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Diversity and clonotypic composition of influenza-specific CD8$^+$ TCR repertoires remain unaltered in the absence of Aire

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TCR repertoire diversity is important for the protective efficacy of CD8$^+$ T cells, limiting viral escape and cross-reactivity between unrelated epitopes. The exact mechanism for selection of restricted *versus* diverse TCR repertoires is far from clear, although one thought is that the epitopes resembling self-peptides might select a limited array of TCR due to the deletion of autoreactive TCR. The molecule Aire promotes the expression of tissue-specific Ag on thymic medullary epithelial cells and the deletion of autoreactive cells, and in the absence of Aire autoreactive cells persist. However, the contribution of Aire-dependent peptides to the selection of the Ag-specific TCR repertoire remains unknown. In this study, we dissect restricted ($D^bNP_{366}^+CD8^+$) and diverse ($D^bPA_{224}^+CD8^+$, $K^dNP_{147}^+CD8^+$) TCR repertoires responding to three influenza-derived peptides in Aire-deficient mice on both B6 and BALB/c backgrounds. Our study shows that the number, qualitative characteristics and TCR repertoires of all influenza-specific, $D^bNP_{366}^+CD8^+$, $D^bPA_{224}^+CD8^+$ and $K^dNP_{147}^+CD8^+$ T cells are not significantly altered in the absence of Aire. This provides the first demonstration that the selection of an Ag-specific T-cell repertoire is not significantly perturbed in the absence of Aire.

Key words: CD8$^+$ T cells · Influenza · T-cell receptor repertoire · Viral infection

Supporting Information available online

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Introduction

$CD8^+$ T cells play a critical role in immune defense against viral infections through direct killing of virus-infected cells, production of antiviral cytokines and establishment of immunological memory [1]. T-cell recognition of viral peptides is mediated through the TCR interactions with a viral peptide bound to host MHC molecules. A diverse repertoire of TCR is involved in the recognition of any given viral peptide. The diverse repertoire of TCR is produced in the thymus through random recombination of germline TCR genes, involving both nucleotide deletion and insertion. The majority of TCR produced in this way are eliminated before maturity during the process of thymic selection. During thymic selection, candidate TCR interact with self-peptide-MHC (pMHC) on the surface of thymic epithelial cells. Only TCR with a sufficient avidity for self-pMHC to mediate useful peptide recognition are allowed to survive the positive selection process. TCR with high avidity for self-pMHC pose a risk of self-reactivity and are eliminated through negative selection [2].

The transcription factor Aire mediates a role in central tolerance by promoting the promiscuous expression of tissue-restricted antigens (TRA) in the thymus, which is associated with the negative selection of autoreactive T cells [3–6] (our unpublished observations). The absence of the AIRE gene in humans is associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which is thought to arise when autoreactive T cells specific for TRA escape negative selection in the thymus. Aire may also play a role in the generation of regulatory T cells [7], thus participating in the generation of a specific TCR repertoire. The expression of Aire in the periphery is less clear. Some studies have reported Aire expressed in lymph nodes, in the peripheral monocyte/DC lineage, and a subset of lymph node stromal cells [8–13], while others suggest that it is rather unlikely that Aire is present in DC or lymph node epithelial cells [14].

The development of autoimmunity in AIRE-deficient patients and Aire-deficient mice provides evidence for the importance of AIRE in eliminating autoreactive T cells in the thymus through the expression of TRA. However, whether the absence of Aire alters the peripheral TCR repertoire available to respond to foreign pathogens is an issue of debate. An early study [15] comparing the features of TCRβ repertoires between wt and Aire-deficient mice found differences in the immunoscopes of three of the 24 Vβ families, suggesting that Aire may significantly perturb the repertoire. A more recent study [16] of the Vβ gene usage and CDR3β lengths of naive $CD4^+$ T cells suggests minimal differences in the TCRβ repertoires between the wt and Aire-deficient mice. Thus, it is unclear whether Aire deficiency significantly alters the selection of the Ag-specific TCR repertoire.

