# The self-peptide repertoire plays a critical role in transplant tolerance induction

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## Conflicts of Interest

E.T.S., M.P-H., A.F.S. (University of Sydney) and N.A.M., A.W.P., P.F., N.L.D. (Monash University) are named as co-inventors in a patent application filed by the University of Sydney and Monash University (PCT/AU2020/051221) covering the identification and use of certain peptides described in the publication.

**Abstract**

While direct allorecognition underpins both solid organ allograft rejection and tolerance induction, the specific molecular targets of most directly-alloreactive $CD8^+$ T cells have not been defined. In this study, we used a combination of genetically-engineered MHC I constructs, mice with a hepatocyte-specific mutation in the class I antigen-presentation pathway and immunopeptidomic analysis to provide definitive evidence for the contribution of the peptide cargo of allogeneic MHC I molecules to transplant tolerance induction. We established a systematic approach for the discovery of directly-recognised pMHC epitopes, and identified 17 strongly immunogenic H-$2K^b$-associated peptides recognised by $CD8^+$ T cells from B10.BR (H-$2^k$) mice, 13 of which were also recognised by BALB/c (H-$2^d$) mice. As few as five different tetramers used together were able to identify almost 40% of alloreactive T cells within a polyclonal population, suggesting that there are immunodominant allogeneic MHC-peptide complexes that can account for a large proportion of the alloresponse.

## Introduction

Allorecognition may result in either graft rejection, graft versus host disease or transplantation tolerance, depending upon the context in which the recipient immune system encounters the allogeneic major histocompatibility complex (MHC). When donor MHC class I (MHC I) molecules are expressed in the hepatocytes of recipient mice, subsequent skin or pancreatic islet grafts bearing the same donor allomorph are accepted indefinitely[1-3]. Insights from this model can inform our understanding of allorecognition and transplant tolerance induction more broadly. MHC I molecules are ubiquitously expressed, and display a range of endogenous peptides (the class I immunopeptidome) reflecting protein turnover and normal cellular processes[4]. While tolerance induction depends upon recognition of intact donor MHC I molecules by recipient $CD8^+$ T cells[3], the contribution of the self-peptide cargo of these molecules to tolerance induction in this setting is unknown. Many alloreactive T cell clones recognise epitopes comprising allogeneic MHC I molecules complexed with self-peptides[5-12]. Conversely, peptide-independent direct allorecognition is also described[7, 13, 14], and the ability of peptide-independent cytotoxic T lymphocyte (CTL) clones to bring about rapid destruction of allogeneic skin grafts has been demonstrated[15]. At the level of a polyclonal alloresponse *in vivo*, there is limited information about the role of the donor's tissue-specific class I immunopeptidome in allorecognition and consequent immune responses that impact on graft survival.

Here, we examined the role of the liver immunopeptidome in transplantation tolerance induction using two different approaches. Firstly, we engineered liver-specific adeno-associated viral (AAV) vectors encoding H-$2K^b$ or H-$2K^d$ as a single chain trimer (SCT) comprising the polymorphic heavy chain (HC), β2 microglobulin (β2m) light chain and a defined covalently-bound peptide species[16, 17], thus excluding presentation of endogenous peptides by the allogeneic MHC I when these constructs were expressed in recipient hepatocytes (Figure 1a). In parallel, we introduced a global shift in the repertoire of peptides bound to allogeneic H-$2K^d$. To accomplish this, we generated recipient mice in which the transporter associated with antigen processing (TAP1) protein was deficient only in hepatocytes (TAP1KOHep, H-$2^b$), and designed a construct expressing the H-$2K^d$ HC with a modification (YCAC) which stabilises the molecule and permits occupancy by lower affinity peptides[18]. Induction of tolerance to subsequent donor skin grafts was blocked

by these manipulations, establishing that the endogenous peptide repertoire of hepatocytes makes an essential contribution to transplant tolerance induction.

Differential responses by alloreactive T cells to MHC I allomorphs expressed by various target tissues is cited as support for peptide-specific allorecognition[6, 19, 20]. Given that expression of allogeneic donor MHC I HC in recipient hepatocytes attenuates responses against both donor skin grafts and splenocyte stimulators[1, 3], we hypothesised that peptides critical for tolerance induction would be found within a subset shared by these tissues. We identified these peptides and assessed their recognition by activated alloreactive B10.BR T cells. Of 100 peptides selected for screening, 17 were bound by more than 5% of this T cell population, and 13 of these peptide-MHC (pMHC) epitopes were also recognised by BALB/c mice. As few as five different pMHC tetramers used together were able to identify around 40% of alloreactive T cells within a polyclonal population, suggesting that there are immunodominant allogeneic MHC-peptide complexes that can account for a large proportion of the alloresponse. The findings of this study represent a significant advance in our understanding of the role of endogenous peptides in direct T cell alloreactivity, and a springboard for further knowledge gain and technological development.

## Results

### Single chain trimer constructs exclude presentation of endogenous peptides.

In preceding studies[1, 3], we have used liver-specific AAV vectors encoding the donor MHC I HC. Within transduced hepatocytes, allogeneic HC associates with native β2m and the resulting heterodimers are loaded with a repertoire of endogenous peptides (Figure 1A). To express allogeneic MHC I at high levels on recipient hepatocytes, while excluding the presentation of naturally processed peptides, we engineered SCT constructs, each encoding the HC of H-2K$^b$, β2m and a single, defined H-2K$^b$-restricted peptide [SIINFEKL (SIIN) or KIITYRNL (KIIT), Figure 1A], and packaged them in hepatocyte-specific AAV2/8 vectors. Sequences are shown in supplementary Figure 1. Transgene expression in hepatocytes was close to maximal by d7 following intravenous (iv) inoculation, and persisted through to at least d100, no significant increases in serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels were observed, and minimal cellular infiltration was detected by histology (Figure 1B-C). SCT molecules were expressed on transduced hepatocytes at equivalent levels to the heterotrimer formed by transgenic H-2K$^b$ HC with native β2m and peptide (Figure 2A). To demonstrate exclusion of naturally processed peptides, we co-transduced B10.BR (H-2$^k$) hepatocytes with AAV vectors encoding full-length chicken ovalbumin (OVA) and either HC-K$^b$ or SCT-K$^b$-KIIT, and stained them with a monoclonal antibody, 25D-1.16, which is specific for the OVA peptide SIINFEKL complexed with K$^b$. K$^b$-SIINFEKL was only detected at the surface of cells co-transduced with HC-K$^b$ and not those expressing SCT-K$^b$-KIIT (Figure 2B-C). We extended this analysis to the broader endogenous peptide repertoire of K$^b$-transduced hepatocytes using immunoaffinity purification with the H-2K$^b$-specific antibody K9-178, followed by reverse phase high performance liquid chromatography (RP-HPLC) to collect peptide-containing fractions that were then analysed using liquid chromatography with tandem mass spectrometry (LC-MS/MS) to identify bound peptides (Figure 2D). The K$^b$ binding motif of peptides eluted from HC-K$^b$ transduced hepatocytes mirrored that obtained from C57BL/6 (H-2$^b$) hepatocytes (Figure 2E). While there was greater diversity among the unique peptides isolated from HC-K$^b$ transduced hepatocytes (Figure 2F), there was substantial overlap between the peptide repertoires, with > 90% of the peptides from C57BL/6 hepatocytes also identified in HC-K$^b$ transduced B10.BR (Figure 2G). In contrast, almost no K$^b$-bound peptides could be identified in association with SCT-K$^b$-KIIT, confirming exclusion of the

endogenous peptide repertoire from presentation by this molecule (Figure 2F). Hepatocytes transduced with SCT-$K^b$-KIIT did not manifest any generalised defect in antigen processing, with both the numbers and repertoire of peptides isolated from the native allomorph H-$2K^k$ being similar across all groups (Figures 2H-I). The full peptide dataset can be found in supplementary data 1.

**SCT-$K^b$-KIIT induces tolerance in alloreactive T cells expressing the cognate T cell receptor but not in a polyclonal alloreactive population.**

We first verified that SCT molecules were recognised by cells bearing their cognate TCRs in a manner analogous to the native epitope. Pulsing RMA-S cells with synthetic peptides KIITYRNL, SIINFEKL and AAAAFAAL (minimum binding requirement for H-$2K^b$) stabilised $K^b$ expression on the cell surface (Supplementary Figure 2A-B). Similar levels of surface expression were achieved by transient transfection of RMA-S cells with the corresponding SCT constructs (Supplementary Figure 2C). Using interferon-gamma (IFN-$\gamma$) ELISPOT, we found a strong, specific response to SCT-$K^b$-KIIT by Des-RAG TCR-transgenic T cells which express the cognate TCR; SCT-$K^b$-SIIN was similarly recognised by OT-I-RAG T cells (Supplementary Figure 2D). Following adoptive transfer into B10.BR mice transduced with SCT-$K^b$-KIIT, Des-RAG T cells proliferated vigorously, expanding more than those transferred to the positive control 178.3 (H-$2^k$ + $K^b$) mice after 2 days ($p < 0.0001$, Supplementary Figure 2E-F). The polyclonal responder population of liver leukocytes from B10.BR mice also contained $CD8^+$ T cells which were activated in response to transduction with SCT-$K^b$-KIIT. Naïve or primed B10.BR mice were inoculated with SCT-$K^b$-KIIT, and liver leukocytes isolated 7 days later (Supplementary Figure 3A). Activated $CD8^+$ T cells, defined as $CD44^+PD\text{-}1^{hi}$, increased upon priming or transduction with SCT-$K^b$-KIIT, with a further augmentation when primed mice received SCT-$K^b$-KIIT vector (Supplementary Figure 3B). Activated $CD8^+$ T cells from transduced mice recognised $K^b$-KIIT dextramers but not dextramers of the self-pMHC complex $K^k$-EEEPVKKI, while PD-$1^-$ bystander $CD8^+$ T cells did not bind either dextramer (Figure 3C-D).

Next, we determined that Des-RAG T cells alone were capable of rejecting $K^b$-bearing 178.3 skin grafts upon adoptive transfer to B10.BR-RAG recipients (Figure 3A-B). Graft survival was inversely related to the T cell

dose; 50,000 transferred cells yielded a median graft survival comparable to that in B10.BR mice with a polyclonal $K^b$-reactive T cell repertoire and this dose was used in subsequent experiments. All grafts to reconstituted B10.BR-RAG mice treated with SCT-$K^b$-KIIT survived indefinitely, whereas no graft survival prolongation was observed in reconstituted mice receiving the control vector SCT-$K^b$-SIIN (Figure 3C). Surviving grafts appeared normal macroscopically (Figure 3D) and upon histology (Figure 3E) with continued expression of H-2$K^b$. In contrast, transduction of wild-type B10.BR mice with either SCT-$K^b$-KIIT or SCT-$K^b$-SIIN only briefly delayed graft rejection (Figure 3F).

**The YCAC mutation alters the repertoire of H-2$K^d$-bound peptides in both TAP1KOHep and C57BL/6 mice.**

Mice with a conditional deletion of *Tap1* in hepatocytes (TAP1KOHep, H-$2^b$) express trace amounts of the native MHC I allomorphs H-2$K^b$ and H-2$D^b$ at the hepatocyte surface (Figure 4A-D). Importantly, expression of these molecules on most other cell types within the liver, in other tissues (such as spleen, thymus and lymph node), and in $TAP1^{fl/fl}$ control mice was normal (Figure 4A-D and supplementary Figure 4A). Transduction of TAP1KOHep mice with a vector encoding the H-2$K^d$ HC (HC-$K^d$) resulted in surface expression of H-2$K^d$ which was clearly positive with respect to untransduced controls, yet was reduced in comparison to that in C57BL/6 or $TAP1^{fl/fl}$ mice (Figure 4E-F). Increasing the vector dose did not yield an appreciable increase in surface expression (data not shown), most likely because of instability of suboptimally-loaded H-2$K^b$ molecules and their rapid recycling from the cell surface[21-23]. To counter this, we designed a construct where the point mutations Y84C and A139C (YCAC) in the H-2$K^d$ HC result in the formation of a disulphide bridge, which stabilises the molecule when empty or loaded with lower affinity peptides[18]. This modification does not interfere with TCR recognition of the bound peptide[18]. The construct sequence is shown in supplementary Figure 1. Comparable strong expression of H-2$K^d$ on the surface of hepatocytes was achieved in TAP1KOHep mice treated with HC-$K^d$-YCAC and in $TAP1^{fl/fl}$ mice receiving either the HC-$K^d$ or HC-$K^d$-YCAC vectors (Figure 4E-G). Immunoaffinity purification and tandem LC-MS/MS were used to characterise the bound self-peptide repertoire of transduced hepatocytes. 9570 unique peptides were identified from C57BL/6 hepatocytes transduced with HC-$K^d$ (B6/HC), compared with 7690, 7776, and

6417 unique peptides respectively, from C57BL/6, TAP1$^{fl/fl}$ and TAP1KOHep transduced with HC-K$^d$-YCAC (*i.e.* B6/YCAC, TAP1$^{fl/fl}$/YCAC and TAP1KO/YCAC). The full dataset can be found in supplementary data 1, while distribution of peptide lengths and spectral intensities is shown in supplementary Figure 5A-B, along with the gene ontology analysis of the subcellular location and function of the source proteins. Whilst the sequences of eluted 9-mer peptides from B6/HC, B6/YCAC and TAP1$^{fl/fl}$/YCAC corresponded to the canonical motif for H-2K$^d$, with tyrosine (Y) residues predominant at position 2, and leucine (L) and isoleucine (I) most frequently found at position 9, this motif was not observed for the peptides eluted from TAP1KO/YCAC (Figure 5A-B). Compared to B6/HC, just under 45% of peptides were common to B6/YCAC or TAP1$^{fl/fl}$ /YCAC, while 22% were shared with TAP1KOHep/YCAC (Figure 5C). Similarity across peptide repertoires was increased when the comparison was weighted for peptide abundance (Figure 5C). The peptide SYFPEITHI (SYFP) was common to all K$^d$ repertoires, and comparable proportions of CD8$^+$ T cells recognising K$^d$-SYFP could be detected among the liver leukocytes isolated from primed TAP1KO/YCAC as well as from primed B6/YCAC and B6/HC (Figure 5D) consistent with published reports that the YCAC mutation alters the peptide repertoire but does not interfere with TCR recognition of presented peptides[18, 24].

