Discovery and Humanization of High Affinity Therapeutic Antibodies Against Transmembrane Proteins

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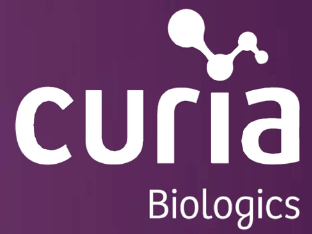


ABSTRACT

Therapeutic antibodies targeting type 1 transmembrane proteins (T1P) are among the most successful biologic drugs yet developed. Monoclonal antibody OKT3, which targets T cell-expressed T1P CD3, became the first approved antibody therapeutic in 1986 and was used to prevent graft rejection. Blinatumomab, which binds CD3 as well as CD19, was the first approved bispecific T cell engager (BiTE, approved in 2014) and is used to treat leukemia. Nivolumab and Pembrolizumab (both approved in 2014) were the first immune checkpoint antagonist mAbs to target T1P PD-1 and are used to treat various cancers. For the next generation of therapeutic mAbs targeting transmembrane proteins, a reliable and robust mAb discovery and humanization workflow is paramount. Here we describe an effective hybridoma-based antibody discovery and humanization approach for a T1P target using PentaMice® immunization to obtain mAbs with picomolar binding potency and affinity and human/pig/cyno (preclinical species) cross-reactivity.

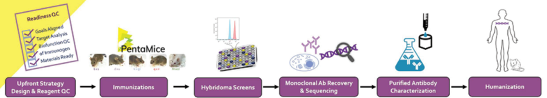
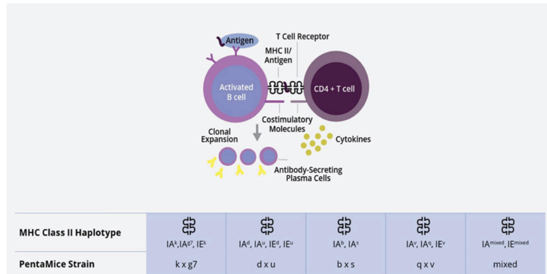


Figure 1. Curia's reliable and robust hybridoma-based mAb discovery workflow. An immunization and screening strategy is designed based on the desired features of the discovered mAbs. Highly immunologically diverse PentaMice strains are immunized and in-life plasma titer checks are used to monitor antibody generation and inform subsequent booster injections. Once sufficient titers are reached, the B cells are harvested and fused with a high efficiency myeloma partner to generate hybridomas, which are typically plated and screened at a scale of at least 10 x 384-well plates. The variable heavy and light chains sequences are obtained for the leads, and additional characterization (e.g. kinetics, functional neutralization) is performed on purified mAbs. The top therapeutic candidates are then humanized.

BACKGROUND

Membrane proteins are the targets of over 60% of current approved drugs and comprise ~23% of the human proteome. Type 1 transmembrane proteins (T1P) represent a large group of 1,412 membrane proteins that are characterized as having an extracellular amino-terminus, single transmembrane-spanning helix and intracellular carboxyl-terminus. The extracellular domain (ECD) functions to sense the cellular microenvironment and to act as a signaling receiver or transmitter to enable cell-cell communication. Given the role of T1P in numerous physiological processes and their accessible ECD, T1P are prime targets for antibody therapeutics.

Most therapeutic antibodies on the market today were derived from hybridomas. The conventional approach of using a single inbred mouse strain for immunization fails to generate the diversity and antibody titers needed to maximize the discovery of high-quality leads. The PentaMice® platform is a collection of five WT mouse strains bred for increased MHC II diversity (**Fig. 2**). B cells use MHC II to present target antigen peptides to T cells, activating them and causing them to express co-stimulatory molecules and secrete cytokines. These signals converge to stimulate clonal B cell amplification and high affinity antibody production. Maximizing this response requires CD4 T cell help, which is driven by T cell recognition of peptides presented by MHC II (**Fig. 2**). Whereas only a single MHC II molecule is possible with certain inbred strains like C57Bl/6, 42 MHC II molecules are possible with the PentaMice platform. This 42-fold increase in MHC II diversity enables a greater diversity of peptides to be presented by B cell to T cells during immunization, increasing the probability of a robust antibody response and high-quality lead generation.



MHC Class II Haplotype	IA^k, IA^{g7}, IE^k	IA^d, IA^u, IE^d, IE^u	IA^b, IA^s	IA^v, IA^q, IE^v	IA^{mixed}, IE^{mixed}
PentaMice Strain	k x g7	d x u	b x s	q x v	mixed

Figure 2. The concept behind the PentaMice platform. Strong interactions between T and B cells during immunization are essential to obtaining high antibody plasma titers. Because there are strain-dependent differences in plasma titers for most targets, using several mice representing different MHC II haplotypes improves the chances for maximal engagement of T cell help and, consequently, high affinity antibody generation.

