

LILRB2 Promotes CAR T-cell Dysfunction and Represents a Therapeutic Target in Acute Myeloid Leukemia

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**LILRB2 Promotes CAR T-cell Dysfunction and Represents a Therapeutic Target
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LILRB2 and CAR T cells in Acute Myeloid Leukemia

Abstract

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy with poor outcomes. Although chimeric antigen receptor (CAR) T-cell therapies have shown major clinical benefit in other cancers, their efficacy in AML has been limited by the lack of targets that can be safely engaged without causing myeloablation, and by an immunosuppressive tumor microenvironment (TME). Leukocyte immunoglobulin-like receptor-B2 (LILRB2) has emerged as an immunosuppressive receptor, but its impact on CAR T-cell function and suitability as a therapeutic target remains unclear.

We found that LILRB2 is overexpressed in 40% of AML blasts, highly expressed on tumor-associated myeloid cells, and largely absent from healthy hematopoietic stem and progenitor cells. Using knockout and overexpression models, we showed that LILRB2 impairs anti-CD33 directed CAR T-cell killing, expansion, and cytokine production, and increases exhaustion, effects which are partially reversible by antibody-mediated LILRB2 blockade. Transcriptomic analyses revealed suppression of immune-related pathways and induction of T-cell senescence. Hence, we designed anti-LILRB2 CAR T cells, which showed potent cytotoxicity in vitro and robust antitumor activity in vivo, including tumor eradication in xenograft models without detectable toxicity in humanized mice.

Collectively, our results identify LILRB2 as a key regulator of CAR T-cell dysfunction and support LILRB2 targeting as a promising therapeutic strategy warranting further study in AML.

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Introduction

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by the uncontrolled proliferation of immature myeloid precursors with impaired differentiation and consecutive failure of healthy hematopoiesis (1). Despite significant advances in our understanding of AML biology and the approval of novel therapeutics, long-term survival remains poor, underscoring a critical need for more effective and durable therapies .

CAR T-cell therapies have reshaped the treatment of B- and plasma- cell malignancies. However, translating this success to AML has proven considerably more challenging, and clinical responses have remained limited. AML presents a unique set of biological obstacles, including its origin from HSPCs, which results in a high phenotypic similarity between leukemic and healthy hematopoietic cells. In AML, engineered T cells can induce remission, but responses are often not durable. This is largely due to antigen heterogeneity leading to immune escape, the need for consolidation strategies such as allogeneic hematopoietic stem cell transplantation (HSCT), and treatment-related toxicities including marrow aplasia (2). Importantly, emerging evidence indicates that engineered T cells in AML become dysfunctional even when they expand (3, 4). AML drives T cells into a short-lived, ineffective state, characterized by loss of persistence and skewing toward terminal or NK-like state, rather than durable anti-leukemic responses (5). The immunosuppressive TME, generated by AML cells, plays a central role in this process, and blockade of established immune checkpoints such as PD-1 or CTLA-4 has thus far yielded only modest clinical benefit, likely reflecting both the low mutational burden of AML and the involvement of additional immune-evasion pathways (6).

We and others have identified the inhibitory leukocyte immunoglobulin-like receptor B (LILRB) family as promising alternative checkpoints associated with the myeloid compartment and important mediators of immune suppression in AML. LILRBs are expressed on AML blasts and tumor-associated myeloid cells, where they likely contribute to leukemic cell maintenance and immune evasion (7-11).

While several approaches targeting LILRB family members have demonstrated promising activity in AML (12-16), their role in regulating CAR T-cell function remains unknown. In particular, LILRB2 remains underexplored despite its strong expression on AML blasts and leukemia initiating and maintaining cells (LIC) (17). We hypothesized that LILRB2 contributes to CAR T-cell dysfunction in AML and that targeting LILRB2 could enhance CAR T-cell efficacy without inducing hematologic toxicity. To test this, we pursued two complementary approaches: investigating the role of LILRB2 in modulating CAR T-cell function and developing anti-LILRB2 CAR T cells to simultaneously target AML cells and overcome microenvironment-mediated immunosuppression. We further provide evidence that LILRB2 CAR T cells may be used either as monotherapy or in combination with additional AML-directed CAR constructs, including our previously developed and validated non-viral anti-CD117 CAR T-cell platform (18).

Methods

Patient Samples

AML patient samples at disease onset were obtained from the biobank of the Department of Medical Oncology and Hematology at the University Hospital Zurich (Switzerland). Written informed consent was available from all donors, and ethical approval was received from the Cantonal Ethics Board Zurich (approval number: 2009-0062, Switzerland). The study cohort comprised 25 patients, including 16 men and 9 women, aged 29-82 years (mean age 58 years) at diagnosis. Additional patient characteristics are summarized in Table S1.

Generation of SB LILRB2.CAR Plasmid

LILRB2.EGFRt CAR vectors were generated using the pTMNDU3 plasmid as backbone (58). The construct incorporates a human EF-1-alpha short promoter, a LILRB2-targeting CAR

cassette (MK-4830, JTX-8064), and a huEGFRt sequence serving as both a selection marker and safety switch . The truncated human epidermal growth factor receptor (huEGFRt) sequence is linked to the CAR construct via a T2A self-cleaving peptide. The CAR construct consists of a light- and heavy-chain scFv derived from an anti-LILRB2 antibody, connected by a linker sequence and followed by a CD8 hinge- and transmembrane domain, a 41BB co-stimulatory domain, and a CD3ζ signaling domain. The CAR-EGFRt expression cassette was synthesized and inserted into the backbone plasmid by restriction enzyme cloning (GenScript, Piscataway, NJ, USA).

Statistical Analysis

Statistical analyses and graphical representations were done using GraphPad Prism (Version 10.3.1, GraphPad Software Inc., San Diego, CA, USA). Data is represented as mean ± standard deviation (SD). Statistical tests used for individual analyses are specified in the respective figure legends. Statistical significance was defined as follows: n.s. (non-significant) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Procedural flowcharts were designed with BioRender (Toronto, Canada).

Further methodological details are described in the Supplementary Methods.

Results

AML patient sample and healthy tissue expression profiling identify LILRB2 as a therapeutically relevant target

Members of the LILRB inhibitory receptor family have been identified as important myeloid immune checkpoints across multiple cancer types. To evaluate their suitability as CAR T-cell targets, we first characterized the expression patterns of LILRB family members in AML cell lines and primary patient samples. Nine AML cell lines were analyzed by flow cytometry for

LILRB1-4 expression, using healthy donor peripheral blood mononuclear cells (PBMCs) as control. LILRB1 exhibited broad expression across all cell lines, whereas LILRB2 and LILRB4 displayed more restricted expression patterns. LILRB3 did not show considerable expression under our conditions (Figure 1A). To assess expression in healthy immune subsets, we performed detailed analyses in healthy donor PBMCs. LILRB1 showed limited specificity, with expression on a substantial fraction of B cells, whereas LILRB2–4 expression was largely restricted to myeloid populations, predominantly monocytes and a subset of granulocytes showing lower levels (Figure S1A-C). Based on these findings, we next examined the expression of LILRB1, LILRB2, and LILRB4 in 23 primary AML patient samples at first diagnosis, in the context of established AML-associated antigens CD33, CLL1, CD123, CD117, and ADGRE2 (Table S1). LILRB2 was expressed on leukemic blasts (hCD45^{dim}) with a mean frequency of 39% across the patient cohort, with increased expression detectable in up to 50% of patient samples when assessed by median fluorescence intensity (MFI), whereas LILRB1 or LILRB4 expression was observed only in a minority of cases (Figure 1B-C, Figure S1D). Using an expression threshold of $\geq 20\%$, 65% of patients were classified as LILRB2-positive, highlighting substantial patient coverage. Importantly, LILRB2 positivity was associated with numerically reduced overall survival in this cohort, confirming its association with adverse clinical outcomes (Figure S1E, (7)). Analysis of AML-infiltrating immune populations revealed abundant expression of LILRB1, LILRB2, and LILRB4 within myeloid compartments, suggesting that targeting LILRB2 may simultaneously impact leukemic blasts and the immunosuppressive microenvironment (Figure 1B). In contrast, LILRB expression was largely absent from patient-derived T cells, indicating a low risk of fratricide by LILRB-directed CAR T cells. Importantly, healthy donor BM CD34⁺CD38⁻ hematopoietic stem and progenitor cells (HSPCs) expressed only minimal levels of LILRB family members, suggesting a favorable therapeutic window (Figure 1B). LILRB2 expression was further assessed in leukemic cells recovered from six independent AML patient-derived xenograft (PDX) models, in which it was detected in 30-85% of leukemic blasts, comparable to CD33 expression levels. Notably, LILRB2 and CD117 showed distinct expression patterns across samples, with high

expression of one marker often accompanied by low levels of the other, indicating complementary target potential to increase coverage of heterogeneous leukemic populations (Figure 1D). Finally, to evaluate potential off-tumor expression, we analyzed LILRB2 distribution across healthy tissues and cell types using the Tabula Sapiens atlas (19). As expected, LILRB2 expression was enriched in hematopoietic organs such as blood, BM, spleen, and liver, while also being present at considerable levels in the lung and skin. However, cell-type-resolved analysis demonstrated that LILRB2 expression was largely restricted to myeloid immune cells, particularly monocytes, macrophages, dendritic cells (DC), and neutrophils, with minimal expression in granulocytes and hematopoietic progenitor cells, and absent expression in hematopoietic stem cells (HSCs) (Figure 1E-F). Collectively, these findings identify LILRB2 as a therapeutically relevant target in AML, characterized by expression on leukemic blasts and tumor-infiltrating myeloid cells, limited expression on healthy tissues and hematopoietic stem cells, thereby minimizing potential on-target off-tumor toxicity.

