

Article

Trans-Presentation of IL-15 by IL15R α Attenuates Tumor Immune Surveillance and Is Dispensable for IL-15-Dependent Tumor Growth Control

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Simple Summary

IL-15 is required for the development and homeostasis of innate and adaptive lymphoid cells, mediated by IL-15 receptor alpha, beta and gamma chains. However, certain functions of IL-15, such as the activation of innate immune cells early during infections, do not require IL-15 receptor alpha. Whether IL-15 receptor alpha is required for antitumor functions of IL-15 remains unclear. Our findings suggest a regulatory role for IL-15 receptor alpha in tumor immunosurveillance, as its deficiency reduces the development of carcinogen-induced endogenous tumors. The proteomes of MCA tumors from IL-15R α -deficient mice displayed reduced antigen presentation pathways. However, tumors expressing NLRC5 that enhances tumor antigen presentation to CD8⁺ T lymphocytes are not impacted by IL-15 receptor alpha deficiency, whereas loss of IL-15 promotes tumor growth. Our findings indicate that IL-15 receptor alpha is not critical for IL-15-dependent anti-tumor functions.

Abstract

Background: IL-15 is a promising cytokine for cancer immunotherapy. IL-15 promotes differentiation and homeostasis of innate and adaptive immune cells, and their cytolytic effector functions. The IL-15 receptor is composed of IL-15R α , IL-15R β and the common γ_c chains. IL-15 is also trans-presented as IL-15R α :IL-15 complex to IL-15R β : γ_c on neighboring cells. IL-15R α is dispensable for early immune response to infections and in autoimmune diabetes. The role of IL-15R α in antitumor immune responses remains unclear. **Methods:** In WT, *Il15*^{−/−} and *Il15ra*^{−/−} mice, we studied the growth of syngeneic tumor cell lines and tumor immune surveillance against endogenous fibrosarcoma induced by methylcholanthrene (MCA). Immune gene signature and total proteome analysis were performed on MCA-induced tumors. **Results:** Lack of IL-15 or IL-15R α did not enhance the growth of implanted tumor cell lines, despite reduced immune cell infiltration. MCA-induced tumor incidence was reduced in mice lacking IL-15R α but not IL-15, although

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both are required for efficient tumor immunoediting. *Il15^{-/-}* and *Il15ra^{-/-}* tumors showed reduced *Ifng* expression but displayed differential modulation of *Ifng*-responsive genes. Proteome profiles of *Il15^{-/-}* tumors, but not tumor-derived cell lines, showed significant reduction in antigen presentation pathways. B16-F10 melanoma cells expressing NLRC5, the IFN γ -induced transcriptional activator of tumor antigen presentation, still required IL-15 but not IL-15R α for efficient tumor control. Conclusions: Our findings show that IL-15 plays a negligible role in immunosurveillance against spontaneous tumor development, whereas IL-15R α restrains immunosurveillance. Neither IL-15 nor IL-15R α have a significant impact on implanted tumor models, although IL-15 facilitates efficient control of highly immunogenic tumors for which IL-15R α is dispensable.

Keywords: IL-15; IL-15R α ; antitumor immunity; tumor immunosurveillance; tumor immunoediting; proteomics; NLRC5

1. Introduction

The IL-15 receptor (IL-15R) is composed of ligand-binding IL-15R α and signal-transducing IL-15R β and γ_c chains, the latter two being shared with IL-2. In producer cells, IL-15 binds to IL-15R α during biosynthesis and this complex is ‘trans-presented’ to responder cells expressing the IL-15R $\beta\gamma_c$. This ‘trans’ signaling is needed for the activation of CD8⁺ T cells, NK cells and other IL-15-dependent immune cell subsets. *Il15^{-/-}* mice are susceptible to viral, fungal and bacterial infections [1–7]. IL-15 has a pathogenic role in autoimmune diseases [8–13]. While many studies using IL-15-deficient mice showed the importance of IL-15 in protective immune responses during infections, very few studies directly explored the role of IL-15 trans-presentation by IL-15R α in this protection. Unlike IL-15-deficient mice, IL-15R α -deficient mice were resistant to infections [5,14]. Similarly, while IL-15 deficiency protected from certain autoimmune diseases, the absence of IL-15R α did not confer protection [5,15].

Given the ability of IL-15 to activate the cytotoxicity of CD8⁺ T, NK, ILC1s and $\gamma\delta$ T cells, IL-15 is a promising cancer immunotherapeutic agent that can enhance antitumor immunity. Transgenic expression of IL-15 contributes to the control of B16 tumors through the activation of NK and CD8⁺ T cells [16,17]. MHC-I expression on tumor cells promotes IL-15-dependent, CD8⁺ T cell-mediated tumor control [16,18]. Overexpressed IL-15 controlled tumor progression in genetic models of oncogene-induced tumors [19,20]. In line with the role of NK cells in controlling tumor metastasis and hematopoietic cancers but not solid tumors [21], IL-15-deficient mice exhibited increased metastasis while transgenic expression of IL-15 diminished metastasis [17,19]. Despite the promising antitumor efficacy of IL-15 predicted from its biological roles of activating cytotoxicity in NK and CD8⁺ T cells, incorporation of IL-15 in tumor immunotherapy regimens has been plagued by toxicity issues and insufficient responses in solid tumors [22]. Based on promising results obtained with a combination of BCG and IL-15 superagonist, Nogapendekin alfa inbakicept (NAI), also known as N-803 [23], N-803 has been approved by FDA for bladder cancer in 2024.

While the role of IL-15 in promoting antitumor responses by activating CD8⁺ T cells and NK cells are well established, it is not clear whether IL-15 and trans-presented IL-15 play different roles in tumor immunosurveillance. In this study we analyzed the role of IL-15 and trans-presented IL-15 in Methylcholanthrene (MCA)-induced carcinogenesis. Our observations show that incidence of tumors is comparable between WT and *Il15^{-/-}* mice, while *Il15ra^{-/-}* mice had reduced incidence of MCA-induced tumors. Furthermore, we show that increasing the immunogenicity of B16-F10 cells by expressing NLRC5 [24]

that results in better clearance of the tumors, requiring IL-15 but not the trans-presented IL-15.

2. Materials and Methods

2.1. Mouse Strains

Mice strains used in this study were bred in our animal facility to avoid major differences in microbiome and environmental influences. Wild type C57Bl/6 (WT) mice, *Il15^{-/-}* and *Il15ra^{-/-}* in the C57Bl/6 background, have been described previously [14]. All experimental protocols on mice were approved by Université de Sherbrooke Ethics Committee for Animal Care and Use in accordance with guidelines established by the Canadian Council on Animal Care (protocols #2021-3144 and 2026-5225).

2.2. Cell Lines and Tumor Growth

EL4, a lymphoma cell line, EG7-OVA (EG7) a lymphoma cell line derived from EL4 and expressing Ovalbumin (OVA) and B16-F10 murine melanoma cells were obtained from American Type Culture Collection (Manassas, VA, USA). EL4 and EG7 cell lines were cultured in Roswell Park Memorial Institute (RPMI, Sigma-Aldrich, Saint Louis, MO, USA) medium containing 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin cocktail, 1% HEPES, and 1% Sodium Pyruvate. For EG7-OVA, 0.4 mg/mL G418 was added to media. B16-F10 melanoma cell line was cultured in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 5% heat-inactivated fetal calf serum and 1% penicillin/streptomycin cocktail. B16-F10-expressing NLRC5 protein [24] were maintained with the addition of blasticidin (8 µg/mL). All cells were grown in a humidified atmosphere of 5% CO₂ at 37 °C.

For tumor growth 2.5×10^5 cells in 50 µL of phosphate-buffered saline (PBS) were injected subcutaneously in the right hind flank of WT, *Il15^{-/-}* and *Il15ra^{-/-}* mice. Tumor dimensions (length and width) were recorded every three days using a digital Vernier caliper. Tumor volume was calculated using the ellipsoid formula: $V = \frac{1}{2} \times \text{Length} \times \text{Width}^2$. At the endpoint (2 cm in diameter in any one direction), mice were euthanized, and tumor samples were collected and analyzed for histopathological features.

2.3. MCA-Induced Fibrosarcoma for Tumor Immunosurveillance

Groups of WT, *Il15^{-/-}* and *Il15ra^{-/-}* mice were injected subcutaneously in the right hind flank with 100, 200 or 400 µg of 3-Methylcholanthrene (MCA, Sigma-Aldrich, Saint Louis, MO, USA, Cat # 212942) in 50 µL of corn oil as previously described [25,26]. The growth of MCA-induced fibrosarcoma was monitored daily over the course of 80–200 days and measured by a digital Vernier caliper along the perpendicular axes of the tumors. Tumors > 2 mm in diameter and demonstrating progressive growth were recorded as positive. Tumor samples were collected for histopathology, immunofluorescence, and proteomics analyses, and to generate MCA-induced tumor cell lines as previously described [26].

2.4. MCA-Induced Fibrosarcoma Cell Lines and Cancer Immunoediting

MCA-induced fibrosarcoma were collected from WT, *Il15^{-/-}* and *Il15ra^{-/-}* mice. Tumor tissues were washed, minced, and mechanically dissociated using gentleMACS C tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) and gentleMACS Dissociator, and then incubated with Collagenase IV (1 mg/mL) and DNase I (40 µg/mL) for 1 h at 37 °C for chemical digestion. Digested tissue was passed through a 40 µm filter and the collected cells were washed with PBS. Cells were cultured in RPMI medium containing 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin cocktail, 1% HEPES, and 1% Sodium Pyruvate for three months in a humidified atmosphere of 5% CO₂ at 37 °C. Cell

proliferation was assessed using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The cells were labeled with fluorochrome-conjugated antibodies for MHC I and PD-L1 antibodies for flow cytometry. For immunoediting assays *in vivo*, 2×10^5 MCA tumor-derived cells from WT, *Il15^{-/-}* and *Il15ra^{-/-}* mice were implanted subcutaneously in naive WT, *Il15^{-/-}* and *Il15ra^{-/-}* mice. Tumor growth was monitored from the tumor onset until the ethical limits were reached. Tumor samples were collected and analyzed as described above.

2.5. RNA Isolation and Gene Expression Analysis

RNA from frozen WT, *Il15^{-/-}* and *Il15ra^{-/-}* tumors and MCA-derived cell lines were extracted using RNeasy Mini kit (Qiagen, Toronto, ON, Canada). cDNA was synthesized from 500 ng of purified RNA using QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada). Quantitative RT-PCR amplification reactions were carried out in QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Mississauga, ON, Canada) using SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada). The expression of indicated genes was measured using primers listed in Supplementary Table S1. Gene expression levels between samples were normalized based on the Cycle threshold (Ct) values compared to the housekeeping gene 36B4 (*Rplp0*).

