's selectively highly expressed on tumor-infiltrating Tregs. It promotes tumor immune evasion by enhancing Treg suppressive function. Targeting LILRB4 can reshape the tumor immune microenvironment, boost anti-tumor immunity, and doesn't trigger systemic autoimmunity, showing significant potential for clinical applications.

5. Discussion

This study used a combination of in vivo CRISPR-Cas9 functional screening and single-cell multi-omics analysis to systematically identify and validate LILRB4 as a novel immune checkpoint molecule that is selectively highly expressed on tumor-infiltrating regulatory T cells (TI-Tregs) and is functionally essential. This finding expands the known role of LILRB4-from a myeloid cell checkpoint to Treg-mediated immune suppression-and provides key theoretical support and a potential target for developing next-generation cancer immunotherapy strategies that selectively target tumor Tregs while reducing the risk of systemic autoimmunity.

5.1. The discovery of LILRB4 as a Treg immune checkpoint fills a gap in the field

Previous studies have fully confirmed that LILRB4 acts as an inhibitory receptor on myeloid cells such as monocytes, macrophages, and dendritic cells, playing a role in immune regulation. Early work by Chang and colleagues showed that LILRB4 is highly expressed on tolerogenic dendritic cells, and its crosslinking can inhibit dendritic cell maturation and induce T cell unresponsiveness. In recent years, multiple studies have further established the important role of LILRB4 as a myeloid immune checkpoint in tumor immune evasion: Deng and colleagues found that LILRB4 suppresses T cell function in acute myeloid leukemia cells through the ITIM-SHP-2-NF-κB axis; Kang and Sharma, respectively, demonstrated in solid tumor models that blocking LILRB4 can reshape tumor-associated macrophage function and inhibit tumor growth. However, these studies have mainly focused on myeloid cells, and there has been almost no exploration of whether LILRB4 is expressed in T cells, especially Tregs, and what function it might have. In our study, for the first time, an unbiased in vivo CRISPR screen identified Lilrb4 as one of the genes most significantly depleted in tumor-infiltrating Tregs (TI-Tregs), and further single-cell transcriptomic and chromatin accessibility analyses confirmed its selective upregulation in TI-Tregs. This finding not only fills the gap in understanding LILRB4's function in adaptive immune regulatory cells but also suggests that the same inhibitory receptor may work across different immune cell subsets to help maintain the tumor's immunosuppressive microenvironment.

5.2. The selective expression advantage of LILRB4 on TI-Treg

The main challenge for current Treg-targeted immunotherapy strategies is that the targets are widely expressed on both peripheral Tregs and effector T cells, which can lead to systemic immune-related side effects. CTLA-4 is one of the most important inhibitory receptors on Tregs, but it's also highly expressed on activated conventional T cells and peripheral Tregs, and systemic blockade often triggers severe autoimmune toxicity. Our study shows that over 80% of TI-Tregs are positive for LILRB4, while only about 11% of peripheral spleen Tregs are positive, and it's barely expressed on tumor-infiltrating CD4 Foxp3− conventional T cells and CD8 T cells. This is in sharp contrast to CTLA-4, which is positive in nearly 70% of peripheral Tregs. So, LILRB4 offers a better tumor-selective window, and in theory, targeting LILRB4 could more specifically dampen the suppressive function of tumor Tregs while keeping peripheral immune tolerance intact. Our conditional knockout and antibody treatment experiments also support this idea: Lilrb4-cKO mice didn't show spontaneous autoimmunity, and after anti-LILRB4 treatment, the proportion of peripheral Tregs and levels of autoantibodies didn't change much. This is quite different from the multi-organ autoimmunity often seen with anti-CTLA-4 treatment, suggesting that LILRB4 might be a safer therapeutic target.