LILRB2 expression by leukemia cells impairs CAR T-cell functionality in vitro and compromises tumor control in vivo

As LILRB2 represents a myeloid immune checkpoint candidate implicated in AML immune evasion, we sought to determine whether LILRB2 expression by leukemia cells directly contributes to CAR T-cell dysfunction. To this end, LILRB2^{KO} and LILRB2^{high} THP1 cell lines were generated (Figure 2A, S2A) and evaluated alongside THP1 wildtype (wt) in co-culture assays with anti-CD33 CAR or non-transfected (NT) T cells at different effector-to-target (E:T) ratios. After one day of co-culture, LILRB2^{high} target cells induced significantly lower TNF- α and IL-2 concentrations in the supernatant without affecting target-cell killing or CAR T-cell proliferation (Figure S2B-D). In contrast, prolonged exposure (5 days) revealed a marked functional divergence, with co-cultures containing LILRB2^{high} cells exhibiting significantly increased target-cell numbers and reduced effector-cell expansion, particularly at low E:T ratios (Figure 2B-C). Furthermore, IFN- γ abundance was reduced, while IL-2 levels were

increased, likely reflecting diminished IL-2 consumption secondary to reduced CAR T-cell proliferation (Figure 2D).

Repeated stimulation assays of anti-CD33 CAR T cells with THP1 target cell lines confirmed these findings (Figure S2E), showing that exposure to LILRB2^{high} targets progressively impaired CAR T-cell cytotoxicity, proliferation, and IFN- γ secretion (Figure S2F-H).

To determine whether these observations translate in vivo, we sub-lethally irradiated and intravenously (i.v.) transplanted NSG mice with 1×10^6 THP1 variants transduced to express green fluorescent protein (GFP) and luciferase. When all the mice had measurable signs of tumor engraftment, 1×10^6 CAR⁺ T cells or NT controls were injected (Figure 2E). Mice bearing LILRB2^{high} leukemia displayed significantly accelerated disease progression following CAR T-cell treatment, as demonstrated by the flux signal (Figure S3A-B), and reduced survival compared with mice engrafted with LILRB2^{KO} cells (Figure 2F). According to the mouse study license defined endpoint for well-being, or at pre-defined study termination on day 50, tumor burden in CAR T-cell treated mice was significantly increased across BM, peripheral blood (PB), and spleen in the LILRB2^{high} group, whereas near-complete leukemia control was achieved in most LILRB2^{KO}-engrafted mice (4 out of 5). Importantly, hCD3⁺ cells remained detectable in all treatment groups at endpoint (Figure 2G-H, S3C-F). Moreover, LILRB2 expression was maintained throughout the experiment irrespective of treatment condition (Figure S3G). Taken together, our data demonstrates that LILRB2 expression by AML cells directly impairs anti-CD33 CAR T-cell function and limits anti-tumor efficacy in vitro and in vivo.

Exposure to LILRB2^{high} leukemia cells induces a dysfunctional CAR T-cell state

To gain mechanistic insight into the reduced CAR T-cell functionality observed in response to LILRB2 expression, we performed complementary in vitro and in vivo analyses (Figure 3A). T-cell phenotypes were characterized following co-culture with THP1 LILRB2 expressing variants. While short-term exposure (1 day) did not alter T-cell differentiation or exhaustion

(Figure S4A-B), prolonged stimulation (5 days) induced a significant increase in terminally differentiated CAR T cells exposed to LILRB2^{high} targets (Figure 3B). This was accompanied by elevated expression levels of the exhaustion markers LAG-3 and PD-1 (Figure 3C). The increase in cell differentiation and exhaustion in CAR T cells exposed to LILRB2^{high} target cells was confirmed in repeated stimulation assays (Figure S4C-D). Given previous reports linking LILRB2 signaling to IL-10-mediated immunoregulation (11), we assessed cytokine production and observed significantly elevated IL-10 levels in co-cultures of either NT or CAR T cells with THP1 LILRB2^{high} cells compared to co-cultures with THP1 LILRB2^{KO} cells (Figure 3D). In vivo, CAR T cells recovered from the BM of THP1 LILRB2^{high} engrafted NSG mice treated with anti-CD33 CAR T cells similarly showed increased PD-1 expression and a trend towards elevated LAG-3 and TIM-3 expression, compared with T cells isolated from THP1 LILRB2^{KO} engrafted mice. This was accompanied by a more differentiated phenotype, although the difference did not reach statistical significance (Figure 3E-F). These findings suggest that LILRB2-expression impairs CAR T-cell function by promoting a dysfunctional, exhaustion-prone CAR T-cell phenotype.

LILRB2^{high} leukemia induces transcriptional programs related to immune suppression and CAR T-cell senescence

To further elucidate the mechanisms underlying impaired CAR T-cell function, we performed bulk RNA sequencing on leukemia and sorted effector cells following repeated stimulation of anti-CD33 CAR T cells with THP1 LILRB2^{KO} or THP1 LILRB2^{high} cell lines. Overrepresentation-analyses revealed significant downregulation of multiple immune response-related gene sets in both CAR T cells exposed to LILRB2^{high} leukemia cells and in the leukemia cells themselves (Figure 4A-B). In CAR T cells, these changes were accompanied by upregulation of genes associated with senescence, cell cycle arrest, and DNA damage responses, including CDKN1A (p21/CIP1), CDKN2A (p16INK4A), CDKN2B (p15INK4B), CHEK1/2, H2AX, and BRCA1/2 (Figure 4C-D). Together, these transcriptional changes

suggest activation of a DNA damage-associated senescence program as a potential mechanism underlying CAR T-cell dysfunction (Figure 4E). Consistent with this hypothesis, CAR T cells exposed to prolonged stimulation with LILRB2^{high} target cells exhibited elevated senescence-associated beta-galactosidase (SA-β-Gal) expression (Figure 4F). Importantly, these CAR T cells also displayed significantly reduced killing activity, as observed in our previous functional assays (Figure S4E). Taken together, these data support a model in which leukemia-expressed LILRB2 promotes a transcriptional program characterized by impaired effector function, exhaustion, DNA damage signaling, and premature senescence in CAR T cells.

Antibody-mediated LILRB2 blockade partially restores CAR T-cell function

To evaluate whether targeting LILRB2 could reverse the detrimental effects exerted on CAR T cells by LILRB2^{high} leukemia, THP1 LILRB2^{high} target cells were co-cultured with anti-CD33 CAR- or NT T cells in the presence or absence of two independent LILRB2-blocking antibodies. Treatment with either antagonistic antibody significantly increased effector-cell numbers in 1-day- co-cultures, without affecting PD-1, LAG-3 and TIM-3 expression as well as the memory phenotype (Figure 5A-D and S5A-D). Reduced target-cell numbers were observed in 5-day co-cultures across multiple E:T ratios, accompanied by decreased frequency of PD-1⁺ and terminally differentiated CAR T cells (Figure 5E-H). Comparable results were obtained using CAR T cells generated from an additional independent donor (Figure S5E-H). These results indicate that LILRB2-mediated CAR T-cell impairment is at least partially reversible and therefore therapeutically actionable.

LILRB2-directed CAR T cells exhibit potent and antigen-specific activity in vitro and in vivo

Based on the observed overexpression of LILRB2 on primary AML blasts and its role in immune evasion, we designed three anti-LILRB2 single-chain variable fragments (scFv) by combining the variable heavy (VH) and variable light (VL) chain sequences of LILRB2 targeting antibodies in either VLVH or VHVL orientations (C1 VHVL, C1 VLVH, C2 VLVH) and fused in frame to a CD8 hinge and transmembrane domain, a 4-1BB co-stimulatory domain, and a CD3 ζ signaling domain. The resulting CAR cassette, linked via a T2A peptide to the truncated human epidermal growth factor receptor (huEGFRt) as a marker to enable CAR T-cell tracking and potential elimination (20), was cloned into our Sleeping Beauty (SB) transposon vector backbone, which we demonstrated to enable clinically-suitable stable, safe and cost-effective non-viral gene transfer (Figure 6A, (21, 22)). CAR T cells were generated by electroporation of healthy donor-derived primary T cells followed by stimulation with anti-CD3/CD28 DynaBeads and IL-2 (Figure 6B). Importantly, CAR transfection, either detected as EGFR or direct CAR receptor staining using a biotinylated LILRB2 protein, did not significantly alter CD4/CD8 composition, T-cell differentiation status, or exhaustion profiles relative to NT controls (Figure S6A-D).

Among constructs tested, the C1 VLVH design showed superior cytotoxicity, specificity and cytokine production against THP1 variants (Figure S6 E-F), MOLM14 wt cells, which express low endogenous LILRB2 levels, and a MOLM14 cell line modified to overexpress LILRB2, GFP and luciferase (MOLM14 LILRB2^{high}, Figure 6C-E) and was selected for further studies. In vivo, anti-LILRB2 CAR T-cell treatment significantly reduced tumor burden as measured by bioluminescent imaging, and prolonged overall survival in NSG mice engrafted with MOLM14 LILRB2^{high} (Figure 6F-J). Analysis of BM, spleen, and PB at sacrifice demonstrated complete disease clearance with persistent CAR T-cell engraftment (Figure 6K-P). Importantly, the efficacy of anti-LILRB2 CAR T cells was comparable to that achieved with established anti-CD33 CAR T cells. Comparable antitumor activity was observed in THP1 LILRB2^{high} engrafted mice, while no activity was observed in THP1 LILRB2^{KO} models, confirming antigen specificity

(Figure S7A-D). These data established LILRB2 as a CAR T-cell target capable of mediating robust anti-leukemic activity in vitro and in vivo.