2.6. Histopathological and Immunofluorescence Analysis

Formalin-fixed paraffin-embedded tissue sections of 4 μ m thickness were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Digital images of the stained sections were acquired using a Nanozoomer Slide Scanner (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan) and analyzed by the Nanozoomer Digital Pathology software NDPview2.7.52 (Hamamatsu Photonics). For immunofluorescence, deparaffinized and rehydrated tumor sections were incubated in Buffer A (Electron Microscopy Sciences, #62707-10, Morgantown, PA, USA) for antigen unmasking using Retriever 2100 (Electron Microscopy Sciences, #R2100-US). Tumor sections were then blocked using 5% BSA in Tris-buffered saline (TBS) containing 20% Tween-20 (TBS-T) followed by overnight incubation with the primary antibody at 4 °C. After washing, tissue sections were incubated with the appropriate secondary antibody for 2 h and washed, and nuclei were stained using Hoechst 33342 DNA staining dye (Thermo Fisher Scientific, Mississauga, ON, Canada). After washing, tissue sections were mounted with a coverslip with Dako Fluorescence Mounting Media (Agilent, Santa Clara, CA, USA, Cat#S3023) and images were taken using fluorescent microscope Zeiss AxioScope 2.

2.7. Mass Spectrometry

The MCA tumor samples and tumor-derived cell lines were processed for proteomic analysis as previously described [26]. The tumors and the tumor-derived cell lines are not paired samples. Briefly, 25 to 50 mg of frozen tumors or cell pellets were lysed with a Lysis buffer (5% sodium dodecyl sulfate (SDS), 50 mM triethylammonium bicarbonate (TEAB), pH 8.5), incubated at 95 °C for 3 min and homogenized using Qiagen TissueLyser (Qiagen, Toronto, ON, Canada). The DNA was sheared by sonication before protein quantification (Pierce BCA Protein Assay Kit, Thermo Fisher, ref#23227). Then, 100 μ g of proteins were reduced in 100 μ l of Lysis buffer by adding Tris(2-carboxyethyl)phosphine (TCEP) to 5 mM final concentration, followed by a 55 °C, 15 min incubation. Proteins were alkylated by adding chloroacetamide (Sigma-Aldrich, Saint-Louis, MO, USA) to a final concentration of 20 mM and incubated for 10 min at room temperature. Complete denaturation of proteins was done by adding phosphoric acid to a final concentration of 2.5%. A binding/wash buffer was then added to protein samples to a final concentration of 100 mM TEAB in 90% methanol before being transferred to a S-trap column. The column was then

washed three times. Proteins were digested by adding 1 µg of Pierce mass spectrometry (MS)-grade trypsin (Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight at 30 °C. Peptides were then eluted using 50 mM TEAB, 0.2% formic acid, and 50% acetonitrile before being concentrated by centrifugal evaporation and then resuspended in 1% formic acid. Peptides were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

For mass data acquisition in data-independent acquisition (DIA) mode, 250 ng of peptides from each sample were injected into an HPLC (nanoElute, Bruker Daltonics, Bremen, Germany) and loaded onto a trap column with a constant flow of 4 µL/min (AcclaimPepMap100 C18 column, 0.3 mm id × 5 mm, Dionex Corporation, Sunnyvale, CA, USA), and then eluted onto an analytical C18 Column (1.9 µm beads size, 75 µm × 25 cm, PepSep) heated at 50 °C. Peptides were eluted for 2 h over gradient of acetonitrile (5–37%) in 0.1% FA at 400 nL/min while being injected into a TimsTOF Pro ion mobility mass spectrometer equipped with a Captive Spray nano electrospray source (Bruker Daltonics). Data were acquired using diaPASEF mode. Briefly, for each single Trapped Ion Mobility Spectrometry (TIMS; 100 ms) in diaPASEF mode, 1 mobility window consisting of 27 mass steps (m/z between 114 and 1414 with $\Delta m/z$ width of 50 Da) per cycle (1.27 s duty cycle) was used. These steps cover the diagonal scan line for +2 and +3 charged peptides in the m/z -ion mobility plane.

2.8. Proteomics Data Analysis

Peptide mass spectra were analyzed using the DIA-NN, an open-source software [27] suite for DIA/SWATH data processing (<https://github.com/vdemichev/DiaNN>, version 1.8.1, accessed on 01-06-2024), installed in an Apptainer container (<https://apptainer.org/>, version 1.3.5 accessed on 01-06-2024) using docker image provided on the docker hub [28]. Analysis was performed using default parameters, except for the following options: two missed cleavages were allowed; trypsin digestion was performed for K/R; and protein N-term methionine excision was used as a variable modification for the in silico digest as described [26].

The *Mus musculus* reference proteome UP000000589 was downloaded from the UniProt website (<https://www.uniprot.org/proteomes/UP000000589>). The reference proteome contained 63,367 proteins. For the FASTA search, DIA-NN was instructed to perform an in silico digest of the sequence database. A mass tolerance accuracy of MS1 and MS2 of 20 ppm was used for the precursor and fragment ions, respectively. Minimum and maximum values were set for peptide length (7–30 amino acids), precursor charge (1–5), precursor m/z (100–1700), and fragmentation m/z (100–1500) for in silico library generation or library-free search. For re-analysis, match between runs (MBR) was enabled and smart profiling was chosen when creating a spectral library from DIA data. Carboxyamidomethylation (unimod4) and oxidation (M) (unimod35) were set as fixed modifications, and N-terminal protein acetylation was set as a variable modification. DIANN protein group matrix was filtered using a custom Perl script to extract protein groups with a single protein.

The proteomic data were analyzed using in-house customized R (4.6.0) scripts for data import, data quality check, analysis of differential proteins expression using the Limma package, generation of volcano plots, gene set enrichment analyses (GSEA) using KEGG and Hallmark gene sets, and pathway enrichment heatmaps. The complete analysis workflow is available on GitHub, commit hash 4da4ecc (<https://github.com/MohammadMoradzad/tumor-proteomics-analysis-pipeline>, V1).

2.9. Flow Cytometry

Tumor-infiltrating lymphocytes (TILs) were isolated using Tumor Dissociation Kit, mouse (#130-096-730) from Miltenyi Biotec, Bergisch Gladbach, Germany. Single-cell

suspensions of TILs, draining lymph nodes (DLN), and non-draining lymph nodes (NDLN) were washed with PBS containing 1% FCS and aliquoted up to 1×10^6 cells/100 μ L into FACS tubes for antibody staining for flow cytometry. The cells were labeled with fluorochrome-conjugated antibodies for flow cytometry as described [24]. Cells were analyzed on a Cytoflex 30 flow cytometer (Beckman Coulter, Brea, CA, USA) or BD FACSDiscover™ S8 cell sorter (BD Biosciences, Milpitas, CA, USA) and data were analyzed using the FlowJo software versions 10 or 11 (BD Biosciences). Antibodies used for flow cytometry are listed in Supplementary Table S2 for Cytoflex 30 and Supplementary Table S3 for BD FACSDiscover™ S8. The phenotype markers of different cell subsets are listed in Supplementary Table S4.

2.10. Data Analysis

Graphpad Prism software (Version 10) was used for data analysis and to generate illustrations. The data are shown as mean $\pm$ standard deviation (SD). One- or two-way ANOVA with Tukey's or Bonferroni's post hoc test was used as indicated in figure legends to compare datasets and to calculate statistical significance.

3. Results

3.1. Lack of IL-15 or IL-15R α Does Not Enhance the Growth of Implanted Syngeneic Tumors

Innate immune responses that contribute to the control of pathogens and promote autoimmunity require IL-15 signaling without the need for IL-15 trans-presentation by IL-15R α [5,14,15]. Even though NK cells are implicated in IL-15-mediated control of an oncogene-driven breast cancer model [19], their role in solid tumors is not clear [29]. To investigate how endogenous levels of IL-15 and its trans-presentation via IL-15R α impact tumor growth, we implanted in wild type (WT), *Il15*^{−/−} and *Il15ra*^{−/−} mice in C57Bl/6 background syngeneic tumor cell lines that vary in MHC class-I (MHC-I) expression and in the ability to activate antitumor immune cell subsets. EL4 is a T-cell lymphoma that expresses high levels of MHC-I and EG7 is an EL4-derived line expressing chicken ovalbumin as a surrogate tumor antigen that render it highly immunogenic [30]. Control (WT), *Il15*^{−/−} and *Il15ra*^{−/−} mice implanted with EL4 developed tumors between 7 and 19 days post-implantation with no significant differences in tumor size or volume (Figure 1A). EG7 cells elicit a strong CD8⁺ T cell response resulting in efficient tumor control, requiring larger inoculum ($>3 \times 10^6$ cells) for tumor formation following subcutaneous implantation [31]. To determine if the loss of IL-15 or IL-15R α would allow tumor growth by sub-optimal dose of EG7, 3×10^5 cells were implanted in WT, *Il15*^{−/−} and *Il15ra*^{−/−} mice. While 3/7 WT (57%), 1/4 *Il15*^{−/−} (25%) and 3/6 *Il15ra*^{−/−} (50%) mice developed tumors between 22 and 50 days, the rest remained tumor-free (Figure 1B), indicating that loss of IL-15 or IL-15R α did not increase tumor formation by EG7 cells. B16-F10 melanoma cells express low levels MHC-I and are inefficient in activating CD8⁺ T cells but can be targeted by NK cell-mediated killing [32,33]. As trans-presented IL-15 is required for NK cell homeostasis [34], we assessed the growth of B16-F10 melanoma in WT, *Il15*^{−/−} and *Il15ra*^{−/−} mice. The growth of B16-F10 melanoma in *Il15*^{−/−} and *Il15ra*^{−/−} mice was comparable to that in WT mice (Figure 1C), even though the number of NK cells within tumor-infiltrating leukocytes (TILs) was significantly reduced in *Il15*^{−/−} mice (Supplementary Figure S1). NKT cells, CD8⁺ T cell subsets and MHC class-II⁺ dendritic cells were also significantly reduced in B16-F10 tumor-draining lymph nodes (DLN) of *Il15*^{−/−} mice (Supplementary Figure S1), suggesting inefficient activation of these cells in the absence of IL-15. A similar pattern was observed in *Il15ra*^{−/−} mice, although the reduction was modest. Overall, the lack of IL-15 or IL-15R α did not result in increased growth of B16-F10, EL4 or EG7 tumors, suggesting that these

tumor cell line models may not be robust enough to investigate the impact of basal levels of IL-15 and IL-15 trans-signaling via IL-15R α on the immune control of solid tumors.

