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Angela Chen April 8th, 2025

Exploring Biomechanical Regulation on Anti-tumor Immunity of CD8 T^+ Cells by Using *Ptpn21* Knock-out Mouse Model

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Abstract

Exploring Biomechanical Regulation on Anti-tumor Immunity of CD8 T+ Cells by Using Ptpn21 Knock-out Mouse Model

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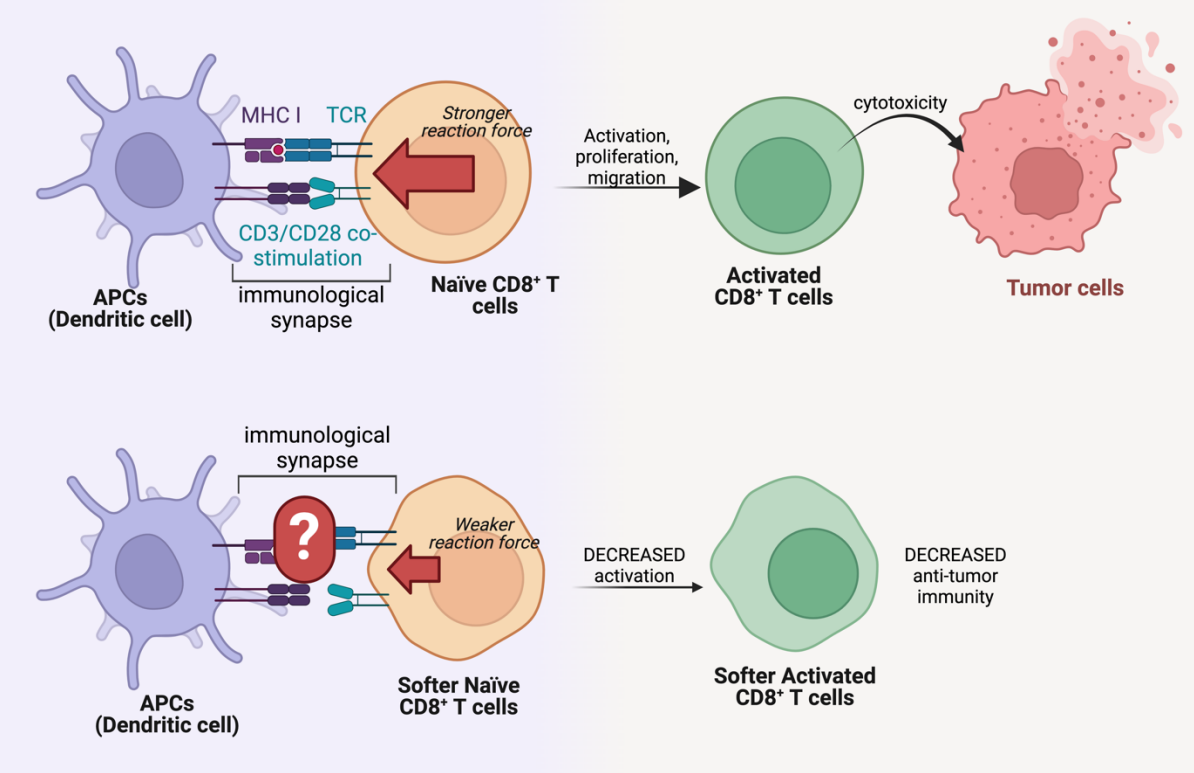
Visual Abstract:



The role of biomechanical regulation in CD8+ T cell function has not been well characterized. In this study, we aim to examine the biomechanical properties of CD8+ T cells by using wildtype (+/+, WT) and knock-out Ptpn21 (-/-, KO) mouse models. Firstly, we observed marked differences in stiffness by using atomic force microscopy, where Ptpn21 KO CD8+ T cells exhibited reduced biomechanical rigidity compared to the WT ones. Due to this reduced stiffness, KO CD8+ T cells demonstrated an enhanced capacity to deform and transmigrate through narrow pores in Transwell migration assay.

Mechanical softness in Ptpn21 KO CD8+ T cells correlates with functional impairments, evidenced by reduced activation responses following acute αCD3/CD28 stimulation. Additionally, lower proportion of Ptpn21 KO CD8+ T cells differentiates into central memory T

cells, suggesting compromised immune memory formation. Analysis of exhaustion markers revealed a decreased level of exhaustion markers in Ptpn21 KO CD8+ T cells, highlighting a link between mechanical softness and reduced cellular effector functions. Supporting in vivo experiments demonstrate impaired circulation of Ptpn21 KO CD8+ T cells within the lymphatic system, with significantly fewer cells found in circulation and primary lymphoid organs post-transfer. In conclusion, the mechanical softness may hinder effective antigen presentation and subsequent activation of CD8+ T cells in vivo under tumor bearing microenvironment as well.

Our findings underscore the importance of biomechanical integrity in preserving the optimal function of CD8+ T cells. The loss of this integrity initiates a cascade of functional deficiencies, notably impaired activation. The results reveal the role of biomechanical regulation in CD8+ T cell functionality and suggest potential therapeutic directions for enhancing T cell responses.

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Introduction

The biomechanical characteristics of cells are critical as they provide the fundamental shape, structure, and are essential for basic cellular response mechanisms to external signals[1,2]. The cytoskeleton structure, which is a dynamic network of protein filaments of actin and microtubules, supports cellular architecture, enables movement, and facilitates cellular processes such as cell division and intracellular transport[1,3]. In stem cells, the upregulation on FAK/ERK and YAP/TAZ signaling pathways, dependent on cell actin filament, enhances cell migration and proliferation by increasing nuclear translocation, initiating transcription and gene expressions[4,5]. Additionally, in $CD4^+$ T cells, the cytoskeletal infrastructure acts as an active mechanical rheostat, modulating cellular activation signals via the RhoA-ROCK-LIMK-cofilin pathway, which regulates shear stress activation upon integrin engfagement[6]. Upregulation of downstream signaling pathway enhances cytoskeletal rigidity, optimizing conditions $CD4^+$ T cell activation. However, the role of cell mechanics in regulating $CD8^+$ T cell function and its associated immune responses remain underexplored.