Anti-LILRB2 CAR T cells mediate antitumor activity against primary AML samples and support combinatorial strategies to overcome heterogeneity

Following the encouraging activity observed in AML cell lines, we next evaluated the efficacy of anti-LILRB2 CAR T cells against AML patient samples. Anti-LILRB2 CAR T cells mediated potent cytotoxicity against an AML PDX sample, with similar activity as anti-CD33 CAR T cells in vitro (Figure 7A), and significantly reduced the leukemic burden in vivo, compared with control NT T cells (Figure 7B-C). At study termination on day 30, blast counts were markedly reduced in the BM, spleen, and PB of treated mice, while hCD3+ cells remained detectable in both treated and control groups (Figure 7D-E).

To further evaluate the clinical applicability of LILRB2-directed CAR T cells, co-cultures with primary AML patient samples were performed. Combined targeting analyses using an OR-gating strategy in the previously analyzed cohort of 23 AML patient samples revealed that the expression patterns of LILRB2 and CD117 were largely non-overlapping, indicating complementary targeting of distinct leukemic compartments. Consequently, combining these targets substantially increased patient coverage. In contrast, the combination of LILRB2 and CD33 did not provide any additional increase in coverage (Figure 7F). Consistent with AML intra-patient heterogeneity, monotherapy responses varied across primary samples, where maximal overall killing achieved with individual CAR T-cell products was approximately 50% for anti-LILRB2 CAR T cells, 55% for anti-CD117 CAR T cells, and 70% for anti-CD33 CAR T cells. Importantly, combinatorial CAR T-cell approaches significantly improved overall leukemia clearance compared with single CAR constructs across all three primary patient samples analyzed (Figure 7G, S8A-E). Within target-positive subsets, all CAR T-cell constructs achieved highly efficient and selective target-cell elimination, with killing rates

approaching 90% (Figure 7H-K, S8F-H, S8J-L). Remarkably, anti-LILRB2 and anti-CD117 CAR T cells exhibited minimal overlap in target-cell recognition, with anti-LILRB2 CAR T cells showing very limited activity against CD117⁺ blasts and vice versa (Figure S8M).

These results demonstrate the ability of LILRB2-targeted CAR T cells to mediate robust anti-leukemic activity against AML PDX and primary patient samples and highlight combinatorial CAR T-cell targeting as a strategy to overcome AML heterogeneity and maximize therapeutic coverage.

Anti-LILRB2 CAR T cells exhibit limited hematopoietic toxicity

To evaluate potential on-target off-tumor toxicities of anti-LILRB2 CAR T-cell therapy, we co-cultured CAR T cells with healthy donor BM and PBMCs. Treatment resulted in moderate depletion of monocytes in both tissues, but spared B cells, T cells, NK cells, and granulocytes. Importantly, treatment did not affect CD34⁺CD38⁻ HSPCs (Figure 8A-B). Killing activity toward monocytes by anti-LILRB2 CAR T cells was comparable to the one observed with established anti-CD33 CAR T cells (Figure 8C, (4)). In hCD34⁺ HSPC humanized mice, anti-LILRB2 CAR T-cell infusion reduced LILRB2⁺ cells within the BM without affecting CD34⁺CD38⁻ HSPCs, CD33⁺ cells, or CD117⁺ populations, and without causing systemic toxicity (Figure 8D-E). Overall, these findings suggest a manageable on-target toxicity profile, with moderate monocytopenia as the primary predictive effect, while sparing other major hematopoietic populations, including healthy HSPCs, supporting the clinical feasibility of this approach.

Discussion

Despite considerable advances in understanding AML biology and the implementation of engineered cell therapies, their application has not translated into durable clinical responses as achieved in B- and plasma-cell malignancies (23). This is largely attributable to

mechanisms of immune evasion that promote premature T-cell dysfunction (24, 25), as well as extensive antigen heterogeneity and overlap between leukemic and healthy hematopoietic compartments, which limit the availability of safe and comprehensive antigen-targeting strategies. In this study, we addressed both challenges by demonstrating that LILRB2 acts as a mediator of AML-induced CAR T-cell dysfunction and as a potential target for CAR T-cell-based immunotherapy, sparing most healthy immune subsets, including healthy HSPCs.

Our expression analysis identified LILRB2 as the most promising candidate among the LILRB family members investigated. LILRB2 was overexpressed on leukemic blasts in a substantial proportion of primary AML samples, supporting its suitability as an AML-directed immunotherapy target. These findings are consistent with previous studies linking elevated LILRB2 expression to monocytic AML biology (8, 26). In addition, comprehensive transcriptomic and proteomic studies have identified LILRB2 among the most enriched AML-associated surface antigens and demonstrated expression within the LIC-enriched population (17). Although LIC expression was not directly assessed in our study, these observations are particularly relevant given the central role of LICs in disease propagation, therapeutic resistance, and relapse. Indeed, reduced expression of established AML targets such as CD33 on LICs has been proposed as one mechanism underlying treatment failure despite efficient elimination of bulk leukemic blasts (27). Compared with currently established AML targets, including CD33, CD123 and CD117, which are being evaluated in increasingly sophisticated CAR T-cell platforms such as adaptor-based or epitope-edited approaches to mitigate on-target hematopoietic toxicity (18, 27-31), we found LILRB2 expression to be largely absent from healthy HSPC-enriched CD34⁺CD38⁻ cells, suggesting a more favorable therapeutic window (17). Likewise, CD371-based approaches, including cytokine-armed CAR platforms, have demonstrated encouraging preclinical activity but remain limited by severe pancytopenia and chronic agranulocytosis due to high expression on healthy granulocytes, requiring HSCT to overcome off-tumor toxicities (3, 32). Unlike several emerging AML targets such as Siglec-6, LILRB2 appears not only to be an AML-associated surface antigen but also to contribute to

AML pathogenesis through the maintenance of leukemic stemness and activation of pro-survival signaling pathways (8), potentially reducing the likelihood of antigen-loss-mediated immune escape. While IL1RAP and FLT3 are expressed in LIC-enriched populations and contribute to AML progression, their expression in healthy HSPCs raises concerns about hematopoietic toxicity. CD70, despite encouraging preclinical activity and AML-supporting functions, is generally expressed at relatively low antigen density on AML cells, increasing reliance on highly sensitive CAR designs for efficient target recognition (33). Collectively, these features position LILRB2 among a small group of promising AML antigens that combine broad leukemic expression, limited expression on healthy HSPCs, and functional relevance to disease maintenance.

Interestingly, we also observed abundant LILRB2 expression on AML-associated myeloid populations. Together with the reported overexpression of LILRB2 on monocyte-derived suppressor cells (MDSCs) and M2-like macrophages, these findings suggest that LILRB2-targeted therapies may simultaneously eliminate leukemic cells while modulating the immunosuppressive TME (34). In cancer, activation of the LILRB2 pathway promotes MDSC- and M2-like polarization, impairs antigen presentation and T-cell priming, and facilitates T_{reg} induction (11, 35, 36). Consistent with this, high LILRB2 expression in AML was shown to correlate with inferior survival and a profoundly immunosuppressive microenvironment characterized by increased monocytic and M2-like macrophage infiltration and reduced abundance of effector lymphocytes (7). Early clinical studies of LILRB2-blocking antibodies have further demonstrated favorable safety profiles and preliminary anti-tumor activity in patients with advanced solid tumors (37, 38), although expression on normal myeloid cells underscores the need for careful evaluation of potential on-target off-tumor effects.

Overall, these observations suggest that LILRB2 may represent a promising component of future combinatorial immunotherapeutic strategies designed to overcome antigen heterogeneity, target the immunosuppressive TME, and preserve normal hematopoiesis.

Clinical adoptive T-cell therapies in AML have thus far demonstrated limited and often non-durable anti-leukemic efficacy. While prophylactic WT1 TCR-transgenic T-cell therapy after HSCT achieved durable disease control, administration of the same product after AML relapse failed to improve survival and was associated with pronounced terminal differentiation of transferred T cells, suggesting AML-mediated T-cell dysfunction through largely unknown mechanisms (5, 39). Our findings identify LILRB2 as a potential contributor to this phenomenon and further support the concept that AML actively shapes an immunosuppressive TME capable of impairing therapeutic T-cell products.

Mechanistically, LILRB2 expression on AML cells impaired CAR T-cell functionality predominantly during prolonged antigen exposure, consistent with progressive loss of T-cell fitness rather than immediate inhibition of cytotoxicity. Through ITIM-dependent recruitment of SHP phosphatases (40, 41), LILRB signaling contributes to physiological immune tolerance, including maternal-fetal tolerance and protection against autoimmunity (42-44). Malignant cells may hijack this pathway to evade anti-tumor immunity, analogous to mechanisms employed by certain pathogens (40, 45-47). Consistent with this concept, LILRB4-positive AML cells suppress T-cell proliferation via a SHP2-uPAR-ARG1-dependent signaling pathway (9), while LILRB3 impairs T-cell-mediated leukemic control by activating cFLIP and NF- κ B and by promoting metabolic reprogramming (10, 15). Likewise, in our study, CAR T cells exposed to LILRB2^{high} AML acquired a more terminally differentiated and exhausted state. Given the well-established association between T-cell differentiation and CAR T-cell efficacy (48), these changes likely contribute to the impaired antitumor activity observed in our models (49). Increased IL-10 production further aligns with previous studies (15, 50, 51) and supports the induction of an immunosuppressive program by LILRB2-expressing AML.