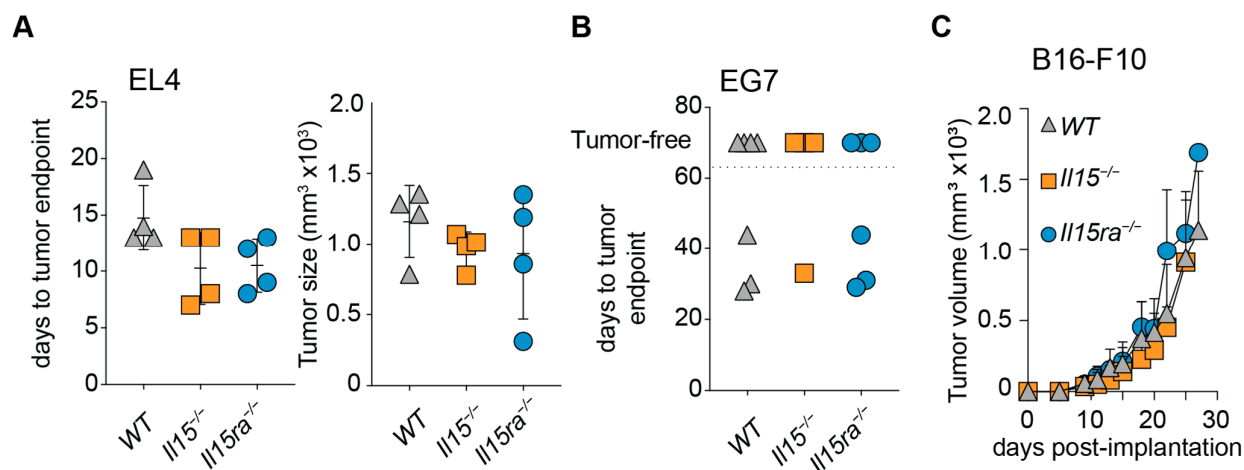


Figure 1. Loss of IL-15 or IL-15R α does not affect the growth of established tumor cells lines. WT, *Il15*^{-/-} and *Il15ra*^{-/-} mice were implanted in their right flank with (A) EL4 lymphoma or (B) EG7 cells (EL4 expressing chicken ovalbumin) or (C) B16-F10 melanoma, and tumor growth was measured every 2 days from day 8 onwards. When the tumor reached the endpoint (20 mm in diameter in any one direction) in more than one of the recipient mice, all mice were euthanized. 4–7 mice per group.

3.2. IL-15R α Restrains Immunosurveillance of Endogenously Arising Tumors

As IL-15 signaling regulates innate immune responses of NK, NKT and $\gamma\delta$ T cells, in addition to regulating some CD8⁺ T cell subsets, we assessed the requirement of IL-15 and IL-15R α in the control endogenously arising tumors that are restrained by immunosurveillance mechanisms [35]. A widely used model of tumor immunosurveillance is the 3-methylcholanthrene (MCA)-induced fibrosarcoma, which demonstrated the requirement of CD8⁺ T cells and the effector molecules perforin and granzyme B for tumor immunosurveillance [36–38]. Earlier studies using anti-NK1.1 and anti-NKG2D antibodies indicated a requirement for NK and NKT cells in the immunosurveillance of MCA-induced tumors [39,40]. However, a more recent study using mice with targeted deletion of NK and ILC1s showed that NK and ILC1 cells are dispensable for immunosurveillance against MCA tumors and even suggested an inhibitory role of these cells [41]. As the MCA dose required to induce MCA tumors vary with genetic background and in different mice colonies [37,38], we used 100, 200 or 400 μ g MCA in WT, *Il15*^{-/-} and *Il15ra*^{-/-} mice (Supplementary Figure S2A–C). Data from both sexes were pooled as male and female mice show no significant difference in tumor incidence [42]. Only a few mice in all three genotypes developed tumors at 100 μ g MCA dose, whereas IL-15R α -deficient mice showed significantly reduced tumor incidence at 200 μ g MCA dose, when compared to WT and *Il15*^{-/-} mice (Supplementary Figure S2B). Cumulative tumor incidence at all MCA doses revealed that mice lacking IL-15R α displayed resistance to MCA-induced tumor development compared to WT and *Il15*^{-/-} mice (Figure 2A). These results indicate that IL-15 plays little role in immunosurveillance of endogenously arising tumors, whereas IL-15R α negatively impacts tumor immunosurveillance.

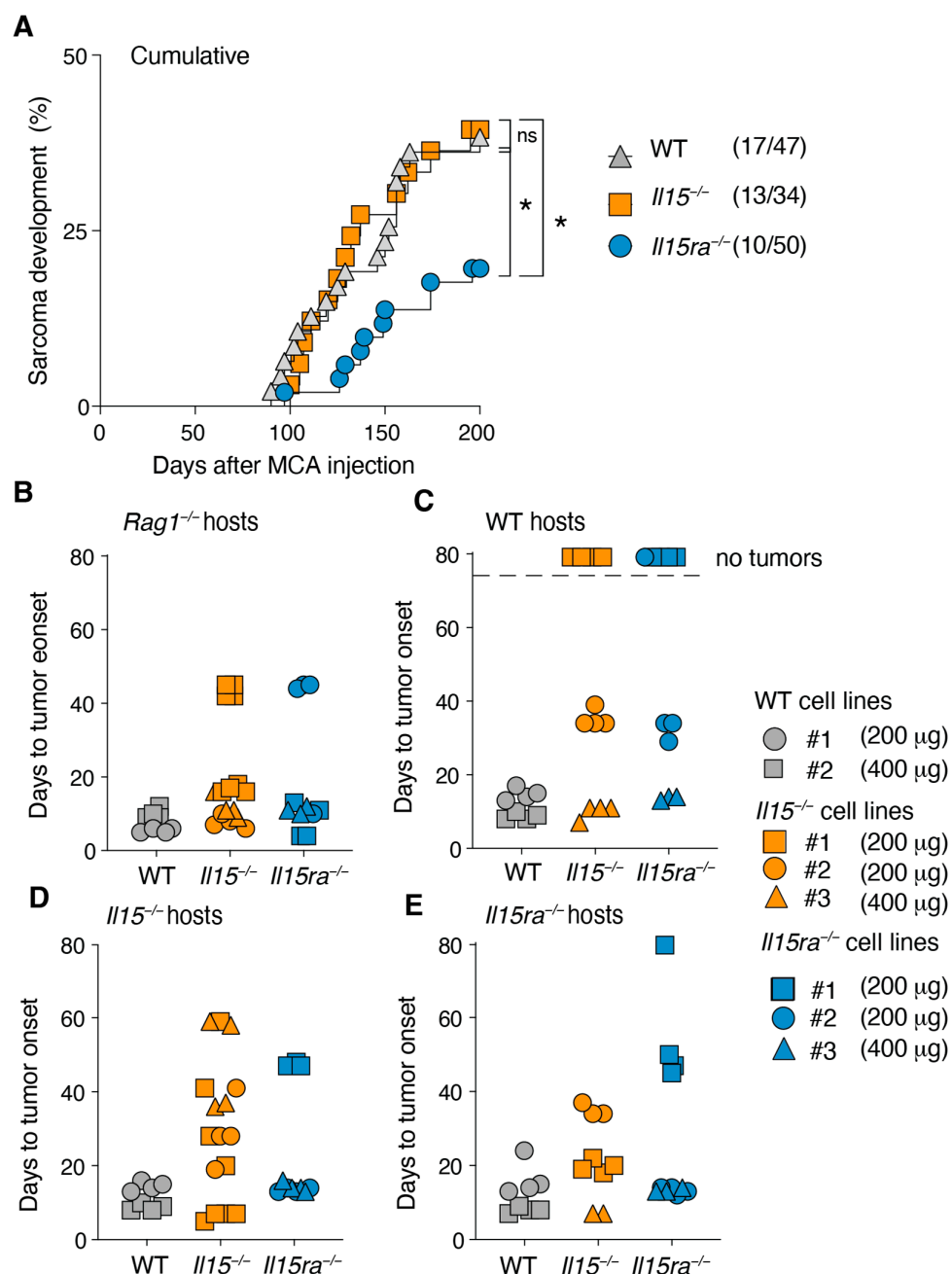


Figure 2. Role of IL-15 or IL-15R α deficiency on tumor immunosurveillance and immunoediting. (A) WT, *Il15*^{-/-} and *Il15ra*^{-/-} mice were injected with the chemical carcinogen 3-ethylcholanthrene (MCA) subcutaneously. Development of fibrosarcoma was monitored visually and by using a digital vernier caliper until the endpoint (tumor diameter of 20 mm in any direction, or ulceration at any diameter), when the animals were euthanized. Kaplan–Meyer curve for the cumulative number of mice that developed MCA-induced fibrosarcoma. Log-rank (Mantel–Cox) test. * $p \leq 0.05$. Numbers in parenthesis adjacent to mice genotype indicate number of mice that developed tumors over the total number of mice tested. (B–E) MCA-derived tumor cell lines from WT, *Il15*^{-/-} and *Il15ra*^{-/-} mice were implanted in *Rag1*^{-/-} (B), WT (C), *Il15*^{-/-} (D) and *Il15ra*^{-/-} (E) mice and followed for tumor development. The dose of MCA associated with the tumor origin is indicated adjacent to the cell line numbers.

3.3. IL-15 or IL-15R α Is Not Required for Tumor Immunoediting

MCA-induced tumors developing in mice with impaired antitumor immune mechanisms are not efficiently immunoedited, and thus cell lines established from these tumors are effectively controlled in immune-sufficient hosts upon transplantation but grow unhindered in immunodeficient hosts [26,43]. As MCA-induced tumor incidence was comparable between WT and *Il15*^{−/−} mice but decreased in *Il15ra*^{−/−} mice, we assessed whether the MCA tumors from *Il15*^{−/−} and *Il15ra*^{−/−} mice differed in their immunoedited status as depicted in Supplementary Figure S3. MCA tumor cell lines established from the WT, *Il15*^{−/−} and *Il15ra*^{−/−} mice showed comparable growth in vitro and were equally responsive to IFN γ in upregulating MHC-I and PD-L1 (Supplementary Figure S4A–C). These cell lines were then tested for in vivo growth in *Rag1*^{−/−}, WT, *Il15*^{−/−} and *Il15ra*^{−/−} mice. All the tested cell lines formed tumors in *Rag1*^{−/−} mice that do not have the adaptive immune system, as expected (Figure 2B). The WT tumor cell lines rapidly formed tumors in WT, *Il15*^{−/−} and *Il15ra*^{−/−} recipients, confirming their immunoedited and thus immune escape status (Figure 2C–E). Of the three cell lines derived from *Il15ra*^{−/−} and *Il15*^{−/−} tumors, one *Il15*^{−/−} and one *Il15ra*^{−/−} cell lines failed to form tumors in WT hosts and two others formed tumors, suggesting that IL-15 or IL-15 trans-signaling is not required for tumor immunoediting. However, *Il15*^{−/−} and *Il15ra*^{−/−} tumor cell lines formed tumors in WT, *Il15ra*^{−/−} and *Il15*^{−/−} hosts, possibly due to the paucity of IL-15-dependent adaptive and innate lymphocytes during tumor development and progression.

