Dissecting the Role of Y4474 and Y4508 on CD91 in AXL/Fgr Binding and Cancer Immunosurveillance Signaling

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Abstract

The recognition of danger-associated molecular patterns (DAMPs) released by tumor cells is a critical early step in recognizing emerging tumors, and these include heat shock proteins (HSPs) that bind to receptors like CD91 on immune cells. In this study, we investigated whether specific tyrosine residues within the CD91 β chain are essential for CD91-directed cancer immunosurveillance signaling. We used CRISPR-Cas9 gene editing to investigate these tyrosines by replacing them with phenylalanine. Our results revealed distinct and residue-specific effects on downstream signaling, the most notable being Y2F clone 4, which contained mutations in the NPxY phosphotyrosine-binding motif, a crucial docking site for signaling proteins. This clone showed significantly reduced cytokine production in response to HSP stimulation, confirming that this phosphotyrosine-binding domain is important for CD91-mediated signal transduction and that these CD91 tyrosine residues are essential for CD91 signaling and cytokine induction. By establishing the functional significance of specific tyrosine residues within CD91, this study deepens our understanding of certain molecular mechanisms underlying cancer immunosurveillance, and allows for further research regarding immunotherapeutic approaches that target the HSP-CD91 axis.

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Introduction

Cancer immunosurveillance is the process by which the immune system detects nascent tumors and eliminates them, and is dependent on tumor-cell-derived molecules. Tumor cells release heat shock proteins (HSPs), which essentially act as signals to alert the immune system. HSPs are an example of danger-associated molecular patterns (DAMPs), which are a category of molecules released from stressed, damaged, or dying cells and activate the immune system. HSPs specifically do this by binding to CD91 on immune cells such as dendritic cells (DCs) and activating them (Nayak & Binder 2023). The activated dendritic cells take up the HSPs and present HSP chaperoned antigens to T cells, which prime the adaptive immune response against the emerging tumor. HSPs chaperone and carry tumor antigens, which are then cross-presented to CD8+ T cells, satisfying the quantitative constraints despite low amounts of available tumor antigens in nascent stages (Mellman et al. 2023). When HSPs bind to CD91, intracellular signaling begins, which releases pro-inflammatory cytokines and upregulates co-stimulatory molecule expression (Binder & Srivastava 2005; Nayak & Binder 2023; Pawaria & Binder 2011).

These findings suggest there is an innate emerging tumor sensing mechanism by dendritic cells involving CD91, which activates the HSP-CD91 axis, leading to anti-tumor responses. CD91 is a central receptor in this process, as it uniquely couples antigen uptake with pro-inflammatory signaling required for effective T cell priming. As such, loss or dysfunction of the HSP receptors, like CD91, could lead to higher cancer incidence. We have previously shown that the expression of CD91 on DCs is necessary for immunosurveillance, as well as established the roles of AXL and Fgr as essential kinases that phosphorylate CD91. It is important to note that CD91 has no intrinsic kinase activity, and inhibition of these protein kinases eliminates the HSP-mediated signaling pathway. These signaling events also include the phosphorylation of two tyrosine residues (Y4474 and Y4508) on the intracellular β chain of CD91 (Harkness et al. 2024).

In this study, we investigate the functional significance of these two tyrosine residues within the CD91 receptor complex. Using CRISPR-Cas9 gene editing mechanisms to substitute each tyrosine with phenylalanine, potential CD91 phosphorylation sites would be abolished, revealing each residue's distinct contribution to the signal transduction pathway. We hypothesize that phosphorylation of CD91 tyrosine residues Y4474 and Y4508 is required for AXL- and Fgr-mediated signaling (Harkness et al. 2024). Consequently, mutation of these residues will disrupt downstream cytokine production critical for cancer immunosurveillance. As such, the signaling cascade will become abolished, and this will lead to a loss of anti-tumor immune responses. This project aims to define the molecular basis by which CD91 tyrosine residues regulate immunosurveillance signaling.

