The ancestral haplotype of P2RX5 yields a B-cell surface marker and a multi-lineage immunotherapy target

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SUMMARY

While CD19- and BCMA-directed immunotherapies have improved outcomes for B-lymphoid and plasma cell malignancies, frequent relapses with antigen loss/downregulation highlight the need for new targets. Here, using transcriptomic datasets and newly-developed monoclonal antibodies, we show that *P2RX5*, long considered a pseudogene in humans, encodes a stable protein in 80% of individuals of African descent carrying the ancestral haplotype. Like CD19, P2RX5 displays B-cell lineage-restricted expression in normal tissues. Unlike CD19, P2RX5 is expressed not only in B-cell neoplasms, but also in T-cell leukemia (T-ALL) and multiple myeloma (MM). We developed P2RX5-directed bispecific T-cell engagers and CAR T cells, which killed T-ALL cells with no evidence of T-cell fratricide. These agents were non-inferior to FDA-approved CD19- and BCMA-directed immunotherapeutics in cell culture and xenograft models of Burkitt lymphoma and MM, while maintaining potency against CD19- and BCMA-negative variants. Hence, P2RX5 is a unique multi-lineage target for frontline or salvage immunotherapy.

KEYWORDS

Purinergic receptors, RNA splicing, B cell development, hematologic malignancies, cellular immunotherapies

INTRODUCTION

Immunotherapies targeting B-lymphoid lineage markers (e.g., CD19, CD20, CD22, and BCMA) have gained wide acceptance in the clinic for the treatment of B-lymphoid and plasma cell malignancies, due to their proven safety and efficacy [1,2]. A prime example is CD20, which almost 30 years ago, became the first clinically developed immunotherapy target [3] . To date, several anti-CD20 mAbs [with rituximab as the prototype [3,4]] and three anti-CD20xCD3 bispecifics (mosunetuzumab [5], epcoritamab [6], and glofitamab [7]) have gained FDA approvals for the treatment of B-cell lymphoma (BCL).

Because most precursor B-cell acute lymphoblastic leukemias (B-ALL) do not express CD20 uniformly, these patients are routinely treated with blinatumomab, a CD19-CD3 bispecific T-cell engager (BiTE) [8], for relapse/refractory (r/r) disease and, more recently, in the frontline setting (the consolidation phase) [9]. Additionally, for the past 10 years, various iterations of CD19-directed chimeric antigen receptor (CAR)-armed T-cell products [CART-19: tisagenlecleucel [10,11], axicabtagene ciloleucel [12], brexucabtagene autoleucel [13], obecabtagene autoleucel [14], and lisocabtagene maraleucel [15]] have been in clinical use for both B-ALL and BCL.

Despite high initial response rates, up to half of patients with B-ALL or BCL will relapse after CART-19 treatment [16,17], with up to 25% of cases due to CD19 loss [18-23]. For patients experiencing CD19-negative relapses, there is an FDA-approved salvage immunotherapy with the CD22-targeting antibody-drug conjugate (ADC) inotuzumab ozogamycin (InO) [24-27]; while autologous CAR T cells (CART-22) are currently undergoing clinical trials [28,29]. Although initially quite effective, CD22 downregulation has been observed following immunotherapy with both CART-22 and InO [29-35]. Once CD22 loss has occurred, no 'third-line' immunotherapy options are readily available.

The scarcity of validated targets is especially acute in the case of multiple myeloma (MM), the malignant cancer of plasma cells, which despite their B-cell origin, express little to no CD20, CD19, or CD22. Consequently, immunotherapies for MM are directed against a different set of surface proteins, most frequently B-cell maturation antigen (BCMA) [36,37] and G-protein coupled receptor family C group 5 member D (GPRC5D) [38]. In fact, two anti-BCMA CARs [ciltacabtagene autoleucel (cilta-cel) [39] and idecabtagene vicleucel (ide-cel) [40]] as well as anti-BCMA and anti-GPRC5D bispecific T-cell engagers [teclistamab [41] and talquetamab [42]] are currently in clinical use in the relapse setting. However, BCMA- and GPRC5D-directed immunotherapies are survival-prolonging but not curative, with frequent cases of epitope loss or downregulation [43-47]. While new targets for cellular immunotherapies [e.g., FcRH5 [48] and SEMA4A [49]] are being developed, these novel approaches are yet to reach the clinic.

In the case of T-cell acute lymphoblastic leukemia (T-ALL), targeting T-cell lineage markers (CD3, CD5 or CD7) is complicated by their presence not only on the cancer cells but also on the healthy T cells, including the CAR T cells themselves. This leads to fratricide during the manufacturing process and after infusion and ultimately to T-cell aplasia, which unlike B-cell aplasia, cannot be remedied with immunoglobulin infusions [reviewed in [50]]. Thus, there is a clinical need for more T-ALL-specific surface antigens.

Here, we show that the ancestral variant of the ATP-gated ion channel known as P2X purinoceptor 5, or P2RX5, is a unique multilineage immunotherapy target. Human *P2RX5* is commonly believed to be a pseudogene due to the pervasiveness of two recent loss-of-function mutations: the rs891746 single nucleotide polymorphisms (SNP) that adversely affects pre-mRNA splicing, and the rs3215407 indel that disrupts the open reading frame [reviewed in [51]]. Our work overturns this paradigm by showing that a stable, full-length P2RX5 (P2RX5-FL) protein is produced in almost 80% of individuals of African descent, who have retained the ancestral haplotype. We find that the *P2RX5* gene is robustly transcribed across BCL, T-ALL, and MM samples; yet its transcription in normal tissues, much like that of CD20-encoding *MS4A1*, is restricted to mature B cells. Furthermore, among MM patients of African descent, the P2RX5-FL haplotype is correlated with worse survival. Thus, we generated two anti-P2RX5 monoclonal antibodies (mAbs), 1D9 and 2F4, and showed that they can be used for flow cytometric and immunohistochemical (IHC) identification of normal B cells and neoplastic BCL, T-ALL, or MM cells. These mAbs were subsequently converted into P2RX5-CD3 bi-specifics and CARs, which displayed *in vitro* and *in vivo* cytolytic activity against various P2RX5-FL-positive blood cancers, including CD19- and BCMA-negative variants. These findings position P2RX5-directed immunotherapeutics as potential frontline or salvage treatments for a subset of patients with the ancestral *P2RX5-FL* haplotype.

RESULTS

Human P2RX5 mRNA is selectively expressed in normal B cells and hematologic malignancies

To uncover novel immunotherapy targets, we surveyed publicly accessible single-cell RNA sequencing (scRNA-seq) data from the Human Protein Atlas [HPA, version 24 [52]], the Tabula Sapiens portal [53], and the MMRF Myeloma Immune Atlas [54]. We found that *P2RX5* mRNA was elevated in B- and plasma cell clusters but was minimal in all other cell types (**Figures 1A and S1A, B**). This B-cell-specific expression pattern was also observed in existing RNA-seq data of various immune cell subsets sorted from human blood (**Figures 1B and S2A**) [52,55], as well as bone marrow (**Figure 1C, right**) and tonsils (**Figure 1C, right**) [56,57]. Specifically, during early B-cell development in the bone marrow, *P2RX5* was absent in early progenitors and CD34+ pro-B lymphocytes but upregulated upon differentiation into pre-B-lymphocytes and immature B cells (**Figure 1C)**.

In bulk RNA-seq of human tissues, the highest *P2RX5* levels occur in B-cell-rich tissues such as the lymph nodes, tonsils, spleen, appendix and small intestines; with little to no expression across other human tissue sites [see **Figure 1D** for the Genotype-Tissue Expression (GTEx) cohort [58], as implemented by the UCSC Toil RNA-seq recompute compendium [59], and **Figure S2B** for the Human Protein Atlas cohort [52]]. This mirrored the tissue expression pattern for other B-cell markers such as CD19, CD20 and CD79B. This elevated P2RX5 expression is retained by neoplastic diffuse large B-cell lymphoma (DLBCL) and ALL cells, relative to non-lymphoid human tissues (**Figure 1D**). Across species, human P2RX5 expression is distinct from its orthologs: murine *P2rx5* is highest in brown adipose and cardiac tissues with minimal splenic expression, while simian *P2rx5* peaks in the brain and cardiac tissues **(Figure S2C)** (GEO dataset GSE219045 [60]).

Aggregate microarray data from the HEMAP portal [61] largely agreed with our RNA-seq findings. Compared to non-cancerous stem, erythroid, myeloid and T-lymphoid cells, *P2RX5* mRNA levels were elevated in non-cancerous B-lymphoid cells as well as B-cell neoplasms [B-ALL, BCL, and chronic lymphocytic leukemias (CLL)] and MM (**Figure 1E**). Surprisingly, there were relatively high *P2RX5* mRNA levels in T-ALL compared to their presumed T-lymphoid cell of origin (**Figure 1E**). Altogether, the transcriptomic data suggest human *P2RX5* expression is B-cell lineage-restricted in healthy tissues, sustained in B-cell neoplasms, but surprisingly reactivated in T-ALL, and MM.

Gene polymorphisms result in multiple variants of P2RX5 mRNA

Given that multiple P2RX5 variants are annotated in GENCODE v49 [62], we re-analyzed Cancer Cell Line Encyclopedia (CCLE) RNA-seq data [63] to determine which variants of P2RX5 were expressed most abundantly. We also parsed previously generated long-read direct RNA sequencing data corresponding to Raji Burkitt lymphoma and Reh B-ALL cells, to obtain end-to-end sequences of *P2RX5* transcripts free of potential cDNA-seq artifacts [64] (**Figure 2A**). In these and other BCL and MM cell lines with high levels of *P2RX5* mRNA expression, we identified up to 5 distinct transcript variants arising from gene polymorphisms and alternative splicing of exons 3 and 10 (**Figure 2B**). The variant dubbed ‘ΔC’ encodes a C-terminally truncated protein due to the rs3215407 delG-allele with a frame-shifting single G nucleotide deletion in exon 3. Theoretically, this loss-of-function mutation could be “rescued” as a result of exon 3 skipping (Δ3); however, exon 3 skipping occurs at low frequencies (<20%) across all cell lines surveyed, limiting functional importance of the Δ3 variant. Cell lines with the frame-preserving G5-allele of rs3215407 fall into two broad categories: some produce the 13-exon full-length (FL) transcript and some exhibit skipping of exon 10 (Δ10) (**Figure 2C**).

To detect exon 10 skipping (Δ10) or inclusion (∇10) via an orthogonal method, we designed exon-specific qPCR primers to discriminate between P2RX5-FL and Δ10 variants, with an additional primer pair amplifying constitutive exon 9 (**Figure S3A**). The use of these reagents allowed accurate quantification of this alternative splicing event in qPCR reactions with single- and mixed-linear dsDNA templates (**Figure S3, B and C**). In RT-qPCR assays, these primers detected exon 10 inclusion in Daudi and Raji cells, as well as exon 10 skipping in Ramos and DOHH2 cells (**Figure S3D**), which was consistent with the RNA-seq data (**Figure 2C**).