**Increasing perturbation of the hepatocyte H-2K$^d$ peptide repertoire correlates with progressive reduction of K$^d$-bearing skin graft survival in transduced mice.**

Mice were inoculated with AAV vectors encoding HC-K$^d$, HC-K$^d$-YCAC, or SCT-K$^d$ with the peptide SYFPEITHI (SCT-K$^d$-SYFP), seven days prior to transplantation with a B6.Kd skin graft. The construct sequence and expression data for SCT-K$^d$-SYFP are shown in supplementary Figures 1 and 6, respectively. The majority of C57BL/6 or TAP1$^{fl/fl}$ mice transduced with HC-K$^d$ accepted K$^d$-bearing B6.Kd skin grafts indefinitely, whereas median survival of B6.Kd skin grafts to C57BL/6 or TAP1$^{fl/fl}$ mice treated with HC-K$^d$-YCAC was reduced to 62.5 $\pm$ 5.3 days and 52.5 $\pm$ 9.2 days respectively ($p = 0.009$ and $p = 0.007$ compared to HC-K$^d$) (Figure 5E). Graft survival in TAP1KOHep mice inoculated with HC-K$^d$-YCAC was further reduced to 20 $\pm$ 1.9 days, similar to that in C57BL/6 mice which received a vector encoding SCT-K$^d$-SYFP (17 $\pm$ 1.2 days). Median graft survival in no vector control mice was 12.5 $\pm$ 0.6 days for C57BL/6 and 10 $\pm$ 0.6 days for TAP1KOHep (Figure 5E). Failure to induce tolerance was not associated with loss of transgene expression

in the transduced livers (supplementary Figure 6). Instead, progressive loss of ability of H-2K$^d$ gene transfer to prolong B6.Kd skin graft survival correlated with increasing disturbance of the hepatocyte K$^d$ peptide repertoire in the various experimental groups. These findings, along with the inability of SCT-K$^b$ vectors to induce tolerance in polyclonal alloreactive T cell populations demonstrate the importance of the hepatocyte immunopeptidome in transplantation tolerance induction following donor MHC I gene transfer to the liver.

**Profiling the tissue-specific immunopeptidomes of hepatocytes, skin and spleen**

Given the ability of H-2K$^b$ or K$^d$ HC expressed in recipient hepatocytes to induce tolerance to allogeneic skin grafts and to downmodulate responses against donor splenocyte stimulators[1, 3], we postulated that the peptides critical for allorecognition and tolerance induction would be found within a subset common to these three tissue types. The self-peptide repertoires of transduced hepatocytes, grafted donor skin and donor spleen were determined using a combination of immunoaffinity purification and RP-HPLC to liberate and collect peptide-containing fractions from MHC I with LC-MS/MS for peptide identification, for both the 178.3 to B10.BR (K$^b$ mismatch, H-2$^k$ background) and B6.Kd to C57BL/6 (K$^d$ mismatch, H-2$^b$ background) strain combinations, as outlined in Figure 6A. The lists of common peptides are found in supplementary Table 2 (H-2K$^d$) and supplementary Table 3 (H-2K$^b$). For H-2K$^d$, 880 common peptides were identified across the three tissue types (Figure 6B), whereas there were 1083 common K$^b$-binding peptides (Figure 6C). The peptide length distributions (Figure 6D and F) and binding motifs (Figure 6E and G) were as anticipated for the respective allomorphs and were similar across tissue types. Of note, the common peptide pool was more limited when TAP-sufficient, or particularly TAP-deficient hepatocytes had been transduced with HC-K$^d$-YCAC (324, 347 and 36 unique peptides respectively), compared to TAP-sufficient hepatocytes transduced with HC-K$^d$ (880 unique peptides) (Figure 6H). Comparison of the different tissue immunopeptidomes showed that in the two settings where skin graft tolerance was achieved in wild-type recipient mice following expression of allogeneic donor MHC I in hepatocytes, the proportion of skin peptides common to hepatocytes was 43% and 45% for H-2K$^d$ (Figure 6B) and H-2K$^b$ (Figure 6C), respectively. Conversely, only 1.6% of skin peptides were also found in TAP1KOHep hepatocytes transduced with HC-K$^d$-YCAC, while in the two groups with intermediate graft survival (TAP1$^{fl/fl}$ or C57BL/6 inoculated with HC-K$^d$-YCAC), the proportion of skin

peptides present in hepatocytes was in the order of 15-17%. Gene ontology analysis of source proteins is shown in supplementary Figure 5C-D.

**H-2K$^b$ peptides from the common peptide pool are recognised by activated alloreactive CD8$^+$ T cells.** A total of 100 peptides were selected for screening (listed in supplementary Table 4). 96 peptides were drawn from the common peptide pool, and their identity was confirmed by a direct comparison between the mass spectra obtained from synthetic and eluted natural peptides. Representative spectra from three peptide pairs, along with the Pearson correlation coefficient and the corresponding p-value between the $\log_{10}$ intensities of identified b- and y-ions in synthetic and sample-derived spectra are shown in Figure 7A. For all peptides, $p < 0.05$. A further four peptides had been previously identified as alloreactive CD8$^+$ T cell epitopes in B10.BR mice[11, 25]. Three of these four epitopes were detected within the common pool. Binding of pMHC tetramers was used to determine which peptides combined with H-2K$^b$ to form immunogenic epitopes recognised by alloreactive B10.BR CD8$^+$ T cells. B10.BR mice were first primed by placement of a K$^b$-bearing 178.3 skin graft. Approximately 30 days after graft rejection, mice were inoculated with HC-K$^b$, and after a further 7 days, liver leukocytes were isolated and stained by flow cytometry (Figure 7B). The gating strategy is shown in Figure 7C. Activated CD8$^+$ T cells were defined as CD44$^+$PD-1$^{hi}$, whereas PD-1$^-$ cells were considered to be an internal control population, which had been exposed *in vivo* to H-2K$^b$ expressed on hepatocytes but were not activated. Peptides were deemed immunogenic when ≥2% of CD44$^+$PD-1$^{hi}$ CD8$^+$ T cells were bound by pMHC tetramer. Representative flow plots demonstrating T cell recognition of immunogenic and non-immunogenic peptides are shown in Figure 7D, and data summarising the results are shown in Figure 8A and supplementary Table 4. Allorecognition of K$^b$-bound peptides was then examined in recipient mice of a second background haplotype (BALB/c, H-2$^d$) (Figures 8A-B and Supplementary Table 4).

Of 100 peptides screened, 17 peptides were recognised by > 5% of activated recipient CD8$^+$ T cells from male B10.BR mice (termed strongly immunogenic), and a further 39 were bound by 2-5% of cells (moderately immunogenic). These responses were mirrored in female B10.BR recipients (Figures 8A-B). A number of pMHC epitopes were recognised by BALB/c mice as well as B10.BR (Figures 8A-B). All peptides recognised

by > 5% of B10.BR responder cells and 42/43 of those binding > 5% of BALB/c cells were 8-mers. For 8-mer peptides, there was a strong correlation between overall peptide abundance (as estimated by the product of the spectral intensity across the three tissue types) and the percentage of T cells with specificity for a given pMHC ($r = 0.52$, $p < 0.0001$, Figure 8C). No such relationship was observed for 9-mers. Predicted peptide binding affinity for H-2K$^b$ (measured by $IC_{50}$) did not differ significantly between strongly, moderately or non-immunogenic peptides (Figure 8D, $p = 0.098$ by one-way ANOVA), nor was a correlation observed between $IC_{50}$ and spectral abundance (Figure 8E). Simultaneous staining with two different pMHC tetramers was used to evaluate the proportion of T cells recognising more than one pMHC specificity (Figure 9A), with a total of six peptides being evaluated. A substantial proportion of T cells recognised two peptides (SGYIYHKL and/or SVYVYKVL) in addition to SNYLFTKL. 86.7% of T cells recognising SGYIYHKL-PE could recognise SNYLFTKL-APC and 66.8% of T cells recognising SVYVYKVL-PE could also recognise SNYLFKTKL-APC, whereas cross-reactivity between VGPRYTNL, INFDFPKL and RTYTYEKL was considerably lower (Figure 9A-B). When 5 of these 6 peptides (excluding SGYIYHKL), each binding between 7.2 and 15.2% of T cells, were used together as a panel, the proportion of alloreactive CD8$^+$ T cells bound increased to 39.1% (Figure 9C-D, $p = 0.002$ compared with SNYLFTKL). This cumulative increase in binding is consistent with alloreactive T cell recognition of epitopes comprising both a self-peptide and allogeneic MHC I molecule, and suggests that the development of pMHC multimer panels for the identification and tracking of alloreactive T cell populations is feasible.

## Discussion

Direct allorecognition refers to the engagement of recipient TCRs by intact allogeneic MHC molecules on the surface of donor cells. In some settings, intact, unprocessed donor MHC is transferred to the surface of recipient APC, a variant known as the semi-direct pathway of allorecognition[26]. While direct/semi-direct allorecognition is a critical driver of both solid organ allograft rejection[26] and tolerance induction[3], the specific molecular targets of most directly-alloreactive $CD8^+$ T cells have not been defined, limiting our understanding of these phenomena. In this study, we provide definitive evidence for the contribution of the peptide cargo of allogeneic MHC I molecules to transplant tolerance induction. Moreover, we demonstrate the feasibility of a systematic approach incorporating mass spectrometry-based identification of alloantigen-presented peptides, alloreactive T cell enrichment and pMHC multimer screening for the discovery of pMHC epitopes that are directly recognised by alloreactive $CD8^+$ T cells. To our knowledge, this represents the first example of such an approach in the context of direct allorecognition.

Studies over many years using T cell clones suggested that most directly alloreactive $CD8^+$ clones recognise allogeneic MHC I molecules complexed with an endogenous peptide, while peptide-independent direct allorecognition was also reported. Our recent findings that direct recognition of hepatocyte-expressed donor MHC I by recipient $CD8^+$ T cells was required for donor skin graft tolerance induction provided us with a unique opportunity to examine the role of the hepatocyte immunopeptidome in transplant tolerance induction, and by extension, in alloresponses more broadly. We used two complementary approaches to separate expression of donor MHC I molecules from presentation of their usual peptide cargo. In the first approach, we utilised an AAV vector encoding H-$2K^b$ HC, β2m and the single peptide KIITYRNL to express high levels of $K^b$ on the surface of recipient hepatocytes, while excluding presentation of naturally-processed endogenous peptides. Tolerance to $K^b$-bearing skin grafts was able to be induced in reconstituted mice with a monoclonal population of T cells all recognising the $K^b$-KIIT epitope, but not in wild-type mice possessing a full TCR repertoire. Secondly, we expressed a mutated version of the H-$2K^d$ HC, which is permissive to loading with suboptimal peptides in a purpose-bred mouse strain (TAP1KOHep) with a hepatocyte-specific deletion of *Tap1*. This combination resulted in a significant alteration of the $K^d$-bound peptide repertoire and

prevented tolerance induction. Graft survival was shortened to a lesser extent in TAP1-sufficient mice transduced with HC-$K^d$-YCAC, in keeping with the lesser perturbation of the $K^d$ peptide repertoire observed in these mice. The point mutations Y84C and A139C in the $K^d$ HC permit the creation of a disulphide bridge which stabilises the peptide binding groove when empty or populated by a low-affinity peptide[18]. The presence of these mutations alters the composition of the $K^d$-associated peptide repertoire but does not impede T cell responses to a given pMHC ligand[24]. Accordingly, the ubiquitous peptide SYFPEITHI was present within the repertoires of all hepatocytes transduced with either HC-$K^d$ or HC-$K^d$-YCAC and comparable proportions of T cells isolated from the livers of these mice bound $K^d$-SYFP dextramers.

Having demonstrated the importance of the peptide cargo of allogeneic MHC I molecules for transplant tolerance induction, we set about identifying the subset of peptides responsible for modulating the anti-graft alloresponse, using an immunoproteomic approach to identify and characterise the immunopeptidomes of relevant tissues. We hypothesised that hepatocyte expression of peptides that were also presented by cells in the grafted skin underpinned tolerance induction. Consistent with this hypothesis, in conditions where tolerance was achieved, 43% of $K^d$-associated peptides and 45% of $K^b$-bound peptides from transduced hepatocytes were also expressed in donor skin. When survival prolongation was minimal, only 1.6% of hepatocyte $K^d$-bound peptides were common to donor skin, and this shared proportion increased to 15-17% in the groups with intermediate graft survival. This data in combination with our previous observations that expression of allogeneic MHC I in recipient liver reduces IFN-$\gamma$ ELISPOT responses against donor splenocyte stimulators, implied that the critical set of self-peptides would be concentrated amongst those common to all three tissue types.

We next set out to determine which peptides from this shared subset would be recognised by alloreactive T cells using tetramer binding assays. We generated an enriched population of alloreactive $CD8^+$ T cells in the liver by priming mice with a skin graft expressing H-$2K^b$, followed by boosting with a liver-specific AAV vector encoding HC-$K^b$. Co-expression of CD44 with high levels of PD-1 allowed us to distinguish between activated cells and bystander cells which had been exposed to the alloantigens, but had not responded. Of the 100

$K^b$-binding peptides selected for tetramer screening, 17 were recognised by > 5% of the enriched alloreactive T cell population. INFDFNTI, previously reported to be recognised by a large proportion of the polyclonal alloreactive T cell population of B10.BR mice[25] was bound by an average of 3.7% of male B10.BR cells.