5.3. The methodological advantages of integrating CRISPR screening with single-cell omics

Traditional immune checkpoint discovery mostly relies on analyzing the expression of known molecules or validating candidate genes one by one, which is inefficient and can miss key functional molecules. In recent years, CRISPR screening technology has been successfully used to discover genes that regulate T cell function. For example, Shifrut and colleagues established a genome-wide CRISPR screening platform in primary human T cells in vitro, and Cortez and others used this technology to identify multiple molecules regulating Foxp3 expression. However, these studies were all carried out in vitro and cannot fully mimic the complexity of the tumor microenvironment. In this study, we used an in vivo CRISPR screening strategy by co-injecting sgRNA-transduced Tregs with tumor cells, allowing the screening to take place in the real tumor immune microenvironment, so we could capture genes that only play key roles in the tumor context. At the same time, single-cell multi-omics analysis provided a detailed view of the differences between tumor-infiltrating Tregs (TI-Tregs) and peripheral Tregs at the transcriptional and epigenetic levels, and integrating the two significantly improved the reliability of candidate molecules. Notably, in our cross-analysis, only three genes—Lilrb4, Ccr8, and Tnfrsf9-met all criteria of being functionally essential, transcriptionally upregulated, and having increased chromatin accessibility, with Lilrb4 showing the most significant loss in screening and the most selective expression. CCR8 has previously been reported as highly

expressed in TI-Tregs and is considered a potential target, which indirectly validates the effectiveness of our screening strategy, while also highlighting LILRB4 as a new target.

5.4. Potential molecular mechanisms by which LILRB4 regulates TI-Treg function

This study preliminarily reveals the phenotypic effect of LILRB4 in enhancing the suppressive function of TI-Tregs: conditional knockout of Lilrb4 significantly reduces Treg's ability to inhibit Tconv proliferation and decreases IL-10 secretion. This result aligns with the signaling mechanisms of LILRB4 in other cell types. LILRB4 contains two ITIM motifs that are known to recruit SHP-1/SHP-2 phosphatases, negatively regulating activation signals. In Tregs, ITIM signaling may strengthen suppressive function by enhancing Foxp3 stability, promoting expression of inhibitory cytokines, or regulating metabolic adaptation. Through motif enrichment analysis using scATAC-seq, we found that the Lilrb4 locus is enriched with HIF-1α and STAT3 binding motifs, suggesting that hypoxia and IL-10 signals in the tumor microenvironment may drive selective expression of LILRB4 in TI-Tregs. This finding is consistent with previous reports that the hypoxic tumor microenvironment promotes Treg functional adaptation, but the specific signaling pathways still need to be experimentally validated. Future studies could focus on how the downstream SHP-1/2 signaling axis of LILRB4 affects Treg transcription programs and effector functions, as well as whether LILRB4 has synergistic or redundant relationships with other inhibitory receptors like CTLA-4 and PD-1.

5.5. Clinical translational potential

Analysis of human tumor samples shows that LILRB4 is co-expressed on tumor-infiltrating Foxp3+ Tregs at a rate of over 68%, and high expression is significantly associated with shorter overall survival in patients, consistent with previously reported prognostic value of LILRB4 in myeloid cells. Notably, we observed similar trends across multiple tumor types (melanoma, non-small cell lung cancer, colorectal cancer), suggesting that LILRB4 may have broad significance across different cancers. Anti-LILRB4 antibodies effectively inhibited tumor growth and enhanced CD8+ T cell effector functions in both B16F10 and MC38 models without notable toxicity, providing promising preclinical data for further clinical development. Several anti-LILRB4 antibodies or fusion proteins have already entered early clinical trials, mainly targeting myeloid cells, but our findings suggest these drugs may also exert effects through Tregs, or that there may be a need to develop bispecific antibodies that can block LILRB4 on both myeloid cells and Tregs.