$CD8^+$ T cells, the key effectors of the adaptive immune system, protect against both exogenous pathogens or toxins, and endogenous malfunctioning cells[7]. Typically, $CD8^+$ T cells are activated by antigen-presenting cells (APCs), primarily dendritic cells, initiating an immune response. The $CD8^+$ cytotoxic T cells recognize antigens presented on major histocompatibility complex (MHC) class I molecules by APCs, differentiate into effector $CD8^+$ T cells, and directly cancerous cells. Upon recognizing tumor-specific antigens, $CD8^+$ T cells release cytotoxic granules containing perforin and granzymes to induce apoptosis in tumor cells[8,9]. $CD8^+$ T cells also release inflammatory cytokines interferon-gamma (IFN-γ) to inhibit tumor growth[10], as well

as activating other immune cells like macrophages[11]. APCs also promote the activity of cytotoxic $CD8^+$ T cells and support the development of immune memory by converting them into memory T cells[12,13].

In the presence of tumor, the mechanisms of regulating $CD8^+$ T cell activity are crucial as immune system is the first line of responders. Distinctive metabolite profiles can create either immune-supportive or immunosuppressive conditions, affecting T cell differentiation and function. For example, hypoxia in tumors results in an immunosuppressive environment that reduces $CD8^+$ T cell proliferation and infiltration to the tumor, and induces T cell exhaustion[14–16]. The lack of oxygen in the tumor microenvironment also leads to Warburg's effect, where tumor cells predominantly undergo anaerobic glycolysis, resulting in a decreased glucose level and increased lactate production and acidity[16]. These alterations in microenvironment impair $CD8^+$ T cell functions, aiding tumor cells evasion from the immune surveillance[17,18]. Moreover, alterations in amino acid profiles within tumor microenvironment, such as changes in glutamine and alanine level, also affect T cell functions[19–21]. Those intrinsic characteristics of $CD8^+$ T cells are well studied and show promising results from extensive research on therapeutic targets, and scientists are putting effort into engineering T cells as a new means for immunotherapy treatment to cancer patients. Various FDA approved CAR T therapies focusing on blood cancer have shown good outcomes[22].

In this study, *Ptpn21* knock-out (KO) mouse model is used to explore the biomechanical regulation on $CD8^+$ T cells. *Ptpn21*, as known as Protein Tyrosine Phosphatase Non-Receptor Type 21, is a critical regulator for cell rigidity and cytoskeletal integrity, interacting with actin

filaments via its FERM domain and influencing downstream FAK pathways[23]. Loss of *Ptpn21* in hematopoietic stem cells leads to a significant reduction in cell stiffness, thus resulting in decreased ability to remain in the niche and differentiate[24]. Investigating how alterations in cell rigidity due to Ptpn21 deficiency affect $CD8^+$ T cells function may elucidate its impact on anti-tumor immunity. A decrease in cellular stiffness, leading to softer $CD8^+$ T cells, could impair their normal function and weaken anti-tumor responses due to inefficient stimulation reception from tumor microenvironment.

Results

Ptpn21 KO CD8⁺ T cells are biomechanically softer, with a greater deformability, compared to WT

Atomic force microscopy (AFM) was performed to determine the biomechanical properties of CD8⁺ T cells. By using a nanoscale probe attached to a flexible cantilever, mechanical responses such as elasticity, stiffness, and viscoelastic behavior were measured[25]. The force–distance curves generated during the indentation process as analyzed to quantify the Young's modulus (E*), which serves as an indicator of cellular stiffness.

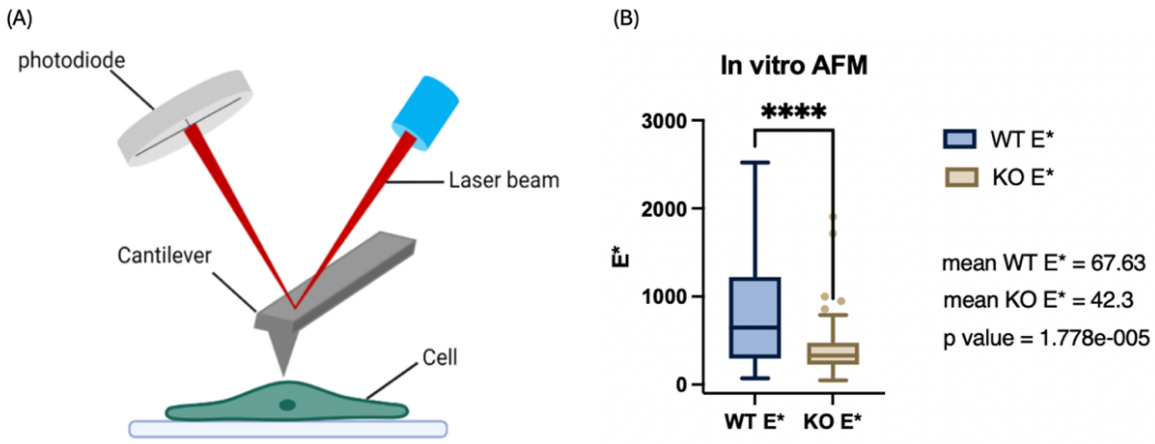

Figure 1. KO CD8⁺ T cells are biomechanically softer with a slower reaction towards applied force. *(A) Schematic representation of AFM adopted from Bally Lab[26]. (B) Cells are harvested from Ptpn21 KO or WT mice (n=3 per group, with 30-50 rounds of measurements in each run) and activated in vitro for 3 days by peptide ovalbumin. AFM measurement on E*, the elastic modulus, is presented in the bar graph. Data are presented as means ± SD of biological replicates. ****P < 0.0001.*

According to the AFM results (Fig. 1), *Ptpn21* KO CD8⁺ T cells exhibit significantly lower elastic modulus, indicating a softer cell structure compared to WT CD8⁺ T cells. These findings align with the hypothesis that the KO cells are softer compared to the WT ones, impacting their functional responses to environmental physical cues. The softer CD8⁺ T cells from *Ptpn21* KO

mouse models can be used as the study tool in this project to provide insight into how T cell mechanical changes affect their immune response towards tumor cells.

As *Ptpn21* KO CD8$^+$ T cells are softer, they also exhibit an enhanced ability to deform and navigate through constricted spaces compared to WT ones. The trans-well migration assay with B16F10 melanoma cells seeded in the lower chamber is performed. As B16F10 melanoma cells express ovalbumin (OVA), which is a specific antigen being recognized by OT1 receptor on T cells, the CD8$^+$ T cells seeded in the upper chamber will migrate to lower chamber in response to those cancer cells.

