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LETTER TO THE JOURNAL

Acquired RD3 loss regulates immune surveillance in high-risk and therapy defying progressive neuroblastoma

Neuroblastoma (NB) is the most common extra cranial solid tumor in children and comprises one tenth of all childhood cancer deaths. More than half of infants presented with NB, a designated "cold tumor" with low immune cell repertoire in the tumor microenvironment (TME) [1], develop progressive disease (PD). The low numbers of tumor infiltrating lymphocytes (TILs) and the limited anti-tumorigenic potential; low expression of major histocompatibility complex (MHC) class I molecules; limitations in the tumor suppressive immune cell infiltration in TME; and the presence of immune-suppressive cytokines are the critical reasons for poor prognosis (< 10% long term overall survival [OS]) in high-risk NB that contributes to about 10% of all childhood cancer deaths [2]. Immune cell components of both the innate and adaptive immune response recognize tumor specific antigens expressed on neoplastic cells and promote an immune response to eliminate cancer cells and to develop immune memory to prevent recurrence [2, 3]. However, these protective responses can take an impromptu turn in favor of tumor progression in immune-compromised individuals, and those tumors with lower immunogenicity [4]. This establishes cancer immune editing within the TME leading to acquired tumor immune evasion (TIME) that substantially contributes to cancer evolution and poor outcomes [2, 4, 5]. Hence, it is of great interest to unearth the drivers and the mechanisms that coordinate TIME, so as to develop effective therapeutic strategies for high-risk and for therapy defying progressive tumors. Our recent studies sequentially identified the availability and abundance of Retinal Degeneration protein 3 (RD3) in human adult and fetal tissues beyond retina [6, 7]; de novo loss of RD3 expression under therapy pressure; its predictive/prognostic relevance to NB clinical outcomes and; defined its novel NB evolution stabilization function [8, 9]. Assessing the function of RD3 in NB TIME (Figure 1), here we recognized the unique requirement for RD3 to maintain NB immune surveillance.

The immune microenvironment enclosed within the TME plays a discrete role in tumor immune surveillance. CIBERSORTx analysis ($P < 0.05$) employing "gene surrogate strategy" in whole genome RNA sequencing (RNA-seq) profiles from our bed-to-bench study identified 22 immune cell-types in NB-TME (Supplementary Figure S1). Differential gene expression analysis within CIBERSORTx [10] in RD3 reverse engineered (RD3-knockout) three unique models inflicted a "model-dependent" loss (vs. $RD3^+$) of naïve B cells, CD8-cells, naïve and memory resting CD4-T cells, follicular as well $\gamma\delta$ T-cells, resting and activated natural killer (NK) cells, M_0, M_1, and M_2 macrophages, resting and activated mast cells, eosinophils and, a "model-independent" loss (vs. $RD3^+$) of neutrophils (Supplementary Figure S2A). The decreased infiltration of these crucial immune cells that normally protect against tumor initiation and development suggests that RD3 negatively regulates TIME within the NB-TME. Identifying the mechanism(s) how RD3 regulates TIME in NB, the effector role of RD3, if any, on the 532 immune-related transcripts (42 of 574 CIBERSORTx identified relevant transcripts were excluded for their low copy number in sequencing) were investigated. Log_2 fold-change coupled with False Discovery Rate (FDR) computed from RNA-seq in three exclusive models identified a RD3-dependent, "model-independent" 27-gene signature (Supplementary Figure S2B-C; Supplementary Tables S1-S2): 8 downregulated, *LTB, SEC31B, MMP9, QPCT, NTN3, MYB, CD4*, and *STXBP6*; 19 upregulated, *ZNF222, HRH1, CYP27B1, PTGER2, NR4A3, CSF1, IL4R, CCL7, IL2RB, SMPDL3B, NOD2, MSC, PRF1, FOSB, CD27, BIRC3, NPL, ZNF442*, and *BFSP1*. RD3 regulated immune cell related transcriptome pertaining to immune surveillance, immune escape and inflammation combined with our documented evidence of

Abbreviations: CD, cluster of differentiation; Dx, diagnosis; FDR, false discovery rate; KO, knockout; MHC, major histocompatibility complex; NB, neuroblastoma; NK, natural killer cell; OS, overall survival; PD, progressive diseases; RD3, retinal degeneration protein 3; RNA-Seq, RNA Sequencing; TIME, tumor immune evasion; TME, tumor microenvironment.