3.4. Immune Response Gene Expression in MCA Tumors of *Il15*^{−/−} and *Il15ra*^{−/−} Mice

Hematoxylin and eosin staining of MCA-induced tumor tissues sections did not reveal any infiltrating immune cell clusters (Figure 3A). Nonetheless, immunofluorescence staining of tumor sections indicated scattered distribution of CD45⁺ cells in all the three genotypes (Figure 3B) and increased staining of CD31, an endothelial cell marker for tumor angiogenesis, in IL-15-deficient tumors (Figure 3C). We analyzed the expression of select set of genes that are characteristic of antitumor immune responses. *Klrc2* (NKG2C), which is expressed on NK cells and some CD8⁺ T cell subsets [44], was reduced in both *Il15*^{−/−} and *Il15ra*^{−/−} tumors (Figure 3D). On the other hand, *Klrb1c* (NK1.1) was reduced in *Il15ra*^{−/−} tumors but not in *Il15*^{−/−} tumors, whereas *Klrk1* (NKG2D) showed an inverse expression pattern (Figure 3D). The expression of *Klrc2* was unaffected in *Il15*^{−/−} or *Il15ra*^{−/−} tumors. Both *Il15*^{−/−} and *Il15ra*^{−/−} tumors displayed reduced *Cd8* expression compared to WT tumors, whereas *Cd4* expression was unaffected (Figure 2E). Notably, *Ifng* but not *Ifna* was greatly reduced in both *Il15*^{−/−} and *Il15ra*^{−/−} tumors when compared to WT tumors (Figure 3F). The expression of *Pdl1*, which is induced by *Ifng* was reduced only in *Il15ra*^{−/−} tumors, whereas the IFN-inducible *Cxcl9* and *Cxcl10* gene expression was reduced in *Il15*^{−/−} tumors but not in *Il15ra*^{−/−} tumors (Figure 3G). These observations suggest that the immune responses towards MCA tumors developing in *Il15*^{−/−} mice and *Il15ra*^{−/−} mice share certain features but show considerable differences.

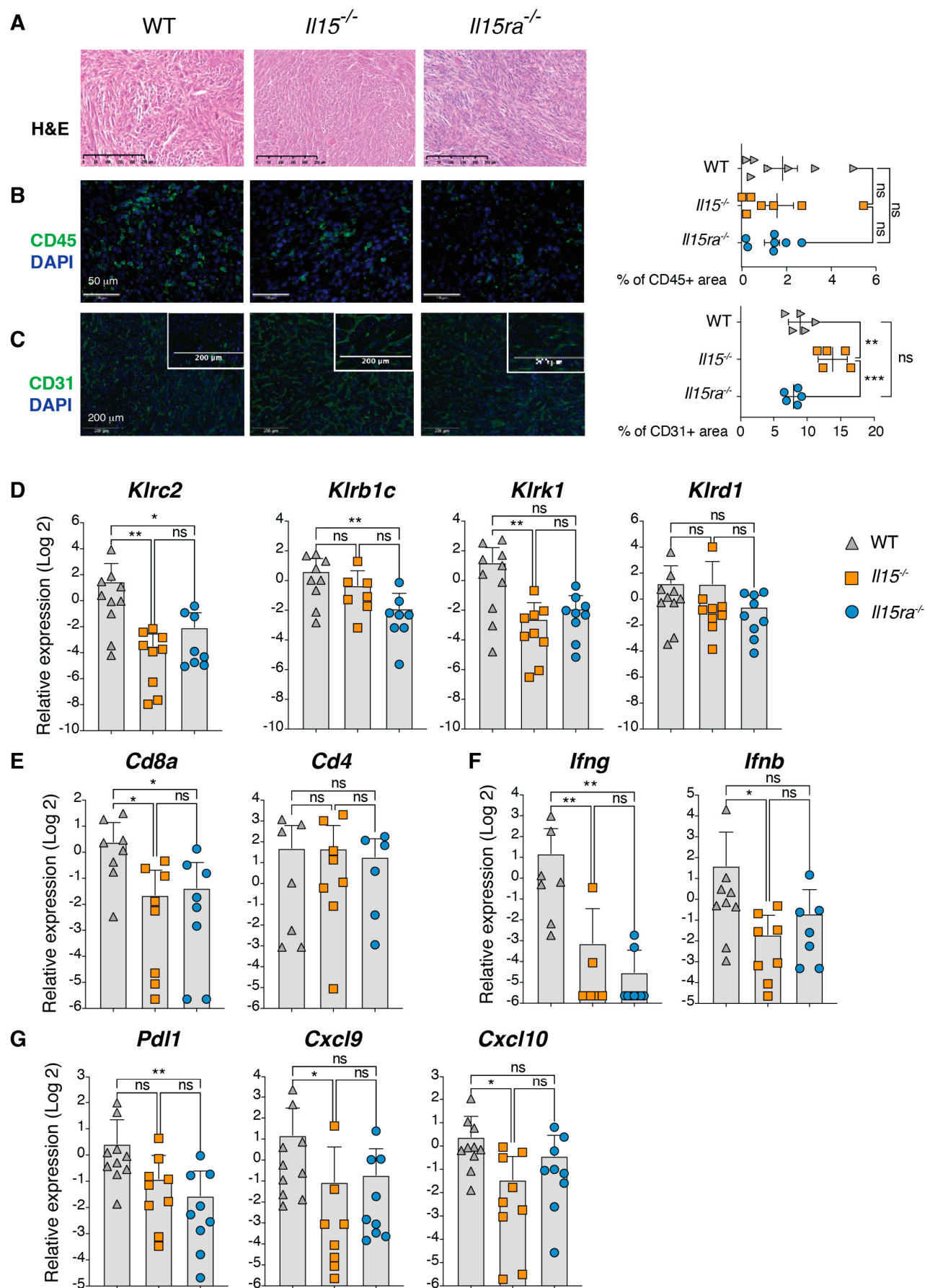


Figure 3. Characterization of MCA tumors. (A) Representative figures of H&E staining (20× magnification) and (B) immunofluorescence staining for CD45/DAPI and (C) CD31/DAPI of MCA tumors. The bar graphs on the right show the % staining for CD45- or CD31- positive area. Gene expression analyses in spontaneous MCA-induced fibrosarcoma was carried out on total RNA extracted from 6 to 8 tumors per group and analyzed by RT-qPCR for the indicated genes. (D) Genes associated with NK cells, (E) *Cd4* and *Cd8*, (F) *Ifna* and *Ifng* and (G) *Ifn* stimulated genes. Statistics: Mean + SD. Ordinary one-way ANOVA with Tukey's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.5. Overlapping and Distinct Proteome Profiles of *Il15*^{−/−} and *Il15ra*^{−/−} MCA-Induced Tumors

Despite the expected heterogeneity of oncogenic drivers in MCA-induced sarcomas, somatic mutation loads in MCA-induced tumors in WT mice are lower when compared to radiation-induced tumors in WT mice [45]. Hence, we performed proteomic analyses of four independently arising MCA-induced tumors from WT, *Il15ra*^{−/−} and *Il15*^{−/−} mice treated with 200 µg MCA to identify signaling pathway alterations associated with the loss of IL-15 or IL-15Rα. Quality control data showed 6000 to 7000 individual proteins in all samples, with comparable level of protein intensity distribution and acceptable levels of missing values in replicates (Supplementary Figure S5A–C). Notably, principal component analysis showed clear separation of *Il15*^{−/−} tumors from WT and *Il15ra*^{−/−} tumors, the latter two showing partial overlap, supported by sample-to-sample correlation (Supplementary Figure S5D–F). Volcano plots and Venn diagrams showed hundreds of differentially regulated proteins (DEPs) unique in tumors arising from *Il15*^{−/−} and *Il15ra*^{−/−} mice compared to WT tumors (Figure 4A–D), suggesting that lack of IL-15 or IL-15Rα differentially affects protein expression across the replicates, notably, a set of proteins that are downregulated in *Il15*^{−/−} tumors but upregulated in *Il15ra*^{−/−} tumors and vice versa, which encompass proteins involved in general cellular homeostasis (Figure 4E). These observations suggest that IL-15 signaling modulates signaling pathways within tumors depending on the availability of IL-15Rα.

To determine how IL-15 and IL-15Rα impact signaling pathways in tumors, gene set enrichment analyses (GSEA) using Hallmark and KEGG pathways were performed on the DEPs in MCA tumors from *Il15*^{−/−} and *Il15ra*^{−/−} mice (Figure 5). Among the Hallmark pathways, IFNα and IFNγ responses were significantly downregulated in both IL-15-deficient and IL-15Rα-deficient tumors when compared to WT tumors (Figure 5A,B). A heatmap analysis of proteins modulated in these two overlapping pathways is shown in Figure 5A. IL-2-Stat5 signaling pathway, which is shared between IL-15 and IL-2 [46], was downregulated in *Il15*^{−/−} tumors when compared to WT and *Il15ra*^{−/−} tumors as expected, indicating towards the possibility of IL-15Rα independent IL-15 signaling (Supplementary Figure S6). Analysis of the KEGG pathways revealed significant downregulation of leukocyte trans-endothelial migration, and antigen processing and presentation pathways in IL-15-deficient tumors when compared to WT tumors (Figures 5D and 6B,C). Notably, IL-15Rα-deficient tumors showed significant downregulation of neutrophil extracellular trap formation, and upregulation of the Notch signaling pathway, polycomb repressive complex, and basal transcription factors compared to WT tumors (Figures 5D,E and 6E,F). These pathways modulated in IL-15Rα-deficient tumors likely require the presence of IL-15, as they were not represented among the DEPs between *Il15*^{−/−} and *Il15ra*^{−/−} tumors (Figure 5C,F).