Immune TCR repertoires can be either diverse, consisting of several clonotypes expressing different CDR3β aa sequences and Jβ regions, or restricted to a few clonotypes with similar Jβ and CDR3β patterns. The diversity of the Ag-specific $CD8^+$ TCR repertoire directed at a particular antigenic peptide plays an important role in efficient protection against invading/emerging pathogens [17], limiting the possibility of viral escape [18] and cross-reactivity between epitopes derived from unrelated pathogens [19]. The exact mechanism for selection of restricted *versus* diverse TCR repertoires is unclear, although one thought is that the epitopes resembling self-peptides might select a limited array of TCR due to the deletion of autoreactive TCR. In this study, we have investigated the role of Aire in shaping the diversity and clonal composition of T-cell repertoire by comparing the $CD8^+$ T-cell repertoires responding to three influenza A virus epitopes *in vivo*. We have utilized a well-characterized non-TCR-transgenic model of influenza A virus infection of mice for dissecting $CD8^+$ T-cell responses and TCR repertoires. This respiratory challenge model is characterized by an acute, transient, localized pneumonia, with virus clearance by day (d) 10 after infection [20, 21]. Although influenza virus immunity has been investigated in both C57Bl/6J (B6, $H2^b$) and BALB/c ($H2^d$) mice, most studies, including those by our group, have used predominantly the B6 model due to the larger number of identified, $H2K^b$-and $H2D^b$-restricted viral epitopes and the extensive characterization of B6 $CD8^+$ TCR repertoires. To assess whether Aire affects TCR repertoire diversity of Ag-specific $CD8^+$ T cells, we investigated the role of Aire on well-characterized TCR repertoires of distinct natures, the restricted $D^bNP_{366}{}^+CD8^+$ [22, 23] as well as diverse $D^bPA_{224}{}^+CD8^+$ [24] T-cell populations in B6 mice. Additionally, we have characterized the TCR repertoires of $K^dNP_{147}{}^+$ $CD8^+$ T-cell responses in BALB/c mice for comparative studies with $Aire^{-/-}$ BALB/c mice. These comparisons suggest that loss of Aire does not cause major alterations in the thymic selection program of influenza-specific $CD8^+$ T cells.

Results

Normal magnitude of influenza-specific $CD8^+$ T-cell responses in $Aire^{-/-}$ B6 and BALB/c mice

To determine whether influenza-specific TCR repertoires are under the control of *Aire*, we first assessed the magnitude of the epitope-specific $CD8^+$ T-cell response at the acute phase (d10) of influenza virus infection in $Aire^{-/-}$ mice. We examined three influenza-specific $CD8^+$ T-cell populations, $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+$ $CD8^+$ T-cell sets in $Aire^{-/-}$ B6 mice and $K^dNP_{147}{}^+CD8^+$ T-cell responses in $Aire^{-/-}$ BALB/c mice. $CD8^+$ T-cell populations in $Aire^{-/-}$ and wt mice in the spleen (Fig. 1A) and at the site of viral infection (bronchoalveolar lavage (BAL); Fig. 1B) were stained with tetramers and enumerated. It was immediately apparent that both splenic and BAL $CD8^+$ T-cell responses in $Aire^{-/-}$ mice following HKx31 infection were comparable with the wt $CD8^+$ T-cell responses. Both $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T-cell responses in $Aire^{-/-}$ B6 mice and $K^dNP_{147}{}^+CD8^+$ T-cell sets in $Aire^{-/-}$ BALB/c mice gave the same results in the absence of Aire. The magnitude of all three primary $D^bNP_{366}{}^+CD8^+$, $D^bPA_{224}{}^+CD8^+$ and $K^dNP_{147}{}^+CD8^+$ T-cell populations was comparable in the spleen (Fig. 1A). Interestingly, the numbers of $K^dNP_{147}{}^+CD8^+$