Most people of African descent inherit the ancestral, full-length variant of P2RX5 (P2RX5-FL)

Curiously, exon 10 was constitutively included in all cell lines homozygous for the ancestral A-allele of rs891746 (e.g., Daudi, Raji, MM.1s and RPMI-8226) but completely skipped in those homozygous for the reference C-allele (e.g., DoHH2, Ramos and Reh) (**Figure 2C and S3D**). This may be due to the A nucleotide (and the complementary T on the opposite strand) forming part of the canonical 5' splice site dinucleotide, GT, in the downstream intron 10, which would be disrupted by the C-allele (**Figure 2A**). While the A-allele had been previously linked to exon 10 inclusion in LCL samples [65], we extended this linkage to all normal tissues, including splenocytes, using Splicing Quantitative Trait Loci (sQTL) analysis implemented in GTEx [66] (**Figure 2D**). Additionally, the effect of the rs891746 SNP on exon 10 splicing was experimentally validated with minigene reporters in Raji and OCI-Ly8 cells (**Figure 2E, top**). Under all conditions, the C-allele produced shorter, exon 10-skipping transcripts; while the A-allele produced FL, exon 10-including transcripts (**Figure 2E, bottom**).

Another striking pattern was that all P2RX5-FL-expressing cell lines were from donors of African ancestry. This prompted us to investigate the prevalence of the ancestral rs891746 A-allele in various human populations. According to aggregate data from the NCBI Allele Frequency Aggregator (ALFA) project [www.ncbi.nlm.nih.gov/snp/docs/gsr/alfa/], the rs891746 A-allele is exceedingly rare in European, Asian, and Latin American individuals. However, up to 80% of African donors are either homo- or heterozygous for the rs891746 A-allele. They also preserve the P2RX5 open reading frame by being simultaneously homozygous for the rs3215407 [G]5-allele (**Figure 2F**). In fact, owing to linkage disequilibrium, rs891746 A-allele and rs3215407 [G]5-allele have a 97% co-occurrence rate (**Figure 2G**).

The resulting P2RX5-FL haplotype was detected in over 75% of MM patients of African ancestry from the MMRF CoMMpass study [67] and endemic Burkitt's lymphoma (eBL) patients from West Kenya [68] and Uganda [69,70] (**Figure 2H**). In healthy tissues, P2RX5 expression pattern in samples homozygous for P2RX5-FL (**Figure S3E**) was indistinguishable from that seen in the broader GTEx population (**Figure 1**), both displaying elevated transcript levels in spleen, EBV-transformed lymphocytes, and small intestine, thus maintaining B-cell lineage-restriction. Of note, for MM patients of African ancestry in the CoMMpass study, Kaplan-Meier analysis revealed an inverse relationship between the number of P2RX5-FL copies and survival probability, with homozygous (+/+) patients showing the shortest overall survival (**Figure 2I**). This suggests that the P2RX5-FL haplotype might be a risk allele in plasma cell malignancies.

P2RX5-FL-encoded protein is stable and properly N-glycosylated

Existing anti-P2RX5 antibodies failed to recognize the protein's native extracellular domain. To generate flow cytometry-compatible mAbs, we immunized mice with syngeneic NIH-3T3 cells overexpressing P2RX5-FL and successfully established multiple P2RX5-reactive hybridoma lines. Of these lines, 1D9 produce an antibody that detected, in both immunoblotting and flow cytometric assays, human and simian P2RX5 proteins overexpressed in Ramos BCL and Jurkat T cells, but not their murine ortholog (**Figure S4A and B**). This species-specificity suggested that 1D9 recognizes amino acids shared by human and simian P2RX5 but not murine P2rx5. After an extensive mutagenesis screen, we discovered that substituting lysine-131 in human P2RX5 to the glutamate found in murine P2rx5 (K131E) was sufficient to abolish 1D9 recognition (**Figure S4C)**. Conversely, introducing the reciprocal E131K substitution into murine P2rx5, "rescued" 1D9 binding to the mutant protein (**Figure S4D**). The likely linear nature of the 1D9 epitope makes the corresponding antibody suitable for a variety of assays, including glycosylation and protein stability studies.

We first used 1D9 to perform immunoblotting on four cancer cell lines with endogenous P2RX5-FL mRNA expression (Daudi, Raji, MM.1s, and RPMI-8226) (**Figure 2C and S3D**). In these cells, 1D9 detected a ~60 kDa protein (**Figure 3A, left**). The specificity of detection was established by knocking out P2RX5 using CRISPR-Cas9 (**Figure S4E and 3A, right**) and through the overexpression of various P2RX5 variants (**Figure 3B**). While the predicted molecular mass of P2RX5 is 49.3 kDa, glycosidase treatment (**Figure S4F**) and N77A/N202A mutagenesis (**Figure S4G**) revealed that the apparent molecular weight was increased by N-linked glycosylation at N77 and N202.

Surprisingly, despite high Δ10 *P2RX5* mRNA levels in DoHH2 cells (**Figure 2C and S3D**), little to no P2RX5 protein was detected using immunoblotting (**Figure 3A, left**). Similarly, Raji cells overexpressing the FL variant made markedly more protein than those overexpressing Δ10 or Δ3Δ10 (**Figure 3B, right**),

despite comparable levels of pan-P2RX5 mRNA (**Figure 3B, left**). Since amino acids encoded by exon 10 constitute one of the two transmembrane, pore-forming domains of P2RX5 (**Figure 2B, right**), we reasoned that exon 10 skipping could destabilize P2RX5 protein, leading to its rapid degradation. Indeed, once new protein synthesis was inhibited by cycloheximide, flow cytometric assays detected more rapid loss of the green fluorescent protein (GFP)-tagged version of Δ10 relative to the FL variant (**Figure 3C**).

P2RX5-FL protein is selectively expressed in normal and malignant B cells

Using 1D9, we also performed IHC staining of formalin-fixed paraffin-embedded (FFPE) sections to spatially profile P2RX5 protein expression in normal human tissues and BCL samples of defined P2RX5 genotypes. Across sections of *P2RX5-FL/FL* and *FL/Δ10* lymph nodes and spleens, P2RX5 protein was detectable strictly in the B-cell compartment of germinal centers, co-localizing with CD20 (**Figure 3D**). No staining was apparent in the skeletal muscle used as a negative control, fully consistent with RNA-seq data (**Figures 1 and S1**). 1D9 also robustly stained xenografts of P2RX5-FL-positive Raji cells but not their P2RX5-knockout (KO) derivatives (**Figure 3E**). Importantly, uniform P2RX5 staining was apparent on two archival BCL samples with the *P2RX5- FL/FL* and *FL/ΔC* variants, but not on the BCL sample with the ΔC/ΔC variants (**Figure 3F**).

P2RX5-FL is robustly expressed on the surface of BCL, MM, and T-ALL cells

To establish that P2RX5-FL localizes to the cell surface in blood cancer cells, we inserted a FLAG tag into predicted extracellular loops of various P2RX5 variants (**Figure 4A**) and overexpressed the recombinant constructs in Raji and Ramos cells, confirming expression by RT-PCR (**Figure 4B, top**). Only in the case of P2RX5-FL were live, non-permeabilized cells strongly positive for cell-surface FLAG in flow cytometry (**Figure 4B, bottom**). We also generated a knock-in (KI) of the FLAG-tag coding sequence into the endogenous *P2RX5* gene of Raji cells using CRISPR-Cas9. After confirming insertion via RT-PCR and amplicon sequencing (**Figure 4C**, **top**), we performed flow cytometric analysis and once again observed strong FLAG-tag positivity of the Raji-KI cells, with parental and P2RX5-KO cells serving as negative controls (**Figure 4C**, **bottom**).

The cell-surface expression of P2RX5-FL was additionally verified through direct staining with another anti-P2RX5 hybridoma clone, 2F4. In live-cell flow cytometry assays, both 1D9 and 2F4 mAbs stained cells with P2RX5-FL expression, either endogenous (Raji and MM.1s) or ectopic (Ramos-P2RX5-FL) (**Figure 4D**). Of note, these antibodies were non-competing, as preincubation of Raji and Daudi cells with unlabeled 1D9, did not inhibit subsequent staining by fluorophore-labelled 2F4, and vice versa (**Figure 4E**). We also used flow cytometric staining to calculate binding affinities of 1D9 and 2F4. These values were marginally higher for 1D9 (EC50 ≈45 nM) relative to 2F4 (EC50 ≈65 nM) across both endogenous (**Figure 4F**) and overexpression (**Figure S5A**) models. No measurable binding to Raji-P2RX5-KO and parental Ramos cells was detected (**Figures 4F and S5A**). Nor did these antibodies stain HEK293T cells overexpressing other P2RX family members (**Figure 4G**), further attesting to their specificity.

Using the higher-affinity 1D9 clone, we performed antibody binding capacity (ABC) assays to quantify P2RX5 surface density. Cell lines homozygous for P2RX5-FL (including Raji, Daudi, MM.1s, RPMI-8226, and a lymphoblastoid cell line [LCL] from an African donor) averaged 3,000–6,000 P2RX5 molecules per cell (**Figure 4H)**. This was lower than the 40,000-100,000 CD19 molecules per cell detected for the LCL, Raji and Daudi cells but comparable to ≈6,000 BCMA molecules per cell expressed by MM.1s and RPMI-8226 cells (**Figure 4H**). We also detected ≈1,000 P2RX5 molecules on the heterozygous P2RX5 FL/ΔC MOLT-16 T-ALL cells, and less than 300 P2RX5 molecules – on P2RX5 Δ10/Δ10 DoHH2 and ΔC/ΔC Ramos cells (**Figure 4H)**. These P2RX5 surface densities were consistent with our earlier immunoblotting data (**Figure 3A**).

In addition to cell lines, we stained live cells from bone marrow aspirates from T-ALL and MM patients with 1D9 and 2F4. All 5 aspirates from African donors were found by RT-qPCR and TaqMan SNP genotyping assays to express at least one copy of P2RX5-FL. Consistent with this finding, we readily detected $CD7^{+}P2RX5^{+}$ T-ALL cells and $CD138^{+}P2RX5^{+}$ MM cells in these five cases (**Figure 4I**) – but not in the case of one P2RX5 ΔC/ΔC donor (MM-3).

The loss of other immunotherapy targets or exposure to common blood cancer therapeutics does not reduce P2RX5-FL expression

To investigate P2RX5-FL expression in lymphoid and multiple myeloma models of antigen escape, we knocked out CD19, CD20, CD22, and CD79B in Raji and Daudi cells; as well as BCMA and CD38 in MM.1s and RPMI-8226 cells. We then confirmed epitope loss by flow cytometry and simultaneously stained KO cells for P2RX5. In none of these cell lines was P2RX5 surface expression lost or diminished (**Figure S5B**). Conversely, P2RX5 KO didn't affect positivity for any of these markers in parental cells (**Figure S5C**). This suggests that potential P2RX5-directed immunotherapeutics would neither be affected by antigen loss due to prior immunotherapies nor jeopardize the efficacy of subsequent ones.