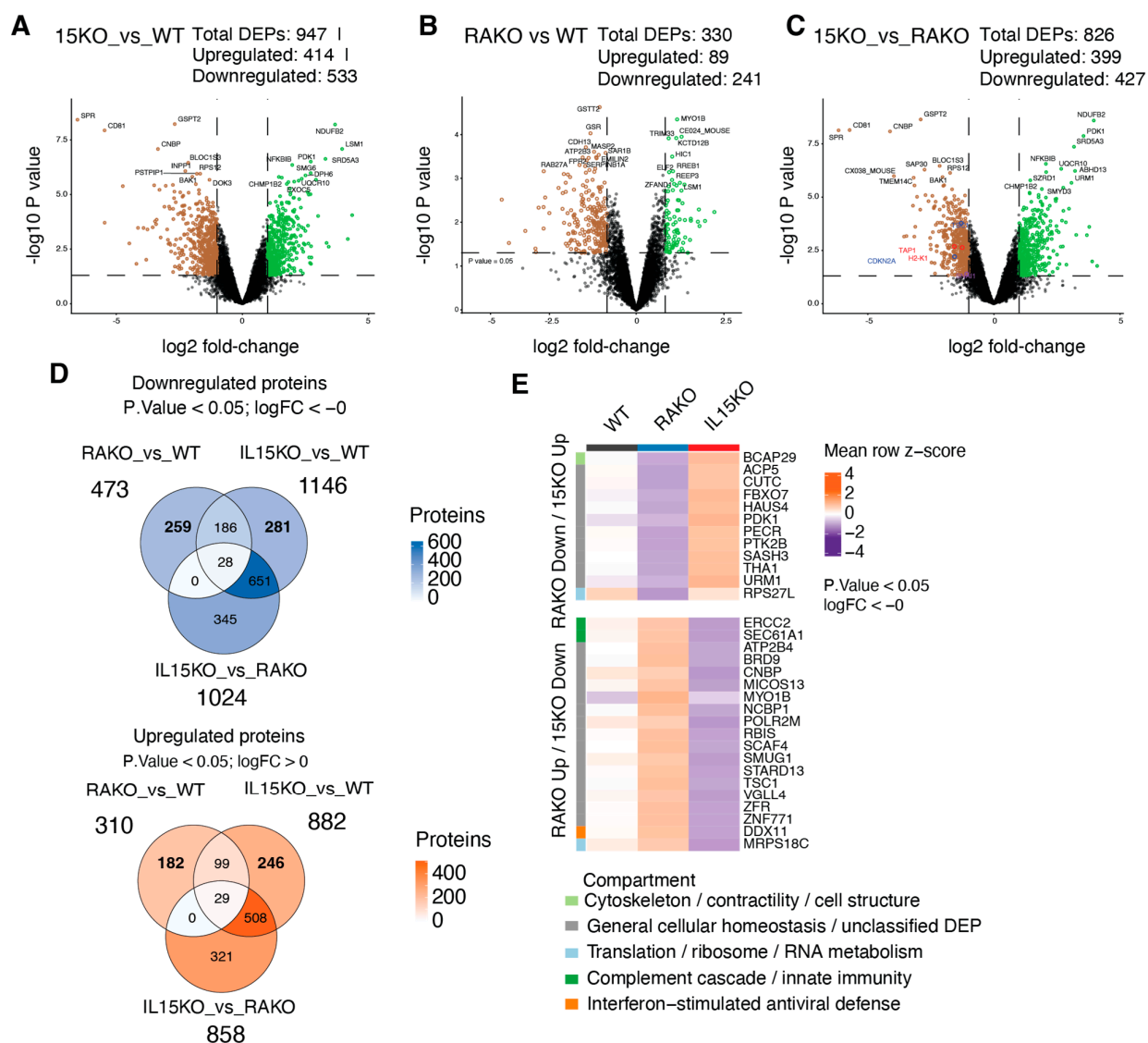


Figure 4. Proteomic analysis of MCA tumors. Proteins extracted from four independent MCA tumors for each genotype were analyzed using mass spectrometry. (A–C) Volcano plots of proteins detected in *Il15*^{-/-} (15KO) tumors (A) and *Il15ra*^{-/-} (RAKO) tumors (B) compared to WT tumors, and 15KO tumors compared to RAKO tumors (C). Upregulated proteins are indicated in green color and downregulated proteins in brown color. (D) Venn diagrams showing the overlap between the pairwise comparisons shown in (A–C) within the upregulated and downregulated proteins. (E) Heatmap analyses of DEPs that are differentially expressed between 15KO and RAKO tumors.

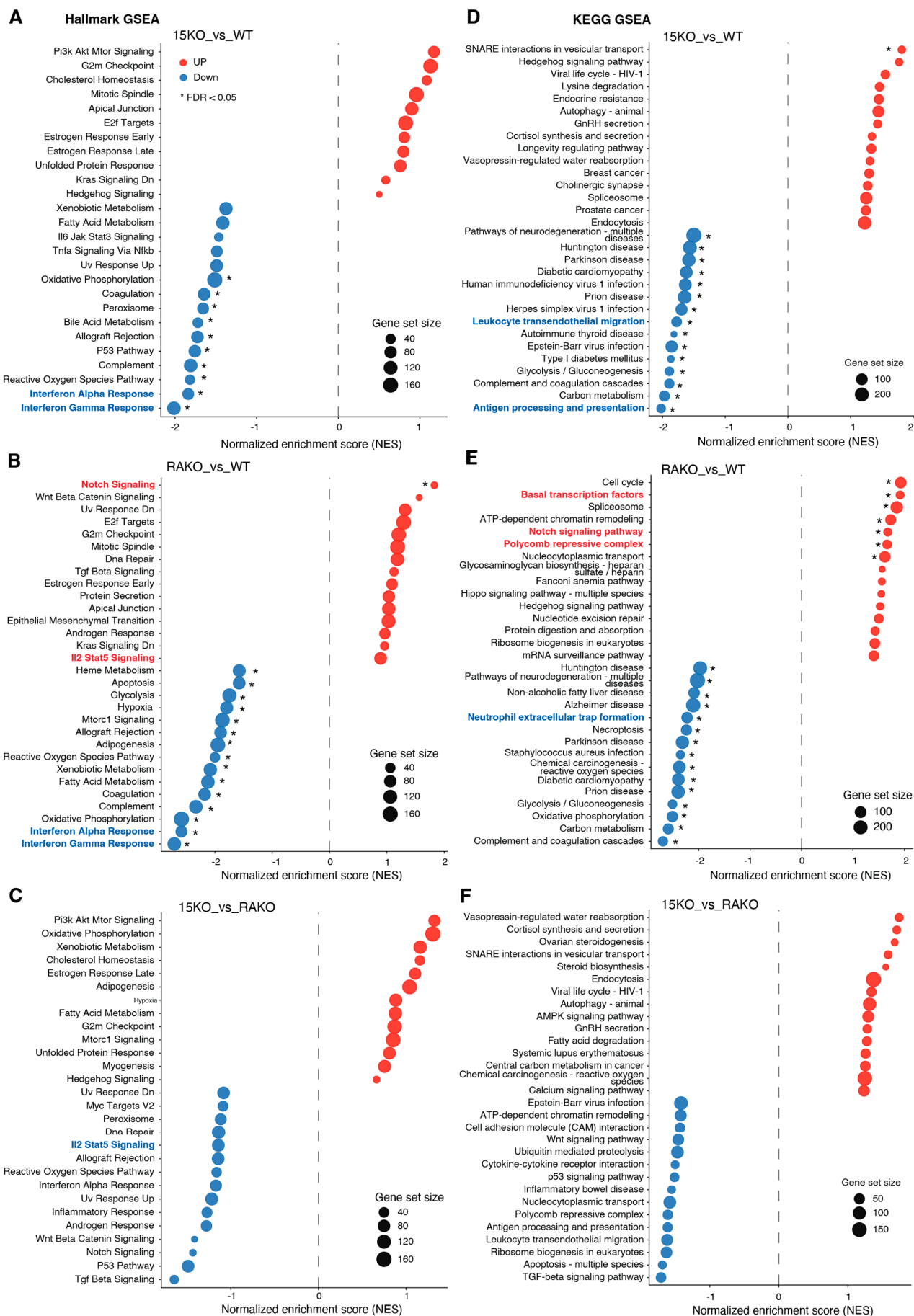


Figure 5. Differentially expressed pathways in the proteomes of MCA-induced fibrosarcoma from WT, *Il15^{-/-}* and *Il15ra^{-/-}* mice. Top 15 upregulated (in red) and downregulated (in blue) pathways in the Hallmark (A–C) and KEGG (D–F) gene set enrichment analyses that are different between 15KO tumors (A,D) and RAKO tumors (B,E) compared to WT tumors, and 15KO tumors compared to RAKO tumors (C,F) are shown.