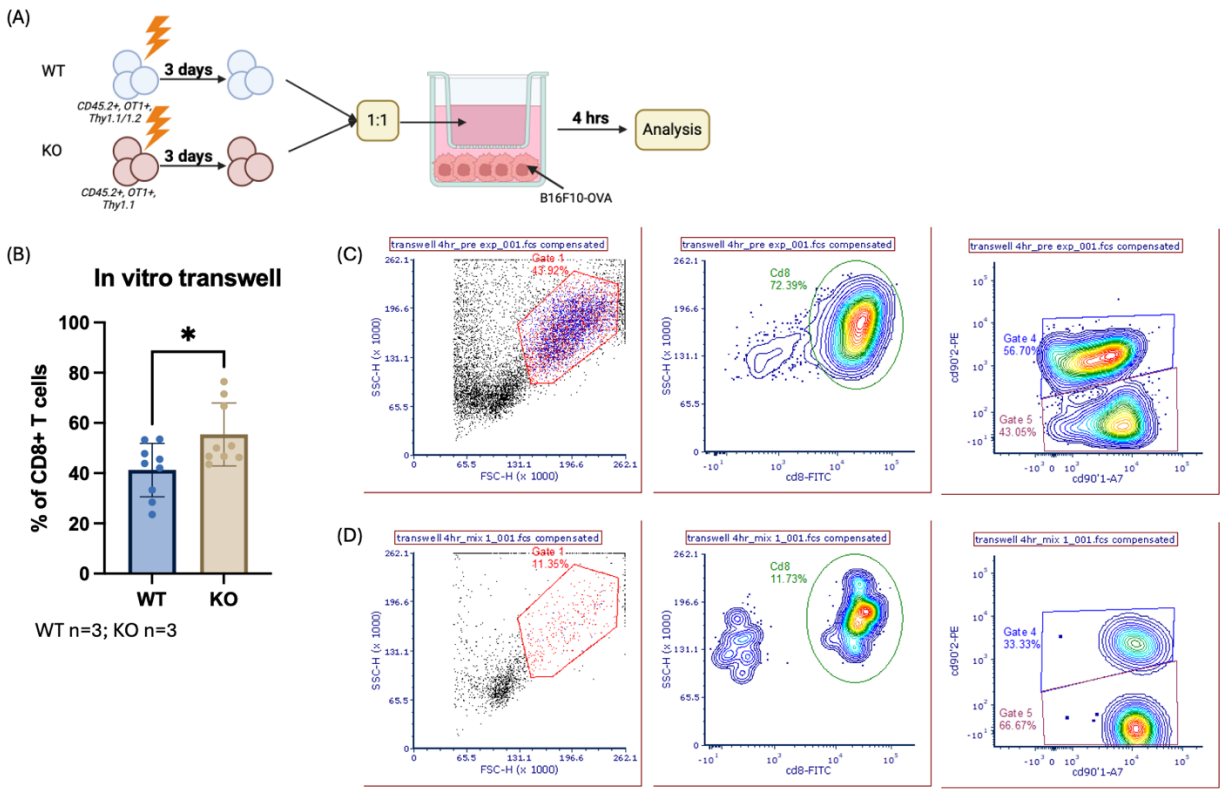

Figure 2. Transwell migration assay confirms that KO CD8$^+$ T cells are more deformable.
(A) Schematic experimental design. (B) Bar graph representing the percentage of total T cells migrated to the lower chamber of Boyden Transwell coculturing well with a pore size of 5μm (n=3 with 3 technical replicates

*each run). (C) and (D) represent the gating of WT and KO cell populations before and after Transwell migration assay, respectively. Data are presented as means ± SD of biological replicates. *P < 0.05.*

Shown in Fig. 2B, KO $CD8^+$ T cells have a significantly higher percentage of infiltration into the lower chamber compared to WT, which suggests that KO $CD8^+$ T cells are more efficient in migrating through the membrane, which may mimic their ability to infiltrate tumor sites. Cell integrity enhances the deformability and infiltration abilities of $CD8^+$ T cells, providing further evidence to show that $CD8^+$ T cells become softer.

Softer $CD8^+$ T cells have weaker response to nonspecific stimulation, showing decreased activation

By examining cell surface markers on $CD8^+$ T cells, their functions, including activation, differentiation, and exhaustion, can be accessed. Besides getting activated specifically by antigens presented on MHC I, $CD8^+$ T cells can also get activated non-specifically through co-stimulatory signals such as CD3 and CD28 antibodies (αCD3/CD28), which together activate downstream cellular signaling pathways[27]. In our *in vitro* experimentations, when $CD8^+$ T cells are softer, their response to external signals is altered. Percentage of $CD25^+$, $CD69^+$, and $CD44^+$ T cells are checked to confirm the immune activation status of WT and KO $CD8^+$ T cells.