Materials and Methods

All methods below were carried out in accordance with the relevant guidelines and regulations as outlined by the University of Pittsburgh. All experiments were performed using immortalized cell lines. No live animals or human subjects were used in this study, and therefore no additional ethical approval was required.

Isolation and Culture of iBMDMs

Immortalized bone marrow-derived macrophages (iBMDMs) were cultured in complete RPMI containing 1% pen-strep, 1% sodium pyruvate, 1% L-glutamine, 1% nonessential amino acids, 0.1% 108 2-mercaptoethanol, and 10% heat-inactivated FBS (GIBCO). The cells were cloned in soft agar and immortalized using the J2 recombinant retrovirus containing v-raf/mil and v-myc oncogenes (Roberson & Walker 1988).

CRISPR-Cas9

Targeted gene editing using the CRISPR-Cas9 system in suspension cells adhered to the IDT CRISPR HDR protocol. Cells were cultured in an appropriate growth medium at 37°C. Before electroporation, cells were washed with phosphate-buffered saline (PBS) to remove any residue. Initially, CRISPR reagents needed to be prepared. gRNA was prepared by combining 5 µL of 100 µM Alt-R CRISPR-Cas9 crRNA and 5 µL of 100 µM Alt-R CRISPR-Cas9 tracrRNA to achieve a final concentration of 50 µM. The mixture was incubated at 95°C for 5 minutes and then cooled to room temperature. For each electroporation well, 3.0 µL of gRNA and 2.0 µL of Alt-R Cas9 enzyme were mixed to prepare the RNP complex with a final Cas9:gRNA ratio of 4:4.8 µM. The mixture was left at room temperature for 10–20 minutes. HDR donor oligos were prepared by resuspending in Nuclease-Free IDTE buffer (pH 7.5) to obtain a final concentration of 100 µM. The cells were suspended in 20 µL Nucleofection Buffer. For the nucleofection procedure, the nucleofection mix was prepared by combining the following components: 5 µL of the RNP complex, 1.2 µL of 100 µM Alt-R HDR Donor Oligos, 1.2 µL of 100 µM Alt-R Cas9 Electroporation Enhancer, 20 µL of cell suspension, and 2.6 µL of PBS. The total volume of the transfection mix was 30 µL. After mixing, 25 µL of the nucleofection mix was transferred to a 96-well Nucleocuvette module. Air bubbles were gently tapped out, and cells were transfected following the specifications of the Nucleofector system. Following electroporation, 75 µL of prewarmed culture media was added to each well. 25 µL of cells were resuspended and transferred to culture plates containing prewarmed 175 µL of culture media supplemented with HDR Enhancer V2. Cells were incubated in a tissue culture incubator for 12–24 hours, after which the media was changed to fresh media without HDR Enhancer V2. gDNA was isolated from the transfected cells 48–72 hours after electroporation to check gene editing efficiency.

RNA Extraction

Following the CRISPR protocol, RNA was extracted from iBMDMs using the QIAGEN RNeasy Mini Kit, following the standard protocols outlined in the RNeasy Mini Handbook (QIAGEN).

iBMDMs were cultured under standard conditions until reaching approximately 80% confluency. Cells were detached using trypsin-EDTA, collected by centrifugation at 300 × g for 5 minutes, and washed twice with phosphate-buffered saline (PBS). To ensure complete lysis, the cell pellet was resuspended in 600 µL of Buffer RLT and homogenized using a QIAshredder spin column. 600 µL of 70% ethanol was added to the lysate and mixed thoroughly by pipetting. Up to 700 µL of the mixture was then transferred to a RNeasy Mini spin column placed in a 2 mL collection tube and centrifuged at 8000 × g for 15 seconds. The flow-through was discarded, and the column was washed with 700 µL of Buffer RW1 to remove contaminants. The column was then washed with 500 µL of Buffer RPE and centrifuged at 8000 × g for 15 seconds. This step was repeated with a centrifuge cycle of 2 minutes to dry the membrane. RNA was subsequently eluted by adding 40 µL of RNase-free water directly onto the membrane and centrifuging at 8000 × g for 1 minute. A NanoDrop spectrophotometer was lastly used to quantify the concentration and quality of the extracted RNA.

cDNA Synthesis

RNA extracted from the previous step was then converted into cDNA following the SuperScript III First Strand Synthesis system (Invitrogen).