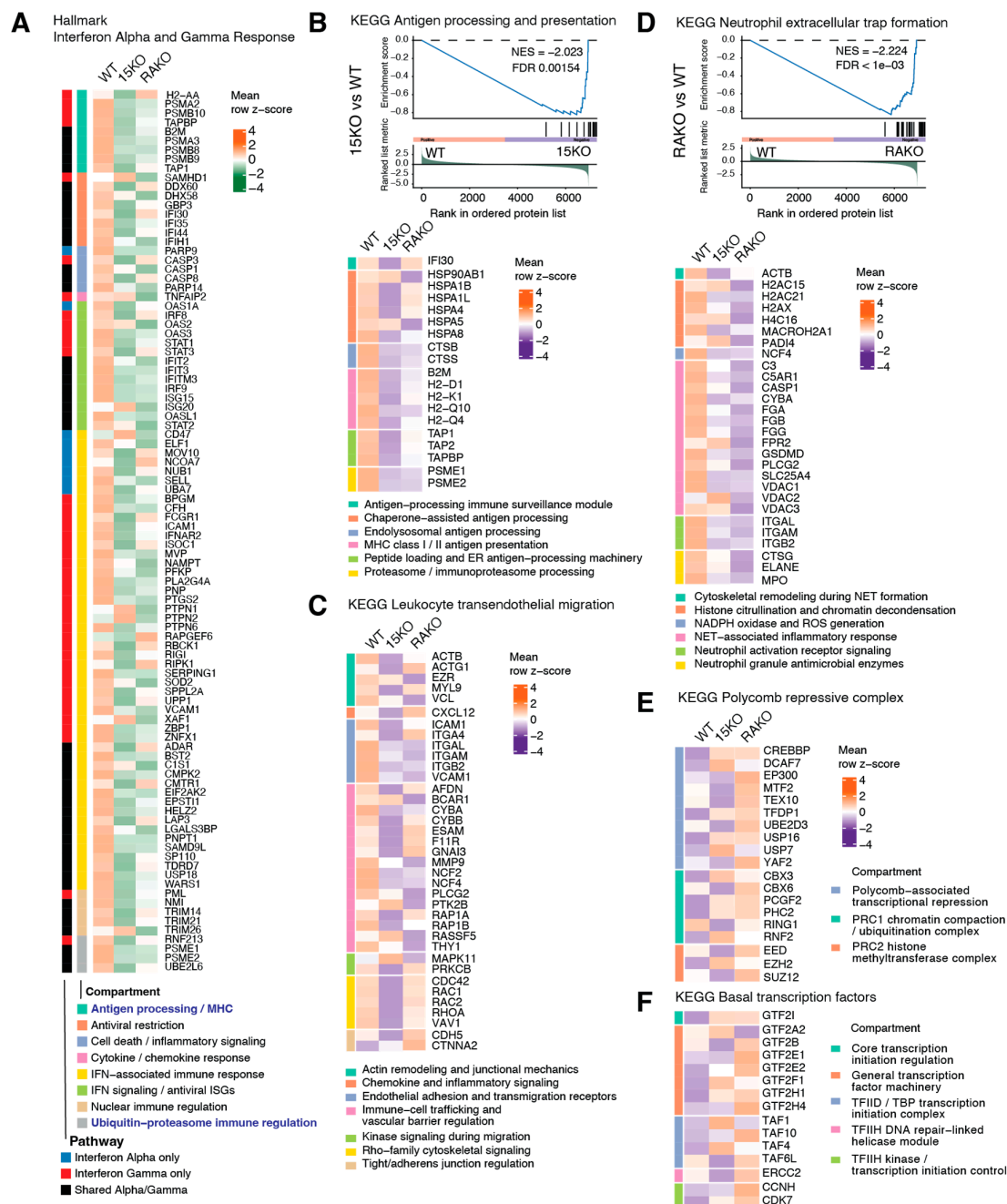
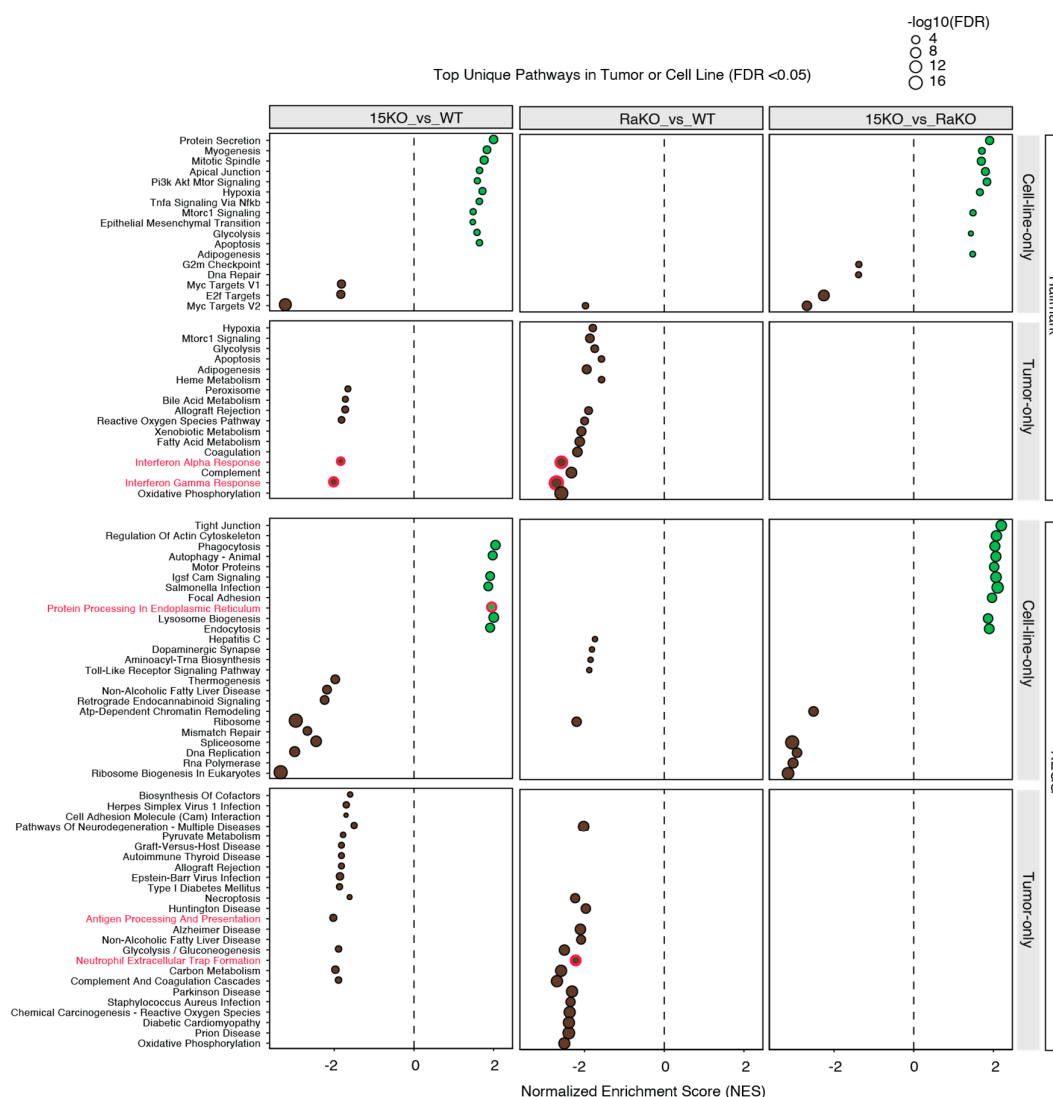


Figure 6. Heatmap analyses of immune-related differentially expressed proteins in MCA tumor tissues. (A) Interferon alpha and gamma responses in Hallmark GSEA; (B,C) antigen processing and presentation and Leukocyte trans-endothelial migration in KEGG GSEA; (D–F) neutrophil extracellular trap formation, polycomb repressive complex and Basal transcription factors in KEGG GSEA.

3.6. Comparison of the Proteomes of MCA-Induced Tumors and Tumor-Derived Cell Lines

The above proteomic analyses of the MCA-induced tumor tissues comprise pathways associated with the tumor cells, as well as the tumor microenvironment of the host, including the infiltrating immune cells. To dissociate the tumor cell-intrinsic pathways modulated by the lack of IL-15 or IL-15R α , we performed proteome analysis of MCA tumor-derived cell lines that grew in culture (Supplementary Figure S7). Hallmark and KEGG pathway analyses showed that processes associated with immune responses such as interferon signaling and antigen processing and presentation were not represented among the pathways modulated in MCA tumor-derived cell lines (Supplementary Figure S8). Comparison of the Hallmark and KEGG pathways modulated by IL-15 or IL-15R α deficiency in tumor tissues and in tumor-derived cell lines revealed several shared pathways (Supplementary Figure S9). However, downmodulation of interferon responses, antigen processing and presentation, and neutrophil extracellular trap (NET) formation were observed only in MCA tumors but not in cell lines (Figure 7). These findings indicated that IL-15 signaling within tumor cells, as well as the tumor microenvironment, modulates host immune responses, some of which are differentially impacted by IL-15R α -dependent regulation.



lines. (B) Tumor growth in WT, *Il15*^{−/−}, *Il15ra*^{−/−} and *Rag1*^{−/−} *Il15*^{−/−} mice following subcutaneous implantation of B16-NLRC5 cells. Statistics: Mean + SEM. Ordinary one-way ANOVA with Tukey's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.8. Loss of IL-15 or IL-15R α Cause Similar Paucity of Cytolytic Lymphoid Cells in TILs

Phenotypic analysis of B16-NLRC5 TILs isolated on day 18 post-implantation (using gating strategies shown in Supplementary Figure S10) showed a significant reduction in CD45⁺ cells in *Il15*^{−/−} mice but not in *Il15ra*^{−/−} mice, compared to WT hosts (Figure 9A). Among the innate lymphoid cell populations, NK, NKT and ILC1-like cells were almost completely absent in the TILs from *Il15*^{−/−} and *Il15ra*^{−/−} mice, whereas the latter hosts also showed significant reduction in $\gamma\delta$ T cells (Figure 9B–E). B16-NLRC5 TILs from *Il15ra*^{−/−} and *Il15*^{−/−} mice also showed significantly reduced numbers of CD4⁺ and CD8⁺ T cells when compared to WT mice (Figure 9F,K). Naïve CD4⁺ and CD8⁺ T cells were minimal in TILs but were higher in DLNs of all three genotypes (Figure 9G,L, Supplementary Figure S11). CD4⁺ T central memory (Cm; CD44⁺CD62L⁺) cells and CD4⁺ T effector (Eff; CD44⁺CD62L[−]) cells were reduced in the TILs of *Il15*^{−/−} and *Il15ra*^{−/−} mice compared to WT, but not statistically significant (Figure 9H,I). CD4⁺CD25⁺ cells, presumably Tregs, were enriched in TILs compared to DLNs but were comparable in all three genotypes (Figure 9J, Supplementary Figure S11). CD8⁺ T Cm cells and CD8⁺ T Eff cells were significantly reduced in TILs from *Il15ra*^{−/−} and *Il15*^{−/−} hosts when compared to WT TILs (Figure 9M,N). However, TILs of *Il15ra*^{−/−} mice had significantly more CD8⁺ T EFF cells than *Il15*^{−/−} mice. Eff CD8⁺ T of WT and *Il15ra*^{−/−} but not *Il15*^{−/−} TILs contained a higher proportion of PD-1⁺ CX3CR1⁺TIGIT⁺ cells representing exhausted phenotype, but the expression of CX3CR1 suggest that they may not be terminally exhausted and may still maintain effector phenotype (Figure 9O–Q) [48–50]. Collectively, despite comparable paucity of different cytolytic innate and adaptive lymphocyte subsets in *Il15ra*^{−/−} and *Il15*^{−/−} mice, *Il15ra*^{−/−} mice controlled B16-NLRC5 tumor growth more efficiently than *Il15*^{−/−} hosts (Figure 8B), indicating that IL-15R α is dispensable for IL-15-dependent control of tumors with competent antigen processing and presentation machinery.

3.9. Lack of IL-15 or IL-15R α Differentially Impact Myeloid Cell Subsets of TILs

Characterization of myeloid lineage cells in B16-NLRC5 TILs revealed a significant enrichment of neutrophils (Ly6G⁺Ly6C^{lo}CD11b⁺) in *Il15*^{−/−} and *Il15ra*^{−/−} mice compared to WT controls (Figure 10A). On the other hand, tumor-associated macrophages (TAMs: CD11b⁺F4/80⁺CD206⁺), monocyte-derived dendritic cells (MoDCs: CD11b⁺CD11c⁺), and CD11c⁺ DCs in TILs of *Il15*^{−/−} and *Il15ra*^{−/−} mice were comparable to WT mice (Figure 10C,D). The number of monocytes (CD11b⁺) was comparable in TILs of WT, *Il15*^{−/−} and *Il15ra*^{−/−} mice (Figure 10E–H). However, CD11c[−]CD11b⁺ immature monocytes that express Ly6C, MHC-II or CD103 were significantly reduced in TILs of both *Il15*^{−/−} and *Il15ra*^{−/−} mice (Figure 10I–L). These immature monocyte precursors, which are mobilized during inflammatory conditions, are considered pro-resolving macrophages [51]. These data suggest that IL-15R α restrains infiltration of neutrophils and TAMs, and promotes immature monocyte precursors into immunogenic tumors, which are likely to impact the outcome of IL-15-driven antitumor immune responses.