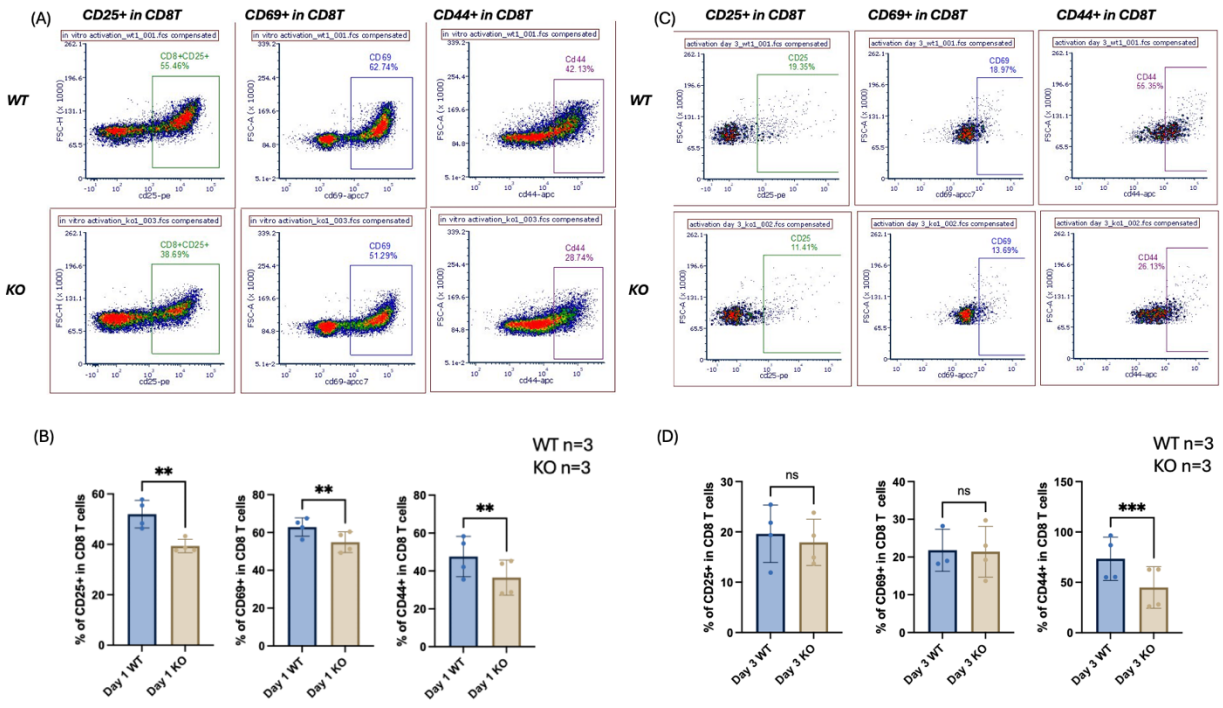

Figure 3. Comparison of activation in WT and KO shows a decreased percentage in CD25$^+$, CD44$^+$, and CD69$^+$ in KO CD8$^+$ T cells by αCD3/CD28 activation.
*(A) Subpopulations in both WT and KO CD8$^+$ T cells under activation for 24 hours. (B) are the bar graphs for percentage of CD25$^+$, CD69$^+$, and CD44$^+$ in WT and KO CD8$^+$ T cells, respectively, under activation for 24 hours. (C) Subpopulations in both WT and KO CD8$^+$ T cells under activation for 72 hours. (D) are the bar graphs for percentage of CD25$^+$, CD69$^+$, and CD44$^+$ in WT and KO CD8$^+$ T cells, respectively, under activation for 72 hours. Data are presented as means ± SD of biological replicates. **P < 0.01 and ***P < 0.001.*

In *Ptpn21* KO CD8$^+$ T cells, activation by αCD3/CD28 antibodies *in vitro* is significantly reduced, especially under acute stimulation (Fig. 3B). However, following 3 days of culture, it seems like the activation status of CD8$^+$ T cells evens out between the WT and KO group, indicating a slower activation but not completely impairment in their ability to get stimulate in the KO (Fig. 3D).

After activation, CD8$^+$ T cells will develop immune memory by differentiating into subtypes, such as effector memory T cells (CD44^{hi} CD62L^{lo}) and central memory T cells (CD44^{hi}

CD62L^{hi}), of which their main function is to format a rapid response or preserve a long-term immunity, respectively[28,29].

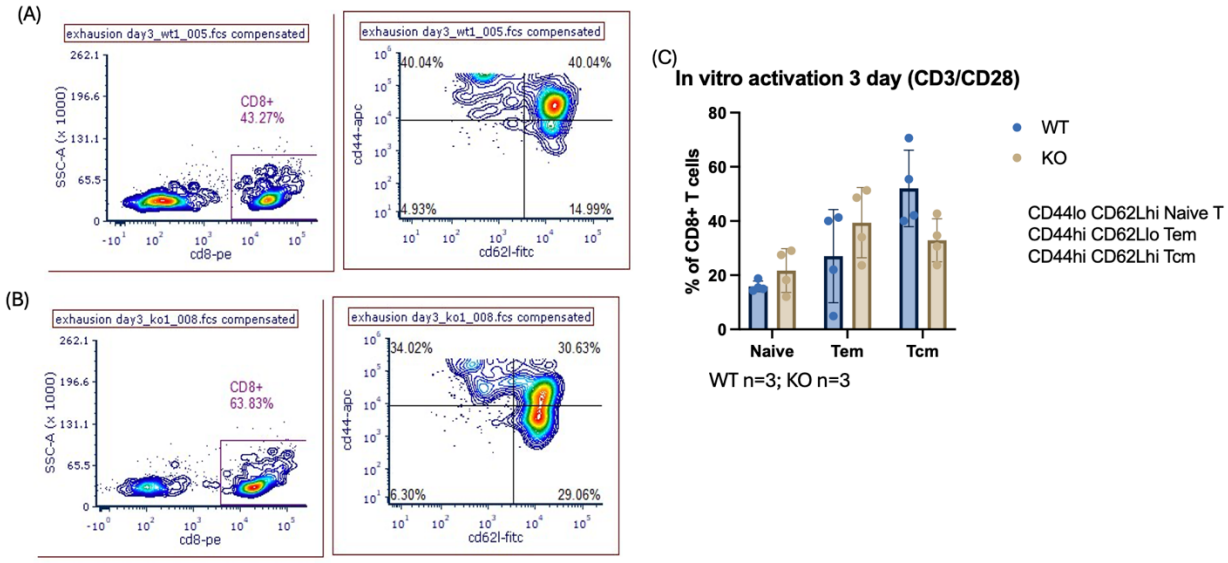

Figure 4. Fewer Ptpn21 KO CD8$^+$ T cells exhibit a memory T phenotype.
(A) and (B) represents different subgroups of CD8$^+$ T cells in WT and KO (n=3 per group). (C) is the bar graph that quantitively compare the level of differentiation into either effector or memory T cells. CD44^{lo} CD62L^{hi} CD8$^+$ T cells are naïve T cells that are not activated, CD44^{hi} CD62L^{hi} CD8$^+$ T cells are central memory T cells, and CD44^{hi} CD62L^{lo} CD8$^+$ T cells are effector memory T cells, as shown in the key. Data are presented as means ± SD of biological replicates.

After activation, fewer CD8$^+$ T cells from the KO group turns into memory phenotypes, and more stay to be in their naïve state, as shown in Fig. 3C. Conversely, it is being noticed that there is a higher percentage of effector memory CD8$^+$ T cells in the KO group, indicating a potential enhancement or modification in the immune system's memory response.

When there is a prolonged stress and stimulation in the microenvironment, CD8$^+$ T cells will get exhausted, as indicated with the markers programmed cell death 1 (PD1) and T cell membrane protein 3 (TIM3). After 3 days of culturing in an environment that induces exhaustion, with prolonged presence of antibodies, it is observed that around 5% of CD44$^+$ population in the KO

CD8$^+$ T cells get express PD1, and around 1% of CD44$^+$ population in the KO CD8$^+$ T cells get express Tim3, compared to 10% and 5% in WT CD8$^+$ T cells, respectively (Fig. 5C). Yet more biological replicates are needed to perform statistical tests, this trend implies that fewer KO CD8$^+$ T cells experienced chronic stimulation and activation, either due to slower or weaker response they can form regarding to αCD3/CD28 antibodies.