Beta Chain Amplification and Sequencing

Sequencing of the cDNA was performed via PCR using gene-specific primer sequences for the beta chain of CD91. The cDNA was used for amplification (in the 5′–3′ direction) by the primer pair CCCCTGCTCAGTGCTCTAGTTGCTGCTCAGATCGACCGCGGAGTCACCCACCTCAATATTTC (CD91NT1) and CGCCAAGGGATCTCCTATCTCGTCTTCAGGTCCCCG (CD91CT). Each 50 µL tube contained 10 µL of reaction buffer, 1 µL of dNTPs, 0.25 µL of each primer sequence, 0.5 µL of Q5 DNA polymerase, 38 µL of nuclease-free water, and 1 µL of cDNA. PCR amplification was performed under the thermocycler conditions of 35 cycles (98 °C for 30 s, 55 °C for 30 s, and 72 °C for 75 s). Following PCR, the products were analyzed using gel electrophoresis. The agarose gel was prepared in a buffer solution with ethidium bromide and poured into a casting tray with the appropriate comb. After the gel had solidified, 10 µL of 6x DNA loading dye was added to each 50 µL PCR reaction, and 12 µL of each sample was loaded into individual wells. Zipruler 2 DNA ladder (ThermoFisher) was also loaded into the first lane as a size reference. Electrophoresis was performed at 100V for 45-60 minutes until the dye migrated to the ends of the gel. The gel was subsequently imaged under the Big Blue imaging system and sent off to PlasmidSaurus for sequencing to determine the β chain sequence.

ELISA

The BioLegend LEGEND MAX™ Mouse TNF-α ELISA Kit was used to carry out the ELISA procedure. 500 µL of the 1,000 pg/mL top standard was prepared by diluting 25 µL of the standard stock solution in 475 µL of Assay Buffer A. Six serial dilutions of the 1,000 pg/mL top standard were made in separate tubes using Assay Buffer A as the diluent. The final mouse TNF-α standard concentrations in the tubes were 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/ mL, 31.3 pg/mL and 15.6 pg/mL, respectively. Assay Buffer A was used as the zero standard (0 pg/mL). The plate was then

washed four times with at least 300 µL of 1X Wash Buffer per well. To perform the assay, iBMDMs were plated overnight and treated with hsp70 and calreticulin in media. After stimulation, the supernatant was removed and cells were spun down. 50 µL of Matrix A was added to the assay wells used for standards, followed by 50 µL of HSP-treated iBMDM supernatant samples into their appropriate wells. 50 µL of diluted standards were also added into the respective wells containing Assay Buffer A. The plate was sealed and incubated at room temperature for 2 hours while shaking at 200 rpm. After incubation, the plate was washed four times with 1X Wash Buffer. Next, 100 µL of Mouse TNF-α Detection Antibody was added to each well, and the plate was incubated for 1 hour at room temperature while shaking. The plate was again washed four times as before. To each well, 100 µL of Avidin-HRP solution was added, and then the plate was incubated for an additional 30 minutes while shaking. After washing the plate five times, 100 µL of Substrate Solution D was added and incubated for 15 minutes in the dark. Following this, the enzymatic reaction was stopped with 100 µL of Stop Solution, leading to a color change from blue to yellow. The absorbance was measured at 450 nm using a microplate reader. TNF-α concentrations were calculated based on the standard curve generated during the assay.

Results

CD91 tyrosine residues are essential to CD91's role in cancer immunosurveillance. We aim to mutate these to reveal their role in downstream signaling and interaction with kinases AXL and Fgr. The β chain sequence was confirmed to be intact via the gel shown in Figure 1. We observed Y1F and Y2F bands, corresponding to the relatively upstream and downstream tyrosine mutations, respectively, of approximately 2000 bp. This indicated that the beta chain was still being expressed and the protein was not disrupted significantly. These bulk CRISPR-treated cells were then cloned to isolate cells containing each mutation.