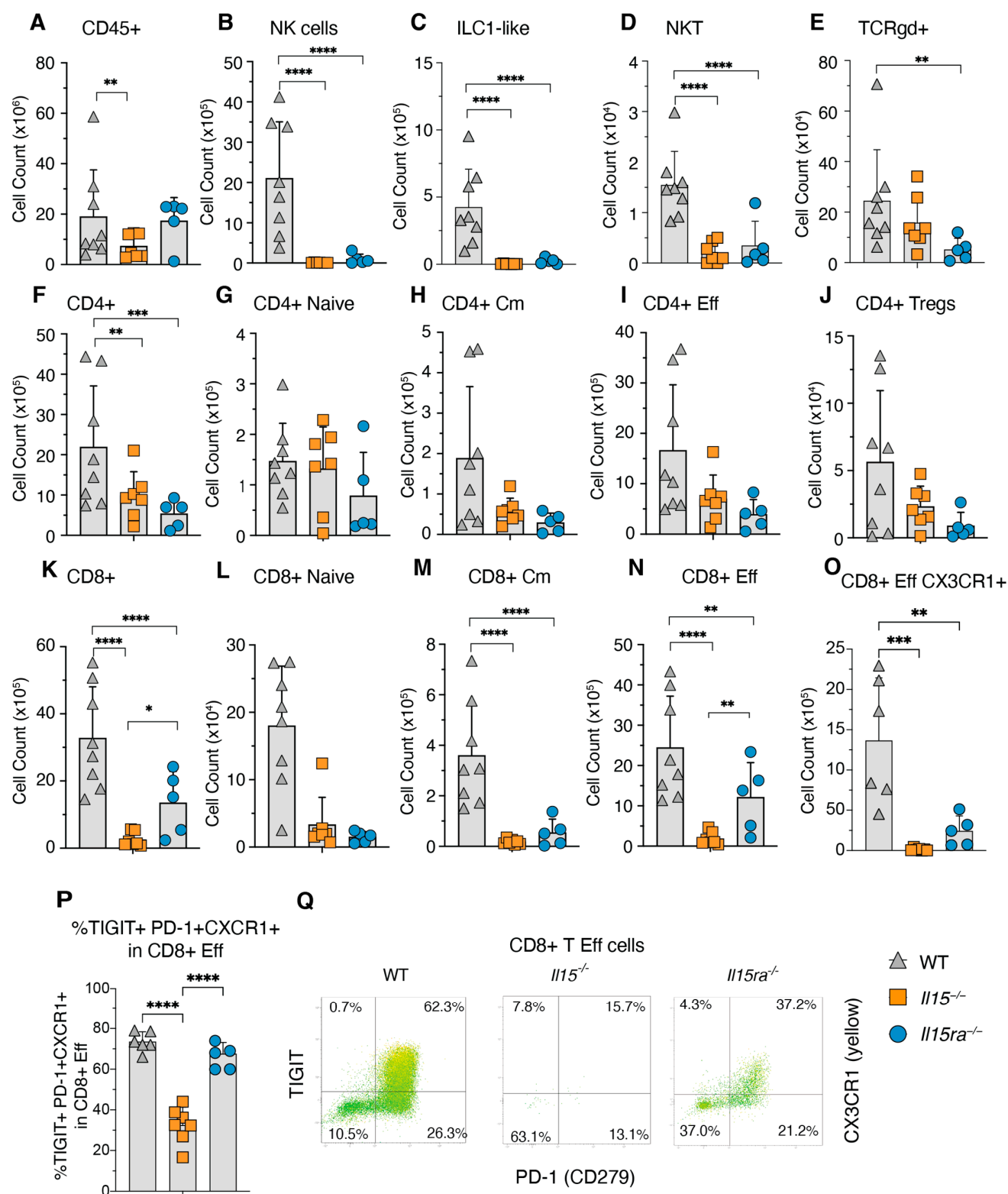


Figure 9. Lymphocyte subsets in TILs of B16-F10-NLRC5 tumors. Tumor-infiltrating lymphocytes (TILs) were isolated at the time of tumor collection from WT, $Il15^{-/-}$ and $Il15ra^{-/-}$ mice hosts implanted with B16-NLRC5 cells. Cells were counted and stained with fluorochrome-conjugated antibodies for lymphoid and myeloid cell markers and analyzed by flow cytometry using gating strategies depicted in Supplementary Figure S10. Data from 7 to 9 mice per group from three independent experiments are shown. The cell numbers in TILs were calculated from their percentages using

the gating strategy shown in Supplementary Figure S10 and were equalized to the weight of the tumor mass (TILs per gram; TILS(g⁻¹)). Absolute number of live (A) CD45⁺ cells, (B) NK cells, (C) ILC1-like cells, (D) NKT cells, (E) TCRγδ T cells, (F) total CD4⁺ T cells, (G) CD4⁺ naïve T cells, (H) CD4⁺ Cm T cells, (I) CD4⁺ Eff T cells, (J) CD4⁺ Treg-enriched cells, (K) total CD8⁺ T cells, (L) CD8⁺ naïve T cells, (M) CD8⁺ Cm T cells, (N) CD8⁺ Eff T cells, and (O) TIGIT⁺ Eff CD8⁺ T cells. (P) Proportion of CD8⁺PD-1⁺CX3CR1⁺TIGIT⁺ Eff T cells and (Q) dot plot of CD8⁺ Eff T cells for TIGIT, PD1, and CX3CR1 (yellow) of representative mice from each group. Phenotype markers of immune cell subsets are listed in Supplementary Table S4. Statistics: Mean + SD. Two-way ANOVA with Tukey's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Cm, central memory; Eff, effector T cells.

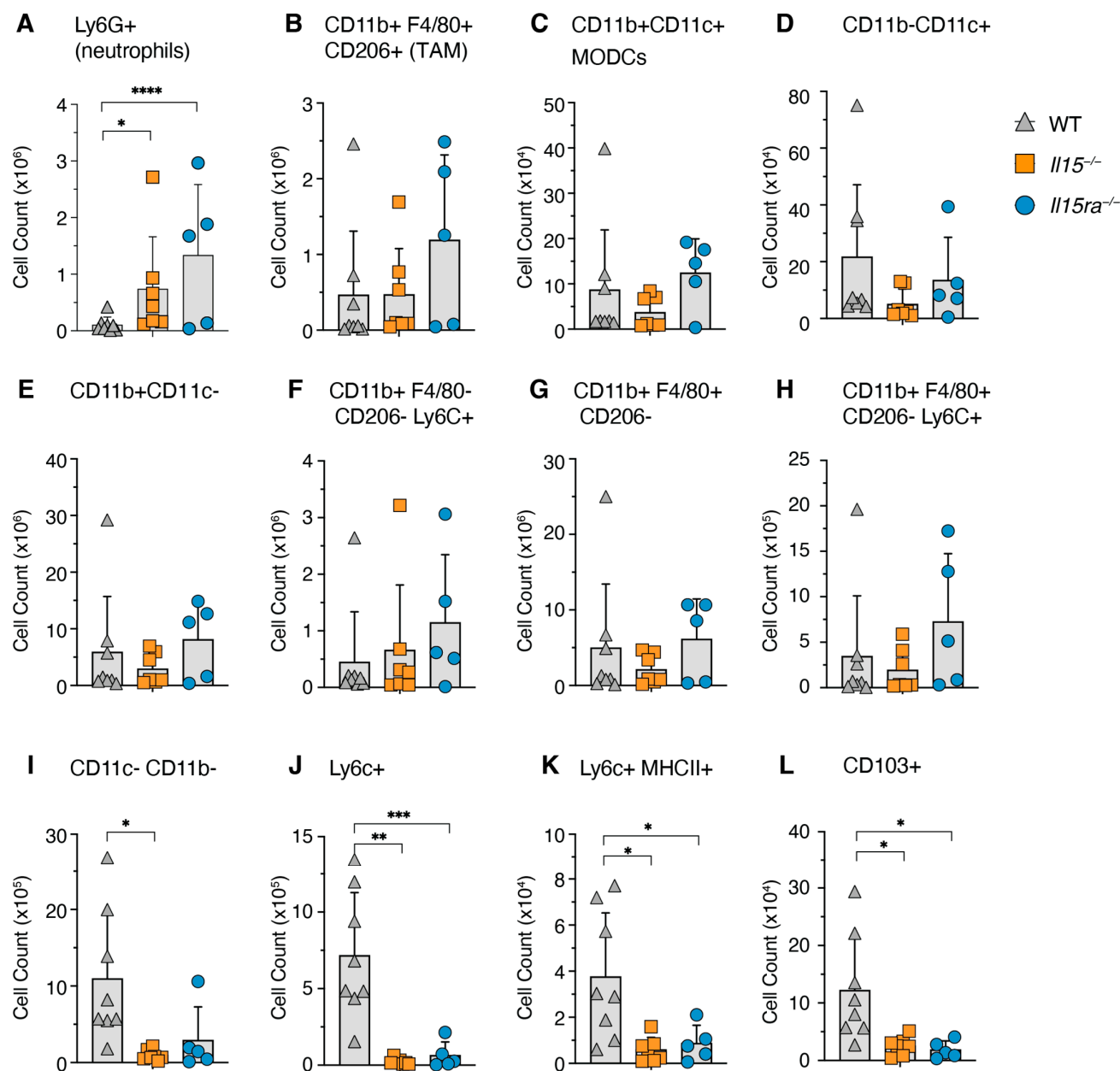


Figure 10. Myeloid subsets in TILs of B16-F10-NLRC5 tumors. Tumor-infiltrating lymphocytes (TILs) and single-cell suspensions from tumor-draining (DLN) and non-tumor-draining (NDLN) lymph nodes were isolated at the time of tumor collection from WT, *Il15*^{-/-} and *Il15ra*^{-/-} mice hosts implanted with B16-F10-NLRC5 cells. Cells were counted and stained with fluorochrome-

conjugated antibodies for lymphoid and myeloid cell markers and analyzed by flow cytometry using gating strategies depicted in Supplementary Figure S9. Data from 7 to 9 mice per group from three independent experiments are shown. The cell numbers in TILs were equalized to the weight of the tumor mass (TILs per gram; TILS(g⁻¹)). Absolute number of (A) Ly6G⁺ neutrophils, (B) TAMs, (C) MODCs, (D) CD11c⁺DCs, (E–H) monocytes expressing F4/80, CD206 and Ly6C or not, and (I–L) CD11c⁺CD11b⁺ immature monocytes expressing Ly6C, MHCII and CD103 or not. Phenotype markers of immune cell subsets are listed in Supplementary Table S4. Statistics: Mean + SD. One-way ANOVA with Tukey's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4. Discussion

Since the cloning of IL-15 a little more than 30 years ago, our collective understanding of the structure, functions and therapeutic applications of IL-15 has resulted in the approval of IL-15-derived biologics in cancer immunotherapy [52]. IL-15 is used in combination with other immunotherapeutic approaches to activate NK and CD8⁺T cells to control tumor growth. While our knowledge on the IL-15-mediated activation of NK and CD8⁺T cells is extensive, other aspects of IL-15 biology is not well understood. Most of the studies related to IL-15 and the development of IL-15 mimics for tumor immunotherapy have centered around the ability of IL-15 to activate ILC1, NK, $\gamma\delta$ T, NKT and CD8⁺T cells, all of which requires trans-presented IL-15. Tissue-specific deletion of IL-15R α revealed the requirement for localized trans-presentation by IL-15R α for the survival of tissue-resident IL-15-dependent lymphocyte subsets [53,54]. Thus, the availability of circulating IL-15 does not replace the membrane bound IL-15:IL-15R α in the maintenance of lymphoid populations in tissues. Additionally, IL-15 administration induces cytokine storm despite its short half-life [55]. On the other hand, soluble IL-15R α that is cleaved from the cell surface acts as an antagonist of IL-15 signaling as it competes with membrane bound IL-15R α for IL-15 [15]. Other than trans-presenting IL-15 to IL-15R β : γ_c , IL-15R α has no identified functions as it does not contain any signaling modules. Taking into consideration the various constraints associated with systemic toxicity and the complexity of IL-15 signaling, approaches to the development of anti-cancer immunotherapeutic agents have evolved over the past three decades, and the first biologic—N-803—has been approved for localized intravesicular administration as part of combination therapy with BCG for non-muscle invasive bladder cancer (NMIBC) in 2024 [52,56].