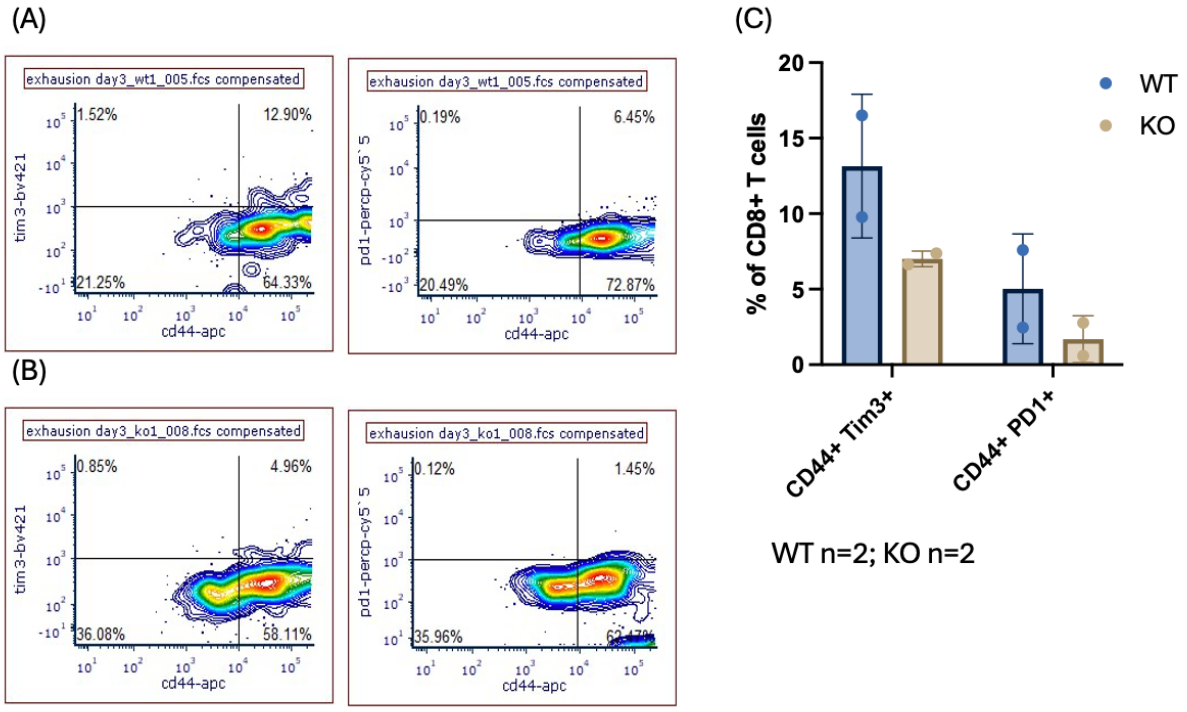

Figure 5. KO CD8$^+$ T cells that get activated are less exhausted under chronic stimulation.
(A) and (B) represent the distribution of expression on PD1 and Tim3 in CD8+ T cells (n=2 per group). (C) is the bar graph comparing CD8+ PD1+ TIM3+ population, which are exhausted T cells between WT and KO. Data are presented as means ± SD of biological replicates.

In vivo Adoptive Transfer reveals the loss of circulation of KO CD8$^+$ T cells in lymphatic system

To evaluate changes in CD8$^+$ T cell behavior *in vivo*, a comparative adoptive transfer of lymphocytes through tail vein injection into the recipient mice is performed. An adjusted 1:1

pooled mix of naïve WT and KO $CD8^+$ T cells derived from donor OT-1 mice is prepared to control the in vivo microenvironment that $CD8^+$ T cells are going into. These cells were introduced into recipients bearing B16F10-OVA tumors, which is a highly invasive and lethal solid tumor cell line. Since the tumor cells express OVA, the selected OT-1 $CD8^+$ T cells can get activated by this peptide antigen. Samples of peripheral blood from tail vein is then collected three days following the procedure to identify the migration and survival characteristics of the transferred $CD8^+$ T cells from both groups, thus providing insights in how $CD8^+$ T cells behave *in vivo*.

A notable difference in the repopulation of donor $CD8^+$ T cells in peripheral blood. Even if the pre-procedure ratio is adjusted to approximately 1:1 (Fig. 6B), there is a lower number of donor KO $CD8^+$ T cells (26.9%) compared to donor WT ones (73.1%), as shown in Fig. 6C. This suggests that KO $CD8^+$ T cells are less likely to participate in lymphatic circulation, likely due to loss of cell integrity and stiffness. The loss of circulation is further confirmed by a second batch of adoptive transfer with multiple replications later. At 7-day endpoint, more substantial differences across different lymphatic tissues as well as the primary tumor site are being observed, as shown in Fig. 6. The donor $CD8^+$ T cells in spleen showed a significant loss of KO population, with nearly 100% of the donor $CD8^+$ T cell population being of WT origin, signifying an inability of KO cells to home to or persist in this primary immune organ. The lymph nodes and peripheral blood presented a reduction in KO cell percentages compared to WT, highlighting a possible impairment in the KO cells' ability to migrate, circulate, or survive in secondary lymphoid organs and circulatory lymphatic system. Loss of KO $CD8^+$ T cells is

relatively milder but still noticeable, which means the cytotoxic T cells are still able to infiltrate to the tumor site but in a less potent and effective manner.