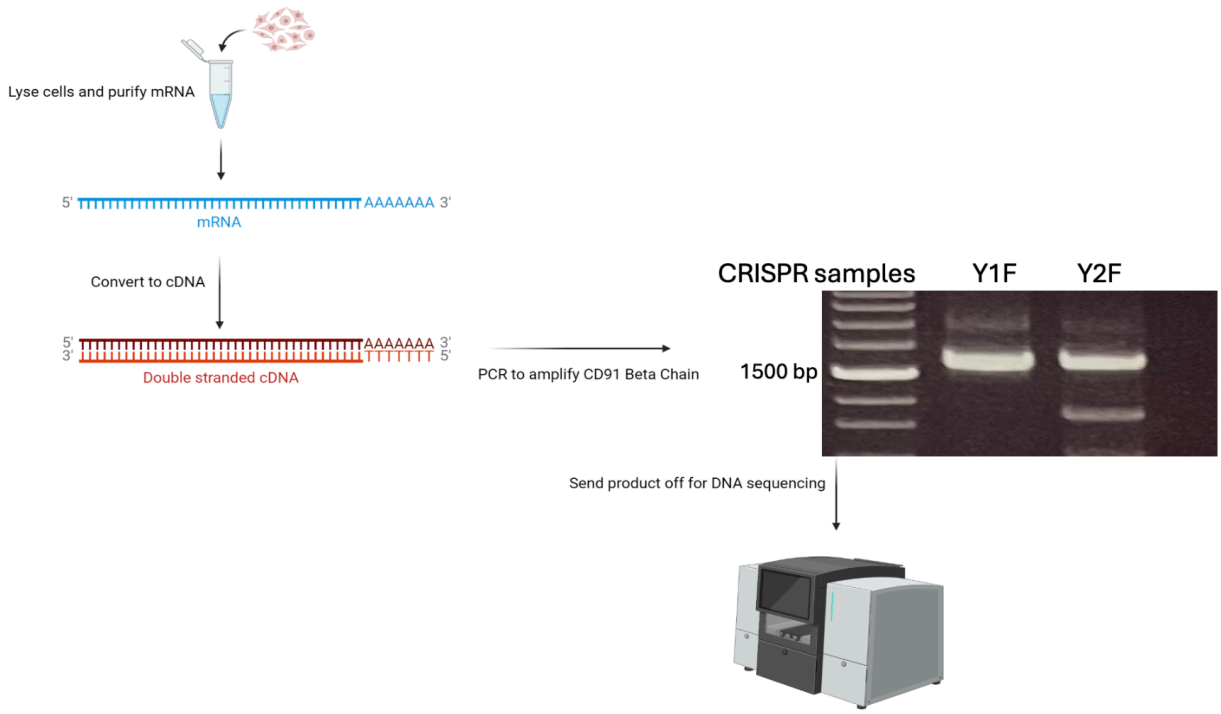

Figure 1. CD91 β chain gel electrophoresis. Cells were lysed and mRNA was purified before being converted to cDNA. PCR was then performed to amplify the CD91 beta chain and gel electrophoresis results show amplified CD91 β chain fragments (~2000 bp) from two CRISPR-edited samples, Y1F and Y2F. DNA sequencing was then performed to confirm if CRISPR worked and whether or not the mutations were induced within the sequence.