To examine factors which might contribute to the immunogenicity of different pMHC, we used spectral intensity as an approximation for relative peptide abundance and $IC_{50}$ to predict binding affinity of the peptide for H-2$K^b$. An overall measure of abundance was derived by multiplying the spectral intensity recorded in each of the three tissues. For 8-mer peptides, a strong correlation was found between abundance and the proportion of $CD8^+$ T cells recognising particular peptides. This is at variance with observations of $CD8^+$ T cell recognition of $K^b$-associated viral epitopes, where the frequency of T cells responding to a given epitope corresponds weakly at best to epitope abundance[27, 28]. In contrast, while there was a tendency for the strongly immunogenic peptides to have high predicted affinity for $K^b$, there was no significant difference in $IC_{50}$ between the strongly-, moderately- and non-immunogenic groups, nor was there an association between $IC_{50}$ and peptide abundance. While a prediction of immunogenicity based on peptide abundance is no substitute for direct measurement, these findings suggest that the likelihood of finding strongly immunogenic peptides is greater if the search is targeted towards the most highly-represented peptide species. The relationship between abundance and immunogenicity did not apply to 9-mer peptides. A staining panel comprising only five strongly immunogenic pMHC epitopes identified nearly 40% of T cells in an enriched alloreactive population; we anticipate that an expanded panel will capture an even greater proportion of alloreactive cells, and that such panels could be used to enumerate, track and phenotype polyclonal alloreactive T cell populations.

One intriguing finding of this study was the broad overlap between strongly immunogenic $K^b$-peptide epitopes recognised by BALB/c and those first identified in B10.BR mice (13/17 peptides). Further studies in mouse and human subjects will be needed to establish the prevalence of such “super-epitopes” recognised by allogeneic T cells across several genetic backgrounds. While extensive cross-reactivity is a feature of self MHC-restricted T cells, the range of possible cross-reactive epitopes is constrained by peptide length, with

T cell clonotypes showing a strong preference for a single length peptide[29]. Alloreactive T cell clones have undergone positive selection during thymic development based on self-MHC. The majority of peptides eluted from H-2K$^k$ are 8-mers ([30] and supplementary data 1) which aligns with the result that in male B10.BR mice (H-2$^k$) 17/79 8-mer peptides but 0/21 9-mers were strongly immunogenic. Most peptides associated with the H-2$^d$ allomorphs D$^d$, L$^d$ and K$^d$ are nonamers[30], yet 42/79 8-mers and only 1/21 9-mers were strongly immunogenic in BALB/c (H-2$^d$) mice. Possible explanations for this asymmetry are that cross-reactivity involving an allogeneic MHC molecule is not subject to the same peptide length limitations as cross-reactivity between self MHC-restricted peptide epitopes, or that a small number of octamer-preferring CD8$^+$ T cells from BALB/c mice expand preferentially in the presence of H-2K$^b$ presenting mainly 8-mers. While the mean H-2K$^b$ $IC_{50}$ among the 9-mer peptides was greater than that of the 8-mers, a number of 9-mer peptides were in the same $IC_{50}$ range as the strongly immunogenic 8-mers, so this is unlikely to fully explain the reduced frequency of recognition of 9-mer peptides.

The findings of this study open a number of avenues for future research. Firstly, they suggest an approach for the systematic discovery of pMHC epitopes for directly alloreactive CD8$^+$ T cells which may be adapted to additional MHC I allomorphs in mice, humans and other species. Identification of large numbers of allogeneic pMHC epitopes will enable the characterisation of alloreactive TCR repertoires, including biophysical and structural studies with many more receptor-ligand pairs than the handful currently known, which in turn will provide further insights into the fundamental basis of alloreactivity. Systematic allogeneic pMHC epitope discovery is complemented by the recent advent of methodologies such as oligonucleotide barcoding permitting the use of pMHC multimers for parallel tracking and enumeration of >1000 individual pMHC specificities, with options for coupling to single cell TCR sequence and gene expression analyses[31, 32]. pMHC multimers conjugated with radionuclides are sensitive tools which could be applied to *in vivo* imaging of specific alloresponses[33]. Beyond their potential as research reagents, allospecific pMHC multimers could enhance post-transplant immune monitoring and immunophenotyping of directly alloreactive T cells in a clinical setting. Finally, pMHC multimers are not only reagents for antigen-specific T cell detection, but potential vehicles for antigen-specific therapy, which may be employed for the depletion or

immunomodulation of alloreactive T cells[34]. In conclusion, the findings of this study represent a significant advance in our understanding of the role of endogenous peptides in direct T cell alloreactivity. They will enable exploration of the alloreactive T cell repertoire and potential translation to clinically-applicable tools.

## Methods

### Peptides, antibodies and reagents

Peptides were synthesised with an average of 98% purity (GL Biochem Shanghai Ltd.). Lyophilised peptides were reconstituted in 10% DMSO and stored at -80°C. Dasatinib (Sigma-Aldrich, catalogue# CDS023389) was reconstituted in DMSO and 5 mM stock was stored at -80°C. Primary and secondary antibodies used in this study are shown in Supplementary Table 5.

### Cell Lines

The *Tap2* deficient T lymphoma cell line RMA-S was cultured in RPMI 1640 medium supplemented with L-glutamine (Lonza, catalogue# 12-702F), penicillin-streptomycin (Invitrogen, catalogue# 15140) and 10% FCS (Sigma-Aldrich, catalogue# 13K179) at 37°C with 5% $CO_2$.

### RMA-S peptide stabilisation and transfection

RMA-S cells were grown to confluence and passaged to a concentration of $3\times10^5$ cells/mL then transferred to flat-bottomed 24 well culture plates. The cells were incubated at 27°C for 20 hours with 5% $CO_2$, then pulsed with peptides at concentrations ranging from 0.0001 – 10 µM. Following 1 hour incubation at 27°C, cells were returned to 37°C for 2 hours. Surface expression of stabilised H-2K$^b$ was quantified using flow cytometry with the conformation-dependent mAb Y-3. For transient transfection of RMA-S cells, $2\times10^6$ cells were nucleofected with 2 µg of pcDNA3.1$^+$ plasmid per reaction (Lonza-AMAXA program X-001, Nucleofector 2b). 24 hours after transfection, transgene expression was evaluated using flow cytometry.

### Mice

Unless otherwise stated, mice were bred at the University of Sydney (Camperdown, Australia). 178.3 mice (originally provided by Drs. W. Health and M. Hoffman, Walter and Eliza Hall Institute, Melbourne, Australia) express a transgenic MHC class I molecule H-2K$^b$ ubiquitously, under the control of its own promoter, on a B10.BR (H-2$^k$) background. Des-TCR mice express an H-2K$^b$-specific TCR, which recognises the peptides KVITFIDL, KVLHFYNV and KIITYRNL restricted by H-2K$^b$ and Des-TCR is identifiable by a clonotypic mAb (Désiré). Des-RAG mice were obtained by crossing Des-TCR mice with CD45.1$^{+/+}$ RAG1$^{-/-}$ mice, which are both on a B10.BR

(H-$2^k$) background. OT-I mice carry a TCR which recognises the peptide SIINFEKL presented by H-$2K^b$. OT-I were crossed with $RAG1^{-/-}$ mice to create the OT-I-RAG line. These mice were bred at the Centenary Institute. $C57BL/6J^{Arc}$ (H-$2^b$) and BALB/c (H-$2^d$) mice (termed C57BL/6 and BALB/c) were purchased from the Animal Resources Centre, Perth, Australia. B6.Kd mice express an H-$2K^d$ transgene ubiquitously on a C57BL/6 (H-$2^b$) background. B6.Kd mice were originally developed by R. Pat Bucy at the University of Alabama (Tuscaloosa, Alabama, USA) and were provided by Robert Fairchild, Cleveland Clinic (Cleveland, Ohio, USA). B6.Kd mice were backcrossed for 4 generations to C57BL/6J, prior to use. TAP1KOHep mice were generated based on the conditional-ready strain 09400, C57BL/6N-*Tap1*<tm2a(EUCOMM)Hmgu>/Ieg, developed as part of the European Conditional Mouse Mutagenesis programme (EUCOMM)[35]. Mice heterozygous for the *Tap1*tm2a allele on the C57BL/6N genetic background were obtained from the European Mutant Mouse Archive, based at Helmholtz Zentrum. These mice were backcrossed to $C57BL/6J^{Arc}$ for 3 generations, then intercrossed with FLPo deleter (B6.129S4-*Gt(ROSA)26SOR*$^{tm2(FLPo)Sor}$/J) mice[36] (imported from the Jackson Laboratory, Bar Harbor, ME) to generate mice carrying the *Tap1*tm2c (floxed) allele. FLPo was bred out by backcrossing to $C57BL/6J^{Arc}$ (2 generations), following which the mice were crossed to Albumin-Cre mice (B6.FVB(129)-Tg(Alb1-cre)1Dlr/J)[37]. TAP1KOHep mice are homozygous for the floxed *Tap1* allele (*Tap1*tm2c) and have one copy of Cre, which is expressed exclusively in hepatocytes resulting in hepatocyte-specific deletion of the floxed *Tap1* allele. Genotyping and genetic background testing was performed on earpunch tissue, isolated hepatocytes or spleen by Transnetyx (Cordova, TN, USA). The genetic background of TAP1KOHep and $TAP1^{fl/fl}$ control mice was at least 91.3% C57BL/6J (91.3-97.9%) and these mice did not reject syngeneic skin grafts from C57BL/6J donors (not shown). Further characterisation of this strain is shown in Figure 4 and Supplementary Figure 3. Animals were randomly allocated to treatment groups. Male and female mice aged between 8 and 12 weeks were used in this study. Male mice were used unless stated otherwise. At the termination of each experiment, tissues were collected under general anaesthesia. Frozen tissues were stored at -80°C.

**AAV vectors**

H-$2K^b$ and H-$2K^d$ were cloned as previously described[3]. SCT constructs consist of a defined peptide sequence, β2m and MHC I HC joined together by flexible linkers[16]. A tyrosine to cysteine substitution at HC position 84 and a cysteine at the second position of the peptide-$\beta_2$m linker form a disulphide trap[17]. SCT (H-$2K^b$) constructs with

peptide sequences KIITYRNL or SIINFEKL and a SCT (H-2K$^d$) construct with peptide sequence SYFPEITHI (termed SCT-K$^b$-KIIT, SCT-K$^b$-SIIN and SCT-K$^d$-SYFP, respectively) were codon-optimised and synthesised by GeneArt (Thermo Fisher Scientific). A further H-2K$^d$ sequence incorporating the Y84C and A139C mutations (termed HC-K$^d$-YCAC), was codon-optimised and synthesised by GenScript. All synthesised genes were delivered in pcDNA3.1$^+$ plasmids. The full-length native chicken ovalbumin (OVA) gene inserted in a pcDNA3.1$^+$ plasmid (clone ID: OGa28271) was purchased from GenScript. Gene inserts from pcDNA3.1$^+$ plasmids were cloned into the pAM2AA backbone incorporating the liver-specific human $\alpha$-1 antitrypsin promoter and human ApoE enhancer flanked by AAV2 inverted terminal repeats. Each gene was then packaged into an AAV2/8 vector, purified, and quantitated as previously described[38]. AAV Vectors were either produced in-house or by the Vector and Genome Engineering Facility, Children's Medical Research Institute, Westmead, Australia. Vector aliquots were stored at -80°C. All vectors were used at a dose of $5\times10^{11}$ vgc except SCT-K$^b$-KIIT which was used at $2\times10^{12}$ vgc. Vectors in 500 µL sterile PBS were administered by intravenous injection under general anaesthesia.

**Skin transplantation**

Skin transplantation was performed as described previously[3]. In short, full-thickness grafts of 1x1cm$^2$ tail skin from donor mice were applied to the dorsum of anaesthetised recipient mice following excision of a 1 x 1 cm$^2$ area of skin to accommodate the donor skin graft. The graft was fixed using cyanoacrylate tissue adhesive (Dermabond, Ethicon, catalogue# ANX12) and bandaged. Mice received analgesia with buprenorphine (Temgesic, Schering-Plough, 0.05 mg kg$^{-1}$ s.c.), prophylactic ampicillin (Alphapharm, 100 mg kg$^{-1}$ s.c.) and 0.5 ml of warmed saline. The bandage was removed 7-10 days later and the grafts were regularly monitored for 100 days post-transplant. Grafts were deemed rejected when less than 20% of the viable skin graft remained. B10.BR and B10.BR-RAG mice (H-2$^k$) received H-2K$^b$ singly-mismatched allogeneic skin grafts from 178.3 strain donor mice. C57BL/6, TAP1$^{fl/fl}$ and TAP1KOHep mice (H-2$^b$) received H-2K$^d$ singly-mismatched allogeneic skin grafts from B6.Kd donor mice. BALB/c mice (H-2$^d$) received fully allogeneic skin grafts from C57BL/6 donor mice. Skin transplant donors and recipients were sex-matched.

### Isolation of hepatocytes and leukocytes from liver, spleen and draining lymph nodes

These procedures were performed as described previously[3]. Hepatocytes for immunoaffinity purification experiments underwent further washing with cold PBS before being stored at -80°C.

### Adoptive transfers

Lymphocytes from portal and mesenteric lymph nodes were collected and processed as above[3]. Cells were resuspended in RPMI 1640 medium containing 10% FCS was labelled with 10 µM CFSE dye (Thermo Fisher Scientific, catalogue# C34570) for 4 minutes at RT. The reaction was quenched by adding more RPMI 1640 medium containing 10% FCS. CFSE-labelled lymphocytes were washed with cold RMPI/FCS10 medium, filtered through 40 µm nylon mesh and resuspended in 500 µL cold sterile PBS. CFSE-labelled lymphocytes were administered via intravenous injection under general anaesthesia. CFSE-labelling and cell viability were assessed using flow cytometry.