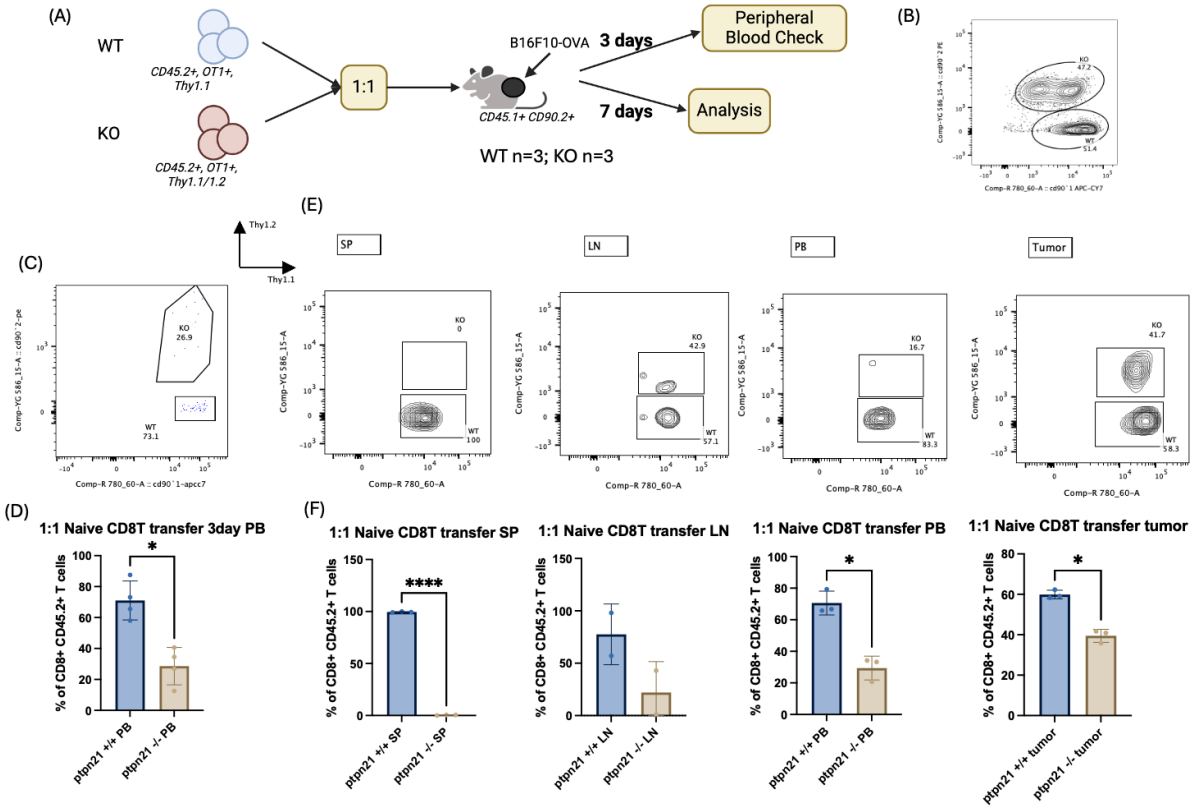

Figure 6. In vivo adoptive transfer of 1:1 pooled mix CD8 T cells suggest loss of circulation of KO CD8 T cells in the peripheral lymphatic system
*(A) Schematic experimental design. (B) Naïve $CD8^+$ T cells isolated from WT mice and KO mice were mixed at the 1:1 ratio. (C) After 3 days of repopulation, peripheral blood samples from tail vein were collected. The distribution of WT $CD8^+$ T cells and KO $CD8^+$ T cells are shown (n=3). (D)The bar graph quantitatively compares the percentage of WT and KO $CD8^+$ T cells in the recipient peripheral blood samples. (E) Naïve $CD8^+$ T cells isolated from WT mice and KO mice were mixed at the 1:1 ratio (F) After 7 days of repopulation, recipient mice were euthanized, and spleen (SP), lymph node (LN), peripheral blood (PB), and tumor samples were collected, and distribution of WT $CD8^+$ T cells and KO $CD8^+$ T cells are shown. Data are presented as means ± SD of biological replicates. *P < 0.05, **P < 0.01, and ****P < 0.0001.*

Since the initial activation and circulation of $CD8^+$ T cells are being confirmed through *in vivo* experimentations, it has been shown in our work that the secondary activation process and circulation of already activated $CD8^+$ T cells is also greatly impacted by loss of cell stiffness. In

another batch of adoptive transfer, an adjusted 1:1 mix of activated WT and KO CD8$^+$ T cells are transferred into mice bearing the B16F10-OVA tumor model through tail vein. Since the cells are activated *in vitro* before transfer, the tumor and the OVA antigen present in tumor microenvironment in vivo will serve as a reactivation.