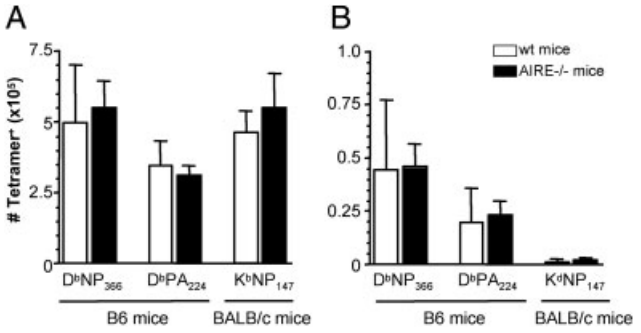

Figure 1. Normal magnitude of influenza-specific $CD8^+$ T-cell responses in $Aire^{-/-}$ B6 and BALB/c mice. The magnitude of $CD8^+$ T-cell responses at the peak (d10) of primary influenza A virus infection in (A) spleen and (B) BAL was determined by tetramer staining. Cells were recovered from either wt (open bars) or $Aire^{-/-}$ (black bars) B6 (H-$2D^b$) or BALB/c (H-$2K^d$) mice. Lymphocytes were stained with D^bNP_{366}-PE and D^bPA_{224}-PE or K^dNP_{147}-PE tetramers for 60 min at room temperature, washed and stained with anti-CD8-FITC mAb. Shown are total numbers of tetramer$^+CD8^+$ T cells for D^bNP_{366}, D^bPA_{224} and K^dNP_{147} tetramers. Mice were infected i.n. with 10^4 pfu of the HKx31 (H3N2) influenza A virus in 30 μL of PBS. Data represent mean ± SD from 4 to 5 mice in each group.

Minimal effect of $Aire^{-/-}$ on $CD8^+$ T-cell function

Does Aire affect the pMHC-TCR avidity and function of virus-specific $CD8^+$ T cells? pMHC-TCR avidity of $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T-cell populations was determined by tetramer dissociation assay [25]. Binding avidities of two main influenza-specific $CD8^+$ T cells in B6 mice were comparable between $D^bNP_{366}{}^+CD8^+$ (Fig. 2A) and $D^bPA_{224}{}^+CD8^+$ (Fig. 2B) T-cell sets for wt and $Aire^{-/-}$ mice. The tetramer dissociation curves for both TCR showed similar trends for off-rate and kinetics. Thus, TCR/pMHCI avidity characteristics of virus-specific $CD8^+$ T-cell responses remain conserved in the absence of Aire.

The functional characteristic that has been explored for $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T-cell responses was the capacity to produce multiple cytokines (IFN-γ, TNF-α and IL-2) simultaneously after short-term (5 h) stimulation with cognate peptide in the presence of Brefeldin A [25]. The frequencies of double (IFN-γ/TNF-α) (Fig. 2C and D) and triple-producers (IFN-γ/TNF-α/IL-2) (Fig. 2E and F) within the $D^bNP_{366}{}^+CD8^+$ (Fig. 2C and E) and $D^bPA_{224}{}^+CD8^+$ (Fig. 2D