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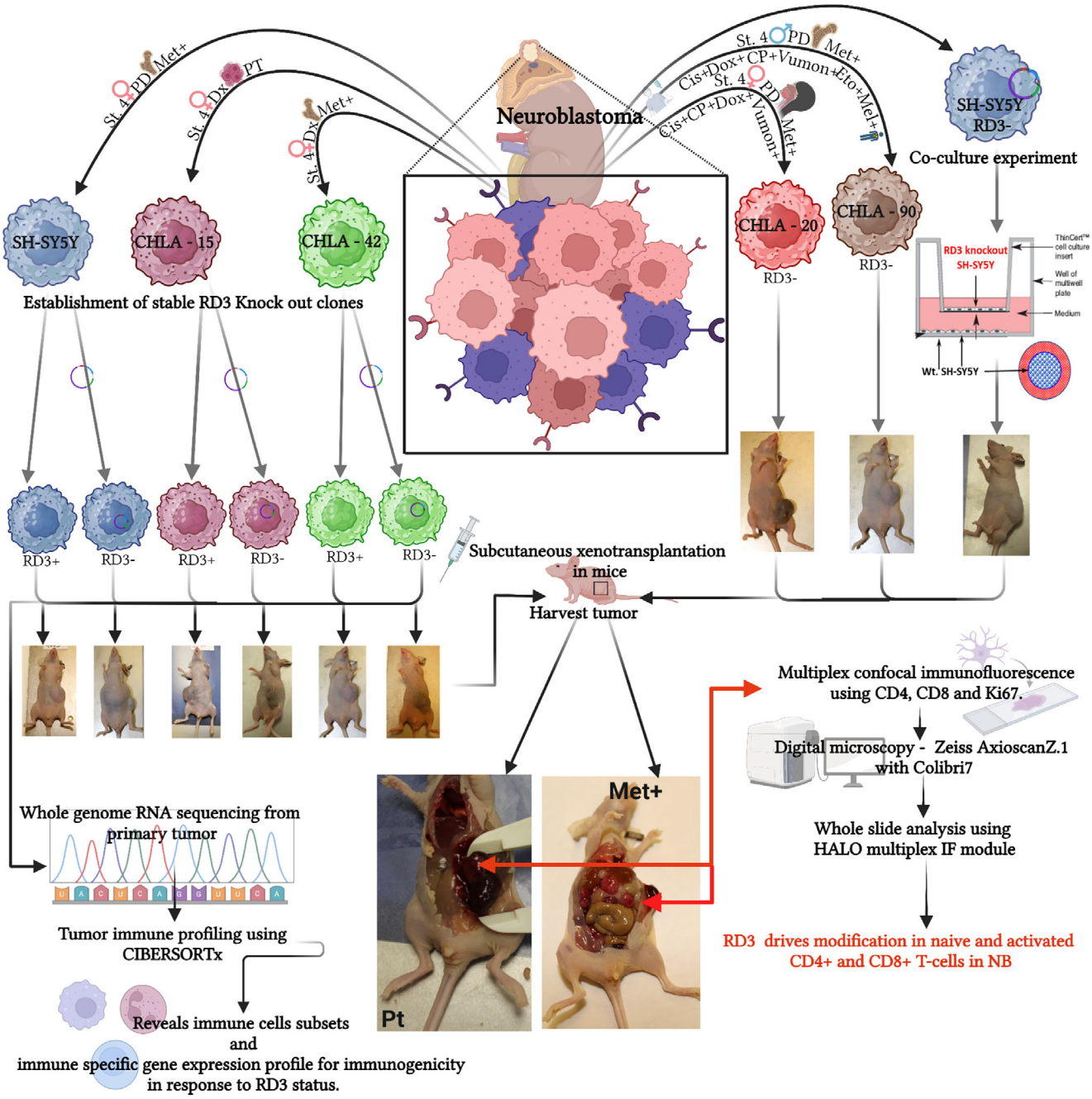