The transcriptional profiling revealed signatures of T-cell dysfunction, DNA damage response, and senescence following exposure to LILRB2^{high} AML cells. Emerging evidence links AML to metabolic reprogramming, including fatty acid accumulation, that promotes T-cell dysfunction, and therapy-resistant LIC populations (52). LILRB2 and its mouse homolog PIRB have

likewise been implicated in fatty acid synthesis, leading to T-cell senescence potentially through cAMP-mediated signaling (53). Together with the known suppressive effects of extracellular adenosine on CAR T cells (54), these observations suggest that LILRB2 contributes to the progressive loss of CAR T-cell fitness through immunoregulatory and metabolic pathways. While further studies are required to establish causality, our findings identify LILRB2 as a previously unrecognized mediator of impaired T cell function in AML and support a model in which LILRB2 promotes progressive CAR T-cell dysfunction associated with T-cell senescence.

Importantly, antibody-mediated LILRB2 blockade partially restored CAR T-cell function by reducing exhaustion and terminal differentiation. Although LILRB-blocking antibodies (9, 10, 12) have shown modest clinical activity in AML (55), their combination with MHC-unrestricted CAR T-cell therapies or T-cell-activating bispecific antibodies may overcome the limited endogenous antileukemic immunity characteristic of AML. Interestingly, LILRB2-targeted CAR T cells incorporating an inhibitory antibody-derived scFv exhibited reduced SA- β -Gal levels compared with anti-CD33 CAR T cells. Whether this reflects concomitant modulation of LILRB2 signaling in addition to direct cytotoxicity warrants further investigation.

Engineered T-cell approaches targeting other members of the LILRB family have previously demonstrated promising preclinical activity (14-16, 56). Here, we show that newly designed anti-LILRB2 CAR T cells exhibit potent antileukemic activity across multiple AML models, including primary patient samples, both in vitro and in vivo. Benchmarking against conventional anti-CD33 CAR T cells demonstrated comparable efficacy. Importantly, anti-LILRB2 CAR T cells exhibited limited toxicity toward healthy hematopoietic populations, preserving HSPCs, despite expected targeting of mature myeloid populations.

Given the marked antigen heterogeneity in AML, combinatorial targeting strategies will likely be required to maximize blast coverage and prevent the outgrowth of antigen-negative subclones (31, 57). Consistent with this concept, Perna et al. identified the LILRB2/CLL1 combination among the most promising target pairs for AML (17). Similarly, dual-targeting

approaches in our study significantly enhanced the elimination of primary AML samples, with the LILRB2/CD117 combination exhibiting particularly complementary expression patterns, suggesting improved coverage of distinct leukemic subpopulations. In this context, CD117-directed therapies are currently being translated into the clinic as a strategy that combines leukemia eradication with non-genotoxic conditioning for HSCT (18). Our findings support incorporating LILRB2 targeting into this therapeutic concept to improve coverage of heterogeneous AML while preserving normal hematopoiesis.

In summary, our study identifies LILRB2 as both a mediator of CAR T-cell dysfunction and a promising therapeutic target in AML. Further studies should define the intracellular signaling pathways downstream of LILRB2 in AML cells, identify the mediators of leukemia-T-cell crosstalk responsible for T-cell dysfunction, and evaluate, in vivo, combinatorial strategies, including CD117/LILRB2-targeted approaches.

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Author contributions

S.B designed and performed all experiments, collected and analyzed the data, prepared the figures, and wrote the manuscript; M.C, N.R, M.P, S.A, L.R, and C.P. helped in performing experiments; J.D. helped in analyzing and interpreting patient data; A.B. and J.M.E provided

a patient-derived xenograft model; M.G.M. provided support in study conceptualization; C.F.M. conceptualized the study, designed the experiments, supervised the study, obtained financial support, and wrote the manuscript. All authors reviewed and approved the final manuscript.

Competing Interest

CFM is an inventor on a patent related to non-viral CAR T-cell therapy (European patent application 15801344), filed by the Tettamanti Foundation, and serves as a mentor, Roche Postdoctoral Fellowship Program (Roche Glycart AG), and a reviewer, Berlin Institute of Health (BIH) SPARK Gene and Cell Therapies (GCT). The remaining authors declare no competing interests.

Data Availability Statement

Data generated in this study are included in the article or uploaded as supplementary information. The transcriptomic datasets generated during the current study have been deposited in GEO repository, accession GSE343739 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE343739>), and will be made publicly available upon publication. During peer review, these materials are available to the editors and reviewers via a private access link provided in the cover letter.

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Figure legends

Figure 1: LILRB2 is highly expressed in adult AML patient samples and myeloid cells while being absent in other healthy cell types and tissues. (A) Histograms showing the fluorescent intensity of indicated LILRB subtypes on cells analyzed by flow cytometry. Shown are different AML cell lines (MOLM13, MOLM14, THP1, HL60, KG1, TF1, OCI-AML3, NOMO1, MARIMO) as well as healthy donor PBMCs as control. (B) Flow cytometry analysis depicting the different LILRB subtypes (left) and AML-associated markers (right) in healthy donor PBMCs (n=4), HDBM (n=4), primary patient AML blasts (n=23), AML-associated T cells and CD33+ myeloid cells (n=23). Every dot represents an individual sample. (C) Histograms showing LILRB expression in three different patient samples analyzed by flow cytometry. Healthy donor PBMCs are shown as control. (D) LILRB2, CD33 and CD117 expression of different patient-derived xenograft samples (n=6) as measured by flow cytometry. (E+F) Percentage of LILRB2 positive cells across depicted compartments (E) and immune cell subtypes (F) of healthy donors. Data are taken from the Tabula Sapiens database. Statistical analysis was performed via two-way ANOVA with Tukey's multiple comparison (B); ****p < 0.0001.

Figure 2: LILRB2 expression by AML impairs CAR T-cell functionality in vitro and in vivo. (A) Number of LILRB2 molecules on THP1 LILRB2 KO and LILRB2 high AML cells, compared to wt, measured by quantitative flow cytometry. The NALM6 B-ALL cell line is shown as control. (B+C) Flow cytometric analysis after a 5-day co-culture of anti-CD33 CAR- or NT T cells with THP1 wt, THP1 LILRB2 KO or THP1 LILRB2 high cells. Shown are target cell counts (B) and effector fold changes (C) at different effector-to-target (E:T) ratios. Data illustrate the means $\pm$ SD from four biological replicates with two technical replicates. (D) Analysis of cytokines in the supernatant of aforementioned co-cultures (E:T ratio 1:15) after 5 days, measured by ELISA. Mean of three biological replicates with two technical replicates $\pm$ SD. (E) NSG mice were sub-lethally irradiated and injected i.v. with 1×10^6 THP1 wt, LILRB2 KO or LILRB2 high cells. After 9 days, mice were treated with 1×10^6 anti-CD33 CAR+ T cells i.v. NT T-cell injection served

as control. (F) Kaplan-Meier survival curves of the depicted treatment groups. (G+H) Total count of target (G) and hCD3+ (H) cells in the BM at humane endpoint or at the end of the experiment (day 50), measured by flow cytometry. Every dot represents a mouse (n=3-5). Data are represented as mean $\pm$ SD. Statistical analysis was performed via one-way ANOVA (D+G+H) or two-way ANOVA with Tukey's multiple comparison (B+C) or via Mantel-Cox test (F); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 3: LILRB2 expression in AML enhances CAR T-cell differentiation and exhaustion. (A) Graphical representation of experiments performed for Figure 3 and 4. (B+C) Flow cytometric analysis after a 5-day co-culture of anti-CD33 CAR T cells with THP1 wt, THP1 LILRB2 KO or THP1 LILRB2 high cells. Shown are (B) the percentage of naïve and stem cell memory ($T_N + T_{SCM}$), central memory (T_{CM}), effector memory (T_{EM}) and terminal effector (T_{TE}) hCD3+ cells, defined by hCD45RA and hCD62L expression, and (C) the percentage of hCD3+ cells expressing PD-1, LAG-3 and TIM-3. Data illustrates the mean $\pm$ SD across four biological replicates with two technical replicates. (D) Analysis of IL-10 in the supernatant of aforementioned co-cultures after 5 days (E:T ratio 1:15), measured by ELISA. Mean of three biological replicates with two technical replicates each. (E+F) NSG mice were sub-lethally irradiated and injected i.v. with 1×10^6 THP1 wt, LILRB2 KO or LILRB2 high cells. After 9 days, mice were treated with 1×10^6 anti-CD33 CAR+ T cells i.v. NT cell injection served as control. Flow cytometry analysis of BM samples harvested at humane endpoint or end of the experiment (day 50). (E) Percentage of naïve and stem cell memory ($T_N + T_{SCM}$), central memory (T_{CM}), effector memory (T_{EM}) and terminal effector (T_{TE}) hCD3+ cells in anti-CD33 CAR T-cell-treated mice bearing LILRB2 wt, KO, and high tumor cells, as defined by hCD45RA and hCD62L expression. (F) Percentage of PD-1, LAG-3, and TIM-3 expression on hCD3+ cells in the BM of anti-CD33 CAR T-cell treated mice (n=4-5). Statistical analysis was performed via one-way ANOVA (D) or via two-way ANOVA (B+C, E+F) with Tukey's multiple comparison; *p<0.05; ***p<0.001.