Since such immunotherapeutics are likely to be tested in patients with prior exposure to chemotherapy, we measured the effect of common anti-cancer drugs on P2RX5 expression. Specifically, we treated Raji and Daudi cells with doxorubicin and vincristine, MM.1s and RPMI-8226 cells with bortezomib and lenalidomide, and all 4 lines with dexamethasone. The drug concentrations were chosen to result in measurable reductions in cell viability (bar graphs in **Figure S5D and E**). We observed that transient exposure to doxorubicin, vincristine, bortezomib, and lenalidomide had no effect on P2RX5 levels (**Figure S5D**). Of note, treatment with the corticosteroid dexamethasone led to elevated P2RX5 expression in all four cell lines tested, with close to a two-fold increase in median fluorescence intensity (MFIs) in MM.1s and Raji cells (**Figures 4J and S5E**).

A P2RX5-CD3-directed bispecific enables killing of BCL and MM cells

To evaluate the potential of P2RX5-directed immunotherapeutics, we tested several prototype bispecifics based on 1D9- or 2F4-derived scFvs in the backbone of blinatumomab, a CD19/CD3-targeting bispecific T-cell engager (BiTE) [71]. Among these, a 2F4-based bispecific dubbed αP2RX5-αCD3 (**Figures 5A and S6**), displayed the most potency *in vitro*. When added to 5-day co-cultures of Raji-CD19-KO target cells admixed with T cells from 3 individual donors, αP2RX5-αCD3 induced robust T-cell expansion and complete lysis of P2RX5-FL-positive Raji cells, regardless of the P2RX5 genotypes of the effector T cells (**Figure 5B, C**). As expected, no T-cell expansion occurred without target cells.

In side-by-side comparisons using 1-day co-culture assays, the αP2RX5-αCD3 BiTE displayed sub-picomolar potency (EC50: 0.24 pM) comparable to that of blinatumomab (EC50: 0.17 pM) (**Figure 5D, right**). As a result, it effectively lysed multiple P2RX5-FL-positive cell lines (Raji, MM.1s, and RPMI-8226, as well as Ramos-P2RX5-FL) while Raji-P2RX5-KO cells and ΔC/ΔC Ramos-Puro cells were spared (**Figure 5D**). Of note, pretreatment of MM.1s target cells with dexamethasone, which upregulates P2RX5 expression (**Figure 4J and S5E**), further enhanced the cytolytic activity elicited by αP2RX5-αCD3 (**Figure 5E**).

To test the feasibility of dual-targeting CD19 and P2RX5, we used a bicistronic lentiviral expression vector (dubbed αCD19-28z.αP2RX5-αCD3) to generate donor T cells that simultaneously express CD19-directed chimeric antigen receptor (CAR) and secrete αP2RX5-αCD3 (**Figure 5F**). These cells were co-cultured for 1 or 5 days with target Raji cells, either wild-type or CD19 KO (**Figure 5G**). Unlike the monospecific αCD19-28z CAR T cells, which efficiently killed only wild-type Raji cells, the αP2RX5-αCD3-armed CAR T cells killed Raji cells without regard for CD19 expression (**Figure 5H and I**).

To test its utility against CD19-negative disease *in vivo*, we intravenously injected luciferase-expressing (luc+) Raji-CD19-KO cells into NSG mice and allowed cell line-derived xenografts (CDXs) to establish for 7 days. Then, the animals were randomized into three groups, to receive either mock-transduced donor T cells, T cells armed with αCD19-28z, or dual-targeting αP2RX5-αCD3.αCD19-28z T cells. Disease spread was assessed over 17 days using optical imaging. We observed that while monospecific αCD19-28z T cells were completely ineffective in controlling growth of CD19-negative Raji cells, dual-targeting αP2RX5-αCD3.αCD19-28z T cells markedly delayed disease progression, even in the absence of normal or neoplastic human B cells capable of triggering CAR T-cell activation and expansion (**Figure 5J**).

P2RX5-redirected CAR T cells demonstrate therapeutic efficacy in MM preclinical models

Because CAR T cells have already proven their utility in MM patients, we designed several 1D9 scFv-based second-generation CARs containing CD3ζ activation domain and either CD28 or 4-1BB costimulatory domains (dubbed αP2RX5-28z and αP2RX5-BBz, respectively) (**Figure 6A**). These

cassettes were inserted into lentiviral vectors and used to transduce donor T cells. As a result, over 30% of donor T cells stained positive for the CAR construct in flow cytometry assays (**Figure 6B**). The choice of CAR did not noticeably affect T-cell expansion over 16 days, which was comparable to that of the control αCD19-28z CAR T cells (**Figure 6C**). Furthermore, in cell avidity assays, more the 70% of both αP2RX5 CAR T cells remained bound to target RPMI-8226 cells at the end of the force curve, relative to the 30% remaining observed for the mock transduced T cells (**Figure 6D**). This suggests that both αP2RX5-BBz and αP2RX5-28z enhance T cell avidity towards P2RX5FL-positive target RPMI-8226 cells.

To assess their cytolytic activity, effector αP2RX5-28z and -BBz CAR T cells were co-cultured with target luc+ cell lines or LCLs derived from the same P2RX5-FL/FL donor as the T cells (**Figure 6E**). After 5 days of co-culture at an effector-to-target or E:T ratio of 1:4, we detected a 60% reduction in viable LCL cell counts, relative to co-cultures with mock-transduced T cells. We also detected a 2-fold increase in the number of co-cultured αP2RX5-BBz CAR T cells, compared to the same T cells cultured alone (**Figure 6F, left**). Reductions in cell viability of 50% or more were also observed across a range of luc+ target cell lines (including MM.1s, RPMI-8226, Raji, Daudi, MOLT-16 and Ramos-P2RX5FL cells), after these cells were co-cultured with αP2RX5 CAR T cells for 4 days at E:T ratios of 1:16 and 1:8 (**Figure 6G**), or for 1 day at a higher E:T ratio of 3:2 (**Figure 6H**). Cell line sensitivity to both αP2RX5 CARs was not affected by BCMA or CD19 knockout, but was abolished by P2RX5 knockout (**Figure 6H, right**). While our tests utilized T cells of different P2RX5 genotypes (FL/FL, Δ10/Δ10, and ΔC/ΔC), we found no difference in the activities of the resulting αP2RX5 CAR T cells (**Figure 6G**). Pretreating MM.1s cells with dexamethasone further enhanced αP2RX5-BBz CAR-mediated cytolysis (**Figure 6I**).

Finally, we evaluated P2RX5-directed CARs *in vivo* using an orthotopic mouse model of MM. In this model, luc+ RPMI-8226 cells were injected into femurs of NSG mice and CDXs were allowed to establish, as described previously [72] (**Figure 6J**). Then 0.8×10^6 CAR-positive T cells were infused intravenously into tumor-bearing mice, and their expansion and persistence were followed by collecting peripheral blood samples at days 9, 17, 27, and 91, and quantifying human T-cell levels with flow cytometry. We observed that both αP2RX5 CAR T-cell products persisted for at least 3 months post-infusion at levels comparable to that of the commercial αBCMA CAR, cilta-cel (**Figure 6K**). Most importantly, compared to the mock T-cell cohort, a single dose of αP2RX5-28z, αP2RX5-BBz, or αBCMA-BBz CAR T cells effectively controlled tumor growth **(Figure 6L)** and extended survival beyond 100 days **(Figures 6M),** with αP2RX5-BBz allowing significantly longer survival times compared to αP2RX5-28z ($p=0.04$) and being non-inferior to the standard-of-care αBCMA-BBz cilta-cel.

DISCUSSION

Since intravenous immunoglobulins can replace normal B-cell function, immunotherapies for lymphoid neoplasms are designed to target not cancer-specific, but rather lineage markers such as CD19 and BCMA [1]. Over the past 15 years, the advent of bispecific T-cell engagers and CAR T cells has raised hopes that even chemotherapy-resistant lymphoid cancers can be cured. Indeed, initial response rates in heavily pre-treated populations often exceeded 80% [2]. However, more recently it became apparent that antigen escape is a major cause of treatment failure, sharply limiting progression-free and overall survival [16,23]. This underscores the need for more diversified target repertoires for both salvage and combination therapies; yet there is paucity of true lineage-restricted surface markers.

It has been proposed that dysregulated RNA splicing could be a rich source of unique proteoforms [73-75]. For example, our earlier work has shown that in pediatric B-ALL there is an abundance of non-canonical transcripts [56,76], including those corresponding to classical immunotherapy targets like CD19 [20-22] and CD22 [35]. We further demonstrated that highly specific antibodies can be generated against such proteoforms, for example the CD22 Δex5-6 variant [35]. Here, by focusing on genetic determinants of alternative splicing, we were able to identify the ancestral variant of P2RX5 as both a novel B-cell lineage marker and a compelling multilineage immunotherapy target.

Research into P2RX5 as a therapeutic target has been limited by the perception that humans express a natural deletion mutant (Δ10) lacking exon 10, which in other species encodes a transmembrane domain. The cause of the P2RX5 Δ10 event is an evolutionarily ‘young’ polymorphism (rs891746) at the 5’ splice site at the beginning of intron 10, replacing the canonical GT dinucleotide with GG. Prior studies had suggested that the resultant Δ10 variant is predominantly intracellular [65,77], while another group reported

upregulation of the Δ10 variant on the plasma membrane during T-cell activation and suggested that it could contribute to immune regulation [78]. Our data shows that at least in B cells, instead of accumulating in the cytosol the truncated Δ10 protein is rapidly degraded, making the underlying event loss-of-function. Thus, although all genetic variants of P2RX5 are transcribed equally well, only the ancestral, exon 10-including full-length variant is likely to be of functional significance.

Furthermore, based on an extensive analysis of published single-cell and bulk RNA-seq data for dozens of human tissues, we found that P2RX5 expression is strictly B-cell lineage-restricted. It begins early, at the pre-B cell stage in the bone marrow, increases as B cells mature, and is suppressed after terminal differentiation into plasma cells. Our findings are consistent with prior RT-PCR-based reports of elevated P2RX5 mRNA in normal and malignant B-lymphoid tissues [79-81]. This diverges markedly from the murine ortholog of P2rx5 which is reportedly expressed in non-B-lymphoid cells such as osteoclasts, macrophages and brown adipocytes [82,8384]. At the tissue level, elevated levels of human P2RX5 mRNA are detectable exclusively in B-cell-rich tissues such as the lymph nodes, tonsils, spleen, appendix and small intestine; while the highest levels of murine P2rx5 were detected in brown adipose and cardiac tissues with minimal expression in the spleen. The expression pattern changes again for simian P2rx5 which had the highest expression in CNS and cardiac tissues. These vastly distinct tissue expression patterns suggest that murine and primate models may not accurately reflect human P2RX5 function and its possible roles in B cells and cancers derived therefrom.