Various lines of evidence from the literature indicate that IL-15 signaling independent of IL-15R α -mediated trans-presentation plays important roles in controlling infections and autoimmunity [5,14,15]. IL-15 activates the innate immune system during infections and, hence, is indispensable for controlling infections, but we showed that trans-presented IL-15 was not essential to control *Listeria* and *Salmonella* infections [14]. In autoimmunity, IL-15-deficient mice are protected from autoimmune type 1 diabetes and certain forms of psoriasis [8,15], but not IL-15R α -deficient mice [5]. In psoriasis, absence of IL-15R α increased the disease severity, while administration of soluble IL-15R α reduced inflammation and improved the clinical status [15]. In contrast, in models of Th-17-mediated autoimmune diseases like EAE, the absence of IL-15 exacerbated the severity, as IL-15 can suppress Th17 development under certain circumstances [57,58]. Thus, in the above models, the mechanisms by which IL-15 and/or trans-presented IL-15 contribute to each of the above pathologies is distinct.

The role of IL-15R α -mediated trans-presentation in mediating IL-15-driven anti-tumor immune responses remains poorly understood. Comparable growth of three different established tumor cell lines in WT, IL-15-deficient, and IL-15R α -deficient mice indicates that the absence of IL-15 signaling directly, or through trans-presentation for the maintenance of IL-15-dependent immune subsets, does not impact tumor growth,

probably due to the aggressivity of the tumor cell lines. In these models, innate lymphocyte subsets play a minimal role in controlling the growth of solid tumors [29]. Constant surveillance by cytotoxic innate immune cells plays an important role in hematological malignancies and viral or oncogene-associated cancers, but not in MCA-tumor model [41,59]. Studies using depleting anti-NK1.1 and anti-NKG2D antibody to determine the relative contribution of innate lymphocyte subsets, such as NK, NKT, and $\gamma\delta$ T cells, and ILCs in immune surveillance of solid tumors, are not very clear as treatment will also deplete specific subsets of effector CD8⁺ T cells that express these markers [60–66]. Deficiency in Rag1, perforin, IFN γ , IFN γ R or trail that severely diminishes the cytotoxicity of CD8⁺ T cells compromises immunosurveillance, resulting in the early onset of MCA-induced tumors [37,67–70]. Our data show that the absence of IL-15 shows negligible impact on the incidence of MCA-induced tumors, indicating that IL-15 is dispensable for immunosurveillance of endogenously arising solid tumors. Nonetheless, the delayed tumor growth or the lack of tumor formation by MCA tumor-derived cell lines from *Il15*^{−/−} and *Il15ra*^{−/−} mice in all the WT recipients suggests that both IL-15 and IL-15R α contribute to tumor immunoediting, possibly from their shared positive impact on CD8⁺ T cell-mediated immune responses.

Strikingly, the reduced incidence of spontaneous MCA-induced tumors in *Il15ra*^{−/−} mice when compared to *Il15*^{−/−} or WT mice reiterates two possible non-mutually exclusive mechanisms: (a) IL-15R α restrains antitumor immune responses that may include both IL-15-driven and non-IL-15-mediated responses, and (b) the enhancement of IL-15R α -independent IL-15 signaling in the absence of IL-15R α -mediated trans-presentation. Previous studies have suggested a negative regulatory role for IL-15R α in controlling infections and autoimmune diseases, but the underlying mechanisms remain unclear [5,14,15]. Our data suggest a similar regulatory role for IL-15R α in tumor immunosurveillance. Our efforts to understand the regulatory functions of IL-15R α using proteomic approaches shed some light on the potential mechanisms involved. Notably, MCA-induced tumors from IL-15R α -deficient mice showed lower levels of proteins implicated in neutrophil net formation (NET), which can be a reflection of reduced NET-induced immune suppression, and hence better tumor responses [71]. In addition, pathways associated with chemical carcinogenesis-ROS was reduced in MCA-tumor proteomes from *Il15ra*^{−/−} mice when compared to WT and *Il15*^{−/−} mice. The high metabolic activity of tumor cells leads to increased ROS production that contributes to cancer progression through many pathways [72]. However, it will be simplistic to assume that these pathways that are differentially regulated are intrinsic to the tumors. Comparison of proteomes of MCA tumors and MCA tumor-derived cell lines shows that many of the changes in the immune-related pathways can be attributed to the host immune system, rather than to the tumors themselves. Thus, IL-15R α seems to impact tumor-associated signaling pathways that are both tumor-intrinsic and modulated by the tumor microenvironment.

Poor immunogenicity of tumors coupled with immunosuppressive tumor environment is a basic feature of tumors with poor prognosis. One of the approaches involves enhancing tumor antigen presentation by expressing the MHC-I trans-activator NLRC5 in tumors [26]. NLRC5-expressing B16-F10 tumors were well-controlled not only in WT mice as we have shown before [24], but also in IL-15R α -deficient mice. These results are surprising as TILs from both IL-15-deficient and IL-15R α -deficient mice had reduced numbers of NK, NKT, ILC1-like, and CD8⁺ T cells, including the different CD8⁺ T cell subsets. These observations open up the possibility that tumor-intrinsic mechanisms play a greater role in efficient antitumor immunity. This is further supported by our previous observations that mice that controlled NLRC5-expressing B16 tumors were able to reject challenges with the parental B16 cell line, possibly due to the expanded repertoire of tumor-derived peptides that are presented [24]. Thus, therapies that target tumors to

become immunogenic may be more effective than trying to target the immune system directly. This point of view may be intuitive as the tumors grow as close to being ‘self’ as possible that the immune system ignores the cold tumors. Breaking this tolerance needs the tumor to become foreign through a plethora of new antigens, and preferably accompanied by innate immune stimulation. Thus, mechanisms that expand the tumor-associated antigen repertoire, rather than targeting activation of specific subsets of immune cells alone, hold promise for developing more efficient antitumor immune therapies.

5. Conclusions

In this study, we dissected the role of IL-15 and IL-15R α in tumor initiation and progression. Our findings show that IL-15 plays a negligible role in immunosurveillance against MCA-induced fibrosarcoma development. However, tumor development was reduced in IL-15R α -deficient mice, suggesting a regulatory role for IL-15R α in restraining antitumor immunosurveillance mechanisms. The proteomes of MCA tumors from IL-15R α -deficient mice displayed reduced antigen presentation pathways. However, the growth of tumors expressing NLRC5, which enhances tumor antigen presentation to CD8 $^{+}$ T lymphocytes, was not impaired by IL-15R α deficiency, whereas IL-15 deficiency promoted tumor growth. As the loss of IL-15 or IL-15R α reduces NK and memory CD8 $^{+}$ T cells, our findings indicate that IL-15R α -independent IL-15 signaling is sufficient to control tumors with robust antigen presentation pathways.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers18152499/s1>, Figure S1: Immune cell infiltration in B16 tumors from WT, *Il15* $^{-/-}$ and *Il15ra* $^{-/-}$ mice; Figure S2: MCA dose-dependent development of fibrosarcomas; Figure S3: Experimental flow for testing the immunoedited status of MCA-induced tumors; Figure S4: Comparable growth of MCA tumor-derived cell lines in vitro; Figure S5: Quality control of mass spectrometry data on MCA tumor proteomes; Figure S6: Differential enrichment of IL-2-Stat5 signaling pathway proteins in WT, *Il15ra* $^{-/-}$ and *Il15* $^{-/-}$ tumors; Figure S7: Quality control of mass spectrometry data on proteomes of MCA tumor-derived cell lines. Figure S8: Differentially expressed pathways from proteome analyses of MCA tumor-derived cell lines. Figure S9: Comparison of differentially expressed pathways from proteome analyses of MCA tumors and MCA tumor-derived cell lines; Figure S10: Gating strategy for data shown in Figures 9 and 10; Figure S11: Distribution of select immune subsets detected in the draining and non-draining lymph nodes of B16-NLRC5 tumor bearing mice; Table S1: List of primers used for RT-qPCR; Table S2: List of antibodies used for flow cytometry on a Cytotflex 30 flow cytometer; Table S3: List of antibodies used for flow cytometry on a BD FACSDiscover™ S8 cell sorter; Table S4: Phenotype markers defining immune cell subsets; File S1: MCA tumors_Proteomics metadata; File S2: MCA tumors_Protein abundance Matrix; File S3: MCA tumor cell lines_Proteomics metadata; File S4: MCA tumor cell lines_Protein abundance Matrix.

Author Contributions: Conceptualization, S.R., S.I. and F.R.; methodology, F.R., S.A.A., A.S., M.M., S.K., E.D., A.A.C. and S.R.; formal analysis, F.R., S.A.A., M.M., S.K., J.-F.L., S.I. and S.R.; resources, H.A.-C., S.I. and S.R.; data curation, F.R., M.M., J.-F.L. and S.R.; writing—original draft preparation, F.R. and S.R.; writing—review and editing, F.R., M.M., J.-F.L., S.I. and S.R.; supervision, S.R. and S.I.; funding acquisition, S.R. and S.I. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board of Université de Sherbrooke (protocols #2021-3144 and 2026-5225, approved on 18 November 2021) for studies involving animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the metadata for proteomic analysis on tumor tissues and cell lines, as well as the protein abundance matrix for all replicates, are given in Supplementary Files S1–S4.

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Abbreviations

The following abbreviations are used in this manuscript:

NLRC5	NLR family CARD domain containing 5
Cm	Central memory
Eff	Effector memory
MCA	3-methylcholanthrene
NET	Neutrophil extracellular trap

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