RESULTS

Desirable features for T1P target X (T1P-X) binding mAbs included (i) human/pig/cyno cross-reactivity; (ii) no cross-reactivity with related T1P-X family member T1P-Y; (iii) < single-digit nanomolar binding affinity, and (iv) binding to a specific subdomain in the ECD that could be modeled by a short 18 residue peptide. To achieve these goals, we immunized 2 cohorts of mice from our PentaMice platform, one with huT1P-X ECD (Cohort 1) and one with huPep18-KLH mixed with pigPep18-KLH (Cohort 2). Mice from both cohorts developed strong plasma titers (Fig. 3).

Immune cells were harvested, hybridomas were generated and plated, and the supernatants (supes) were screened by ELISA for binding to huT1P-X-ECD-HIS, huT1P-X-Fc, huPep18-BSA, pigPep18-BSA, huT1P-Y ECD, and an irrelevant HIS-tagged protein (Fig. 4). A diverse set of binding profiles was observed. 44/55 hybridoma supes showed binding to huT1P-X and hu/pigPep18 with five different binding profiles. Twelve supes had optimal features (Profile 1) (Fig. 4).

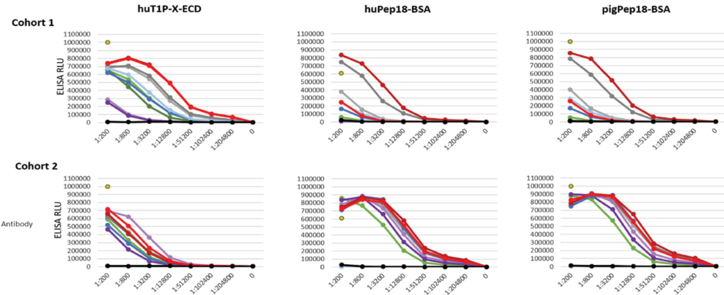
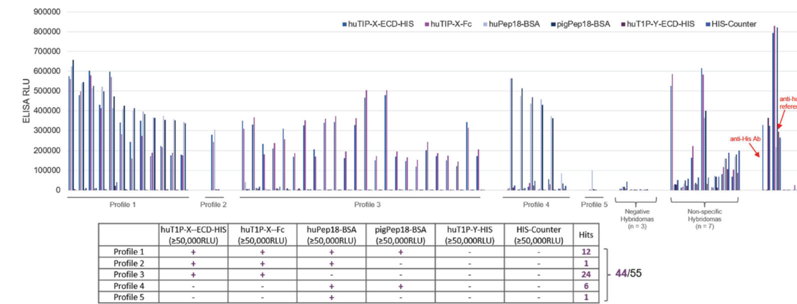


Figure 3. In-life plasma titer checks guide selection of mice with highest target T1P-X titer. Plasma was collected on Day 24 and tested at the indicated dilutions by ELISA [plates coated with huT1P-X ECD (1 ug/ml), huPep18-BSA or pigPep18-BSA (5 ug/ml); secondary anti-mouse IgG-HRP (1:4,000 dilution) and chemiluminescence substrate used for detection]. In Cohort 1 mice immunized with huT1P-X ECD there were clear strain-dependent differences in titer: the q x v and b x s strains have the highest T1P-X ECD titers, while k x g7 was a particularly poor responder. q x v and b x s mice also developed good titers against the domain of interest, huPep18 ,with notable pigPep18 binding. In Cohort 2 mice immunized with huPep18-KLH and pigPep18-KLH, strong titers developed against both hu/pigPep18-BSA largely independent of strain, with some titer against the larger huT1P-X-ECD.

RESULTS (continued)

Off-rate ranking by biolayer interferometry (BLI, Octet® system) was used to identify antibodies with a desirable slow rate of dissociation. 33/55 hybridoma supes showed sufficient response rates to indicate binding to huT1P-X-Fc. The dissociation constant k_{diss} values ranged from 1.2×10^{-4} to 4.9×10^{-3} sec^{-1} (**Fig. 5**).