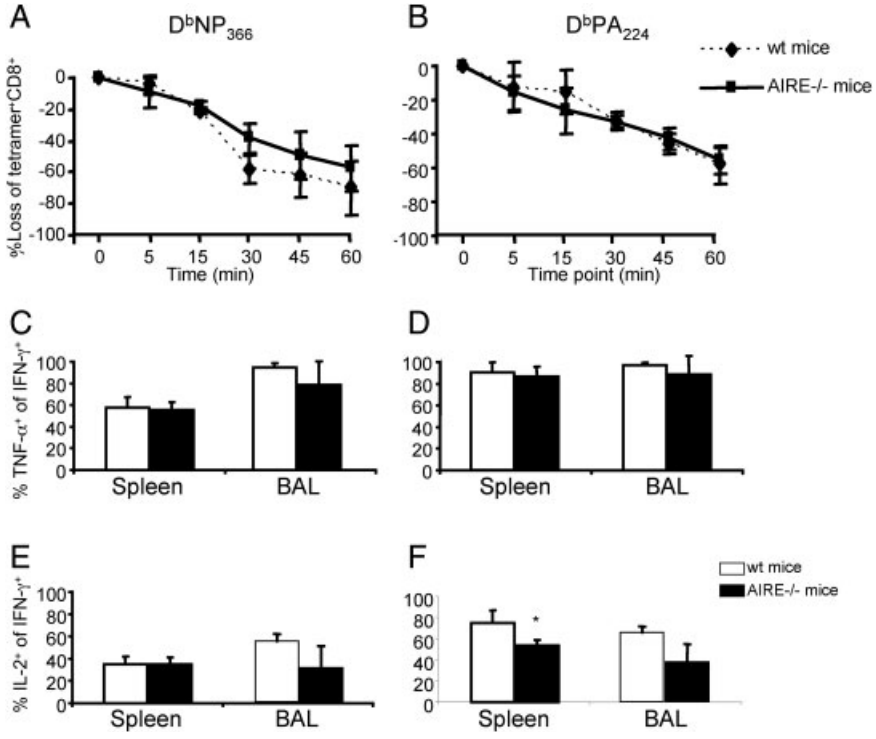

Figure 2. Functional quality of influenza-specific $CD8^+$ T-cell population in $Aire^{-/-}$ mice. $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T cells generated in $Aire^{-/-}$ mice were assessed for (A, B) TCR avidity for the pMHC complex and (C–F) simultaneous production of IFN-γ, TNF-α and IL-2. (A, B) Tetramer dissociation was assessed as a measure of TCR avidity for the cognate pMHCI complex. Splenocytes obtained from influenza-infected wt (open bars) or $Aire^{-/-}$ (black bars) were stained with either the (A) D^bNP_{336} or (B) D^bPA_{224} tetramers, then incubated at 37°C with a mAb to $H2D^b$ to prevent rebinding of dissociated tetramer. The progressive diminution in tetramer staining was measured by flow cytometric analysis. Data represent mean ± SD. (C–F) Compiled data (mean+SD) for hierarchical production of IFN-γ, TNF-α and IL-2 are shown for (C, E) D^bNP_{336} and (D, F) D^bPA_{224} $CD8^+$ T cells obtained from spleens or BAL of mice infected with influenza A virus. *$p<0.05$ was derived using a Student's *t*-test. Data represent mean+SD from 4 to 5 mice in each group.

T-cell sets in BALB/c mice (both wt and $Aire^{-/-}$) were lower at the site of viral infection (BAL) than those for $D^bNP_{366}{}^+CD8^+$ or $D^bPA_{224}{}^+CD8^+$ T-cell responses (Fig. 1B).

and F) sets in $Aire^{-/-}$ mice were equivalent to those found in wt B6 mice, although $D^bPA_{224}{}^+CD8^+$ T cells in the spleen of $Aire^{-/-}$ mice seemed to produce less IL-2 (Fig. 2F). Overall, our data

suggest that the influenza-specific $CD8^+$ T cells are functionally comparable in wt and $Aire^{-/-}$ mice.

Conserved Vβ usage in influenza-specific $CD8^+$ T-cell responses in the absence of *Aire*

Although the magnitude and function of the $CD8^+$ T-cell responses between wt and $Aire^{-/-}$ mice were comparable, this could be a reflection of the Ag load rather than the underlying TCR repertoire. To investigate whether Aire controls the TCR that are recruited into immune $CD8^+$ T-cell responses, we needed to define the nature of the influenza-specific $CD8^+$ T-cell repertoire at the clonal level. Since immunodominant $CD8^+$ T-cell responses to a variety of viruses are characterized by epitope-specific biases in TCR Vβ element usage [26–29], influenza-specific $CD8^+$ T-cell responses were first analyzed for their Vβ bias. Both $D^bNP_{366}{}^+CD8^+$ [22, 30, 31] and $D^bPA_{224}{}^+CD8^+$ [32] T-cell responses in B6 mice are characterized by a dominant Vβ element, Vβ8.3 (Fig. 3A) and Vβ7 (Fig. 3B), respectively. The strong Vβ8.1/8.2 bias was found for $K^dNP_{147}{}^+CD8^+$ T cells (Fig. 3C) by staining with a panel of anti-Vβ mAb (data not shown). Analysis of the T-cell populations responding to all three epitopes ($D^bNP_{366}{}^+CD8^+$ (Fig. 3A), $D^bPA_{224}{}^+CD8^+$ (Fig. 3B) and $K^dNP_{147}{}^+CD8^+$ (Fig. 3C) in the $Aire^{-/-}$ mice showed that the dominant Vβ is conserved. Comparable results were obtained for both the spleen and BAL compartments, suggesting that Vβ bias is consistent in different anatomical sites.