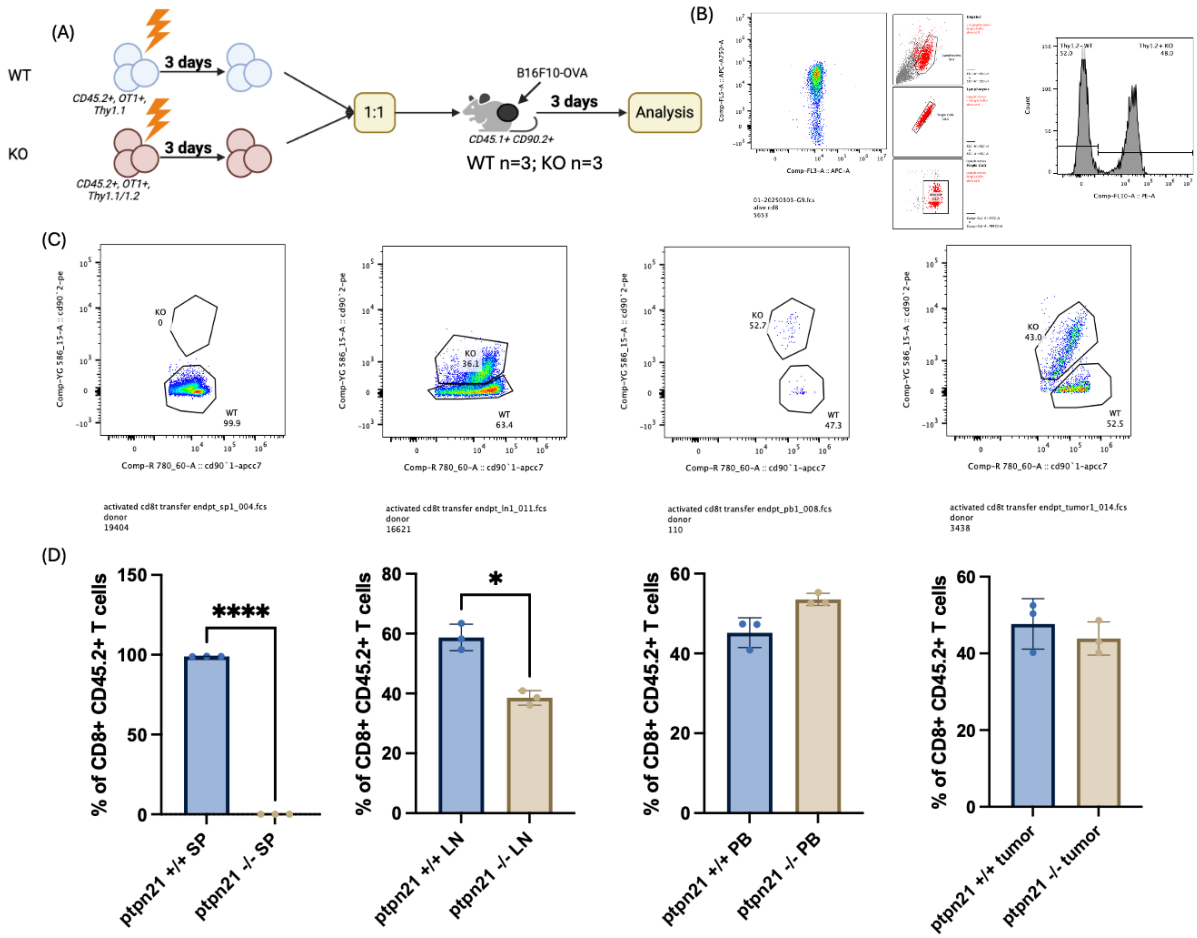

Figure 7. There is a reduced number of activated KO CD8$^+$ T cells circulating in the lymphatic system. *(A) Schematic experimental design. (B) Naïve CD8$^+$ T cells isolated from WT mice and KO mice and activated in vitro by OVA peptides for 4 hours and culture for 3 days. Then the cells were mixed at the 1:1 ratio (C) After 7 days of repopulation, recipient mice were euthanized, and spleen (SP), lymph node (LN), peripheral blood (PB), and tumor samples were collected, and distribution of WT CD8 T cells and KO CD8$^+$ T cells are shown (n=3). (D)The bar graph quantitatively compares the percentage of WT and KO CD8$^+$ T cells in SP, LN, PB, and tumor sites of recipients. Data are presented as means ± SD of biological replicates. *P < 0.05 and ****P < 0.0001.*

Similar trend is observed, where a losing donor KO $CD8^+$ T cells across spleen and lymph nodes is significant (Fig. 7C-7D), which is nearly 100% representation of donor $CD8^+$ T cells being WT in the spleen, and 63.4% representation of donor $CD8^+$ T cells being WT compared to 36.1% of that being from the KO group in lymph nodes. This consistent pattern suggests a pivotal role of cell stiffness level in maintaining $CD8^+$ T cell mobility function in primary and secondary lymphoid organs, critical for effective immune surveillance and tumor response.

Discussions and Future Direction

The results of this study underscore significant differences in the biomechanical properties of $CD8^+$ T cells with and without *Ptpn21* gene. Notably, *Ptpn21* KO $CD8^+$ T cells demonstrate a lower elastic modulus (E*), as measured by atomic force microscopy (AFM), confirming our hypothesis that *Ptpn21* deficiency in $CD8^+$ T cells results in a biomechanically softer cellular structure. This biomechanical softness correlated with greater cellular deformability, suggesting potential alteration in their cytoskeletal arrangement or modifications in the cytoskeletal structure. To identify the precise molecular pathways involved, further investigations using advanced imaging techniques such as confocal microscopy after immunofluorescence staining, such as on F-actin, a major cytoskeleton compartment in cells, are recommended.

The biochemical softness impacts the cells' responses to external signals, primarily through a process called mechanotransduction, where cells are able to sense physical forces, altering protein conformation to generate intracellular signals to activate downstream pathways[30,31]. Literatures suggest that externally applied strain forces can increase cytoskeleton linkages with extracellular matrix, promoting cell cycle progression[31,32]. As antigen presentation occurs when a T cell interacts with APCs, there is a formation of immunological synapses, where regulation of T-cell receptor-mediated actin polymerization need to occur[33–35]. We propose that the reduced stiffness in KO $CD8^+$ T cells diminishes their ability to sense and respond to external forces, impacting the stimulation and activation by APCs within these immunological synapses, and thus resulting in a weaker signaling cascade essential for their normal functionality. This finding aligns with observations in $CD4^+$ T cells, where a certain level of cell stiffness is necessary for effective T cell receptor activation[6].

Functional analysis comparing $CD8^+$ T cells from both WT and KO mice underlines a correlation between reduced cellular integrity and decreased activation in $CD8^+$ T cells. This is evidenced by the decreased percentage of $CD8^+$ T cells expressing CD25 and CD69 markers upon T cell activation, which are pivotal for the transition from a naïve to an activated state, as well as activating immune responses by T cells[36]. Although initial activation levels are lower in KO $CD8^+$ T cells, prolonged antibody exposure eventually equalizes activation level between WT and KO $CD8^+$ T cells, suggesting that while initial activation threshold are higher of KO ones, persistent stimulation can overcome these deficits.

In vivo experiments using adoptive transfer reveal functional impairments in biomechanically softer $CD8^+$ T cells from the KO group in tumor-bearing environment. Specifically, KO $CD8^+$ T cells exhibit reduced survival and trafficking capabilities, which are crucial for effective immunological responses. The marked decrease in the presence of KO $CD8^+$ T cells in circulation and primary lymphoid organs post-transfer, especially in the spleen and lymph nodes, highlights sever impairments in their ability to home to and persist within primary immune response sites. Thus, cytoskeleton integrity in general plays a critical role in maintaining the biomechanical characteristics necessary for optimal $CD8^+$ T cells function. However, more experiments on the direct anti-tumor immunity from WT $CD8^+$ T cells or softer KO $CD8^+$ T cells should be performed through long-term monitor of tumor size and weight in mouse model.

However, several limitations in this study that must be considered. The use of a single knockout mouse model may not fully capture the complexities of T cell biomechanics across varied physiological conditions. Additionally, while atomic force microscopy provides valuable results

to the physical properties at the cellular level, there is a lack of information regarding the biochemical pathways that may be affected by the changes in biomechanical properties. A more detailed investigation into the intracellular signaling pathways is essential, particularly targeting molecular pathways such as Src/FAK signaling pathway, known to be associated with *Ptpn21* and the biomechanical regulation of different cell types[6,23]. Proteomics analysis can also reveal the downstream effectors involved in biomechanical regulation of $CD8^+$ T cells, such as Septin1, which has been previously reported to have increased expression level[24]. Furthermore, the experiments conducted have not yet clarified the fate of KO $CD8^+$ T cells in a tumor-bearing environment. More *in vivo* experimentations are necessary to determine whether these cells undergo apoptosis or suffer from proliferation deficits, resulting in a loss in the circulating lymphatic system as we have observed.

To continue with this project, a comprehensive analysis of T cell mechanical properties using biomechanical engineering devices, such as microfluidic systems, is essential to understand how these cells navigate and respond within the complex tumor microenvironment in a more simplified but direct apparatus Given that activation levels are directly impacted in KO $CD8^+$ T cells, a microfluidic system would enable controlled experiments with minimal direct cell-to-cell interactions is designed, which uses a probe equipped with antibodies or specific peptides to measure activation thresholds and response kinetics of $CD8^+$ T cells from both WT and KO groups. Additionally, the development of a novel perfusion system would allow for controlled exposure of $CD8^+$ T cells to activating agents from limited interaction, facilitating detailed studies on cell adhesion and activation dynamics under defined conditions.