Ultimately, five hybridomas were selected for single cell cloning, variable heavy and light chain (VH/VL) sequencing and mAb purification from saturated hybridoma supes. Highly potent antibodies were discovered, with ELISA binding EC_{50} ranging from 0.4 to 23.5 nM for huT1P-X ; and from 0.3 to 26.9 nM for huPep18 (**Fig. 6**).



	huT1P-X--ECD-HIS (≥50,000RLU)	huT1P-X--Fc (≥50,000RLU)	huPep18-BSA (≥50,000RLU)	pigPep18-BSA (≥50,000RLU)	huT1P-Y-HIS (≥50,000RLU)	HIS-Counter (≥50,000RLU)	Hits
Profile 1	+	+	+	+	-	-	12
Profile 2	+	+	+	-	-	-	1
Profile 3	+	+	-	-	-	-	24
Profile 4	-	-	+	+	-	-	6
Profile 5	-	-	+	-	-	-	1

44/55

Figure 4. Diverse binding profiles identified against huT1P-X and hu/pigPep18 by ELISA. Strong binding was detected by ELISA for 12 hybridoma supes against huT1P-X-ECD (both HIS and Fc-tagged versions), both human and pig Pep18-BSA, and were clean against family member huT1P-Y-ECD-HIS and an irrelevant HIS-counterscreening control protein. These supes comprise Profile 1, which is the desired binding profile. Other supes were ble to bind huT1P-X and huPep18 but not pigPep18 (Profile 2), or just huT1P-X and not Pep18 (Profile 3), or Pep18 but not huT1P-X (Profiles 4,5). Binding of anti-HIS and anti-T1P-X reference mAb controls were as expected.

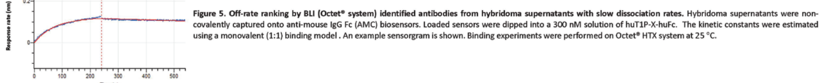


Figure 5. Off-rate ranking by BLI (Octet® system) identified antibodies from hybridoma supernatants with slow dissociation rates. Hybridoma supernatants were non-covalently captured onto anti-mouse IgG Fc (AMC) biosensors. Loaded sensors were dipped into a 300 nM solution of huT1P-X-huFc. The kinetic constants were estimated using a monovalent (1:1) binding model . An example sensorgram is shown. Binding experiments were performed on Octet® HTX system at 25 °C.

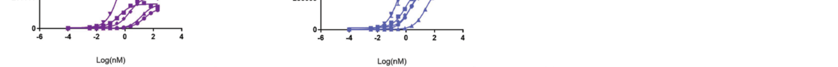


Figure 6. Discovery of potent mAbs that bind to both T1P-X and Pep18. Binding EC_{50} was determined by ELISA, and sigmoid curve fitting (non-linear regression) to estimate EC50s was performed using GraphPad Prism® software.

Antibodies were then selected for humanization. Humanization was performed by (i) homology modeling of the mAb 3D structure; (ii) identification of key positions supporting the CDR loop structure and VH-VL interface (changes to these positions were avoided if possible); and (iii) mutating residues *in silico* to achieve maximal T20 "humanness" scores (algorithm developed by Curia). The top 3 candidate humanized VH and VL chains were then recombinantly produced in our TunaCHO℠ platform as huIgG1 mAbs in all 9 combinations and tested by BLI for binding to human, pig, and cyno T1P-X. Very high affinity humanized mAbs were identified, with K_D below the limit of detection by BLI (≤1 pM) (**Fig. 7**). mAb5 binding to huT1P-X had an on-rate K_a of 1.2×10^5 $M^{-1}s^{-1}$, but a very slow off rate k_{diss} $<1 \times 10^{-7}$ s^{-1}, and remained bound for at least 600 s.

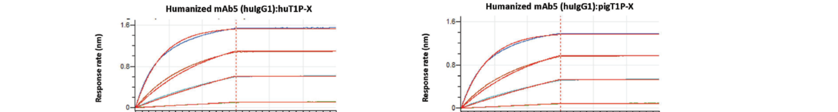


Figure 7. High affinity humanized antibodies identified by BLI. huT1P-HIS (*left*) and pigT1P-X (*right*) was loaded onto anti-penta-HIS (HIS1K) biosensors. Loaded sensors were dipped into a serial dilution of humanized huIgG1 formatted mAb5 (111.1, 37, 12.3, 1.4 nM). Kinetic constants were calculated using a monovalent (1:1) binding model.

CONCLUSIONS

Type 1 transmembrane proteins have a proven track record as highly successful targets for therapeutic antibodies. A reliable and robust mAb discovery workflow is needed to supply the next generation of T1P-targeted antibodies. A strong discovery strategy focused on the desired antibody features, PentaMice immunizations for maximum diversity, hybridoma-based antibody recovery, supe screening, VH/VL sequencing, and humanization provides a fundamentally sound pathway for success.

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