### Histology and immunostaining

For immunohistochemical staining, OCT-embedded frozen tissues were cut into 6 µm thick sections. Sections were allowed to air dry for 1 hour at room temperature (RT) prior to fixation in acetone for 8 minutes at RT. Sections were blocked with 20% normal mouse serum (MilliporeSigma, catalogue# M5905) and 5% normal porcine serum (Thermo Fisher Scientific, catalogue# 31890) for 20 minutes at RT and stained with FITC-conjugated primary antibodies or the corresponding isotype controls for 30 minutes at RT. Sections were then incubated with horseradish peroxidase-conjugated rabbit-anti-FITC secondary antibody before development with diaminobenzidine (DAB) substrate chromogen system (Dako, catalogue# K3468). Sections were counterstained in Mayer's hematoxylin solution (MilliporeSigma, catalogue# MHS16) for 2 minutes and mounted with Fronine safety mount No.4 (Thermo Fisher Scientific, catalogue# FNNII068). Tissue processing and H&E staining were performed by the Histopathology Laboratory, Discipline of Pathology, Sydney Medical School. For H&E staining, 5 µm thick sections from formalin-fixed paraffin-embedded tissues were used. Stained sections were examined by a blinded observer.

### Confocal imaging

The livers of freshly-sacrificed mice were perfused retrogradely via the IVC[3] with 3 ml of PBS and then 10 ml of 2% paraformaldehyde (Sigma, catalogue# 30525-89-4) in PBS. The gallbladder was removed and the liver was fixed in 2% paraformaldehyde in PBS for 8 hours. A section of the liver was embedded in 3% agarose (Fisher Biotec, catalogue# AGR-LM-50) and 150 µm thick sections were cut using a Vibratome 1000 Plus Sectioning System (Harvard Apparatus, Holliston MA). Sections were blocked with 4% bovine serum albumin (Tocris bioscience, catalogue# 9048-46-8), 5% normal goat serum (Invitrogen, catalogue# 31873) and 0.3% Triton-X 100 (Sigma, catalogue# 9002-93-1) in PBS for 20 hours at 4°C. Sections were stained with primary antibodies for 20 hours at 4°C, washed, then incubated with secondary antibodies for 20 hours at 4°C, followed by staining with DAPI (Sigma, catalogue# 28718-90-3) for 1 hour at 4°C. Primary and secondary antibodies were diluted in blocking buffer. Washing buffer comprised 0.1% Triton-X 100 in PBS. Images were acquired using a Leica SP8 confocal microscope at 93x objective magnification with a numerical aperture of 1.35. The images were analysed using Imaris v9.5 (Oxford instruments).

### Flow cytometry

Cells resuspended in cold staining buffer (2% FCS in PBS) were blocked with mouse Fc Block (BD Biosciences, catalogue# 553141) for 10 minutes at 4°C and stained with a panel of antibodies (Supplementary Table 5). Cells were washed twice with PBS before staining with Zombie NIR viability dye (BioLegend, catalogue# 423105) for 15 minutes at RT. Cells were washed with the staining buffer before analysis. The samples were analysed using BD Fortessa X-20 (BD Biosciences) or BD LSR-II (BD Biosciences) instruments and analysis of data was performed using FlowJo v10 (BD).

### ELISpot

IFN-γ ELISpot assays were performed according to the manufacturer's protocol (U-Cytech, catalogue# CT317-PR5). RMA-S cells either pulsed with peptides or transiently transfected with SCT plasmids, were irradiated with a dose of 3000 rad. Responders were either OT-I-RAG or Des-RAG splenocytes. For pre-stimulation, $1\times10^6$ irradiated stimulator cells and $1\times10^6$ responder splenocytes were suspended in 250 µL of RPMI/FCS10 medium with penicillin-streptomycin in each well of a 96-well U-bottom plate (Corning, catalogue# 3788). They were

cultured at 37°C with 5% $CO_2$ for 24 hours and then transferred into an antibody-coated polyvinylidene difluoride (PVDF) plate, serially diluted, and incubated for a further 16 hours. The plates were then developed, and the spots were counted using an AID iSpot plate reader as previously described[1].

### pMHC multimer staining

The pMHC multimer staining method was adapted from that of Dolton *et al.*[39]. Cells were incubated with a protein kinase inhibitor, 50 nM dasatinib, for 30 minutes at 37°C. PE- or APC-conjugated tetramers or dextramers were centrifuged at 16,000 *g* for 1 minute to remove aggregates. The cells were stained with 0.5 µg of pMHC tetramer or pMHC dextramer at 6.4 nM in 50 µL (unless stated otherwise) for 30 minutes on ice. Following pMHC multimer staining, the cells were washed with cold staining buffer twice. Samples were incubated with mouse Fc Block (BD Biosciences, catalogue# 553141) for 10 minutes at 4°C and either or both mouse anti-PE and anti-APC were added at 0.5 µg/100 µL depending on the pMHC multimer conjugates used. The cells were washed and incubated with a cocktail of antibodies against surface markers for 30 minutes on ice. Cells were washed twice with PBS before staining with Zombie NIR viability dye (BioLegend, catalogue# 423105) for 15 minutes at RT. Cells were washed with staining buffer before analysis. The samples were run on a BD LSR-II cytometer and analysis of data was performed using FlowJo v10. Dextramers were purchased from Immudex. QuickSwitch Custom Tetramer Kits (MBL International) were utilised to generate multiple tetramers with selected peptides in order to screen an array of pMHC epitopes. Quantitation of peptide exchange with selected peptides was performed according to the manufacturer's protocol.

### Immunoaffinity purification

Two replicate samples were prepared for each tissue or experimental group. Around $1\times10^8$ purified hepatocytes from 4 - 5 mice were pooled per sample. Hepatocytes were lysed in 0.5% IGEPAL, 50 mM Tris (pH 8), 150 mM NaCl and protease inhibitors (Roche cOmplete Protease Inhibitor Cocktail; Merck, catalogue# 11836145001). Spleens, skin grafts (d7 post-transplant) or tail skins from 5 - 9 donors were pooled per sample. Spleen and skin samples were ground in a Retsch Mixer Mill MM 400 under cryogenic conditions and then lysed in 0.5% IGEPAL, 50 mM Tris (pH 8), 150 mM NaCl, and protease inhibitors. Lysates were incubated for 1 hour at 4°C, then cleared by ultracentrifugation (40,000 rpm, 30 min) and MHC complexes were isolated from supernatant by immunoaffinity

purification using solid-phase-bound monoclonal antibodies SF1-1.1.10 (anti H-2K$^d$), K9-178 (anti H-2K$^b$), Y3 (anti H-2K$^b$/K$^k$) and 28.14.8s (anti H-2D$^b$) as described previously[40]. Peptides were dissociated from the MHC with 10% acetic acid. For purified hepatocyte and spleen samples, the mixture of peptides, class I HC and β2m was fractionated on a 4.6 mm internal diameter × 100 mm monolithic C18 column (Chromolith SpeedROD; Merck Millipore, catalogue# 1021290001) using an ÄKTAmicro RP-HPLC (GE Healthcare) system, running a mobile phase consisting of buffer A (0.1% trifluoroacetic acid; Thermo Fisher Scientific) and buffer B (80% acetonitrile, 0.1% trifluoroacetic acid; Thermo Fisher Scientific), 1 mL min$^{-1}$ with a gradient of B of 2–40% over 4 min, 40–45% over 4 min and 45–99% over 2 min, collecting 500 µL fractions. Peptide-containing fractions were either unpooled or combined into pools, vacuum-concentrated and reconstituted in 0.1% formic acid (Thermo Fisher Scientific) for mass spectrometry analysis. For tail skin samples, the mixture of peptides, class I HC and β2m was purified using Millipore 5 kDa Amicon centrifugal units (Human Metabolome Technologies; catalogue# UFC3LCCNB_HMT) in 0.1% trifluoroacetic acid. Peptides were extracted and desalted from the filtrate using ZipTip C18 pipette tips (Agilent Technologies, catalogue# A57003100K) in a final buffer of 30% acetonitrile, 0.1% trifluoroacetic acid. Peptide samples were vacuum-concentrated and reconstituted in 0.1% formic acid for mass spectrometry analysis.

**Mass Spectrometry**

Reconstituted peptides were analysed by LC-MS/MS using an information-dependent acquisition strategy on a Q-Exactive Plus Hybrid Quadrupole Orbitrap (Thermo Fisher Scientific, Bremen, Germany) coupled to a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) as described previously[31]. Briefly, peptides were trapped on a 2 cm Nanoviper PepMap100 trap column at a flow rate of 15 min using a RSLC nano-HPLC. The trap column was then switched inline to an analytical PepMap100 C18 nanocolumn (75 µm x 50 cm, 3 µm 100 Å pore size) at a flow rate of 300 nL/min using an initial gradient of 2.5% to 7.5% buffer B (0.1% formic acid 80% ACN) in buffer A (0.1% formic acid in water) over 1 min followed with a linear gradient from 7.5% to 32.5% buffer B for 58 min followed by a linear increase to 40% buffer B over 5 min and an additional increase up to 99% buffer B over 5 min. Survey full scan MS spectra (m/z 375–1800) were acquired in the Orbitrap with 70,000 resolution (m/z 200) after the accumulation of ions to a $5\times10^5$ target value with a maximum injection time of 120 ms. For Data Dependant Acquisition (DDA) runs, the 12 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented by higher-energy collisional dissociation (HCD) at 27% with an injection time of 120 ms, 35,000

resolution and target of $2\times10^5$ counts. An isolation width of 1.8 m/z was applied and underfill ratio was set to 1% and dynamic exclusion to 15 sec. For Data Independent Acquisition (DIA) runs, the MS1 survey scan and fragment ions were acquired using variable windows (Supplementary Table 6) at 35,000 resolution with an automatic gain control (AGC) target of 3e6 ions. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE[41] partner repository with accession number PXD022695.

**DDA data analysis**

For peptide identification, the acquired raw files were searched with PEAKS Studio X+ (Bioinformatics Solutions) against the *Mus musculus* (SwissProt) database. The parent mass error tolerance was set to 10 ppm and the fragment mass error tolerance to 0.02 Da. Oxidation of methionine (M) was set as variable modifications and a false-discovery rate (FDR) cut-off of 5% was applied.[42]

**DIA data analysis**

PEAKS Studio X+ was used to generate a spectral library from all DDA data. DIA raw files were imported to Spectronaut 11 Pulsar (Biognosys). The parent mass error tolerance was set to 10 ppm and the fragment mass error tolerance to 0.02 Da. Oxidation of M was set as variable modifications and a peptide list was exported at Q-value = 1%.

**Immunopeptidome analysis**

Known contaminants were removed from the analysis. Unique peptides from the DDA (replicate 1) and DIA (replicate 2) datasets were combined to increase the coverage of the tissue immunopeptidomes. For analysis requiring spectral intensity values, only DDA datasets were used. Binding motifs of 8-mer peptides from samples that were immunoaffinity purified with K9-178 antibody (H-2K$^b$ group) and 9-mer peptides from samples that had been immunoaffinity purified with SF1-1.1.10 antibody (H-2K$^d$ group) were visualised using the GibbsCluster2.0 algorithm (NetMHC4.0)[43]. For comparison of unique H-2K$^b$ peptides between different tissues, 8-mer to 11-mer peptides with a predicted half-maximum inhibitory concentration ($IC_{50}$) of binding to H-2K$^b$ less than 500 nM (NetMHC4.0 database) were selected. For comparison of unique H-2K$^d$ peptides between tissues, all 9-mer peptides were included. The source proteins associated with the eluted peptides were analysed using the

PANTHER Gene Ontology classification system[44]. Function classification analysis and statistical over-representation tests were performed[45].

### Validation of peptide identification using retrospectively synthesised peptides

We validated the identity of a panel of peptides by comparing chromatographic retention and MS/MS spectra of synthesised peptides (GL Biochem, Shanghai) with those of the corresponding eluted peptides. The PKL files of the synthetic and eluted peptides were exported from PEAKS X plus studio software. To evaluate the similarity between two spectra, we predicted all b- and y-ions for each sequence and then extracted the intensity for each ion (with a fragment mass error tolerance of 0.02 Da). The Pearson correlation coefficient and the corresponding p-value between the $\log_{10}$ intensities of identified b- and y-ions in the synthetic and sample-derived spectra were calculated[46]. The closer the correlation coefficient to 1, the greater identity between paired spectra. All tested peptides were found to have a *p*-value of less than 0.05.

### Statistical Analysis and Data Visualisation

Data are represented as mean ± SEM unless otherwise stated. Unpaired Student's t-tests were performed to calculate statistical differences in a single variable between the means of two groups and one-way analysis of variance (ANOVA) in conjunction with Sidak's multiple comparison tests were used to calculate statistical differences between the means of three or more groups. For analysis of 2 variables, two-way ANOVA with Sidak's multiple comparison tests were used. Graft survival curves were compared using Mantel Cox log-rank tests. Synthetic and corresponding eluted peptide spectra were compared using Pearson correlation tests. The relationship between overall peptide abundance and alloreactive T cell binding, and the impact of differences in sexes and strains on alloreactive T cell binding, were analysed using linear regression and Pearson correlation tests. Statistical tests were performed using GraphPad Prism version 8.01 (GraphPad Software, La Jolla CA). Heatmaps were generated using Morpheus software (https://software.broadinstitute.org/morpheus).

### Study Approval

All animal procedures were approved by the University of Sydney Animal Ethics Committee (protocol 2017/1253) and carried out in accordance with the Australian code for the care and use of animals for scientific purposes.

## Author contributions

A.W.P., N.A.M., E.T.S., P.B., D.B. and A.F.S. designed experiments. E.T.S., M.P-H., P.F., S.H.R., M.L., K.E., A.B., N.L.D. and N.A.M. performed experiments. A.W.P., L.L., I.E.A. and P.B. provided reagents and/or samples. E.T.S., P.F., S.H.R., A.W.P., N.A.M. and A.F.S. analysed data. P.F., S.H.R., N.A.M., E.T.S. and A.F.S. wrote the manuscript. All authors read and approved the manuscript.