694

695 Figure 4: LILRB2 expression in AML leads to downregulation of gene sets linked to immune
696 response in both CAR T and target cells and increases CAR T-cell DNA damage response
697 and senescence pathways. (A-D) RNA sequencing data of effector and target cells after re-
698 challenging anti-CD33 CAR T cells four times with THP1 LILRB2 KO or THP1 LILRB2 high
699 cell lines at an E:T ratio of 1:2.5 and subsequent cell purification by FACS. Re-challenge was
700 performed in technical triplicates. Top 10 downregulated biological process-related gene sets
701 in bulk effector (A) or target (B) cells as analyzed by an over-representation analysis. Shown
702 is the change of gene expression in LILRB2 high compared to LILRB2 KO conditions. Color
703 indicates p-value while the x-axis shows the total count of downregulated genes per gene set.
704 (C) Gene ontology (GO) tile plots of significantly dysregulated genes associated with cellular
705 senescence (left, GO:0090398), replicative senescence (middle, GO:0090399) and positive
706 regulation of cellular senescence (right, GO:2000774) in CAR T cells. (D) Significantly
707 dysregulated genes associated with DNA damage response pathways. (E) Proposed
708 mechanism of combined downregulation of immune response-related gene sets, DNA
709 damage induction, and senescence initiation. (F) Presence of β -Gal in CAR T cells after a 5-
710 day co-culture of anti-CD33 (left) CAR- or anti-LILRB2 (right) CAR T cells with THP1 wt, THP1
711 LILRB2 KO or THP1 LILRB2 high cells as measured by flow cytometry. Data illustrate the
712 mean $\pm$ SD of two technical replicates. Statistical analysis was performed via kallisto / edgeR
713 (A-D) or two-way ANOVA with Tukey's multiple comparison (F); *p<0.05; **p<0.01;
714 ***p<0.001; ****p<0.0001.

715

716 Figure 5: Antibody-mediated LILRB2 blockade partially reverses impaired CAR T-cell
717 functionality in vitro. Flow cytometric analysis of effector and target cells after a 1-day (A-D) or
718 5-day (E-H) co-culture of anti-CD33 CAR- or NT T cells with THP1 LILRB2 high cell lines
719 together with LILRB2 blocking antibodies or isotype control (IgG). The 1-day co-culture was
720 performed at an effector-to-target (E:T) ratio of 1:1, and the 5-day co-culture at the E:T ratios

indicated in the graphs. Target (A) and effector (B) cell counts after 1-day co-culture. (C) Percentage of hCD3+ cells expressing PD-1, LAG-3, and TIM-3 in anti-CD33 CAR T-cell conditions treated either with IgG or LILRB2 blocking antibodies during co-culture, as indicated on the Y-axis. (D) Percentage of naïve and stem cell memory (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminal effector (T_{TE}) hCD3+ cells, as defined by hCD45RA and hCD62L expression, in the conditions indicated on the X-axis. Target (E) and effector (F) cell counts at indicated E:T ratios after 5-day co-culture. (G) Percentage of hCD3+ cells expressing PD-1, LAG-3 and TIM-3 in anti-CD33 CAR T-cell conditions at the indicated E:T ratios. (H) Percentage of naïve and stem cell memory (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminal effector (T_{TE}) hCD3+ cells, as defined by hCD45RA and hCD62L expression, in anti-CD33 CAR T-cell conditions at indicated E:T ratios. The data illustrate one representative donor with two technical replicates $\pm$ SD. Statistical analysis was performed via one-way ANOVA (A-D) or via two-way ANOVA (E-H) with Tukey's multiple comparison; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 6: Anti-LILRB2 CAR T cells show potent effector function against AML cell lines in vitro and in vivo. (A) Schematic representation of the anti-LILRB2 CAR cassette, controlled by a human PGK promoter and linked to a truncated human EGFR (huEGFRt) marker via a T2A peptide. The entire cassette is flanked by the SB-pT left and right inverted repeats (LIR/RIR). (B) Representation of the SB-based CAR T-cell production protocol (C) Number of LILRB2 molecules on the AML cell line MOLM14 modified to overexpress (OE) LILRB2, measured by quantitative flow cytometry. (D) Killing activity of T cells engineered to express different anti-LILRB2 CAR constructs (C1 VHV, C1 VLVH, C2 VLVH) after a 1-day co-culture with MOLM14 wt or MOLM14 LILRB2 high cells at the indicated E:T ratios. Analysis was performed by flow cytometry and NT and anti-CD33 CAR T cells served as negative or positive control respectively. Data illustrates the mean $\pm$ SD across three biological replicates and two technical replicates. (E) Analysis of cytokines in the supernatant of anti-LILRB2 C1 VLVH CAR

construct from the aforementioned co-culture at an E:T ratio of 1:1, measured by ELISA. Mean of three biological replicates with two technical replicates each $\pm$ SD. (F) NSG mice were sub-lethally irradiated and injected with 1×10^5 MOLM14 LILRB2 high cells. After 10 days, mice were treated with 2.5×10^6 anti-LILRB2 CAR+ T cells or controls. (G) Total flux (photons / second) of depicted treatment groups at different timepoints, as measured by IVIS. (H) Kaplan-Meier survival curves of depicted treatment groups. (J) IVIS images of mice receiving the indicated treatments. (K-P) Total count of target cells in BM (K), Spleen (M), or PB (O) or hCD3+CAR+ cells in BM (L), Spleen (N), or PB (P) at humane endpoint or end of the experiment (day 28), measured by flow cytometry. Every dot represents a mouse (n=2-4). Data are represented as mean $\pm$ SD. Statistical analysis was performed via one-way ANOVA (E, K-P) or two-way ANOVA with Tukey's multiple comparison (D, G), or via Mantel-Cox test (H); *p<0.05; **p<0.01; ***p<0.001 ****p<0.0001.

Figure 7: Anti-LILRB2 CAR T cells show potent effector functions towards patient samples and patient-derived xenografts in vitro and in vivo. (A) Killing activity of anti-LILRB2 CAR T cells after a 1-day co-culture with a patient-derived xenograft sample at the indicated E:T ratios. Readout was done by flow cytometry and NT and anti-CD33 CAR T cells served as negative and positive controls, respectively. Data illustrate the mean of one patient-derived xenograft sample cultured with two to three different T-cell donors with two technical replicates per donor $\pm$ SD. (B) NSG mice were sub-lethally irradiated and injected i.v. with 1×10^6 patient-derived BM cells. After 9 days, mice were treated with 5×10^6 anti-LILRB2 CAR+ T cells (n=3) or NT controls (n=3) i.v. (C) Blast engraftment in PB over time, shown as a fraction of total cells, as measured by flow cytometry. (D) Total count of AML blasts or (E) hCD3+ cells in BM, Spleen or PB at humane endpoint or end of the experiment at day 30, measured by flow cytometry (n=3). (F) Expression of LILRB2, CD117, CD33 and combinations thereof in a total of 25 AML patient samples as measured by flow cytometry. (G-K) Killing activity of CAR T cells targeting LILRB2, CD33, CD117, and their combinations after a 1-day co-culture with an

AML patient BM sample (PID1) at indicated E:T ratios, normalized to NT controls. Depicted are the total killing activity (G), as well as the specific killing activities against CD33+ (H), LILRB2+ (J) and CD117+ (K) subpopulations. Two technical replicates were prepared and analyzed by flow cytometry. Data are represented as mean $\pm$ SD. Statistical analysis was performed via two-way ANOVA (A,C, G-K) or one-way ANOVA (F) with Tukey's multiple comparison, or via Welch's t test (D-E); **p<0.01; ****p<0.0001.

Figure 8: Anti-LILRB2 CAR T cells spare healthy hematopoietic precursor cells. (A+B) Total cell numbers of cell types depicted on the y-axis after a 1-day co-culture of PBMCs (A) or HDBM (B) with the indicated CAR T cells at an E:T ratio of 1:1, normalized to NT controls. (C) Specific killing activity of anti-LILRB2 and anti-CD33 CAR T cells against monocytes at indicated E:T ratios after a 1-day co-culture with PBMCs. (D) Humanized NSG mice were injected with 2.5×10^6 anti-LILRB2 CAR+ T cells or NT controls upon showing detectable engraftment in the PB. (E) Fraction of CD34+CD38-, CD33+, C117+ and LILRB2+ cells normalized to NT control groups in the BM after 25 days. Readout for all experiments was done by flow cytometry. One biological replicate (HDBM) or two to three biological replicates (PBMC) with two technical replicates each are pooled for the in vitro data. In vivo, four mice were used for the NT control groups and five mice for the anti-LILRB2 CAR T group. Data is represented as mean $\pm$ SD. Statistical analysis was performed via two-way ANOVA (C) or one-way ANOVA (A+B, E) with Tukey's multiple comparison; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 1