Not surprisingly, P2RX5 is expressed in all types of B-lymphoid malignancies of various stages of differentiation, ranging from acute lymphoblastic to chronic lymphocytic leukemia. What was surprising is the P2RX5-positivity of MM and T-ALL– malignancies that typically exhibit no or very low expression of classical B-cell markers, such as CD19, CD20, and CD79B. This expression is of translational significance when paired with the fact that normal T cells of various P2RX5 genotypes (FL, Δ10, and ΔC) remained P2RX5-negative, i.e., non-reactive with the 1D9 and 2F4 mAbs. This suggests that autologous P2RX5-directed CAR T cells could be produced for T-ALL patients without risking fratricide or T-cell aplasia, the phenomena documented for CAR T cells targeting T-cell lineage markers such as CD5 and CD7 [85,86]. Indeed, P2RX5-directed CAR T cells generated from P2RX5-FL/FL donors expanded *in vitro* at rates comparable to CART-19, confirming a lack of self-targeting.

In addition to CAR T cells, we also developed bispecific T-cell engagers and found that both P2RX5-directed therapies were highly potent against P2RX5-FL-positive BCL and MM cell lines, on par with FDA-approved CD19-directed blinatumomab and BCMA-directed cilta-cel. Of note, P2RX5 expression persists after the genetic ablation of common immunotherapy targets (CD19, CD20, CD22, CD79B, CD38 and BCMA). Conversely, the deletion of P2RX5 did not affect positivity for the same common immunotherapy targets. Thus, P2RX5 is a viable co-target in combination therapies to prevent relapses driven by single-antigen loss. Another attractive aspect of targeting P2RX5 is its upregulation during treatment with dexamethasone. This suggests that P2RX5-directed therapies could be most effective when administered alongside corticosteroids which are routinely given to patients with MM or BCL, as a direct chemotherapeutic agent or to manage inflammation, including the toxicities related to CAR T-cell therapy such as CRS or ICANS.

In summary, we have credentialled the ancestral, FL variant of P2RX5 as a promising target for the genotype-guided immunotherapy of lymphoid malignancies. While P2RX5-directed therapies would be feasible only in selected populations of African ancestry, it is worth noting that the very same populations have benefited far less from modern forms of immunotherapy [87]. In the U.S., only 1.3% of participants in CART trials are of African ancestry even though they account for more than 25% of MM cases [88,89]. Compared to non-African populations, African Americans also have two-fold higher incidence and mortality rate for MM [90-92] and worse survival for multiple forms of lymphoid malignancies, including CLL[93,94], DLBCL[95] and follicular and marginal zone lymphoma [95]. It stands to reason that the advent of P2RX5-directed immunotherapies may help address some of these disparities.

STUDY LIMITATIONS

Our study is primarily limited by a relatively small number of samples from donors of African ancestry. While we were able to measure P2RX5 protein levels (via flow cytometry or IHC) in several P2RX5-FL-genotyped normal tissues and tumor samples, the B-cell lineage-restricted expression of P2RX5 protein should be substantiated across a range of different organs from a larger cohort of P2RX5-FL-genotyped donors. Although we have inferred that over 80% of patients of African descent express at least one copy of P2RX5-FL based on transcriptomic data, this inference needs to be validated at the protein level in more BCL, T-ALL and MM samples. Finally, our observation that an increase in P2RX5-FL copy numbers was associated with worse outcomes for African patients with MM, needs to be replicated in a larger cohort of patients.

METHODS: EXPERIMENTAL MODELS

Animal models

Mice studies were carried out in accordance with Children's Hospital of Philadelphia and University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) guidelines. For αP2RX5-αCD3 secreting αCD19-28z CAR T-cell experiments, 6- to 8-week-old female NSG mice were injected intravenously (IV) with $0.2x10^6$ Raji-CD19KO-luciferase-zsGreen cells on day -7. Tumor burden was determined by IVIS spectrum whole animal bioluminescent imaging on day -1. The mice were then randomized to groups with equal mean tumor burdens and a total of $0.3x10^6$ CAR T cells were IV injected into each mouse on day 0. To compare the αP2RX5-28z or -BBz CAR T cells with ciltacabtagene autoleucel, 6- to 8-week-old NSG mice were injected intraosseously with RPMI-8226-luciferase cells as described earlier [72]. After xenografts were established, mice were injected intravenously with a total of $0.8x10^6$ CAR T cells. Whole animal bioluminescent imaging was performed with a using the IVIS Lumina S5 (PerkinElmer) and the images were analyzed using Living Image, v.4.1, software (PerkinElmer). The total bioluminescent signal flux for each mouse (flank) was expressed as average radiance (photons per second per square centimeter per steradian). Survival was recorded and analyzed at the end of the study. To determine the presence of CAR T and tumor cells, peripheral blood was collected from all animals and the absolute numbers of live/human CD45+/murine CD45-/CD3+ T cells were determined by flow cytometry.

Human samples

Archival FFPE samples of donor tumor (lymphoma), lymph node, spleen and muscle tissue were obtained from the Penn Tumor Tissue/Biospecimen Bank (TTAB) under the Penn Institutional Review Board (IRB) approved protocol 856585. Cryopreserved bone marrow aspirates from Black or African American patients with T-ALL were obtained from the CHOP Center for Childhood Cancer Research Biobank and Repository under the CHOP IRB-approved protocol #10-007767. Bone marrow aspirates from Black or African American patients with MM were collected under the approved IRB protocol 842940 through the PCD group at the Hospital of the University of Pennsylvania.

Dataset usage

ScRNA-seq-based normalized protein counts per million (nCPM) for P2RX5 were downloaded from the Human Protein Atlas portal (HPA, version 24) 34321199 [53]. Within each dataset, we calculated weighted mean nCPM counts for all clusters sharing a cell type, weighting by the number of cells per cluster. Clusters with mixed cell type or low-confidence cell type annotations were excluded.

The Monaco PBMC (PRJNA418779) [Ref [55]], Boultwood Bone Marrow (PRJNA473835) [Ref [96]], bone marrow and tonsil subsets (PRJNA475684) [Ref [35,56,57]], mouse and macaque tissue (GSE219045) [Ref [60]], CCLE (PRJNA523380) [Ref [63]], eBL Uganda 1 (PRJNA292327) and BL Uganda 2(PRJNA374464) [Ref [69,70]], eBL West Kenya (phs001282.V2.p1) [Ref [68]], MMRF CoMMpass (PRJNA248539) [Ref [67]] datasets were from the BioProject or dbGaP databases of the National Center for Biotechnology Information (NCBI). For the CoMMpass dataset, samples were categorized as from donors of African ancestry based on prior principal component analysis (PCA) of 4,761 Ancestry Informative Markers (AIMs) SNPs [97].

METHODS DETAILS

RNA seq analyses

RNA-seq reads were first trimmed to remove adapters (BBTools v38.96) and were then aligned using STAR v2.7.9a to the hg38 reference genome while providing known gene isoforms through the GENCODE annotation V32. We used STAR flags "–quantMode GeneCounts" and "–alignSJoverhangMin 8" to quantify genes and ensure spliced reads had an overhang of at least eight bases. Junction spanning reads were obtained from the STAR "*_SJ.out.tab" result files, and each entry was normalized by dividing by the total number of junction-spanning reads and multiplying by a factor of 1 million to obtain the "junctions per million" or JPM. TPMs for all samples were calculated using TPMCalculator [98] version 0.0.3. Sample P2RX5 genotype were classified based on the ratio of [exon-10 to -11 JPM] / [exon-9 to -11 JPM] where values over 0.9 were classified as homozygous for exon-10 inclusion, in between 0.1 and 0.9 as heterozygous and below 0.1 as homozygous for exon-10 skipping. The rs3215407 alleles were determined via manual look-up of the alignments on IGV.

Plasmid constructs

The *P2RX5* minigene sequence (exons 9-11) containing truncated introns and either the rs891746(A or C) alleles was fused upstream of a NanoLuc ORF and cloned into the MXS_CMV::PuroR-bGHpA plasmid, replacing the PuroR ORF. MXS_CMV::PuroR-bGHpA was a gift from Pierre Neveu (RRID: Addgene_62439) [99]. . The pCDH-EF1 vector was a gift from Kazuhiro Oka (RRID: Addgene_72266). The pLC-Luc-IRES-zsGreen-P2A -Blast lentiviral vector was constructed by replacing the CMV promoter sequence (immediately upstream of the zsGreen ORF) in pLC-ZsGreen-P2A-Blast with the EF1a promoter, Luciferase ORF and IRES sequence of pHIV-Luc-ZsGreen. The pLC-ZsGreen-P2A-Blast plasmid was a gift from Eva Gottwein (RRID: Addgene_123322) (PMID: 29954751) while pHIV-Luc-ZsGreen was a gift from Bryan Welm (RRID: Addgene_39196).

Gene knockouts

Alt-R™ CRISPR-Cas9 sgRNAs (Integrated DNA Technologies) were complexed with Alt-R™ S.p. Cas9 Nuclease V3 (Integrated DNA Technologies). The CD38 gRNA sequence was described previously (PMID: 37171449). The resulting ribonucleoprotein complexes were transfected into the target cells using the Neon Electroporation System (Invitrogen).

DNA or RNA extraction.

Total RNAs or genomic DNA were isolated from whole cells or FFPE sample scrolls using the appropriate Maxwell® RSC simplyRNA Cells, RSC RNA FFPE, RSC Cultured Cells DNA or RSC DNA FFPE Kits (Promega).

Reverse transcription and PCR analysis

To generate cDNA, 500 ng of RNA (via NanoDrop™ 2000) were hybridized with a mixture of Oligod$(T)_{20}$ and random hexamer primers and reverse transcribed with SuperScript™ IV VILO™ Master Mix (ThermosFisher Scientific). Traditional PCR was performed using the Q5® High-Fidelity 2X Master Mix (New England Biolabs). Primers include P2RX5 CDS (1332 bp amplicon on P2RX5FL cDNA; forward: ATGGGGCAGGCGGGCTGCAAGG; reverse: TCACGTGCTCCTGTGGGGCTC) and P2RX5 FLAG-KI (forward:GGTTACCAAGACGTCGACAC; reverse:CGACATCCCAGATCCGCTG). Quantitative PCR (qPCR) was performed using PowerSYBR Green PCR Master Mix (Life Technologies) on an Applied Biosystems Viia7 machine. According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [100], all primers used had efficiencies exceeding 95% and amplified single PCR products from cDNA mixtures, which was then validated via Sanger sequencing. Reference gene primers used include RPL27 (123 bp amplicon on NM_000988; forward: ATCGCCAAGAGATCAAAGATAA; reverse: TCTGAAGACATCCTTATTGACG), GAPDH (227 bp amplicon on NM_002046; forward: GAAGGTGAAGGTCGGAGTC; reverse: GGAAGATGGTGATGGGATTTC) and ACTB (121 bp amplicon on NM_001101.5; forward: GTCATTCCAAATATGAGATGCGT; reverse: GCTATCACCTCCCCTGTGTG) [101,102]. P2RX5 primers: exon-10 skipping (Δ10) (93 bp amplicon; forward: TGATGGTGAACGGCAAGGGT; reverse: CCTCGTACTTCTTGTCACGG), exon-10 inclusion (∇10) (135

bp amplicon; forward: AGTTCAGCATCATTCCCACCA; reverse: CCTCGTACTTCTTGTCACGG) and exon 9 (pan) (55 bp amplicon; forward: ATATTACCGAGACGCAGCCG; reverse: CCCGTAGGCTTTCATCAGGG). Expression levels relative to control were calculated using the 2-ΔCT and 2-ΔΔCT methods. The data were normalized to the CT or RPL27 or when indicated, the mean CT of GAPDH, ACTB and RPL27. DNA contamination was routinely assessed via qPCR of negative control reverse transcription reactions which lacked reverse transcriptase (cycle thresholds above 35).