From the sequencing results in Figure 2, we observed that CRISPR/Cas-9 successfully generated mutations at the two tyrosine residues within the CD91 beta chain. In Y1F Clone 3, multiple amino acid changes were visible around the tyrosine residue, which could lead to the potential deletion of the CD91 phosphorylation site, but could have additional effects as well. For Y1F Clone 5, there was a more extensive mutation of the tyrosine and the amino acids surrounding it, but the reading frame was still maintained downstream. For the Y2F mutations, clone 4 successfully mutated tyrosine (Y) to phenylalanine (F), which was our desired result. There were also several other mutations to adjacent amino acids in the NPxY motif. The motif is also known as a phosphotyrosine binding domain, and we predicted that mutating this motif would disrupt protein binding and eliminate phosphorylation at the site. Y2F Clone 13 also had a larger mutation and eliminated most of the target region while maintaining the reading frame, similar to Y1F clone 5. The preservation of reading frames in all the clones, despite some additional sequence alterations, suggests that these mutants will express functional CD91 proteins that lack specific phosphorylation sites.

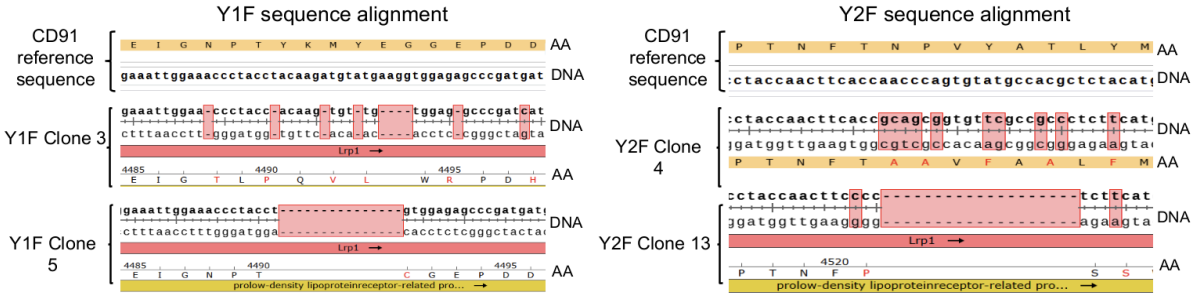

Figure 2. CD91 mutated β chain sequencing results. Left panel: Y1F sequence alignments showing clone 3 with multiple small mutations (pink highlights) and clone 5 with a larger deletion. Right panel: Y2F alignments showing clone 4 with point mutations and clone 13 with a larger deletion. Each alignment displays DNA sequences (middle) and amino acid sequences (top/bottom), with pink highlighting indicating CRISPR modifications confirming successful editing of the CD91 β chain.

To test the impact of these mutations on downstream signaling and cytokine production, we conducted an ELISA to measure TNF-α production in supernatant collected from cells activated with the HSPs calreticulin and HSP70. From the results in Figure 3, the wild-type macrophages showed baseline TNF-α production, with some moderate increase after being treated with HSP70. Y1F mutant clones had altered cytokine secretion patterns when compared to the wild-type cells. Y1F clone 3 showed increased cytokine production in both media and HSP/calreticulin-stimulated conditions. Y1F clone 5 showed the most upregulated baseline cytokine production response compared to all the tested clones, with almost double the concentrations of the wild-type cells; however, HSP70 did not induce more TNF-α production. Y2F mutant clones had varied responses. Interestingly, Y2F Clone 4 showed reduced cytokine levels than the wild-type under all conditions and had a normalized response lower than baseline conditions as well. Y2F Clone 13 also showed reduced concentrations but had a normalized response similar to wild-type cells upon HSP stimulation. These results suggest that mutations at the Y1F site potentially increase baseline TNF-α production in CD91, while Y2F mutations appear to have a more varied effect. Both Y1F and Y2F mutations have the potential to influence TNF-α concentrations in response to HSP70 stimulation. The differences between clones with mutations at the same residue could be from the varying extent of sequence alterations from Figure 2.