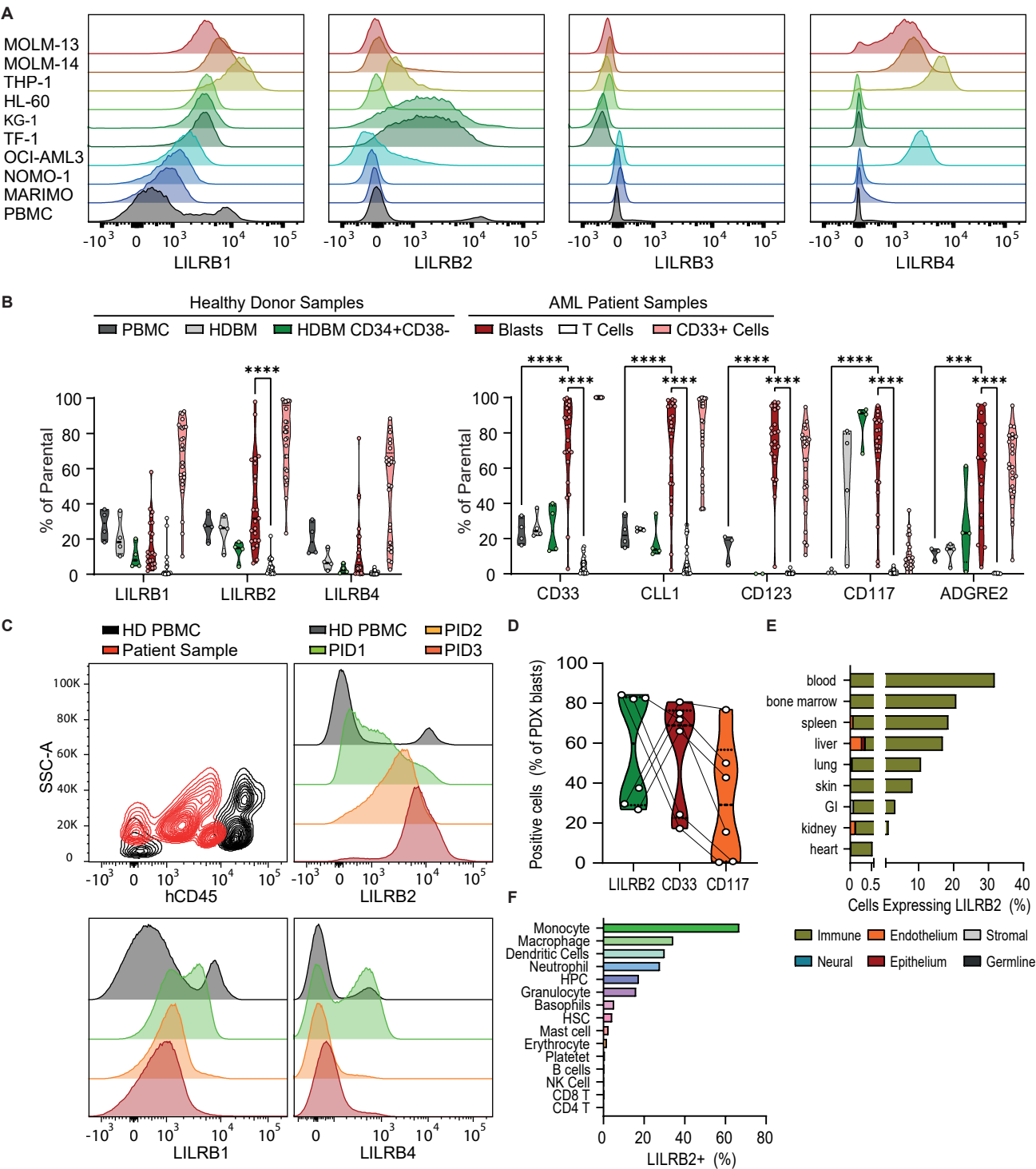


Figure 2

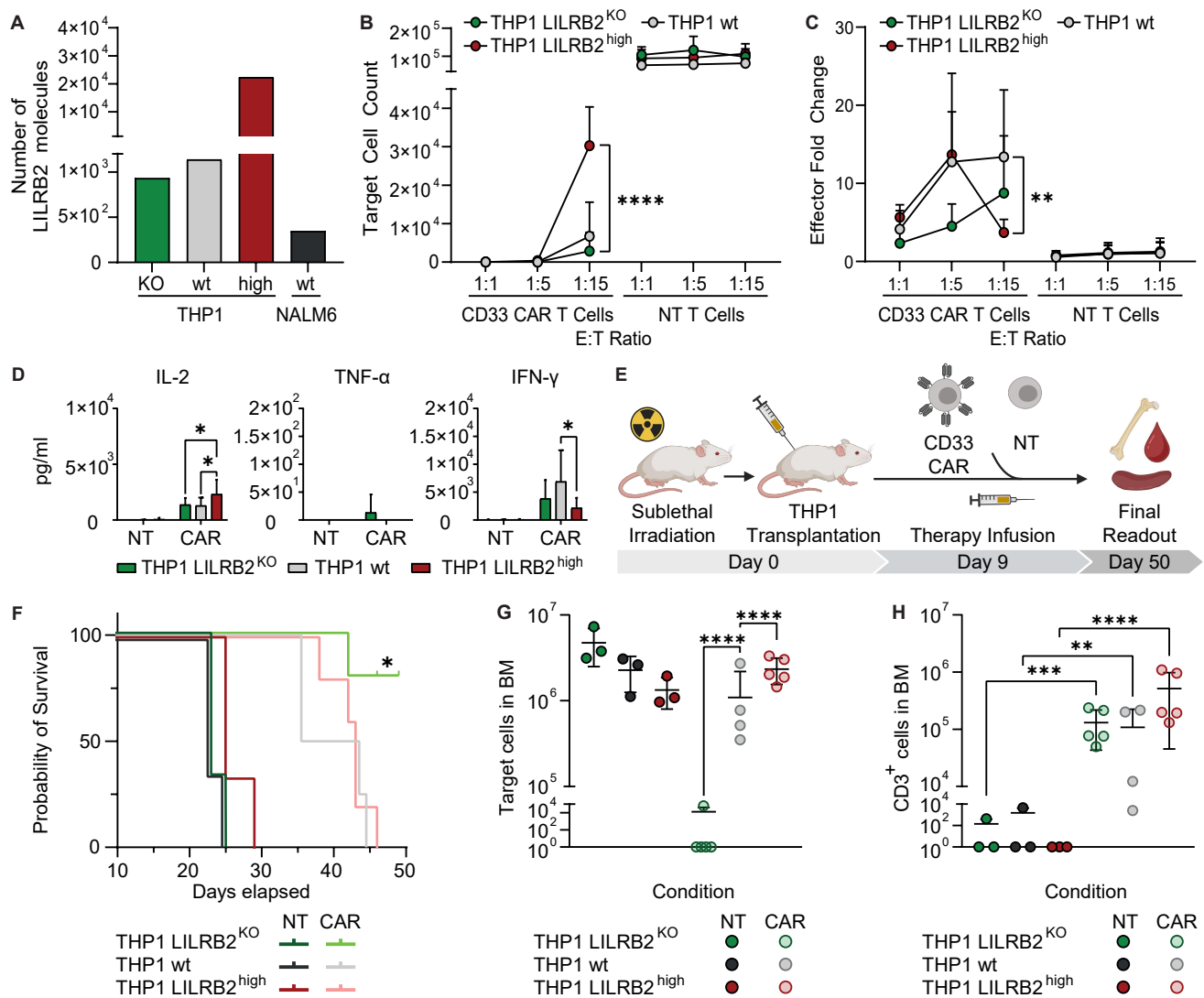


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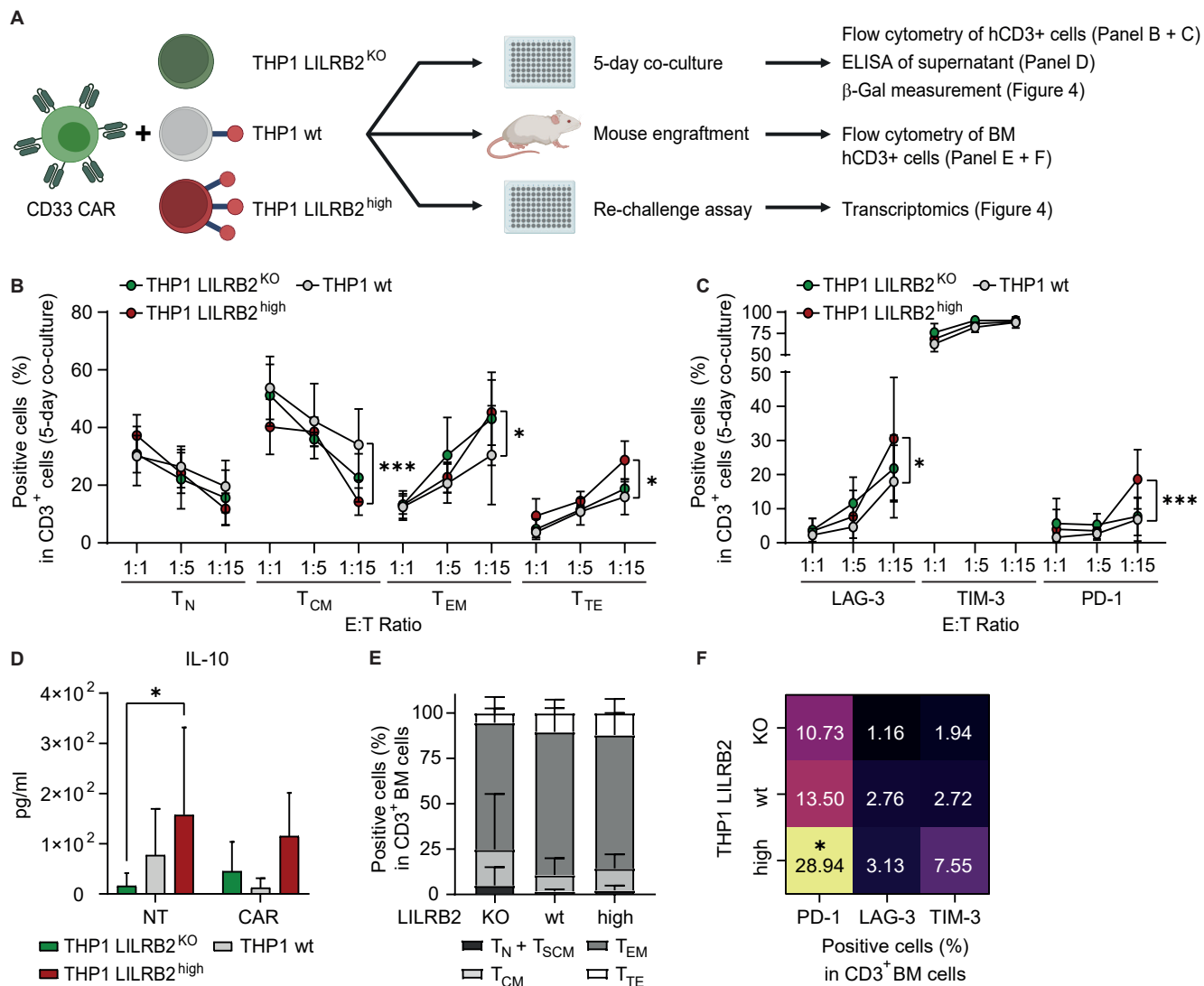