TCR Vβ repertoires of $D^bNP_{366}{}^+CD8^+$ and $D^bPA_{224}{}^+CD8^+$ T-cell responses in $Aire^{-/-}$ B6 mice

Our previous single-cell TCRβ analysis of influenza virus-specific D^bNP_{366} and D^bPA_{224} epitopes defined the extent of clonotypic diversity and composition for those two immunodominant $CD8^+$ T-cell responses. The $D^bNP_{366}{}^+Vβ8.3^+CD8^+$ repertoire is relatively focused and heavily biased toward usage of a nine-aa CDR3β loop and Jβ2.2 [22, 23]. In contrast, the $D^bPA_{224}{}^+Vβ7^+CD8^+$ response [24, 32] is clonotypically more diverse in terms of the CDR3β loop length, Jβ usage and the variety of different clonotypes. The $D^bPA_{224}{}^+Vβ7^+CD8^+$ T cells are characterized by a shorter CDR3β loop of six or seven aa and dominant Jβ1.1, Jβ1.5 and Jβ2.6 elements. Thus, it was of interest to determine whether Aire controls the diversity and composition of influenza-specific TCR repertoires of such distinct natures.

A single-cell RT-PCR approach to the TCRβ repertoire analysis has been previously successfully used to detect differences in TCRβ repertoires between high avidity and low avidity populations [33] as well as $CD62L^{hi}$ and $CD62L^{lo}$ subsets [31] of

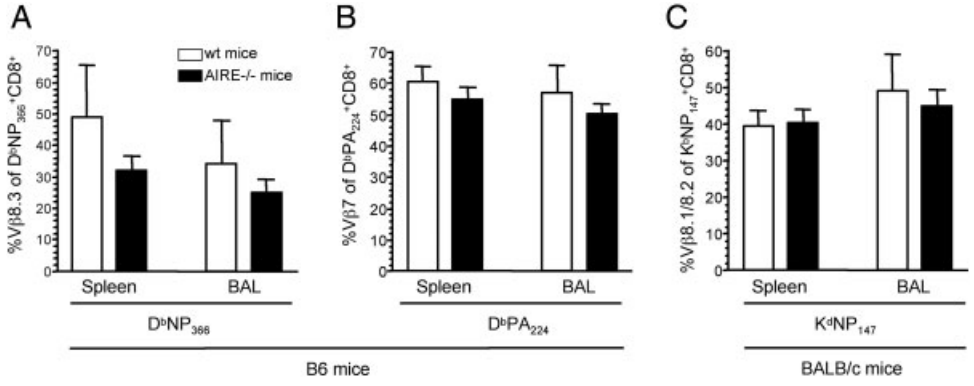

Figure 3. Comparable levels of the dominant Vβ bias in $Aire^{-/-}$ B6 and BALB/c mice. $D^bNP_{366}{}^+CD8^+$, $D^bPA_{224}{}^+CD8^+$ or $K^dNP_{147}{}^+CD8^+$ T cells in $Aire^{-/-}$ mice were assessed for their dominant Vβ bias (Vβ8.3, Vβ7 and Vβ8.1/8.2, respectively). Lymphocytes obtained from spleens of either wt (open bars) or $Aire^{-/-}$ (black bars) B6 (H-$2D^b$) or BALB/c (H-$2K^d$) mice were stained with D^bNP_{366}-PE, D^bPA_{224}-PE or K^dNP_{147}-PE tetramers for 60 min at room temperature, washed and stained with anti-CD8-APC and anti-Vβ-FITC mAb. Data represent mean+SD from 4 to 5 mice in each group.

Table 1. Summary of the $D^bNP_{366}{}^+Vβ8.3^+$ and $D^bPA_{224}{}^+Vβ7^+$ TCRβ repertoires in wt and $Aire^{-/-}$ B6 mice and the $K^dNP_{147}{}^+Vβ8.1/8.2^+$ TCRβ repertoires in wt and $Aire^{-/-}$ BALB/c mice after primary influenza infection

	$D^bNP_{366}{}^+Vβ8.3^+$		$D^bPA_{224}{}^+Vβ7^+$		$K^dNP_{147}{}^+Vβ8.1^+/Vβ8.2^+$	
	wt mice	$Aire^{-/-}$ mice	wt mice	$Aire^{-/-}$ mice	wt mice	$Aire^{-/-}$ mice
Mice analyzed[a)]	8	5	7	5	8	5
No. of TCR sequenced	477	332	386	302	385	316
Range in no. of TCR sequenced	54–67	55–76	39–64	41–75	37–65	58–66
Mean no. of TCR sequenced	60	66	55	61	48	63

[a)] Naive B6 mice were challenged i.n. with HKx31 influenza virus. Spleens were sampled at day 10 for analysis of primary responses.