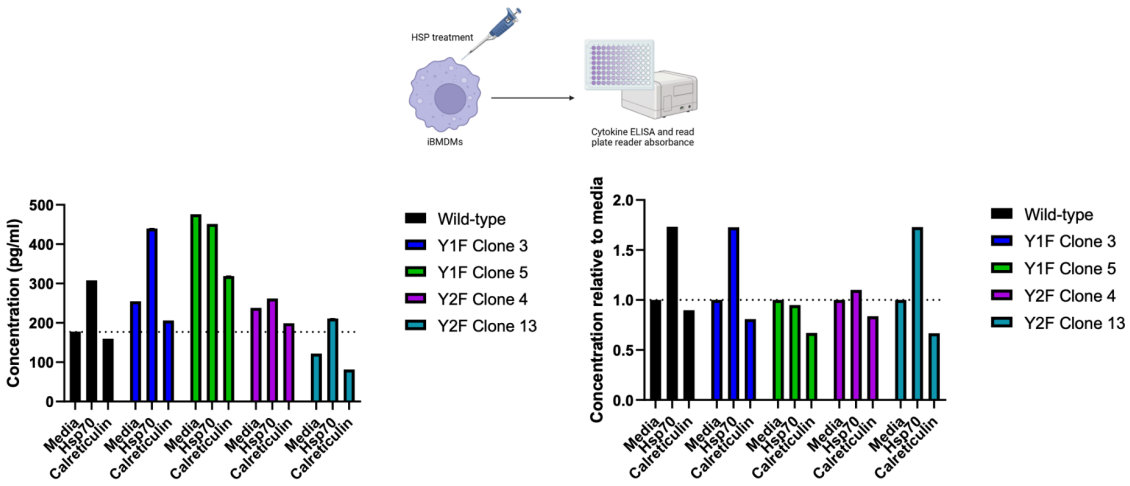

Figure 3. TNF-α ELISA results for each clone. iBMDMs from wild-type and CRISPR-edited CD91 clones were treated with heat shock proteins (HSPs) and analyzed for cytokine production. The left panel shows absolute cytokine concentrations (pg/ml) of TNF-α in media alone or after HSP70/calreticulin treatment. The right panel presents the same data normalized to media control (concentration relative to media). The dotted line represents the baseline response threshold.

Discussion

The most interesting findings emerged from Y1F clone 5 and Y2F clone 4, where we successfully mutated the NPxY motif. The motif is a known conserved tyrosine phosphorylation motif that binds to phosphotyrosine binding domain (PTB), and we predicted that mutating this motif would disrupt protein binding and eliminate phosphorylation at the site. This hypothesis was strongly supported by our results. Both Y1F clone 5 and Y2F clone 4 had significantly reduced TNF-α production in response to HSP70 activation compared to wild-type cells, showing that the NPxY motif is essential for CD91's ability to respond to heat shock proteins that are crucial in cancer immunosurveillance. Our findings make way for multiple future investigations. We are currently working on generating a double CD91 mutant, with substitutions at both tyrosine sites, to examine the interactions between these residues. It would also be helpful to more thoroughly characterize these mutations by testing different cytokine and chemokine production, such as IL-6, CXCL1, and RANTES. Another critical step is translating these results to mouse models for in vivo testing. Testing mice that would contain these Y1F and Y2F mutations would allow us to understand these effects on tumor immunosurveillance as they are happening physiologically. We would also be able to evaluate cytokine production within the tumor microenvironment and how these results ultimately impact tumor growth and the progression of cancer within the mice.

Our findings contribute significantly to the understanding of the HSP-CD91 axis and its role in cancer immunosurveillance. By identifying specific amino acid residues critical for CD91 signaling, we can enhance our understanding of how cells recognize and respond to danger signals associated with cancer. We could potentially target or enhance these amino acids in future therapeutic intervention

projects. For example, in immunotherapy, enhancing CD91 signaling could increase anti-tumor immune responses, but in autoimmune conditions, eliminating this excessive signaling might be helpful. This could relate to AXL and Fgr as well; since these kinases likely mediate the phosphorylation of the tyrosine residues, enhancing their binding to CD91 could be future directions for therapy. Overall, our study provides insights into key tyrosine residues in the CD91 β chain and their roles in regulating immune responses. These findings not only advance our fundamental understanding of cancer immunosurveillance mechanisms but also identify potential targets for therapeutic intervention in cancer and other related disorders.

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