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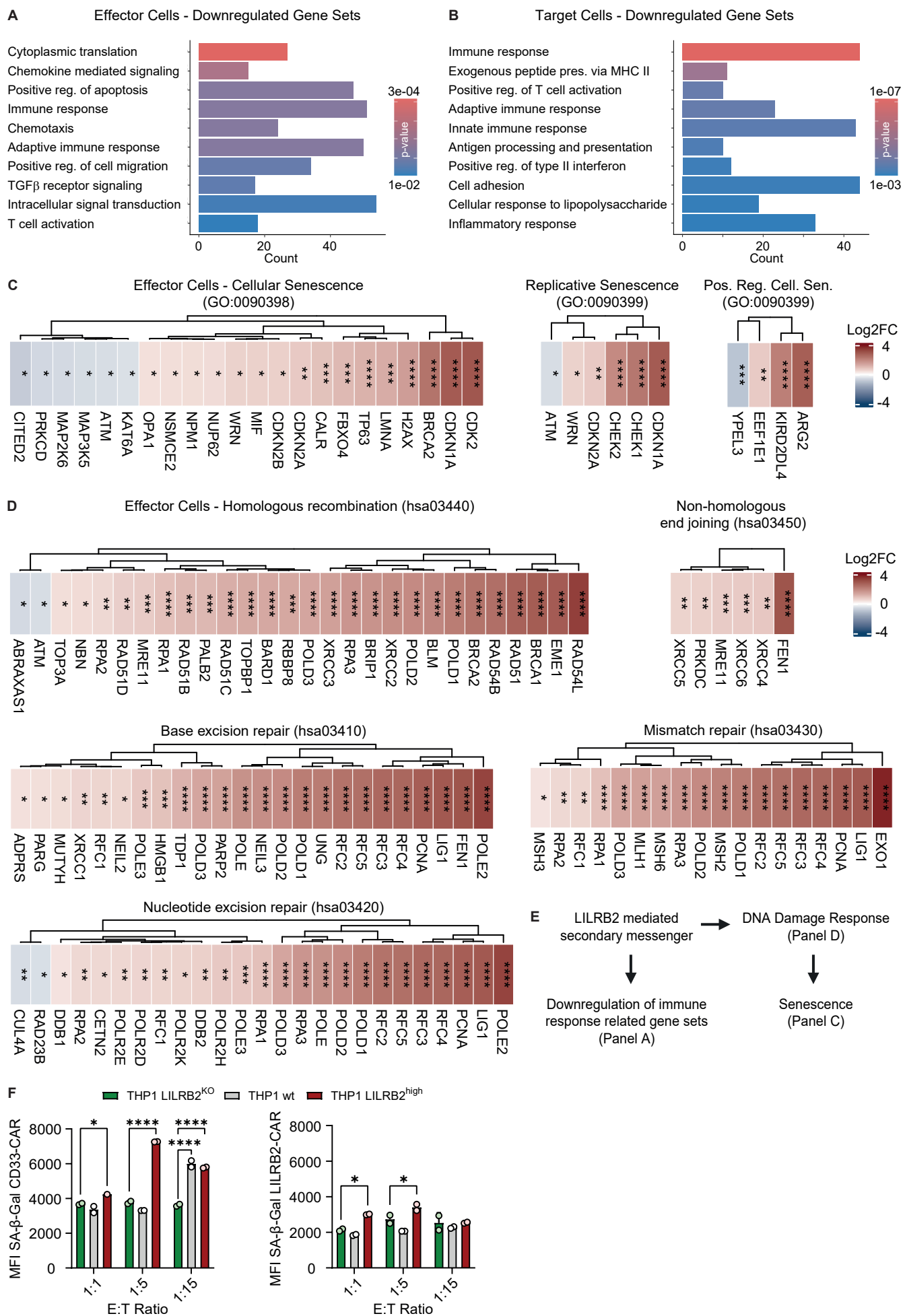