P2RX5 variant determination

For donor samples and cell lines, both genomic DNA and cDNA was genotyped using predesigned Taqman probes (Thermo Fisher Scientific. C_8728453_10 for rs891746.). RT-qPCR was also performed to determine if pan P2RX5 mRNA was upregulated, and if exon-10 was included or skipped. For cell lines, genomic DNA was also genotyped using PCR amplification followed by Sanger sequencing for rs3215407 (251 bp amplicon; forward: GCCCTCTTTCAGGTAACGCT; reverse: GGAGTGGACAGACACCCTTG) and rs891746 (231 bp amplicon; forward:TGGGGGCCCCGCTGTCCAAC; reverse:TCTTTCTGGGGGCACTGGGACAC).

Splicing minigene assay

The minigene plasmids were electroporated into Raji and OCI-ly8 cells using the Neon System (Invitrogen). Two days later, minigene transcription was analyzed via RT-qPCR and Sanger sequencing using the “minigene primers” ((495 bp amplicon; forward: ATATTACCGAGACGCAGCCG; reverse: GCCAGTCCCCAACGAAATCT)).

Antibody discovery

Anti-P2RX5 monoclonal antibodies were generated at the Fred Hutchinson Antibody Technology Core. Briefly, male and female 20-week-old mice were immunized with NIH/3T3 cells that stably overexpressed P2RX5-FL-FLAG. Following a 12+ week boosting protocol, splenocytes were isolated from high-titer-yielding mice and electrofused with FOX-NY myeloma cells. From the resulting hybridoma clones, two unique monoclonal antibody sequences, 1D9 and 2F4, were reactive to cell-surface P2RX5-FL in flow cytometry assays. 1D9 and 2F4 were produced in serum free media and purified using protein A and protein G columns on AKTA pure HPLC.

Western blotting.

Total cell lysates were prepared in Laemmli Sample Buffer (Bio-Rad) with 50 mM DTT, resolved on a 4–15% precast polyacrylamide gel (Mini-PROTEAN® TGX™, Bio-Rad) in Tris/glycine/SDS running buffer (Bio-Rad), and then transferred onto a PVDF membrane (Millipore Sigma, Immobilon-P, 0.45 µm) via the Mini Trans-Blot® Cell system (Bio-Rad). These membranes were then probed with antibodies against P2RX5 (1D9 clone), FLAG (L5 clone, Biolegend), ACTB (8H10D10 clone, Cell Signaling Technology), GAPDH (14C10 clone, Cell Signaling Technology); followed by horseradish peroxidase–linked secondary antibodies (Cell Signaling Technology). Western blot chemiluminescent signals were captured with a G:Box Chemi XX6 Gel Doc (Syngene).

Protein deglycosylation

Raji and MM.1s cell lysates (in RIPA buffer) were deglycosylated with the Protein Deglycosylation Mix II (P6044, New England Biolabs), under denaturing reaction conditions, according to the manufacturer’s protocol. The Protein Deglycosylation Mix II was replaced with water for the negative control reaction. Experimental Model and Subject Details

Immunohistochemistry (IHC)

Formalin fixed, paraffin embedded (FFPE) section staining was performed on a Bond Max automated staining system (Leica Biosystems). Antigen retrieval was performed for 20 mins with the E2 (CD20 staining) or E1 (P2RX5 staining) retrieval solution (Leica Biosystems). For CD20 staining, we used the Bond Refine polymer staining kit (Leica Biosystems) with 1 hour room temperature primary antibody (clone L26, Dako) incubation. For P2RX5 staining, we used the ImmPRESS Excel Amplified Polymer Staining Kit, Anti-Mouse IgG, Peroxidase (MP-7602-15; Vector Laboratories) with 1 hour room temperature primary antibody (clone 1D9) incubation. Slides were rinsed, dehydrated through a series of ascending concentrations of ethanol and xylene, and then cover slipped. Stained slides were then

digitally scanned at 20x magnification on an Aperio CS-O slide scanner (Leica Biosystems). Representative pictures were taken on scanned IHC slides with Aperio ImageScope program v12.2.2.5015.

Cell culture

HEK293T cells were a kind gift from Dr. Peter Choi (Children's Hospital of Philadelphia, Philadelphia, PA). DoHH2, Ramos, MM.1s, RPMI-8226 and MOLT-16 cells were obtained from the ATCC (American Type Culture Collection). Raji cells were authenticated with short tandem repeat profiling. All cell lines were routinely tested to be Mycoplasma free via EZ-PCR Mycoplasma Detection Kit (Sartorius). DoHH2, Raji, Ramos, MM.1s, RPMI-8226 and MOLT-16 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L L-alanyl-L-glutamine dipeptide at 37°C and 5% CO2. The HEK293T cells were maintained in DMEM medium supplemented with 10% FBS, 2 mmol/L L-alanyl-L-glutamine dipeptide at 37°C and 5% CO2. To generate luciferase-zsGreen reporter cell lines, cells were transduced the pLC-Luc-IRES-zsGreen-P2A -Blast lentiviral vector and cultured in 10 ug/mL Blasticidin for 3 weeks.

LCL generation.

B95-8 cells (ATCC # CRL 1612; 13) were seeded at 3 x 10^5 cells/ml in a 75cm^2 tissue culture flask in complete RPMI 1640. After two days, cells were resuspended in fresh complete RPMI 1640 at 1 x 10^6 cells/ml and stimulated with 20ng/ml tetradecanoyl phorbol acetate (TPA; Sigma-Aldrich) for 1 hour to induce virus production and then resuspended in complete RPMI 1640 for 96 hours before centrifugation at 600 g for 10 min at 4˚C. Cell supernatant was then filtered through a 0.45-micron filter and stored at -80°C. For infection with EBV, 500µl of filtered supernatant containing EBV (B95.8) was added to 5X10^6 PBMCs in 5 ml complete RPMI medium in a 25 cm^2 tissue culture flask and placed upright in a CO_2 incubator at 37°C for 2-4 hours. Subsequently, 10 ml additional medium and cyclosporin A (1µg/ml; Sigma-Aldrich, Inc., St. Louis, MO) was added. On days three and six, nine, and twelve, half of the medium was replaced with fresh complete RPMI with cyclosporin A. After two weeks, stable EBV+LCLs are formed and anti-CD19 staining was used to confirm that the culture is 100% B cells by flow cytometry.

Live-cell flow cytometry

Live cells were incubated in blocking buffer (PBS with 2% FBS, 1 mM EDTA and Human TruStain FcX™ (Biolegend)) for 20 min prior to staining with primary antibodies (i.e.: anti-FLAG, L5 clone; anti-CD19, HiB19 clone; anti-BCMA, 19F2 clone; anti-CD7, clone 4H9 and anti-CD138, clone DL-101; all from Biolegend) for 30 min. When unlabeled 1D9 or 2F4 primary antibodies were used, the blocking buffer was supplemented with 10% (v/v) goat serum (Fisher Scientific, 16-210-064) and an additional 30 min secondary antibody staining with Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 (Invitrogen, A-21235), was performed. For competition assays, the blocking buffer was supplemented with 80 ug/mL of unlabeled 1D9 or 2F4 antibodies. All incubations were at 4ºC. Propidium Iodide (PI) was used to exclude dead cells from analysis. Acquisition was done on a BD Accuri™ C6 Cytometer (BD Biosciences). Manual gating was performed on the FlowJo Software v.10 (BD Biosciences). Median fluorescence intensity (MFI).

Cycloheximide chase assay

Cell lines were transduced with lentiviral particles ORFs for the indicated constructs or an empty vector control followed by 2-week selection with 10 µg/mL Puromycin (Invitrogen).
Cultured Raji cells were treated with 60 µg/mL of cyclohexmide (CHX) (Sigma-Aldrich, C1988-1G)

Manufacturing of CAR T-cells

Healthy primary human T cells were obtained from the Human Immunology Core at the University of Pennsylvania. T-cells were isolated from frozen donor PBMCs using the EasySep™ Human T-Cell Enrichment Kit (STEMCELL Technologies). Isolated T-cells were cultured in ImmunoCult™-XF T Cell Expansion Medium (STEMCELL Technologies) supplemented with 100 IU/ml of recombinant human IL-2 (Preprotech), and activated with ImmunoCult™ Human CD3/CD28 T Cell Activator (STEMCELL Technologies). Lentiviral transduction was performed the next day. The number viable cells in culture was assessed every 2 to 3 days using a Countess 3 Automated Cell Counter with tryphan blue staining and the number of population doublings was calculated. At day-7 post CD3/CD28 activation, T-cells were

cryopreserved. The % of CAR positive T-cells was monitored via flow cytometry analysis upon staining with the MonoRab™ Rabbit Anti-scFv Cocktail [iFluor 647] (Genscript).
For the αP2RX5-28z/-BBz CAR T cells and ciltacabtagene autoleucel used in the intraosseous RPMI-8226 mouse xenografts models, the CAR T cells were generated as previously described [72]. Briefly, CD4+ and CD8+ cells were combined at a 1:1 ratio and activated using anti-human CD3/CD28 Dynabeads (Invitrogen, #40203D) at a 3:1 ratio. 24 hours (day 1) post activation, T cells were infected with lentiviral vectors containing the CAR transgenes (multiplicity of infection ~1.5 viral particles / T cell). Magnetic beads were removed from T cells on day 5.

Manufacturing and quantification of BiTEs

HEK293T cells that stably secrete the αP2RX5-αCD3 BiTE were generated via lentiviral transduction followed by selection with 10 µg/mL Puromycin (Invitrogen). These HEK293T-αP2RX5-αCD3 cells were expanded to confluence and left in culture for 5 days before the BiTE-containing media was concentrate with Amicon Ultra Centrifugal Filter, 50 kDa MWCO (Sigma Millipore, UFC9050). The size and concentration of the αP2RX5-αCD3 BiTE was assessed via western blotting with an anti-His-Tag antibody (Cell Signaling Technology, D3I1O clone) and a standard curve generated from Blinatumomab (MedChem Express, HY-P9963).

In vitro co-culture assays

Target luciferase reporter cells were co-cultured with expanded healthy donor T cells at 9-days post CD3/CD28 activation in the indicated effector CAR T cell or T cell to target cell (E:T) ratios for 1 or 4 days before luciferase activity was monitored with the ONE-Glo™ EX Luciferase Assay System (Promega) and a FLUOstar Microplate Reader (BMG Labtech), as a proxy for cell viability. Target cell viability and specific cytotoxicity values (%) were normalized to mock transduced T cells or T cells without bispecifc (NT) controls. In the 5-day co-culture assays, expanded T cells at day-12 post CD3/CD28 activation were co-culture with target cancer cells. Viable cell counts were determined via live-cell flow cytometry, and normalized with CountBright™ Absolute Counting Beads (Thermo Fisher Scientific). Propidium Iodide (PI) was used to exclude dead cells from analysis. APC-CD7 (Biolegend, clone 4H9), iFluor647-scFv (Genscript) and FITC-CD22 (Biolegend, clone HIB22) staining was performed to distinguish T cells (CD7+CD22-) or effector CAR T cells (scFv+CD22-) from Raji or LCL cells (CD7-CD22+).