FIGURE 1 Defining the function of RD3 in the modification of immune surveillance in NB-TME. Experimental design incorporated a reverse engineering bed-to-bench approach constructing in vivo models with stage-4 patient-derived cell lines from primary and metastatic sites during Dx and/or PD that defied multimodal clinical therapy. In vivo models with established RD3 knockout clones were compared to the wild-type RD3 expressing and RD3 null PD models. Further RD3-loss driven modification in TIME was validated with in vivo models established with RD3 expressing clones subjected to co-culture communication with RD3-KO clones. Abbreviations: St.4, stage 4; Dx, diagnosis; PD, progressive disease; PT, primary tumor; Met, metastatic tumor; CD8, cluster of differentiation 8 (surface T-cell receptor on cytotoxic T cells); CD4, cluster of differentiation 4 (T cell receptor on helper T cells); Ki-67, marker of cell proliferation; Cis, cisplatin; Dox, doxorubicin; CP, carboplatin-paclitaxel; Eto, etoposide; NB, neuroblastoma.

de novo acquisition of RD3-loss with therapy pressure and RD3-loss orchestrates NB evolution [8, 9], portray not only the definitive contribution of RD3-loss in TIME but also the RD3-regulated genetic determinants that define TIME in NB.

Substantiating the biological function of RD3-loss dictated immune-related transcriptomic rearrangements, induced changes in the surveillance of naïve and activated $CD4^+$- and $CD8^+$-T cells were investigated. For this, nine unique NB in vivo

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models including SH-SY5Y-Primary-RD3$^+$, SH-SY5Y-RD3$^-$, CHLA-15-Primary-Dx-RD3$^+$, CHLA-15-RD3$^-$, CHLA-42-Primary-Dx-RD3$^+$, CHLA-42-RD3$^-$, CHLA-20-Primary-PD-RD3$^-$, CHLA-90-Primary-PD-RD3$^-$, and SH-SY5Y-Primary-RD3$^+$ co-cultured with SH-SY5Y-RD3$^-$ were used. The homing of immune cells within the NB-TME (multi-plex-IF, Zeiss imaging, and Halo analysis) were classified as resting (CD4$^-$CD8$^-$Ki67$^-$) and proliferating (CD4$^-$CD8$^-$Ki67$^+$) tumor, naïve (CD4$^+$CD8$^-$Ki67$^-$) and proliferating (CD4$^+$CD8$^-$Ki67$^+$) CD4-T cells, naïve (CD4$^-$CD8$^+$Ki67$^-$), and proliferating (CD4$^-$CD8$^+$Ki67$^+$) CD8$^+$-T cells (Supplementary Figure S3A). Across three models, muting RD3 (vs. RD3$^+$) significantly inhibited the surveillance of proliferating CD4$^+$- and CD8$^+$-T cells in both primary and metastatic NB (Supplementary Figure S3B-C). Since our findings indicated that acquired RD3-loss in select tumor cells could initiate bystander metabolic connections and dictate tumor evolution, we compared such response with SH-SY5Y-Primary-RD3$^+$ that was co-cultured with SH-SY5Y-RD3$^-$. NBs with RD3$^+$ bystander cells co-cultured with RD3$^-$ cells not only displayed a marked reduction in proliferating CD4$^+$- and CD8$^+$-T cells (vs. Primary-RD3$^+$ NB) but also mimicked NBs developed with RD3-KO cells (Supplementary Figure S3B-C). Crucially metastatic tumors across NB models displayed a consistent RD3-loss dependent reduction of proliferating CD4$^+$- and CD8$^+$-T cells. RD3-loss inhibited immune surveillance in primary and metastatic disease implies that RD3 impedes aggressive NB evolution by facilitating CD4$^+$- and CD8$^+$-T cells surveillance. Next, we assessed whether de novo acquisition of RD3-loss under therapy pressure impedes immune surveillance within NB-TME. Earlier, we have shown that de novo acquisition of RD3-loss with therapy pressure dictates NB evolution [6, 7]. Relative abundance of infiltrating active CD4$^+$- and CD8$^+$-T cells in NB developed with cells derived from stage-4 patients during Dx (RD3$^+$-CHLA-42, -SH-SY5Y) was compared to their matched RD3$^-$ tumors and in NBs established with cells derived during therapy-defying PD (RD3$^-$-CHLA-20, -CHLA-90). Compared to RD3$^+$ Dx NBs, a marked decrease in the CD4$^+$- and CD8$^+$-T cells surveillance in PD tumors and mimicked RD3-KO NBs (Supplementary Figure S3D-E). The outcomes exclusively recognize the requirement of RD3 for better immune surveillance and, intrinsic and/or therapy pressure-acquired RD3-loss regulates surveillance of activated CD4$^+$- and CD8$^+$- T cells in NB-TME. Comparing the infiltration of proliferating CD8$^+$- and CD4$^+$-T cells off primary and metastatic NB from RD3$^-$ models (RD3-KO CHLA-42, SH-SY5Y; RD3-null CHLA-90 and CHLA-20) to the primary NB of RD3$^+$ SH-SY5Y affirmed the RD3-loss dictated inhibition of immune surveillance (Supplementary Figure S3F-G). Intrinsic RD3-loss in select clones within NB dictating TIME and could contribute to the development of high-risk disease, while clinical therapy pressure driven de novo acquisition of RD3-loss deterred tumor immune cell surveillance could contribute to the evolution of PD.