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**Figure 1** **A**

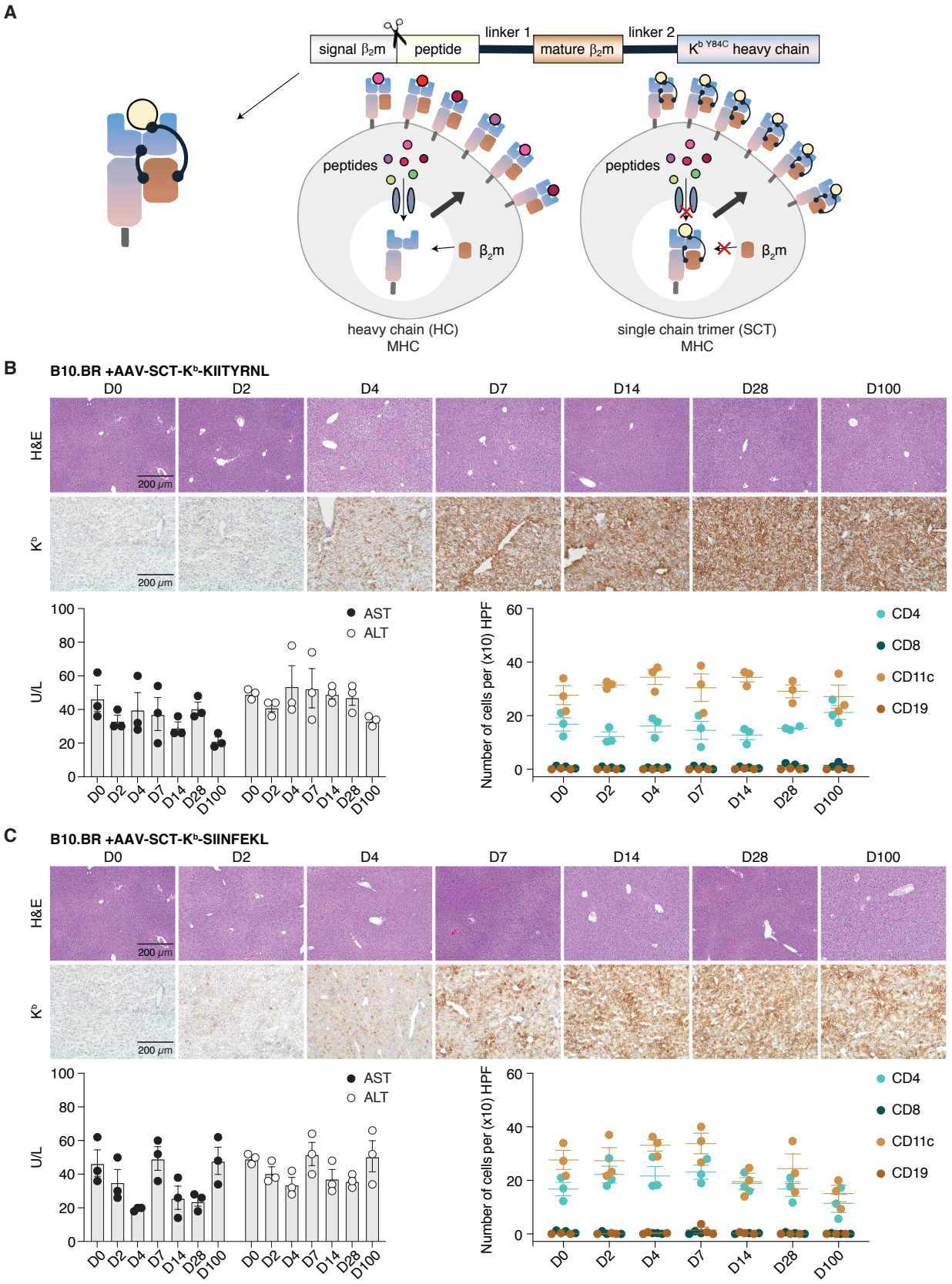



**Figure 1. Expression of SCT-K$^b$-peptide in mouse hepatocytes is robust and persistent**

**(A)** Schematic diagram of SCT pMHC constructs. MHC I HC expressed in hepatocytes can bind endogenous peptides and associate with endogenous β2m to form a wide repertoire of pMHC complexes (left). Conversely, SCT constructs exclude binding of endogenous peptides and present a single type of pMHC epitope on the cell surface (right). B10.BR mice were inoculated with AAV-SCT-K$^b$-KIITYRNL **(B)** or AAV-SCT-K$^b$-SIINFEKL **(C)**. On days 2-100 post-inoculation, tissues were collected for analysis (n = 3 / time point). Representative immunostained (IHC) and H&E images show transduced liver sections (×100 magnification). Robust expression of H-2K$^b$ was present through day 100 post-inoculation. Histologic examination of the liver sections was normal. Levels of aspartate transaminase (AST) and alanine transaminase (ALT) did not increase significantly from baseline in mice treated with AAV-SCT-K$^b$-KIITYRNL (one-way ANOVA, $p = 0.4$ for AST and $p = 0.21$ for ALT) or in mice receiving AAV-SCT-K$^b$-SIINFEKL (one-way ANOVA, $p = 0.13$ for AST and $p = 0.02$ for ALT, due solely to a decrease in ALT on d4). Minimal infiltration with cells expressing the markers CD4, CD8, CD11c or CD19 was detected. **(B,C)** Mean ± SEM are shown.

**Figure 2**

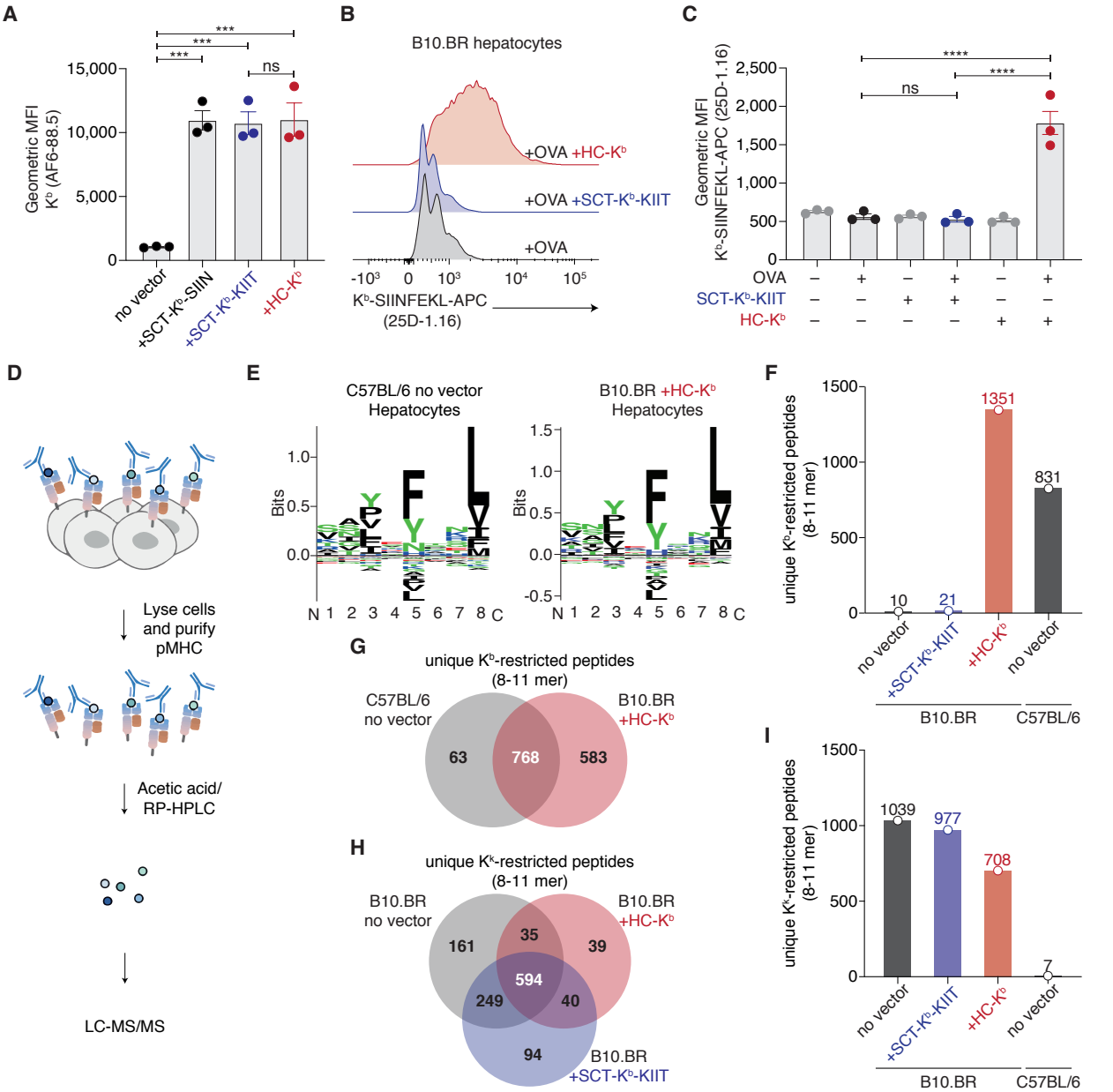



**Figure 2. SCT-Kᵇ-KIIT constructs exclude presentation of endogenous peptides**

**(A-C)** B10.BR mice were transduced with AAV-SCT-Kᵇ-SIIN, AAV-SCT-Kᵇ-KIIT or AAV-HC-Kᵇ with or without AAV-OVA. Flow cytometric analysis of isolated hepatocytes (d7) is shown. **(B)** Equivalent levels of H-2Kᵇ cell surface expression were demonstrated using the mAb AF6-88.5. **(B,C)** of SIINFEKL ($OVA_{257-264}$) peptide bound to H-2Kᵇ was detected using an anti-Kᵇ-SIINFEKL mAb (25D-1.16). Co-transduction with AAV-OVA permitted presentation of SIINFEKL by hepatocytes transduced with AAV-HC-Kᵇ. Conversely, the SCT-Kᵇ-KIIT construct excluded presentation of endogenously-processed SIINFEKL peptide. For **(A)** and **(C)** n = 3 biological replicates per group, mean ± SEM are shown, Statistical analysis comprised one-way ANOVA with Sidak's multiple comparison test: ns, not significant, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *** $p < 0.0001$. Panel **(B)** depicts one representative experiment (from n = 3). **(D)** Control B10.BR hepatocytes, B10.BR hepatocytes transduced with either AAV-HC-Kᵇ or AAV-SCT-Kᵇ-KIIT or control C57BL/6 hepatocytes underwent immunoaffinity purification in order to identify unique H-2Kᵇ and H-2Kᵏ peptides. A false discovery rate (FDR) of 5% was employed. Two replicate experiments were performed. Samples from 4 mice were pooled/group for each replicate, and combined lists of peptides present in either or both replicates were generated. **(E)** H-2Kᵇ-binding motifs were generated from a non-redundant list of 8-11-mer peptides using the GibbsCluster-2.0 algorithm. The binding motif obtained from B10.BR hepatocytes transduced with AAV-HC-Kᵇ closely reflected that from C57BL/6 (H-2ᵇ) hepatocytes. **(F,G)** Abundant Kᵇ-binding peptides were isolated from both C57BL/6 and AAV-HC-Kᵇ-transduced B10.BR hepatocytes, with significant overlap between repertoires, while very few Kᵇ-binding peptides could be eluted from B10.BR hepatocytes transduced with AAV-SCT-Kᵇ-KIIT. **(H,I)** Comparable numbers of Kᵏ-binding peptides were isolated from all B10.BR hepatocyte samples, but not from C57BL/6 mice which lack this allomorph. **(F,I)** Bars represent the cumulative number of unique peptides obtained from replicate experiments.