Cell-to-cell avidity assays

Avidity was measured using the Lumicks Z-Movi assay. Z-Movi-compatible acoustofluidic chips were coated with poly-L-lysine for 3 h prior to attaching a monolayer of RPMI-8226 cells. CellTrace far-red labeled CAR T cells were allowed to incubate on the monolayer of RPMI-8226 for 10 min, and then a ramping acoustic force was applied. Cell-detachment was analyzed using ImageJ and R. Avidity experiments were conducted according to the manufacturer's (LUMICKS™) instructions.

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents may be directed to and will be fulfilled by the lead contact Andrei Thomas-Tikhonenko (andreit@pennmedicine.upenn.edu).

Materials availability

- The murine hybridomas will be available upon request following publication of the patent application.

Data and code availability

- Controlled access GTEx data were retrieved through dbGaP Study phs000424.v10.p2. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health.
- This paper does not report any original code.
- Microphotographs and any additional information required to reanalyze the data reported in this paper are available upon request from the lead contact.

AUTHOR CONTRIBUTIONS

Conceptualization: Z.A. and A.T.T. Data curation and formal analysis: Z.A., A.C., L.P., C.S., K.E.H., F.S., M.T., D.M., P.P. and V.P. Funding acquisition: A.T.T. Investigation and methodology: Z.A., A.C., L.P., C.S., F.S., S.S., M.T., C.K., P.K.S., K.J., P.S., D.M. and P.P. Resources: Z.S.H., S.S., P.J.K, J.F., J.B.S, J.L.R, D.T.V., P.M.L. and D.A. Supervision: M.R. and A.T.T. Writing – original draft: Z.A. and A.T.T. Writing – review & editing: Z.A. and A.T.T.

DECLARATION OF INTERESTS

Z.A. and ATT are listed as co-Inventors on the patent application PCT/US2025/046721 “Antibodies and Chimeric Antigen Receptors binding to P2RX5 and Methods of Use for Treating Cancers.”

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES

During the preparation of this work, no generative AI or AI-assisted technologies have been used.

SUPPLEMENTAL INFORMATION - Supplemental Figures S1-S6

FIGURE TITLES AND LEGENDS

Figure 1. P2RX5 mRNA is selectively expressed in mature B cells and hematologic malignancies.

(A) Heatmap of P2RX5 transcript levels, expressed as normalized transcripts per million or nTPM, in various cell types (*y-axis*) identified from scRNA-seq of multiple human tissues (*x*-axis). Larger circle sizes and darker colors indicate higher P2RX5 nTPM values. Data from the Human Protein Atlas (HPA, version 24).

(B and C) P2RX5 transcript levels (TPM) in various human immune cell subsets. Cells were isolated from the peripheral blood mononuclear cell (PBMC) fraction and bone marrow via fluorescence-activated cell sorting (FACS) prior to RNA-seq.

(D) Plotted RSEM expected counts for P2RX5 in TCGA DLBCL and TARGET ALL patient samples as well as in GTEx normal tissues. Shown are pre-compiled bulk RNA-seq data from the UCSC Xena portal.

(E) P2RX5 expression levels in the collated microarray data of ~10,000 normal and neoplastic leukocyte samples across all lineages, as published on the HEMAP portal.

Figure 2. Four-fifths of the African population with the ancestral haplotype express the full-length P2RX5 protein variant (P2RX5-FL).

(A) TOP: MANE annotations of exon-intron structure of human *P2RX5*. MIDDLE: Sashimi plots showing read densities across all *P2RX5* exons in Raji and Reh cells. Arcs that connect exons represent splice junctions. BOTTOM: Nucleotide changes corresponding to rs3215407 indel and rs891746 SNP.

(B) LEFT: P2RX5 protein variants that arise due to the rs3215407 indel, the rs891746 SNP, and alternative splicing of exon 3. RIGHT: Ribbon diagram of P2RX5-FL protein structure, as predicted by AlphaFold 2. Amino acids encoded by exons 3 and 10 are highlighted in green and blue, respectively.

(C) *P2RX5* variant expression in a selection of cell lines, based on RNA-seq data. The genotype (i.e., rs3215407 and rs891746 alleles), predominant variants detected, expression levels (TPM) and exon-exon junction count per million (JPM) are shown. Exon9-exon11 junction reads are reflective of exon10 skipping while exon10-exon11 junction reads are reflective of exon10 inclusion.

(D) Normalized P2RX5 exon10 inclusion ratios in spleen (y-axis), plotted against the corresponding *rs891746* alleles (x-axis). Adapted from the GTEX portal.

(E) Splicing minigene assay. TOP: Design of minigene expression vectors containing the sequence of *P2RX5* from exons 9 to 11, with truncated introns and either the rs891746 A- or C-alleles. PCR primer bindings sites are shown in green. BOTTOM: RT-PCR analysis of Raji and OCI-ly8 cell mRNA, after transfection with the minigene expression vectors or a GFP control. Control PCRs used the minigene expression plasmids as templates. Amplicons were separated by agarose gel electrophoresis and stained with EtBr.

(F) Aggregate allele frequencies for the rs3215407 indel and rs891746 SNP across populations, as reflected in the NCBI SNP database (ALFA Project Release 4).

(G) Table displaying haplotype counts and allele frequencies for rs3215407 and rs891746 across all 1000 Genomes Project populations, accessed via LDlink 5.8.0 release. Statistics are shown to the right.

(H) P2RX5 transcript levels (TPM) in MM patients of African ancestry (MMRF CoMMpass cohort) and endemic Burkitts lymphoma (eBL) patients from Africa, based on RNA-seq analysis. Samples were stratified according to the P2RX5 variants expressed.

(I) Overall survival of MM patients with African ancestry (RIGHT), stratified based on their P2RX5 variant (LEFT). Data from the MMRF CoMMpass study.

Figure 3. The P2RX5-FL protein has a longer half-life and is detectable in normal and malignant B cells with the 1D9 monoclonal antibody.

(A) Immunoblots of various cell line lysates using the anti-P2RX5 mAb 1D9. The predominant P2RX5 variant in each cell line is noted in the blue brackets.

(B) P2RX5 expression in Raji cells upon transduction with lentiviral expression vectors for the indicated P2RX5 variants or an empty vector negative control. LEFT: P2RX5 mRNA levels via RT-qPCR normalized to negative control cells. Primer binding sites are shown to the right of the graphs. RIGHT:

Immunoblots of P2RX5 protein levels using the 1D9 mAb. The FL-overexpression lane contains 90% less total cellular protein relative to other lanes to avoid signal saturation. Shown in blue are the calculated molecular weights (MW) of each overexpressed variant based on amino acid sequence alone.

(C) Flow cytometric analysis of GFP or P2RX5(Δ10 or FL)-GFP fusion protein levels in Raji cells at various time points following cycloheximide treatment.

(D, E and F) IHC staining for CD20 and P2RX5 (using 1D9 mAb), as indicated by brown DAB chromogen staining, on FFPE sections of normal lymph nodes, spleen and skeletal muscles **(D)**, Raji-WT and P2RX5 KO mouse xenografts **(E)**, as well as BCL tumor samples **(F).** Hematoxylin nuclear counterstaining in blue. * Sample P2RX5-FL, -Δ10 or -ΔC variant, as determined by RT-qPCR and Taqman SNP genotyping assays.

Figure 4. B-cell lymphoma, MM, and T-ALL samples stain positive for cell-surface P2RX5-FL with the 1D9 and 2F7 monoclonal antibodies.

(A) AlphaFold prediction-based ribbon diagram of the P2RX5-FL structure, with FLAG-tag insertion site highlighted in red.

(B) Analysis of Raji and Ramos cells after transduction to overexpress P2RX5 variants with extracellular FLAG tags. TOP: RT-qPCR analysis of pan-P2RX5 mRNA levels, using primers for the constitutive exon 9. Each bar represents a replicate cell sample. BOTTOM: Cell-surface FLAG expression via live-cell flow cytometry. Data from two replicate experiments are shown.

(C) Knock-in of an extracellular FLAG-tag into the *P2RX5* gene (FLAG-KI). TOP: RT-PCR analysis of mRNA from unmodified Raji cells with wild-type *P2RX5* (P2RX5WT) or Raji FLAG-KI cells. PCR amplicons were separated by agarose gel electrophoresis and stained with EtBr. The PCR primer binding sites (which flank the FLAG insertion site) and the expected amplicon sizes are shown to the top-right. BOTTOM: Live-cell flow cytometry analysis of cell-surface FLAG expression in Raji P2RX5WT and FLAG-KI cells, as well as Raji FLAG-KI cells that had undergone *P2RX5* gene knockout (FLAG-KI + P2RX5KO).

(D, E, F, G, H, I and J) Detection of cell-surface P2RX5 via live-cell flow cytometry. The indicated cell lines were stained with unlabeled 1D9 or 2F4 mAbs followed by indirect staining with an Alexa647-labeled secondary antibody **(D)**. In competition assays, Raji and Daudi cells were pre-incubated with unlabeled 1D9, 2F4 or no blocking antibody; followed by staining with Alexa627-labeled 1D9 or 2F4 **(E).** For the antibody titration curves, parental and P2RX5KO Raji cells were stained with unlabeled 1D9 or 2F4 mAbs at the indicated concentrations prior to indirect staining with fixed concentrations of Alexa647-labelled secondary antibody **(F)**. Data from two replicate experiments are shown, with the EC50 values calculated from the median fluorescence intensities (MFI). To test antibody cross-reactivity, HEK23T cells were transfected to express FLAG-tagged P2RX1-to-7 before staining with fluorophore-labelled anti-FLAG, 1D9 and 2F4 mAbs **(G)**. The cell-surface antigen densities on cell lines, as quantified using an Antibody Binding Capacity (ABC) assay paired with fluorophore-labelled 1D9 (anti-P2RX5), anti-CD19 or anti-BCMA mAbs **(H)**. Each peak/spot represents a replicate experiment with the average density shown **(H)**. Bone marrow aspirates from patients with T-ALL **(I)** or MM **(J)**, after staining for P2RX5 with Alexa647-labeled 1D9 or 2F4. Neoplastic cell fractions were identified by positive staining for CD7 **(I)** or CD138 **(J)**, respectively. MM.1s and Raji cell lines after 3-day treatment with the indicated concentrations of Dexamethasone **(K)**.

Figure 5. The 2F4-scFv-equipped bispecific, αP2RX5-αCD3, redirects T cells to lyse P2RX5-positive neoplasms.

(A) The design of αP2RX5-αCD3.

(B) Outline of ex vivo assays, performed in panels C, D and E, to evaluate αP2RX5-αCD3 activity.