Validating such RD3-dependent immune surveillance associated biological response, we assessed tumor growth (Supplementary Figure S3H). Proliferation index (percent Ki67$^+$ tumor cells) was significantly high in both primary and metastatic NBs across all RD3-knockout models when compared with their matched RD3$^+$ tumors. Consistently, therapy defying PD NBs mimicked a profound ($P < 0.01$) increase in the tumor cell proliferation (Supplementary Figure S3I). Exceptionally, these results corroborate the RD3-loss dependent regulation of immune surveillance in NB-TME and, causally indicate that RD3-regulated TIME may serve as a critical determinant of high- risk as well the progressive NB evolution.

Cancer and the immune system have a cause-and-effect relationship and the presence of immune cells in the TME indicates good prognosis (*4*). Conversely, tumors compromise the cytotoxic effects of the immune system, allowing tumor growth, dissemination and evolution, warranting targeted immunotherapy to improve clinical outcomes. With designated "cold-tumor" status, immunotherapies thus far used against NB have been futile. With our understanding on the significance of RD3 in NB evolution and prognosis, here we identified that RD3 determines immune cell type composition in the NB-TME; RD3 modify the transcriptome that prevents prognostic TIME; RD3-dependent 27-gene signature that could serve as a tool for predictive/mechanistic insight; RD3-loss impeded the homing of activated-CD4$^+$ and -CD8$^+$ T cells in NB-TME; therapy pressure driven de novo acquisition of RD3-loss in TIME that could contribute to the NB evolution; and RD3-loss dependent TIME corresponds with NB growth. Acknowledging the limitations (e.g., requirement of clinically translatable models with host immune response), studies are underway in such models aimed at enhancing the arsenal of immunotherapeutic interventions for this deadly cancer in infants. Overall, this study provides compelling evidence recognizing that RD3 stabilizes the intricate landscape of TIME in NB.

AUTHOR CONTRIBUTIONS

Natarajan Aravindan contributed to the conception and design of the experiments. Poorvi Subramanian, Sreenidhi Mohanvelu, Dinesh Babu Somasundaram, and Sheeja Aravindan performed the experiments and contributed to the acquisition of the data. Poorvi Subramanian, Sreenidhi Mohanvelu, Sheeja Aravindan, and Natarajan Aravindan contributed to data analysis and interpretation of the data. Poorvi Subramanian, Natarajan Aravindan drafted

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the manuscript, and Sheeja Aravindan, Dinesh Babu Somasundaram, Sreenidhi Mohanvelu helped in revising it critically. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors have nothing to disclose. No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal studies conformed to American Physiological Society standards and were approved by our Institutional Animal Care and Use Committee (IACUC) (Protocol #23-001-CHIX). All animal studies complied with institutional guidelines on handling laboratory animals as well as all appropriate state and federal regulations.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and as supplemental information.

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