**Figure 3**

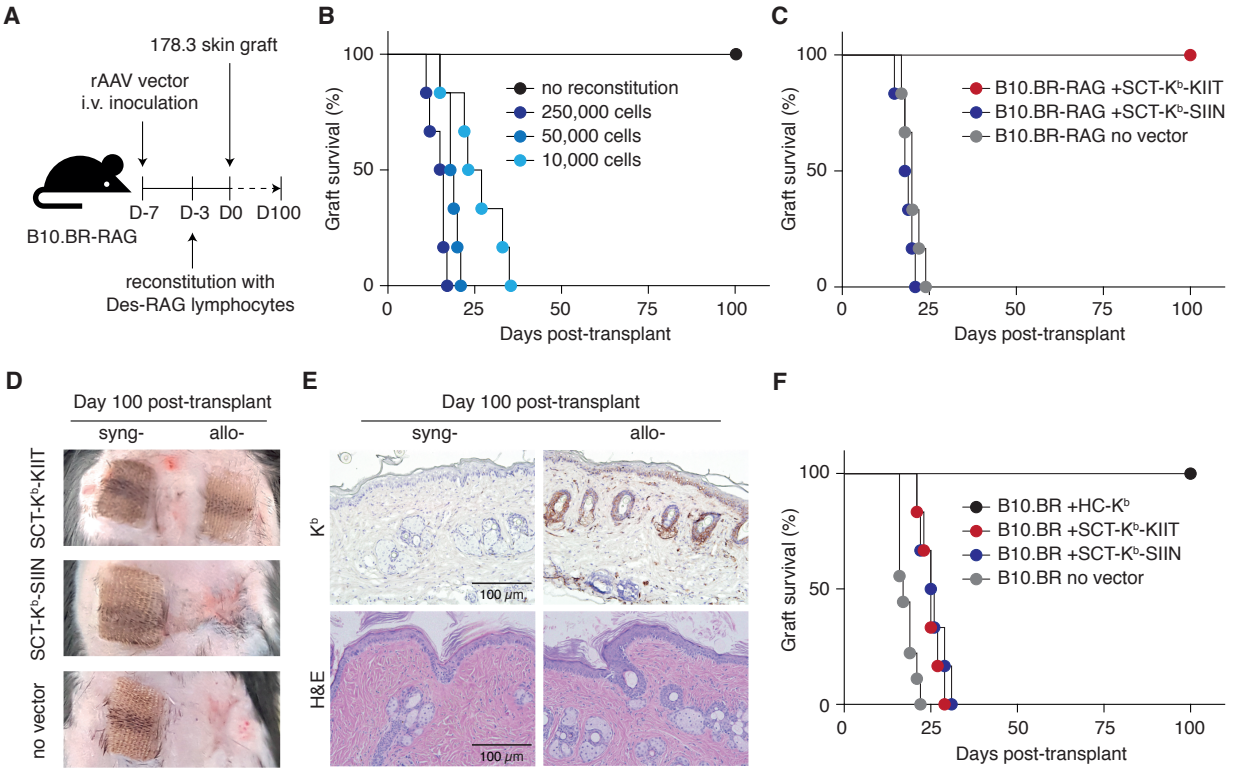
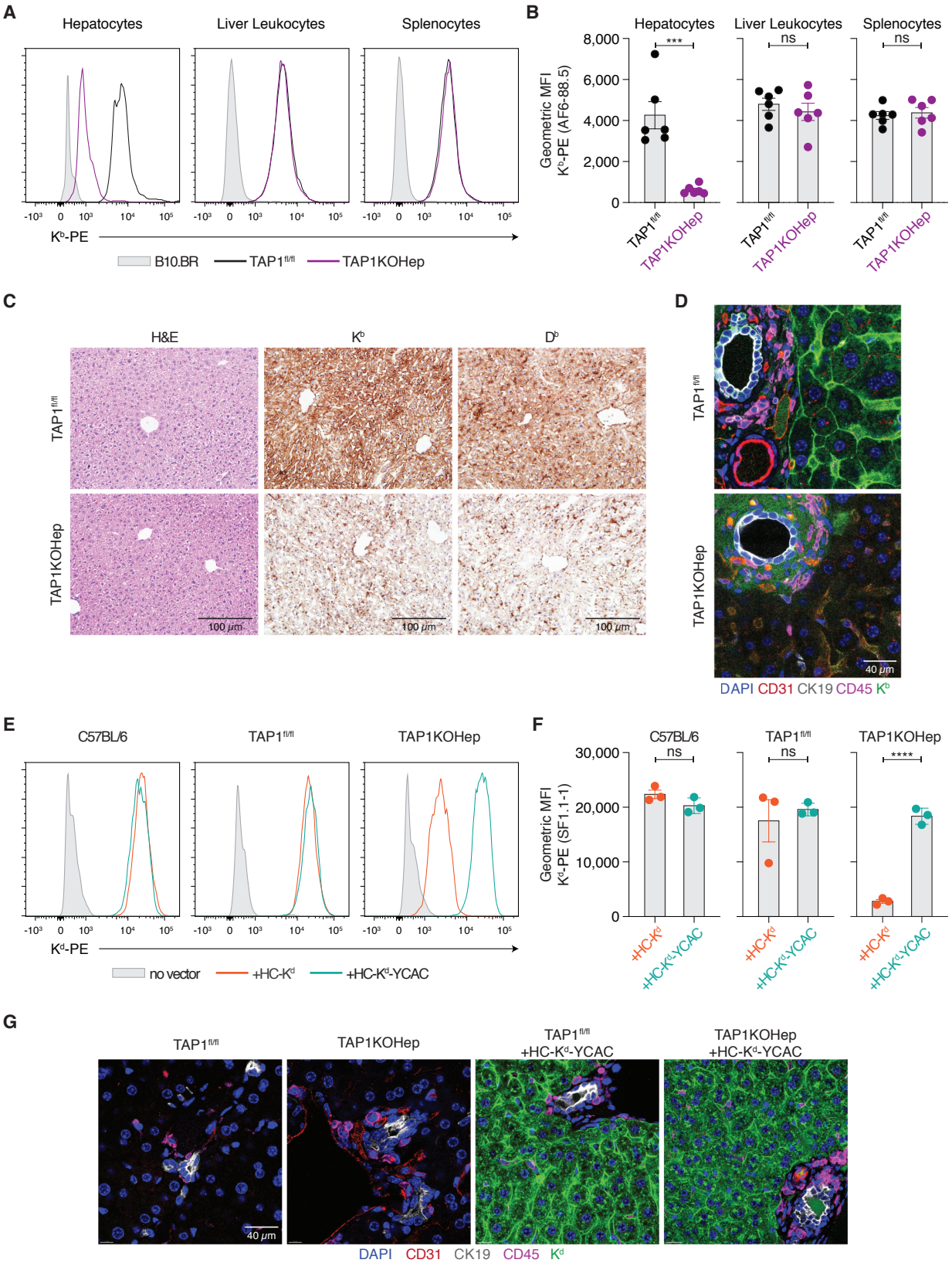



**Figure 3. Expression of the SCT-Kᵇ-KIIT construct in recipient hepatocytes prevents skin graft rejection mediated by alloreactive CD8⁺ T cells expressing the cognate TCR but does not induce tolerance in a polyclonal alloreactive population.** **(A)** B10.BR-RAG mice were reconstituted with $1.0\times10^4$ - $2.5\times10^5$ Des-RAG lymphocytes, 3 days prior to receiving a Kᵇ-bearing 178.3 skin graft. Some mice were also inoculated with AAV vectors. **(B)** 178.3 skin grafts survived indefinitely on immunodeficient B10.BR-RAG hosts. Progressive shortening in survival accompanied adoptive transfer of increasing cell numbers (Mantel-Cox log-rank test for trend: ****$p < 0.0001$, n = 6 per group). A dose of 50,000 cells was used subsequently. **(C)** Inoculation of reconstituted B10.BR-RAG recipients with AAV-SCT-Kᵇ-KIIT resulted in indefinite graft survival, whereas treatment with AAV-SCT-Kᵇ-SIIN offered no survival prolongation compared with that in untransduced recipients; median survival time (MST) of 18.5 days versus 20 days (Mantel-Cox log-rank test: ns, $p = 0.2$, n = 6 per group). ***$p = 0.0005$, Mantel-Cox log-rank test between B10.BR-RAG mice receiving AAV-SCT-Kᵇ-KIIT versus AAV-SCT-Kᵇ-SIIN (n = 6 per group). **(D)** Representative macroscopic images demonstrate continued allograft survival in reconstituted B10.BR-RAG mice treated with AAV-SCT-Kᵇ-KIIT but not other groups at 100 days after transplantation. **(E)** Representative IHC and H&E images showing syngeneic (B10.BR-RAG) and allogeneic (178.3) skin grafts 100 days post-transplant (×200 magnification). Skin transplants are morphologically normal, with persistent expression of H-2Kᵇ in 178.3 grafts. **(F)** Immunosufficient B10.BR mice were inoculated with either AAV-HC-Kᵇ, AAV-SCT-Kᵇ-KIIT or AAV-SCT-Kᵇ-SIIN (n = 6 per group) or were not transduced (n = 9). 7 days post-inoculation, the mice received 178.3 skin grafts. In these mice, expression of either SCT-Kᵇ-KIIT or SCT-Kᵇ-SIIN led to a modest increase in graft survival (MST of 25 days versus 17 days in no vector controls, Mantel-Cox log-rank test: ***$p = 0.0007$). However, only expression of H-2Kᵇ loaded with the endogenous peptide repertoire was able to induce tolerance to 178.3 skin graft. ***$p = 0.0005$, Mantel-Cox log-rank test between B10.BR inoculated with AAV-HC-Kᵇ and B10.BR inoculated with either AAV-SCT-Kᵇ-KIIT or AAV-SCT-Kᵇ-SIIN.

**Figure 4**


A
Hepatocytes
Liver Leukocytes
Splenocytes
Kᵇ-PE
B10.BR
TAP1fl/fl
TAP1KOHep
B
Geometric MFI Kᵇ-PE (AF6-88.5)
8,000
6,000
4,000
2,000
0
***
ns
ns
TAP1fl/fl
TAP1KOHep
C
H&E
Kᵇ
Dᵇ
100 μm
D
40 μm
DAPI CD31 CK19 CD45 Kᵇ
E
C57BL/6
Kᵈ-PE
no vector
+HC-Kᵈ
+HC-Kᵈ-YCAC
F
Geometric MFI Kᵈ-PE (SF1.1-1)
30,000
20,000
10,000
0
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G
TAP1KOHep +HC-Kᵈ-YCAC
DAPI CD31 CK19 CD45 Kᵈ

**Figure 4. Hepatocytes from TAP1KO Hep mice express high levels of H-2K$^d$ following transduction with AAV-HC-K$^d$-YCAC.** **(A-D)**. Expression of K$^b$ on liver leukocytes and splenocytes was equivalent between TAP1KOHep and floxed control mice, while much lower levels were detected on hepatocytes from TAP1KOHep, compared with those from floxed littermates. Representative flow plots from n = 6. **(C)** TAP1KOHep and floxed control livers are morphologically normal. IHC staining demonstrates expression of K$^b$ and D$^b$ in nonparenchymal cells from TAP1KOHep livers, while K$^b$ and D$^b$ are absent from hepatocytes in these mice (×200 magnification, representative images from n = 3). **(D)** Thick sections (150 µm) from TAP1KOHep or floxed control livers, were stained with antibodies against H-2K$^b$, CD31, CK19 and CD45. Confocal micrographs were obtained. K$^b$ is ubiquitously expressed in floxed controls, but absent from hepatocytes in TAP1KOHep livers (×930 magnification, representative images from n = 3). **(E,F)** While H-2K$^d$ was clearly present on hepatocytes from TAP1KOHep transduced with AAV-HC-K$^d$, expression did not reach that in TAP-sufficient mice transduced with the same vector. To achieve robust cell surface expression of K$^d$, we designed a construct where point mutations (Y84C and A139C; YCAC) stabilise expression of K$^d$ when empty or loaded with low-affinity peptides. Inoculation of TAP1KOHep mice with AAV-HC-K$^d$-YCAC yielded comparable expression to that of TAP-sufficient mice transduced with AAV-HC-K$^d$. Representative flow plots from n = 3 are shown. **(G)** Thick sections of TAP1KO/YCAC and TAP1$^{fl/fl}$/YCAC livers were obtained and stained with antibodies against H-2K$^d$, plus CD31, CK19 and CD45 as above. Equivalent strong expression of K$^d$ in hepatocytes, but not other cell types, was observed for both treatment groups (×930 magnification, representative images from n = 3). **(B,F)** Mean ± SEM are shown. Statistical analysis comprised unpaired Student's t-test: ns, not significant, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *** $p < 0.0001$.

**Figure 5**

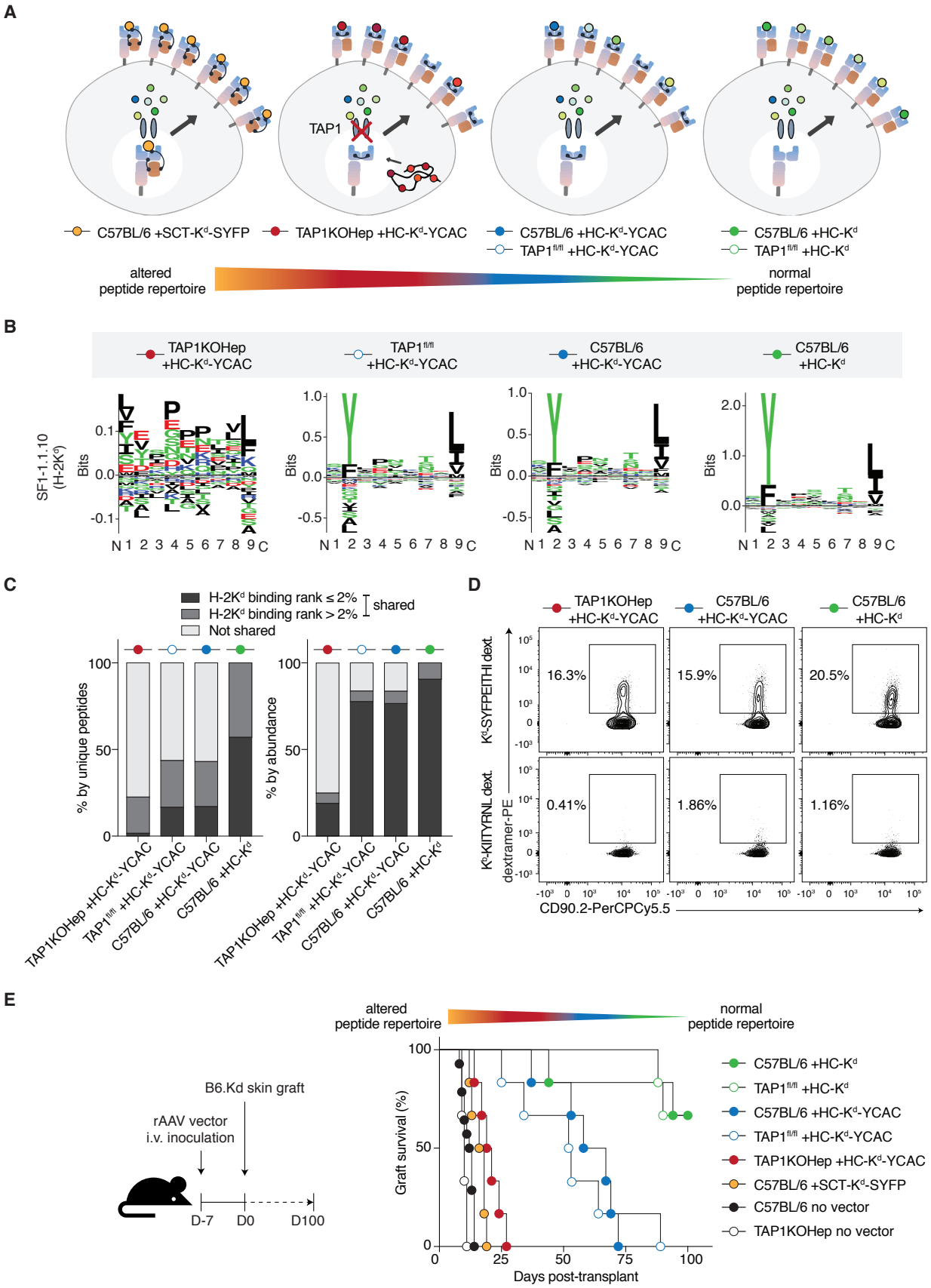
A
TAP1
C57BL/6 +SCT-Kd-SYFP
TAP1KOHep +HC-Kd-YCAC
C57BL/6 +HC-Kd-YCAC
TAP1fl/fl +HC-Kd-YCAC
C57BL/6 +HC-Kd
TAP1fl/fl +HC-Kd
altered peptide repertoire
normal peptide repertoire
B
TAP1KOHep +HC-Kd-YCAC
TAP1fl/fl +HC-Kd-YCAC
C57BL/6 +HC-Kd-YCAC
C57BL/6 +HC-Kd
SF1-1.1.10 (H-2Kd)
Bits
N 1 2 3 4 5 6 7 8 9 C
C
H-2Kd binding rank ≤ 2%
H-2Kd binding rank > 2%
shared
Not shared
% by unique peptides
% by abundance
TAP1KOHep +HC-Kd-YCAC
TAP1fl/fl +HC-Kd-YCAC
C57BL/6 +HC-Kd-YCAC
C57BL/6 +HC-Kd
D
Kd-SYFPEITHI dext.
Kd-KIITYRNL dext.
dextramer-PE
16.3%
15.9%
20.5%
0.41%
1.86%
1.16%
CD90.2-PerCPCy5.5
E
altered peptide repertoire
normal peptide repertoire
B6.Kd skin graft
rAAV vector i.v. inoculation
D-7
D0
D100
Graft survival (%)
Days post-transplant
C57BL/6 +HC-Kd
TAP1fl/fl +HC-Kd
C57BL/6 +HC-Kd-YCAC
TAP1fl/fl +HC-Kd-YCAC
TAP1KOHep +HC-Kd-YCAC
C57BL/6 +SCT-Kd-SYFP
C57BL/6 no vector
TAP1KOHep no vector