(C) Flow cytometric analysis of viable T- and Raji cell counts, 5-days after the addition of the αP2RX5-αCD3 BiTE to cultures of T cells alone or in co-culture with Raji-CD19KO cells at a 2:1 ratio. Data from P2RX5 (ΔC/ΔC, Δ10/Δ10 and FL/FL)-genotyped T cells are shown, each represented by a different datapoint shape and color. Cell counts normalized to 'no BiTE treatment' (NT) controls.

(D) Target cell viability after 1-day treatment with αP2RX5-αCD3 or blinatumomab BiTEs at 0.01 pM concentration (rar graphs on the left) or across a range of concentrations (dose response curves on the

right). Cell viabilities are normalized to the NT controls. Data from 2 different T-cell donors are shown and used to calculate the average EC50 value.

(E) Target MM.1s cell viability, after 1-day in co-culture with T cells and the indicated concentration of αP2RX5-αCD3. The MM.1s cells were pretreated for 3-days with the indicated concentration of dexamethasone (DEX) prior to DEX washout and co-culture. Cell viabilities are normalized to the NT controls.

(F) Design of αCD19-28z.αP2RX5-αCD3 construct. Transduction with this construct leads to the generation of CD19-directed CAR T cells that secrete the αP2RX5-αCD3 BiTE.

(G) Outline of ex vivo assays, performed in panels H and I, to evaluate αCD19-28z.αP2RX5-αCD3 CAR T-cell activity.

(H and I) Mock, αCD19-28z CAR or αCD19-28z.αP2RX5-αCD3 CAR T cells co-cultured with the indicated cell lines at the effector CAR T-cell to target Raji cell (E:T) ratio of 1:1. After 5 days, viable cell counts were assessed via flow cytometry **(H)**. After 1 day, target Raji cell viability was assessed via the luciferase reporter **(I)**. T-cell counts were normalized to control cultures of the same T cells alone (without Raji cells). Raji cell viabilities were normalized to the mock transduced T-cell co-cultures. Data from 2 different T-cell donors are shown.

(J) Bioluminescent imaging of NSG mice after IV infusion with luc+ Raji CD19KO cells followed by $3x10^5$ mock transduced, αP2RX5-αCD3, or αCD19-28z.αP2RX5-αCD3 CAR T cells per mouse.

Figure 6. 1D9-scFv CAR-armed T cells display potent cytolytic activity towards P2RX5-positive neoplastic cells.

(A) Design of 1D9 scFv-based CARs (i.e.: αP2RX5-28z and -BBz).

(B) Representative transduction efficiency of the 1D9-scFv-based CARs into T cells, at 9 days post-transduction, via flow cytometric analysis of cell-surface scFv positivity.

(C) Population doublings of P2RX5-FL/FL-genotype T cells after transduction with lentivirus expression vectors for either the αP2RX5-28z, αP2RX5-BBz, or αCD19-28z CARs. T cells were activated with soluble anti-CD3 and -CD28, one day before transduction.

(D) Cell avidity assays. T-cell detachment from adhered RPMI-8226 targets cells, plotted against force in pN.

(E) Outline of ex vivo assays, performed in panels F, G, H and I to evaluate αP2RX5-28z or -BBz CAR T-cell activity.

(F) Flow cytometric analysis of viable cell counts, after 5-day co-culture of effector T cells (mock transduced, αP2RX5-28z, or αP2RX5-BBz CAR T cells) with LCLs generated from the same P2RX5-FL/FL-genotyped donor. The effector T-cell counts were normalized to control cultures of T cells alone (without target LCL), while LCL counts were normalized to the mock transduced T-cell co-cultures.

(G, H and I) Viability of the indicated luc+ target cells, after 4- **(G)** or 1-day **(H and I)** in co-culture with αP2RX5-28z, αP2RX5-BBz, or αCD19-28z CAR T cells. T cells from P2RX5-FL/FL, -Δ10/Δ10 and -ΔC/ΔC donors were tested **(G)**. In panel **(I)**, MM.1s cells were pretreated for 3-days with the indicated concentration of dexamethasone (DEX), before DEX wash-out and co-culture with αP2RX5-BBz CAR T cells.

(J-M) Testing 1D9-scFv-equipped CAR T cells in a mouse xenograft model of MM. As outlined in panel **(J)**, NSG mice were implanted intratibially with luc+ RPMI-8226 cells several days prior to IV infusion with mock, αP2RX5-28z, αP2RX5-BBz, or αP2RX5-BBz CAR T cells (0.8×10^6/mouse). Disease course was assessed by following T-cell persistence in the peripheral blood **(K),** tumor progression as measured by bioluminescent imaging **(L),** and overall survival **(M)**.

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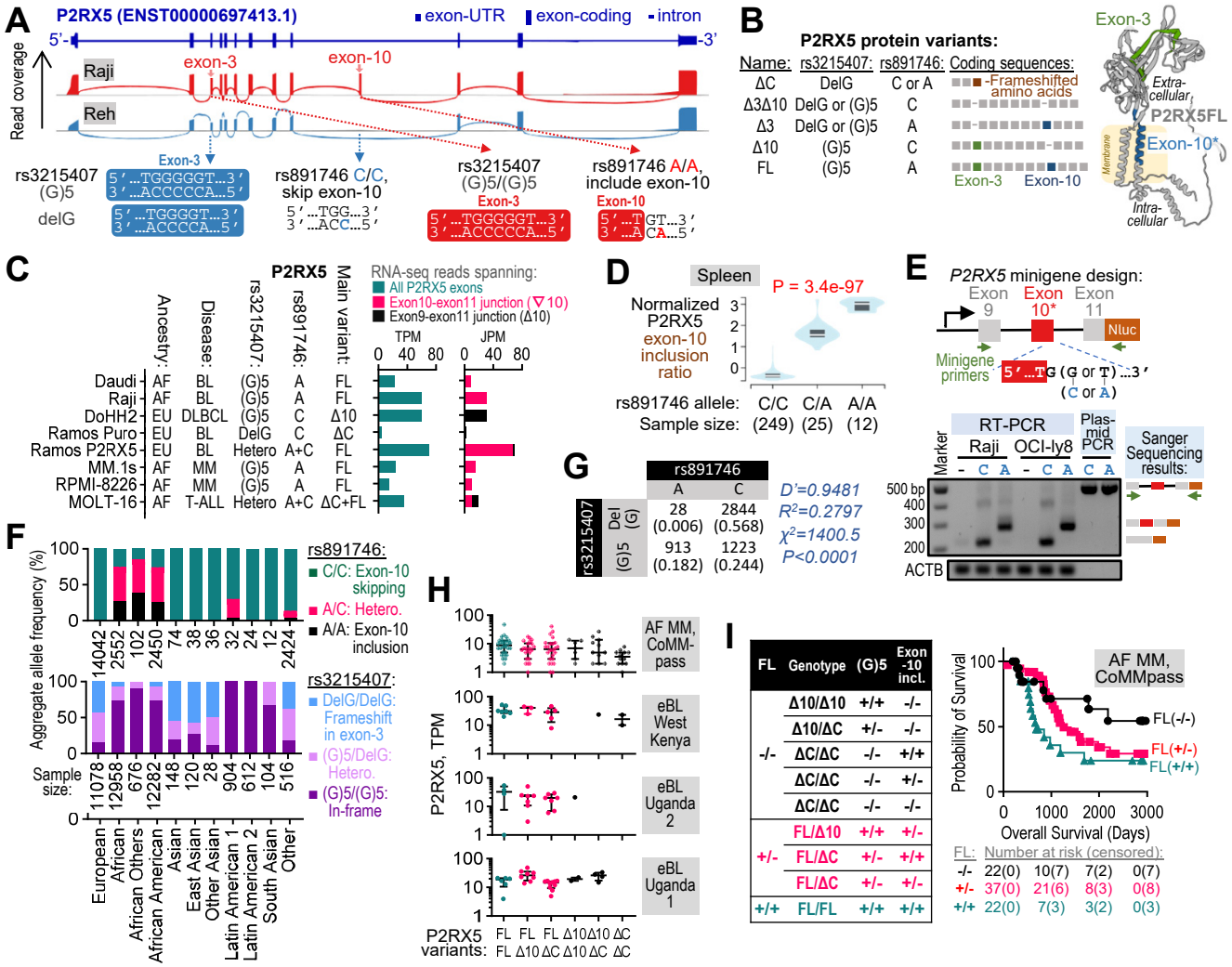
Ang et al, Figure 1



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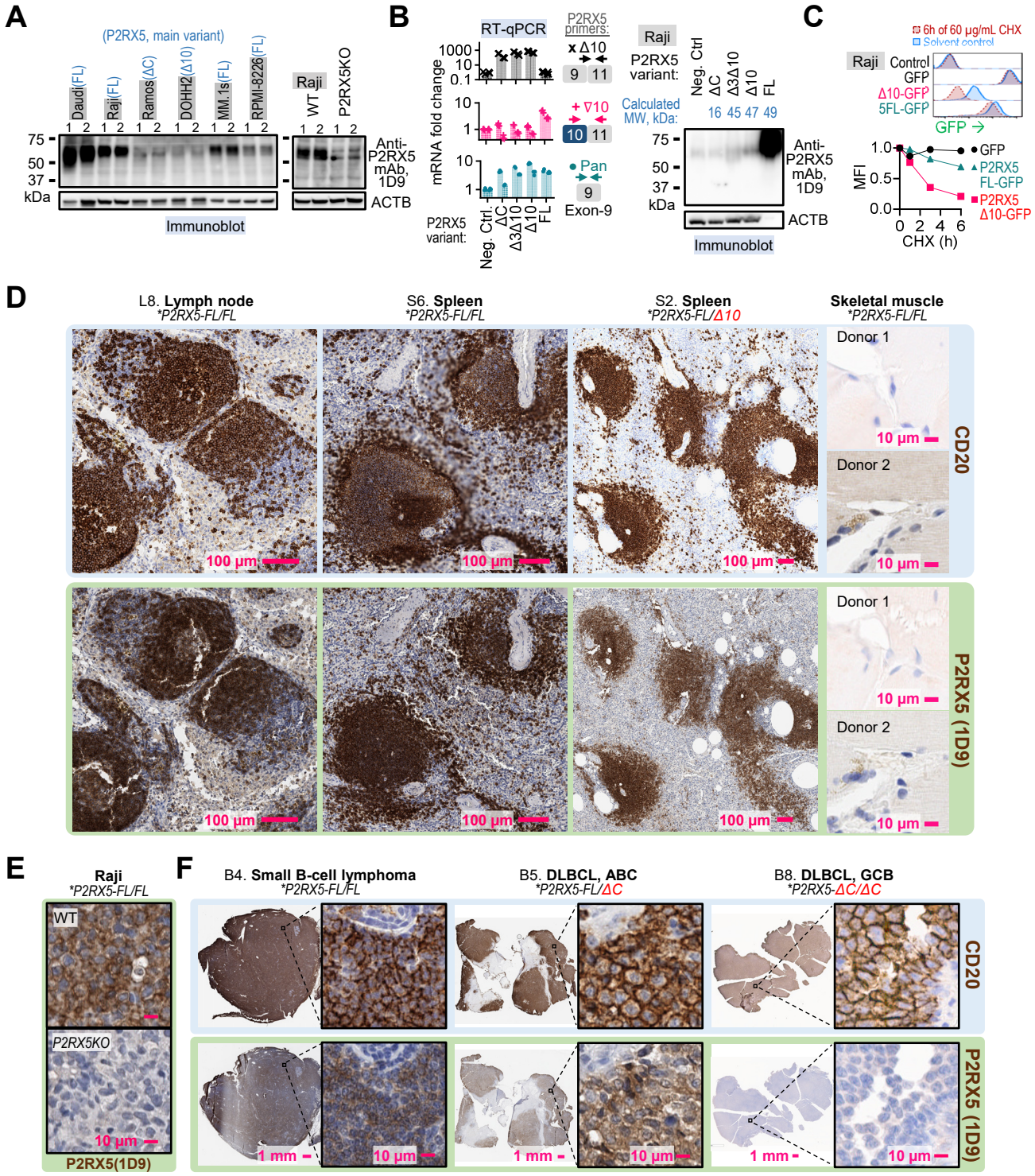