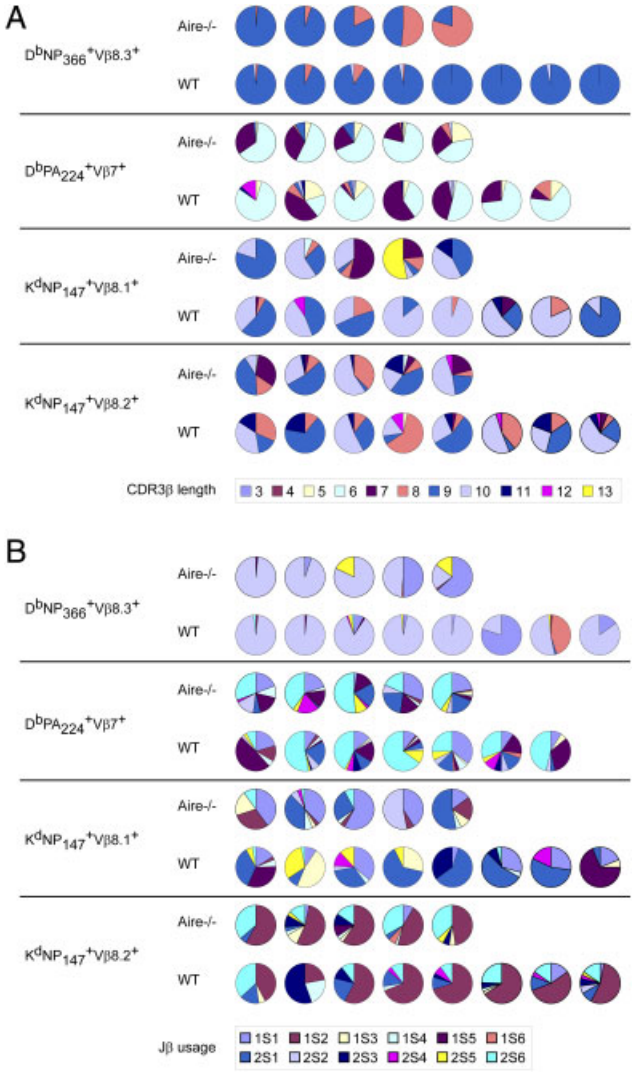

Figure 4. Comparison of the CDR3β lengths and Jβ usage between the Aire$^{-/-}$ and wt mice in the $D^bNP_{366}{}^+V\beta8.3^+$, $D^bPA_{224}{}^+V\beta7^+$ and $K^dNP_{147}{}^+V\beta8.1/V\beta8.2^+$ $CD8^+$ T-cell responses. Shown are the proportion of each epitope-specific and Vβ-specific TCRβ repertoire in each mouse with a particular CDR3 length (A) and using a particular Jβ gene (B).

influenza-specific $CD8^+$ T cells. Using this method, we first defined diversity of $D^bNP_{366}{}^+V\beta8.3^+$ $CD8^+$ T-cell repertoire in Aire$^{-/-}$ mice at the clonal level (Tables 1 and 2). As for the wt mice, $D^bNP_{366}{}^+CD8^+$ T cells in Aire$^{-/-}$ animals selected consistent TCRVβ profiles. The majority (~70% of 332 sequences) of $D^bNP_{366}{}^+V\beta8.3^+CD8^+$ TCRβs were characterized by the usage of Jβ2.2 and a nine-aa CDR3β length. In three of the five mice, the repertoires were dominated by a nine-aa CDR3β length and Jβ2.2 usage, while in two of the mice, TCRβ sequences of length eight aa and usage of Jβ1.1 were dominant (Fig. 4). The XGGXNTQGL motif that is prevalent in the $D^bNP_{366}{}^+V\beta8.3^+$ $CD8^+$ repertoires in wt mice was also prevalent in the Aire$^{-/-}$ mice. The SGGANTGQL and SGGSNTGQL clonotypes that have been previously identified as public clonotypes in wt mice were present in four out of five Aire$^{-/-}$ mice