Overall, this thesis highlights the significant impact of biomechanical regulation on $CD8^+$ T cell function and anti-tumor immunity. The evidence presented confirms that alterations in cellular integrity are linked to changes in $CD8^+$ T cell activation, as well as their behavior *in vivo*. These findings not only underscore the potential for further research in this area but also suggest that understanding biomechanical properties could lead to innovative anti-tumor therapeutic targets and strategies to enhance cancer treatment. This study sets the groundwork for future investigations that could provide more insights into therapeutic approaches for cancer patients through biomechanical interventions.

Methodology

Mouse Models

Ptpn21 $^{-/-}$ (KO) mice were generated in previous study in our lab[24]. C57BL/6 (CD45.2^{+}) (strain no. 000664) and congenic BoyJ (CD45.1^{+}) mice (strain no. 002014) were purchased from the Jackson laboratory. C57BL/6 (CD45.2^{+}) mice are used as *Ptpn21*$^{+/+}$ (WT) model as the control group. All the mice were kept under specific pathogen–free conditions at Emory University Division of Animal Resources and raised with care per facility requirement and protocols. Thy1, a cell surface glycoprotein that is generically expressed in T cells, is used to separate WT and KO mice and primary cells based on the specific genotypes of Thy1. Tumor bearing mice are prepared by subcutaneously injecting 0.5x10^{6} B16F10 melanoma cells to the lower right limb of BoyJ mice, and letting the tumor grow until the mass is palatable, which takes around 7 days.

Primary cell samples preparation

Primary cells are isolated from mice at an age around 6 weeks to 10 weeks. Lymphocytes were harvested from both spleen and lymph nodes (including axillary lymph nodes and inguinal lymph nodes). After grinding and red blood cell lysis, negative selection is performed by using magnetic column and microbeads that are CD8 specific to enrich the CD8^{+} T cell population. Once enriched, cells are cultured in complete T cell culture medium, which is RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), sodium pyruvate (1%), β-mercaptoethanol (50 μM), and human interleukin-2 (50 U/ml).

$CD8^+$ T cell activation

$CD8^+$ T cells can be activated either by nonspecific αCD3/CD28, or specifically by antibodies. As the mice are bred to have OT-1 receptor, they will respond to ovalbumin (OVA) peptide in the tumor microenvironment and get activated. For every $1x10^6$ cells per ml, 1 μL of 200 μM OVA is added to the medium, per pervious optimized conditioning experimentations. After culturing in complete medium for 4 hours in 37°C incubator, OVA are removed and $CD8^+$ T cells are continued to culture in complete medium for 3 days, split when necessary. On the other side, $CD8^+$ T cells can be activated by non-specific antibodies as well, which are direct binding to T cell receptor (TCR) and induce proliferation and activation signals. To activate $CD8^+$ T cells through non-specific binding $CD8^+$ T cells are co-cultured with αCD3/CD28 beads for 24 hours and removed afterwards.

FACS Flow Cytometry Quantification

Fluorescence-activated cell sorting (FACS) flow cytometry was utilized to quantify specific cell populations. Cells were harvested after experimentations, washed with phosphate-buffered saline (PBS), and stained with fluorophore-conjugated antibodies targeting relevant surface markers (e.g., CD8, CD45.1 or CD45.2, Thy1.1 or Thy1.2). After incubation at 4°C in the dark for 30 minutes, excess antibodies were removed by washing with FACS buffer. Data acquisition was performed using a BD Symphony A5. Analysis was conducted using FlowJo, with gating strategies set to exclude non-target signals such as debris and doublets, ensuring accurate quantification of the target populations.

Biomechanistic Characterization

Atomic Force Microscopy (AFM) is used to investigate the mechanical properties of $CD8^+$ T cells at the nanometer scale. Primary cells harvested from mouse models are processed according to the procedure described earlier and then adjusted to a concentration of $1x10^5$ cells per ml in non-FBS contained medium. Multiple force curves per cell will be recorded, and data will be processed using Hertzian contact models.

In Vitro Characterization

In vitro experiments utilize $CD8^+$ OT-1 T cells harvested from the spleens and lymph nodes of mouse models, which are activated by either αCD3/CD28 or OVA, depending on the purpose of experiment. FCAS flow cytometry allows the characterization of cells into either WT or KO (through Thy1.1 and 1.2 expression), and evaluation of basic cell behaviors such as activation, apoptosis, proliferation, cytokine production, exhaustion, and cell-to-cell interactions between WT and KO $CD8^+$ T cells. Markers in our interests vary depending on the experimental design: either activated by peptides or antibodies.

Transwell Migration Assay

Boyden Transwell migration assay can be used to check the tumor infiltration rate under the presence of tumor. The same amount of B16F10 melanoma cells will be seeded in the lower chamber, which will produce chemokines that can attract $CD8^+$ T cells in the upper chamber to migrate through a membrane with pores of limited size of 5 μm. Cells that successfully migrate

will be quantified by flow cytometry, providing a model system to check the migratory response of T cells to tumor stimuli.

In Vivo Repopulation Assay

T cells, whether WT, KO, or 1:1 pooled mix of WT and KO cells, are adoptively transferred through tail vein into the recipient tumor bearing mice, with a cell number around $10x10^6$ cells. As donor $CD8^+$ T cells are genetically labeled based on Thy1 expression into recipient mice (BoyJ $CD45.1^+$). This allows tracking of the repopulation and distribution of the cells *in vivo*. $CD8^+$ T cells can either be naïve or activated, depending on the experimental design. If a competitive 1:1 pooled mix of cells is transferred, a pre-experimental flow cytometry check is needed to adjust the cell number. After repopulation for 3 days (for activated $CD8^+$ T cells) or 7days (for naïve $CD8^+$ T cells), recipient mice are sacrificed and lymphocytes from spleen, tumor draining lymph nodes, peripheral blood (tail vein), and tumor mass are isolated, minced, triturated, and passed through 40-µm filters. If necessary, samples were enriched by density gradient centrifugation using 1x lymphocyte separation medium. Samples are analyzed by flow cytometry.

Statistics and reproducibility

All data are presented as means ± SD of biological replicates with independent animals within independent experiments. Unpaired two-tailed Student's t tests were used for the statistical comparison of the two groups. *P < 0.05 **P < 0.01; ***P < 0.001; ****P < 0.0001.

Accomplishment

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