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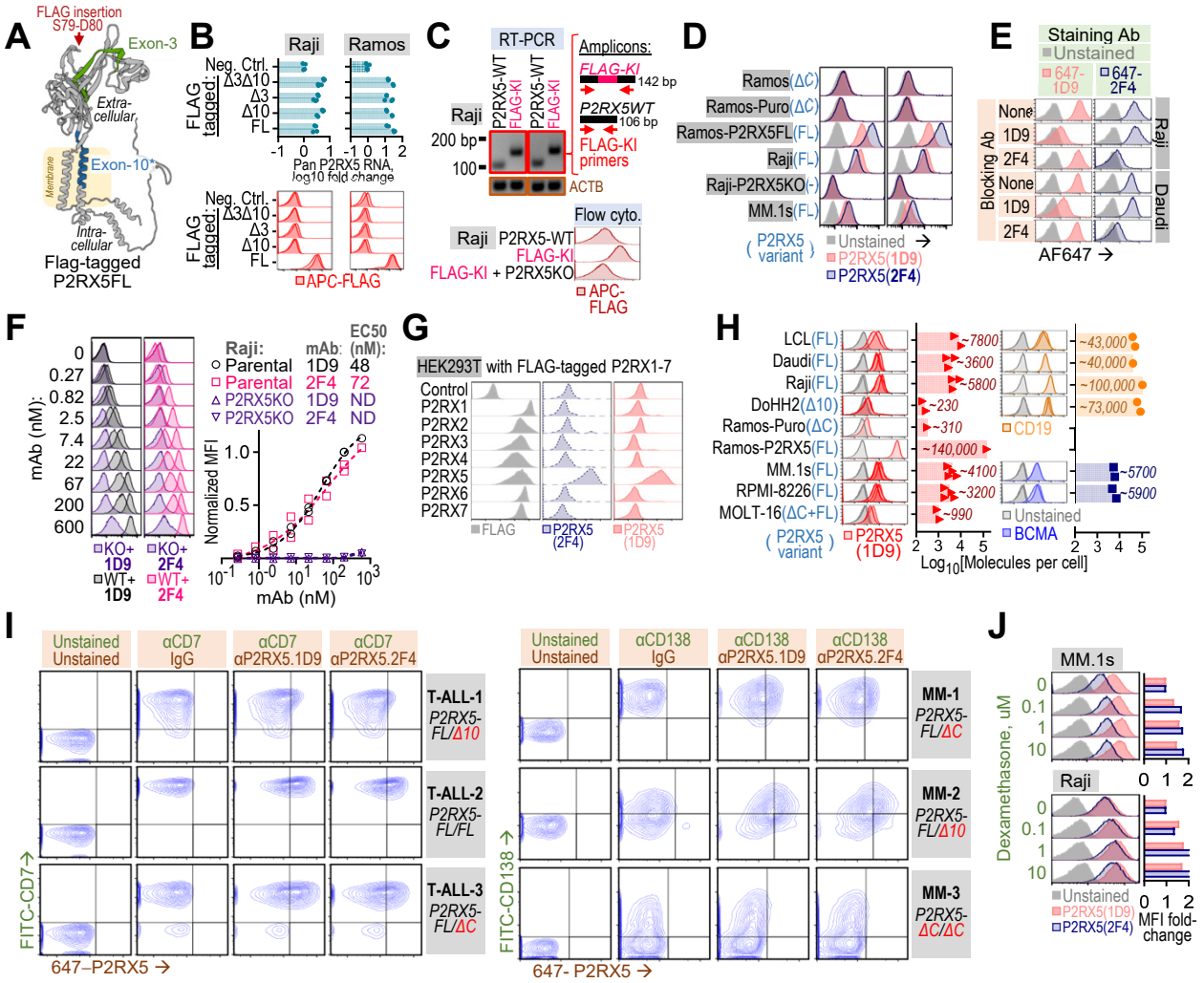

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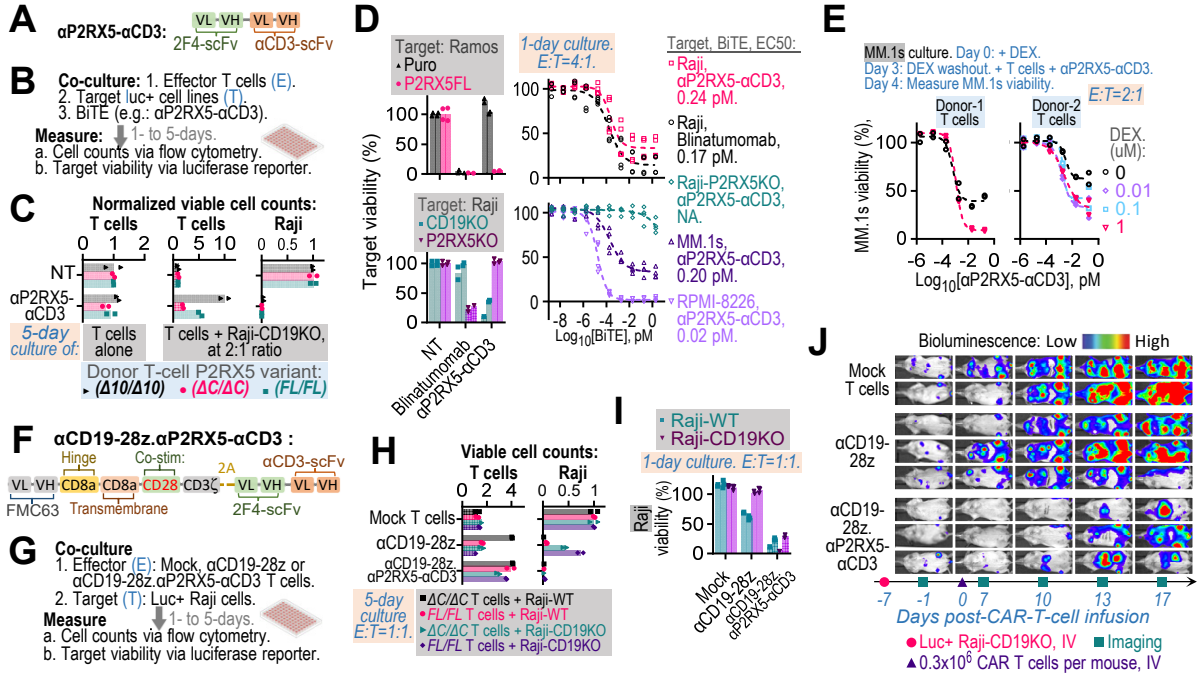

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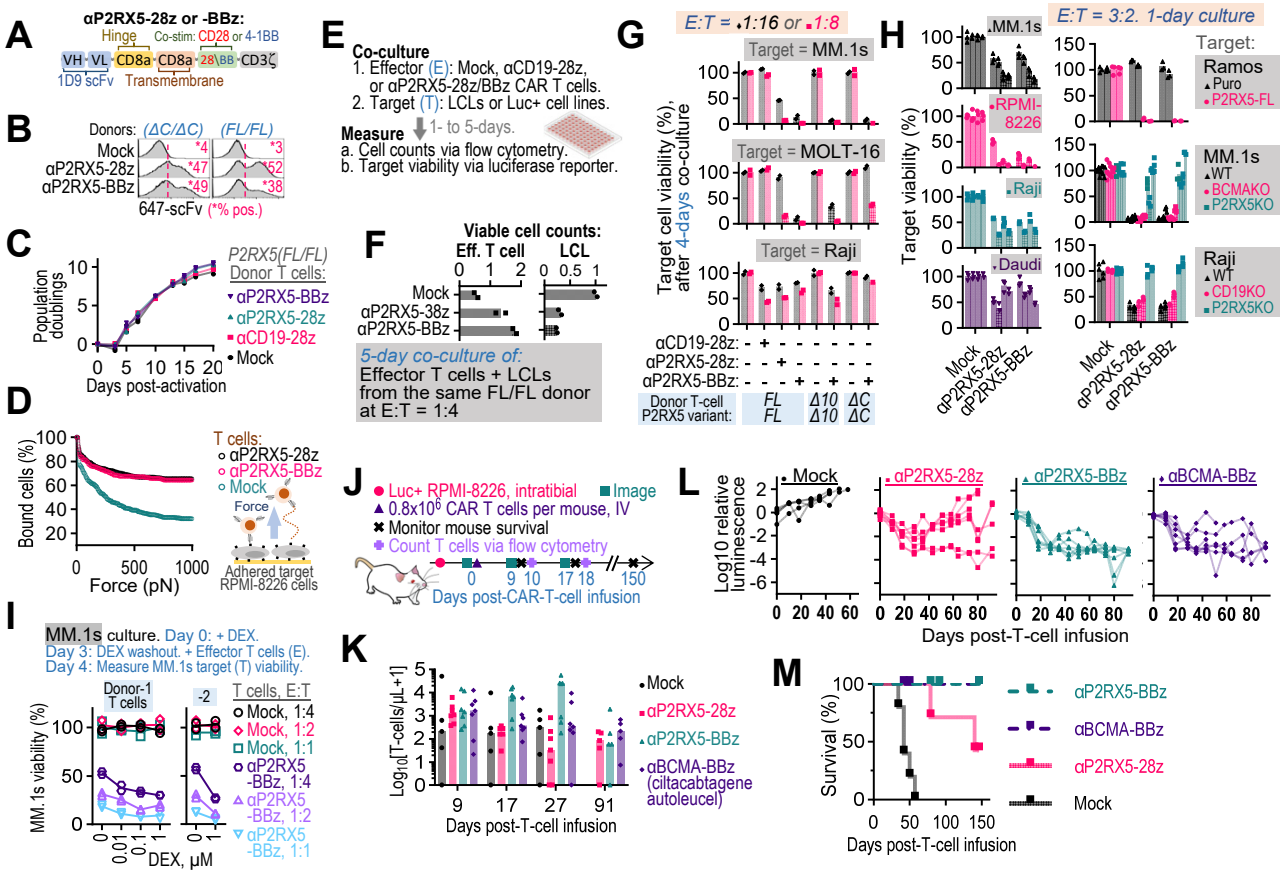

Ang et al, Figure 6