**Figure 5. Alteration in the endogenous peptide repertoire impedes transplantation tolerance induction.**
**(A)** Differences in peptide presentation between TAP-sufficient and deficient hepatocytes transduced with AAV vectors encoding one of three H-2K$^d$ constructs. From left to right, diagrams show a single presented peptide (SYFPEITHI), a substantially-altered repertoire resulting from the combination of K$^d$-YCAC expression and absence of TAP, a moderately altered repertoire when K$^d$-YCAC is expressed in cells where TAP is active, and the repertoire normally associated with K$^d$ in TAP-sufficient hepatocytes. Both empty and peptide-loaded K$^d$-YCAC molecules are expected to be present at the cell surface. **(B)** H-2K$^d$-binding motifs were generated from a non-redundant list of 9-mer peptides using GibbsCluster-2.0. Peptides eluted from B6/HC, B6/YCAC or TAP1$^{fl/fl}$/YCAC, hepatocytes displayed the canonical K$^d$ binding motif of tyrosine (Y) at position 2 and leucine (L) or isoleucine (I) at the C-terminus while no clear binding motif was discernible in the sequences for peptides eluted from TAP1KO/YCAC hepatocytes. **(C)** Just under 45% of peptides eluted from B6/YCAC TAP1$^{fl/fl}$/YCAC or TAP1$^{fl/fl}$/YCAC hepatocytes were common to the B6/HC repertoire, while only 22% of peptides from TAP1KO/YCAC were shared with B6/HC. Similarity across peptide repertoires was increased when the comparison was weighted for peptide abundance. The peptide SYFPEITHI was present in all repertoires. **(D)** Comparable proportions of activated CD8$^+$ T cells able to recognise K$^d$-SYFPEITHI were detected among the liver leukocytes of primed C57BL/6 mice inoculated with AAV-HC-K$^d$ or AAV-HC-K$^d$-YCAC and those of TAP1KOHep mice treated with AAV-HC-K$^d$-YCAC. Representative flow plots from n = 3 are shown. **(E)** TAP1KOHep, TAP1$^{fl/fl}$ or C57BL6 mice were inoculated with AAV-HC-K$^d$-YCAC or were not transduced. Another group of C57BL/6 were transduced with AAV-SCT-K$^d$-SYFP, while C57BL6 and TAP1$^{fl/fl}$ controls received AAV-HC-K$^d$ (n = 6 per group). 7 days post inoculation, the mice were challenged with B6.Kd skin grafts. Expression of HC-K$^d$ loaded with the endogenous peptide repertoire was able to induce tolerance to B6.Kd skin grafts in the majority of In C57BL/6 and TAP1$^{fl/fl}$, recipients. Increasing perturbation of the H-2K$^d$ bound peptide repertoire progressively shortened graft survival of H-2K$^d$ bearing skin grafts. Mantel-Cox log-rank test. ***p = 0.009, between C57BL/6 inoculated with AAV-HC-K$^d$ (MST: indefinite) and C57BL/5 inoculated with AAV-HC-K$^d$-YCAC (MST: 62.5 days), n = 6 per group. ***p = 0.007, between C57BL/6 inoculated with AAV-HC-K$^d$ (MST: indefinite) and TAP1$^{fl/fl}$ inoculated with AAV-HC-K$^d$-YCAC (MST: 52.5 days), n = 6 per group. ***p = 0.0005, between C57BL/6 inoculated with AAV-HC-K$^d$ (MST: indefinite) and TAP1KOHep inoculated with AAV-HC-K$^d$-YCAC (MST: 20 days), n = 6 per group.

**Figure 6**

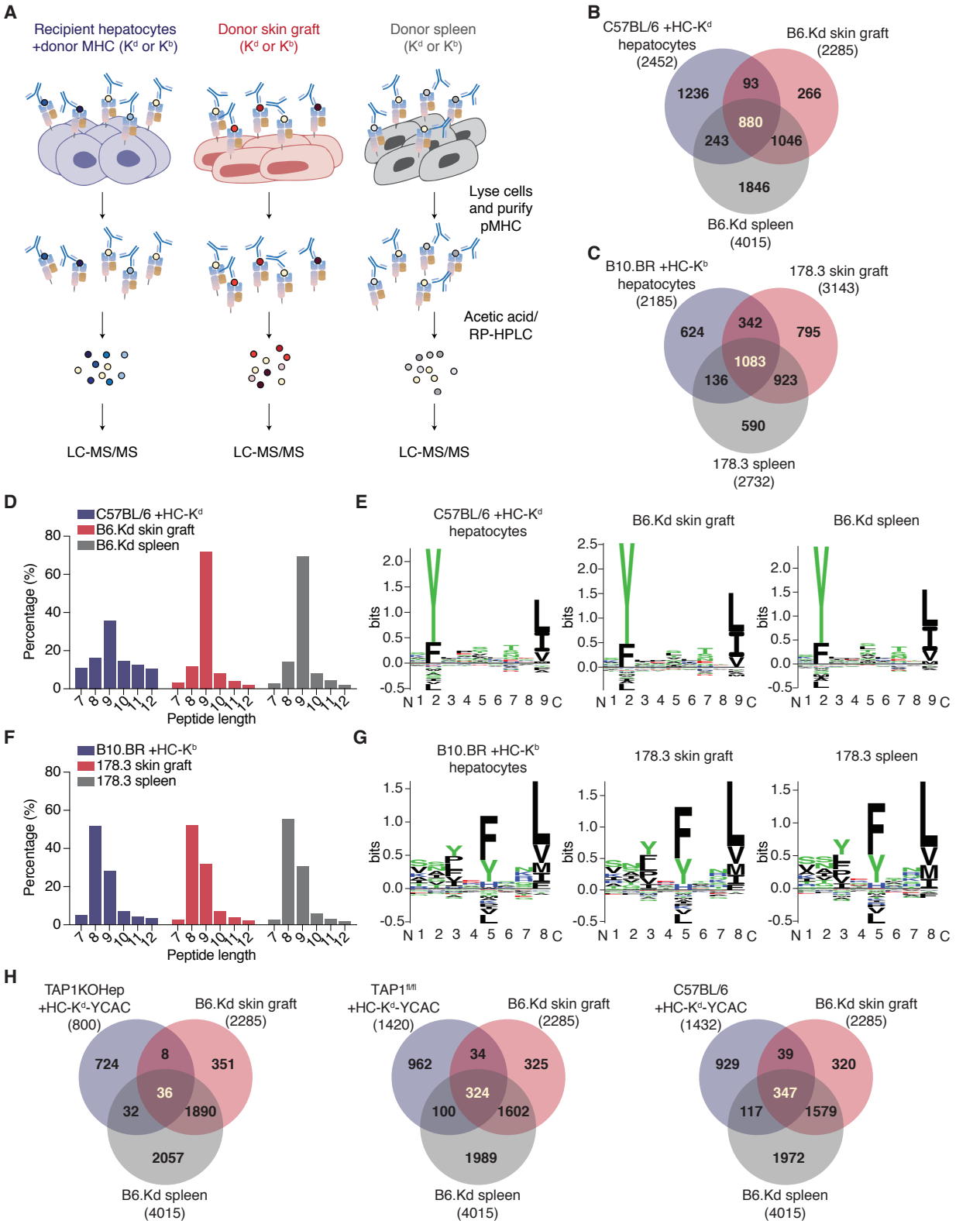


**Figure 6: Profiling of the H-2K$^d$ and H-2K$^b$-associated peptide repertoires of transduced hepatocytes, skin and spleen**
**(A)** A schematic diagram illustrating the immunoaffinity purification workflow. **(B,C)** Unique peptides were identified from transduced hepatocytes, skin and spleen. For H-2K$^d$ the peptide repertoires of C57BL/6 hepatocytes transduced with AAV-HC-K$^d$, B6.Kd spleen and B6.Kd skin grafts collected 7 days after transplantation were determined, while for the H-2K$^b$, the corresponding tissues were B10.BR hepatocytes transduced with AAV-HC-K$^b$, 178.3 spleen and 178.3 skin grafts sampled at 7 days post-transplant. Data from two independent experiments are shown. Within each replicate experiment, samples from 3-4 mice were pooled per condition. For K$^d$, 880 9-mer peptides were found to be shared across all three tissue types, while 1083 K$^b$ peptides (8-11 mer, $IC_{50}$ < 500nM) were common to the three tissues. **(D)** Length distribution of filtered H-2K$^d$ peptides from hepatocytes, spleen and skin graft tissue samples. The number of peptides of each length identified with a 5% FDR cut-off are shown. **(E)** Peptide-binding motifs for H-2K$^d$ peptides generated from a non-redundant list of 9-mer peptides using GibbsCluster-2.0. **(F)** Length distribution of H-2K$^b$ peptides from hepatocytes, spleen and skin graft tissue samples (as for **D**). Most eluted peptides are 8-mers, with 9-mers also relatively frequent. **(G)** Peptide-binding motifs for H-2K$^b$ peptides generated from a list of 8-11-mer peptides using the GibbsCluster-2.0 algorithm as above. The canonical binding motifs were observed for all three tissues for both H-2K$^d$ and H-2K$^b$. **(H)** The extent of peptide sharing between the H-2K$^d$ repertoires of hepatocytes, spleen and skin was substantially reduced when TAP1KOHep, TAP1$^{fl/fl}$ or C57BL6 mice inoculated with AAV-HC-K$^d$-YCAC were substituted for C57BL/6 transduced with AAV-HC-K$^d$. This reduction was particularly striking for the combination of TAP1KOHep with AAV-HC-K$^d$-YCAC.