5.6. Limitations of the study

Although this study provides multilayered evidence, there are still several limitations. First, the animal models used were all subcutaneous transplantation tumor models, whose tumor

microenvironment differs from spontaneous or orthotopic tumors, and may not fully replicate the complex status of Tregs in human tumors. Future studies should further validate LILRB4 function in orthotopic models or genetically engineered mouse models. Second, regarding the molecular mechanisms by which LILRB4 regulates Treg function, this study only offered preliminary phenotypic data and clues about signaling pathways. The specific molecular events downstream of ITIM and their interactions with other signaling pathways have not yet been clarified. A deeper understanding of LILRB4's signaling network in Tregs could help design more precise intervention strategies. Third, the human sample analysis was based on a retrospective cohort with a relatively small sample size (90 cases in total) and did not include patients receiving immune checkpoint inhibitors, so it cannot directly assess the potential of LILRB4 as a marker for ICIs resistance. Fourth, it remains unclear whether the Fc effector function of anti-LILRB4 antibodies clears Tregs through ADCC, which is important for guiding antibody design (such as whether to retain Fc-mediated effects). Finally, the relative contribution of LILRB4 on Tregs versus myeloid cells still needs to be clarified through cell-specific knockout and combination treatment experiments.

5.7. Future outlook

Future research should focus on clarifying the precise signaling mechanisms of LILRB4 in Tregs, evaluating its potential as a companion diagnostic marker for immunotherapy, and advancing therapeutic antibodies that specifically target LILRB4 toward clinical translation. In addition, given the complexity and dynamic evolution of the tumor microenvironment and its profound impact on the effectiveness of immunotherapy (Liu et al., 2026). Integrating explainable spatial AI with multi-omics data will help systematically map the spatial interaction network between tumors and immune cells, further improving the accuracy of treatment outcome predictions and uncovering new combination therapy targets (Liu, 2026a; Liu, 2026b).

6. Conclusion

This study combined in vivo CRISPR-Cas9 functional screening with single-cell multi-omics analysis to systematically identify and confirm LILRB4 as a novel immune checkpoint molecule that is selectively highly expressed and functionally essential in tumor-infiltrating regulatory T cells (TI-Tregs). The main findings include: LILRB4 is specifically upregulated in TI-Tregs across various tumor models, while its expression is very low in peripheral Tregs and effector T cells, offering a tumor-selectivity advantage over existing targets like CTLA-4; LILRB4 promotes tumor immune evasion by enhancing Treg suppressive function and IL-10 secretion; conditional knockout of Lilrb4 in Tregs or treatment with anti-LILRB4 monoclonal antibodies can significantly reshape the tumor immune microenvironment, boost CD8+T cell effector function, inhibit growth of several mouse

tumors, and does not trigger systemic autoimmunity; in human tumor samples, high LILRB4 expression on tumor-infiltrating Tregs is strongly associated with poor prognosis.

These results show that LILRB4 is a key link between tumor microenvironment signals and Treg immune suppression, and it could become a next-generation immunotherapy target specifically aimed at tumor-infiltrating Tregs. Future research should further clarify the precise signaling mechanism of LILRB4 in Tregs, its synergy with other immune checkpoints, assess its potential as a companion diagnostic in patients resistant to immune checkpoint inhibitors, and promote the clinical development of therapeutic antibodies or bispecific agents that specifically target LILRB4.

Acknowledgments

Not applicable.

Author contributions

Conceptualization, Gang Liu; methodology, Jia Wang; validation, Liqiang An; formal analysis, Gang Liu and Jia Wang; investigation, Jia Wang and Liqiang An; resources, Gang Liu; data curation, Liqiang An; writing—original draft preparation, Jia Wang; writing—review and editing, Gang Liu; visualization, Liqiang An; supervision, Gang Liu; project administration, Jia Wang. All authors have read and agreed to the published version of the manuscript.

Funding

Not applicable.

Data availability

Not applicable.

Declarations

Conflict of interest

The authors declare that there is no conflict of interest.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors have given their consent.

Additional information

Injury and Rehabilitation Care
WISDOM ACADEMIC https://doi.org/10.63808/irc.v2i1.525 Volume 2 , Issue 1
Received: 9 September 2026
Accepted: 14 September 2026
Published online: 16 September 2026

Publisher's Note

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