Figure 5

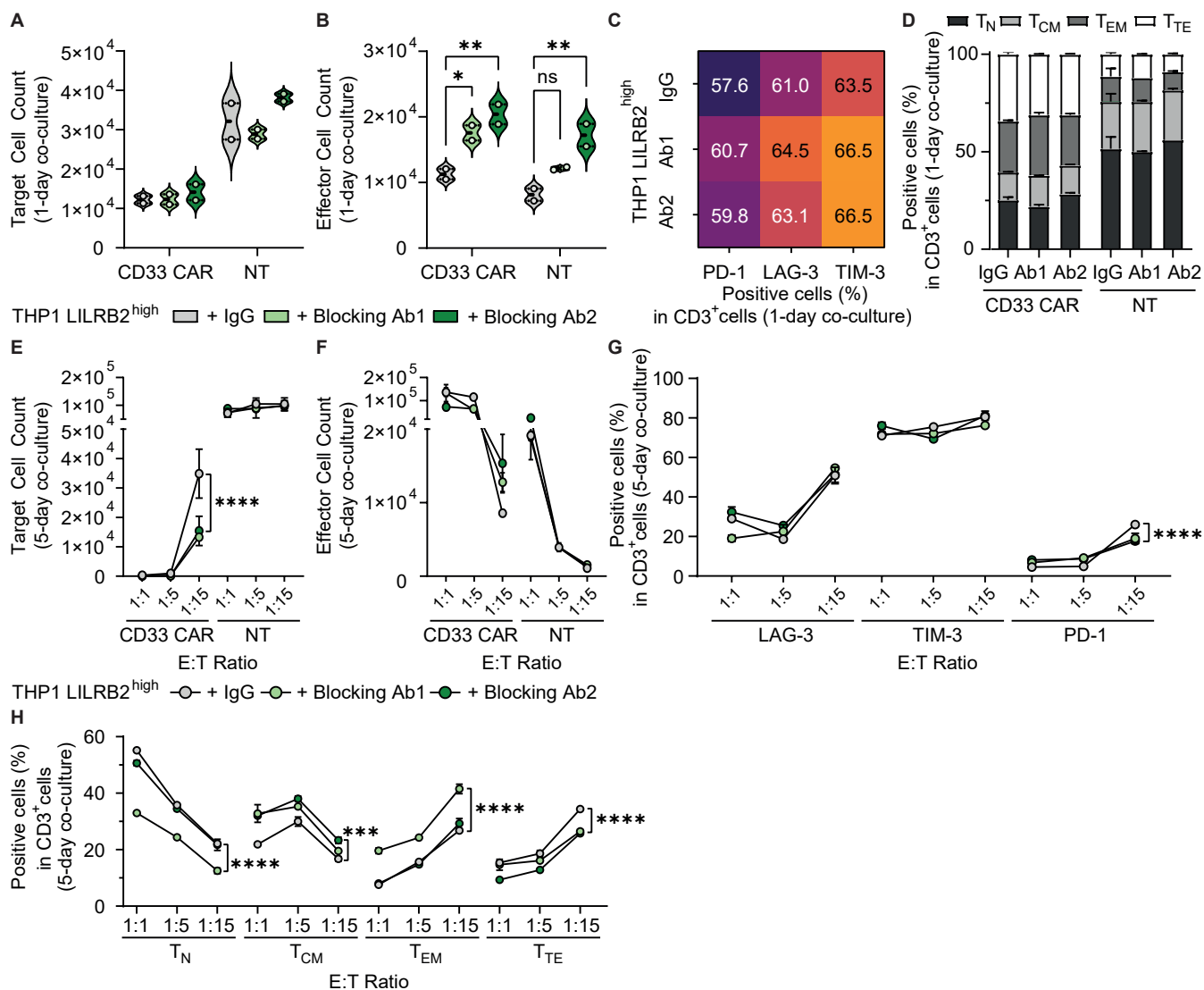


Figure 6

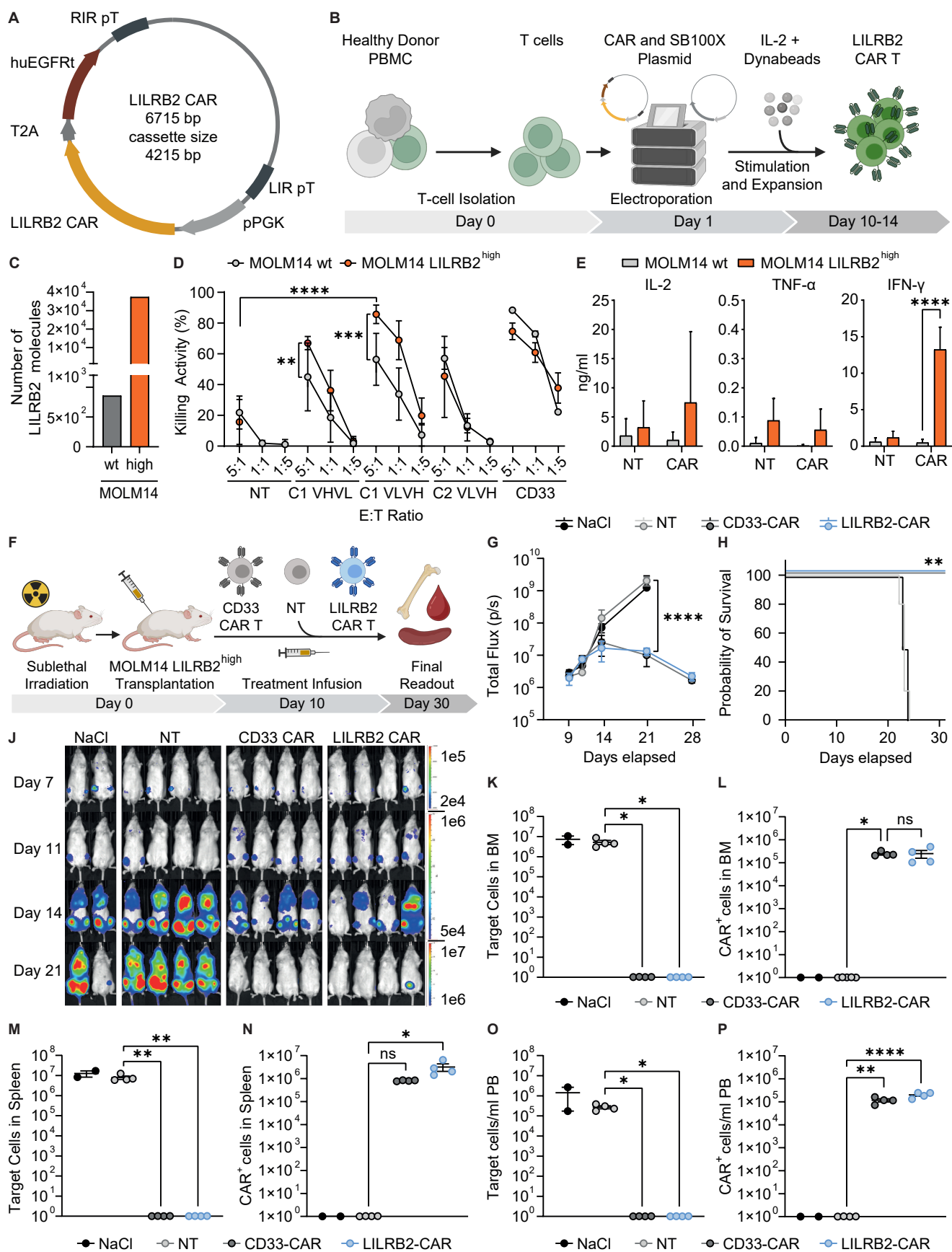


Figure 7

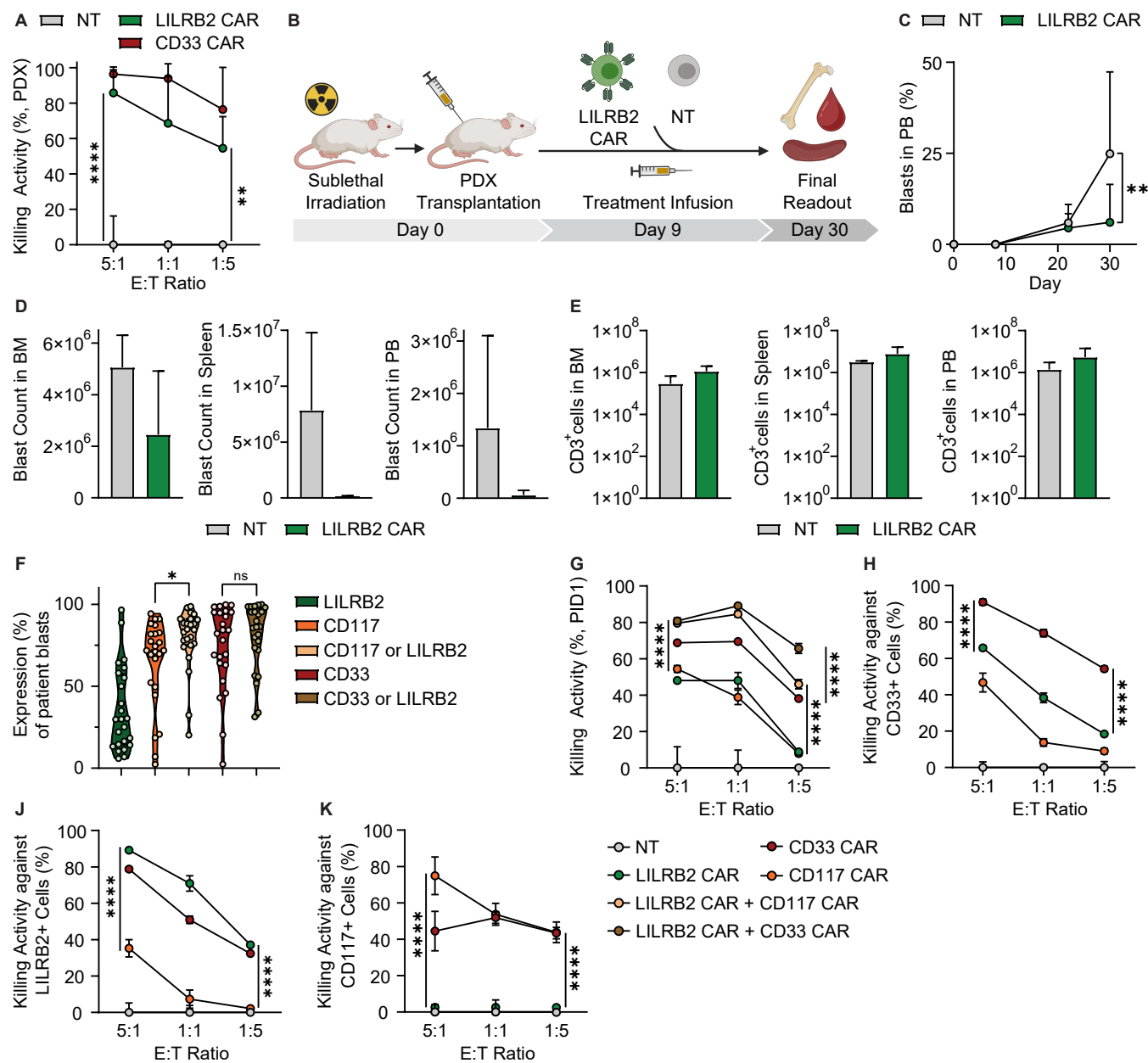
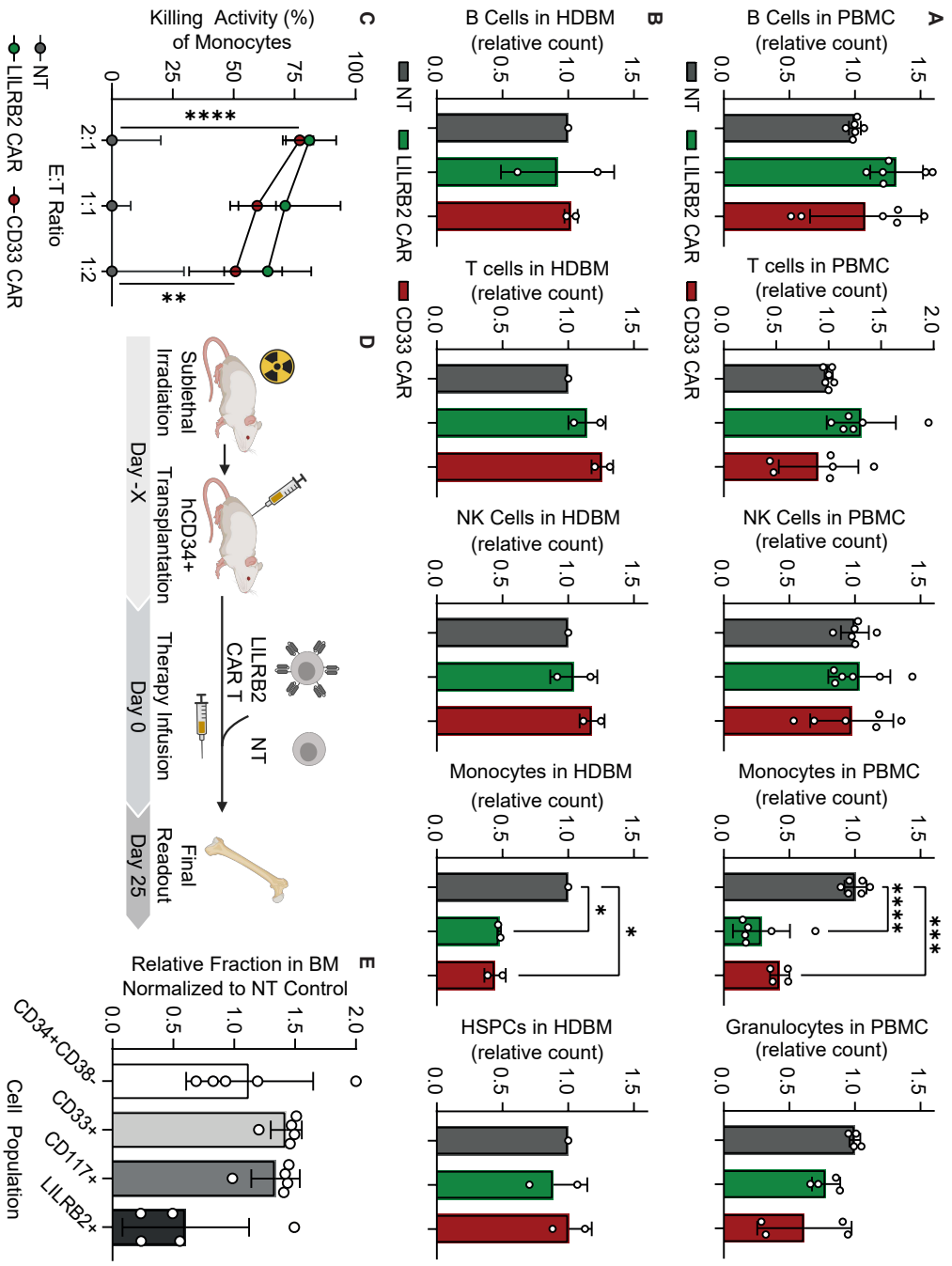


Figure 8



Supplementary Files

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