**Figure 7**

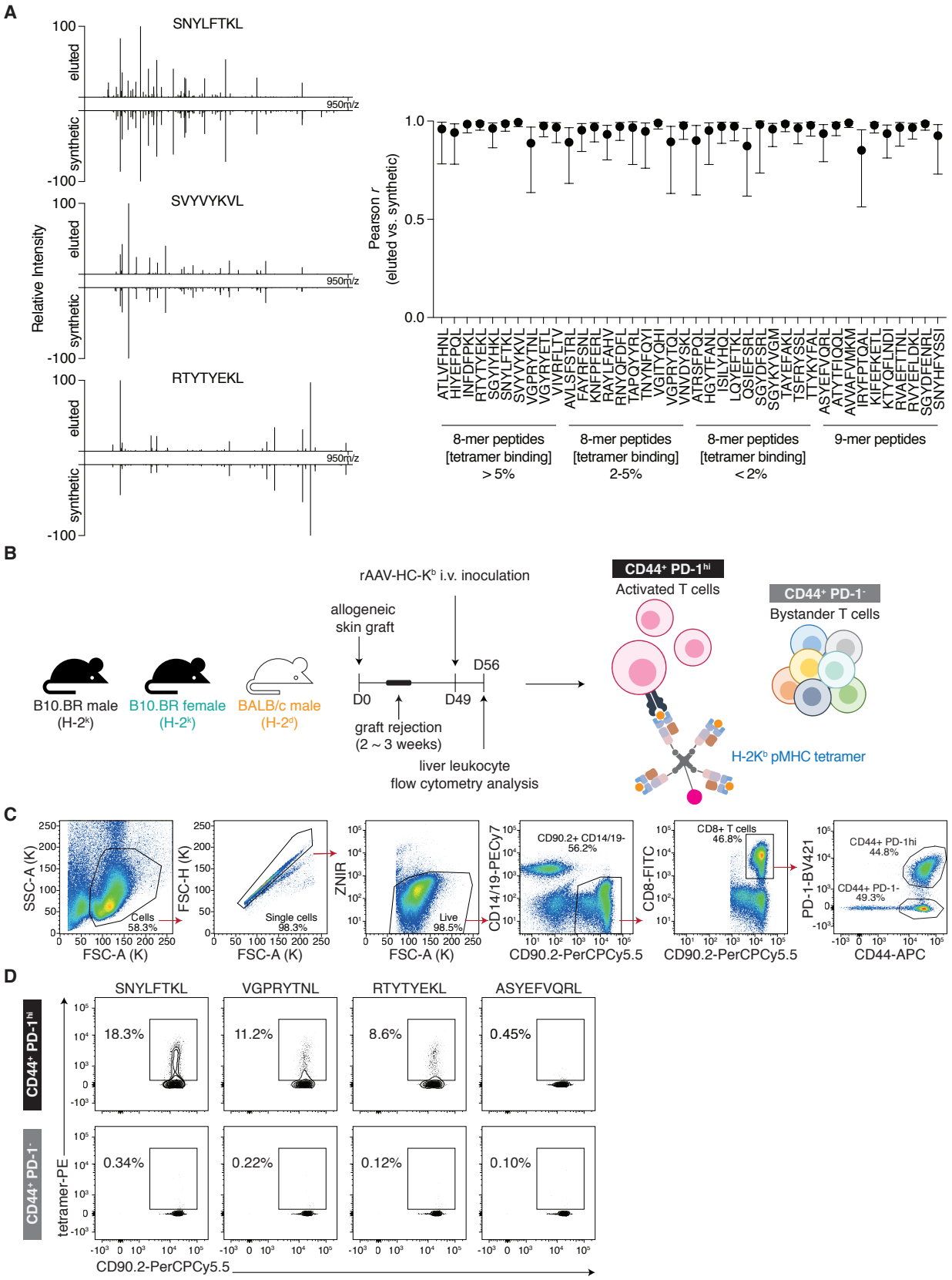


**Figure 7. Identification of pMHC epitopes recognised by alloreactive CD8+ T cells.**
**(A)** We validated the identity of a panel of eluted peptides by comparing chromatographic retention and MS/MS spectra with those of the corresponding synthetic peptides. Example spectra for three pairs of synthetic and eluted peptides are shown at left. Pearson correlation coefficients (*r*) between the $\log_{10}$ intensities of identified b- and y-ions in the synthetic and sample-derived spectra are shown on the right. Error bars represent the 95% confidence intervals. The corresponding p-value was < 0.05 for each peptide pair. **(B)** Male B10.BR mice were primed with male 178.3 skin grafts and 30 days after rejection, mice were transduced with AAV-HC-Kb. Liver leukocytes were isolated and pMHC tetramer binding was analysed using flow cytometry. **(C)** Shows the gating strategy that was employed to identify and stratify CD8+ T cells into two groups; CD44+PD-1hi and CD44+PD-1-. CD44+PD-1hi cells are activated alloreactive CD8+ T cells and CD44+PD-1- cells are non-activated bystander CD8+ T cells which serve as internal controls. **(D)** Examples of pMHC tetramer binding by CD44+PD-1hi and CD44+PD-1- CD8+ T cells. Representative flow plots (from n = 3 biological replicates) for the selected pMHC epitopes are shown. Kb-SNYLFTKL ($Epas_{387-394}$), Kb-VGPRYTNL ($Mapk1_{19-26}$) and Kb-RTYTYEKL ($Ctnnb1_{329-336}$) were recognised by a large proportion of activated alloreactive T cells, whereas few T cells bound Kb-ASYEFVQRL ($Dync1h1_{1379-1387}$).

**Figure 8**

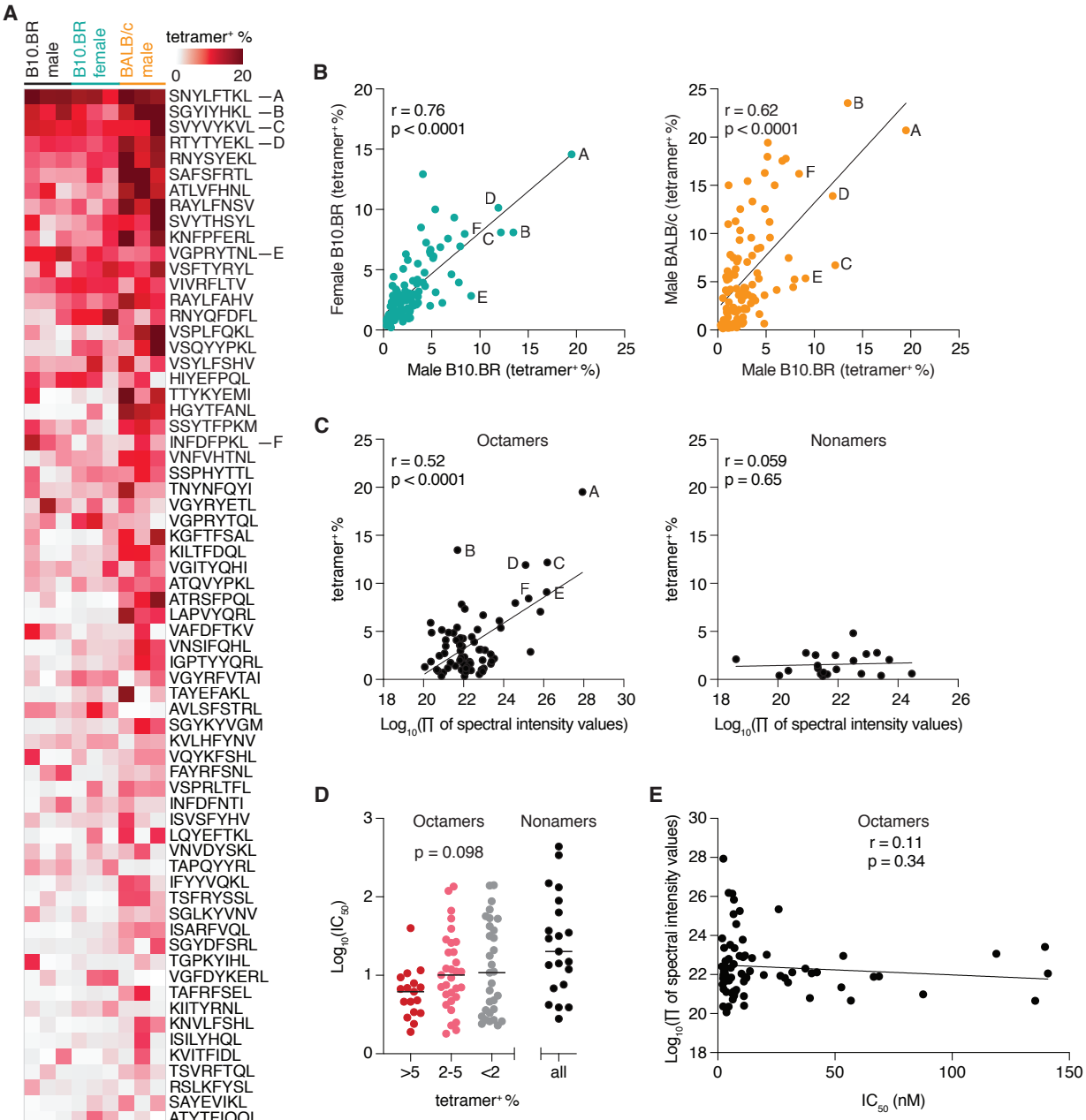


**Figure 8. Features of immunogenic pMHC recognised by mice from different allogeneic backgrounds**

**(A)** 100 peptides were screened for recognition by activated alloreactive T cells. T cells from female B10.BR mice primed with a female 178.3 skin graft and male BALB/c mice primed with a male C57BL/6 skin graft were used in parallel with T cells from primed male B10.BR. A heatmap was generated in order to compare recognition of pMHC epitopes across different sexes and strains. Epitopes are ordered from the top to the bottom by the average of pMHC tetramer binding across all samples. 13 pMHC were recognised by > 5% of activated alloreactive T cells across all three groups. Full peptide screening data can be found in Supplementary Table 4. **(B)** Strong correlation of T cell binding to each pMHC was found between male and female B10.BR [Pearson correlation coefficient (r) = 0.76, ***p < 0.0001] and between male B10.BR and male BALB/c (r = 0.62, ***p < 0.0001). **(C)** For male B10.BR mice, the proportion of T cells binding 8-mer pMHC tetramers was proportional to log10 of the product of the spectral intensity values for each peptide across three different tissues (r = 0.52, ***p < 0.0001). Conversely, binding to 9-mer pMHC tetramers did not correlate with peptide spectral intensity (r = 0.059, p = 0.65). For each pMHC epitope, data was obtained from one - two independent experiments with a total of 3 biological replicates. **(D)** Predicted peptide binding affinity for H-2K$^b$ (measured by $IC_{50}$) did not differ significantly between strongly, moderately or non-immunogenic peptides (p = 0.098 by one-way ANOVA, line at mean), **(E)** nor was a correlation observed between $IC_{50}$ and spectral intensity values (r = 0.11, p = 0.34).

**Figure 9**

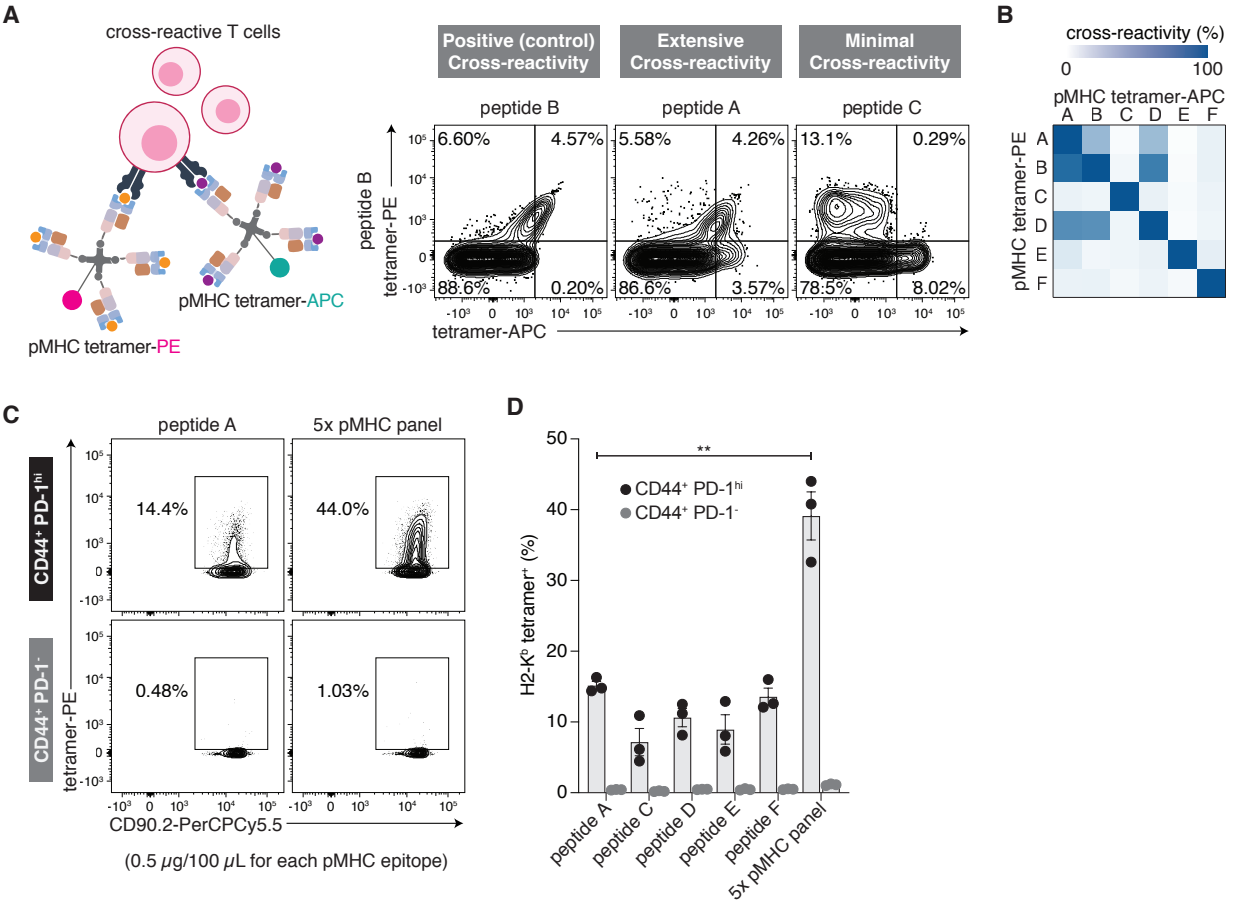


**Figure 9 pMHC multimers can be combined to enhance detection of alloreactive T cells within a polyclonal population.** **(A, B)** Staining with two different pMHC tetramers was used to evaluate the proportion of T cells recognising more than one pMHC specificity. Six strongly immunogenic peptides were tested. 86.7% of SNYLFTKL+ T cells recognised SGYIYHKL in addition to SNYLFTKL, while SVYVYKVL tetramers bound 66.8% of SNYLFTKL+ T cells and SGYIYHKL tetramers bound 75.8% of SVYVYKVL+ cells. Cross-reactivity between VGPRYTNL, INFDFPKL and RTYTYEKL was considerably lower (4.0 and 8.4% of T cells recognised VGPRYTNL in addition to INFDFPKL and RTYTYEKL, respectively). **(A)** Representative flow plots from two independent experiments with a total of 3 - 4 mice pooled per experiment. **(B)** A heatmap was generated in order to compare cross-reactivity between different pMHC epitopes. **(C, D)** When 5 of these 6 peptides (excluding SGYIYHKL), each binding between 7.2 and 15.2% of T cells were used together as a panel, the proportion of alloreactive CD8+ T cells bound increased to 39.1% ($p = 0.002$ compared with SNYLFTKL). Each tetramer was two-fold less concentrated compared to other multimer staining experiments due to the obligatory increase in staining volume (0.5 µg/100 µL versus 0.5 µg/50 µL, respectively). Data were obtained from one experiment with a total of $n = 3$ biological replicates per group. **(C)** Representative flow plots (from $n = 3$). **(D)** Data are shown as mean ± SEM, statistical analysis comprised unpaired Student's t-test: ns, not significant, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *** $p < 0.0001$.