Table 2. Frequency (%) of TCRβ aa sequences of primary $D^bNP_{336}{}^+V\beta8.3^+$ $CD8^+$ T cells following influenza virus infection in Aire$^{-/-}$ B6 mice

CDR3β region	Jβ	Length (aa)	M1	M2	M3	M4	M5
Vβ8.3							
SGGANTGQL	2S2	9	–	4.0	52.7	19.7	5.9
SGGSNTGQL	2S2	9	81.0	80.0	7.3	15.8	–
SGGGNTGQL	2S2	9	–	–	–	–	5.9
KGGGNTGQL	2S2	9	–	–	12.7	9.2	–
KGGAGTGQL	2S2	9	–	–	1.8	1.3	–
KGGANTGQL	2S2	9	–	4.0	–	–	–
KGGSNTGQL	2S2	9	–	–	–	–	5.9
SGGGRTGQL	2S2	9	–	1.3	–	2.6	–
RGGANTGQL	2S2	9	–	–	7.3	–	–
RGGGNTGQL	2S2	9	–	5.3	–	–	–
SGGARTGQL	2S2	9	15.5	–	–	–	–
RGGSNTGQL	2S2	9	–	–	–	–	2.9
SGGSNTGHL	2S2	9	1.7	–	–	–	–
SARTANTEV	1S1	8	–	–	–	–	58.8
SDAAATEV	1S1	8	–	–	–	28.9	–
SDAFKSEV	1S1	8	–	–	–	21.1	–
SDAQNTEV	1S1	8	–	–	–	–	4.4
SDHRNTEV	1S1	8	–	4.0	–	–	–
SDHRYTEV	1S1	8	–	1.3	–	–	–
SPTGASDY	1S2	8	–	–	–	–	1.5
SVGNQAP	1S5	8	1.7	–	–	–	–
SDNYNSPL	1S6	8	–	–	–	1.3	–
SVGGRDTQ	2S5	8	–	–	18.2	–	14.7
Total sequenced			58	75	55	76	68

(Table 2). However, the remaining public clonotype SGGGNTGQL was present only in one Aire$^{-/-}$ mouse. A comparison of the $D^bNP_{366}{}^+V\beta8.3^+CD8^+$ TCRβ repertoire diversity between Aire$^{-/-}$ and wt mice determined no significant differences in the number of different clonotypes (Fig. 5A) or total diversity accounting for the clonal dominance hierarchy (Fig. 5D). We also assessed the similarity of the $D^bNP_{366}{}^+V\beta8.3^+$ $CD8^+$ TCRβ repertoires between all pairs of Aire$^{-/-}$ and wt mice and found that the similarity of the TCRβ repertoires between Aire$^{-/-}$ and wt mice was not significantly less than between pairs of Aire$^{-/-}$ mice or between pairs of wt mice (Fig. 5G).

Clonal analysis of $D^bPA_{224}{}^+V\beta7^+CD8^+$ TCRβ sequences confirmed the diverse nature of this repertoire in Aire$^{-/-}$ mice (Table 1, Supporting Information Table 1). As in wt mice, the $D^bPA_{224}{}^+V\beta7^+CD8^+$ repertoires in Aire$^{-/-}$ mice featured diverse usage of CDR3β loop length and Jβ usage, utilizing predominantly the CDR3β loop of six and seven aa and Jβ2.6 and Jβ1.1 (Fig. 4). The SSYEG clonotype, detected in four out of five Aire$^{-/-}$ mice, was found in six of the seven wt mice used in this study [31]. There were no significant differences in TCRβ diversity between the $D^bPA_{224}{}^+V\beta7^+CD8^+$ T-cell responses in Aire$^{-/-}$ and wt mice (Fig. 5B and E). Moreover, the $D^bPA_{224}{}^+V\beta7^+CD8^+$ TCRβ repertoires between Aire$^{-/-}$ and wt mice were comparably

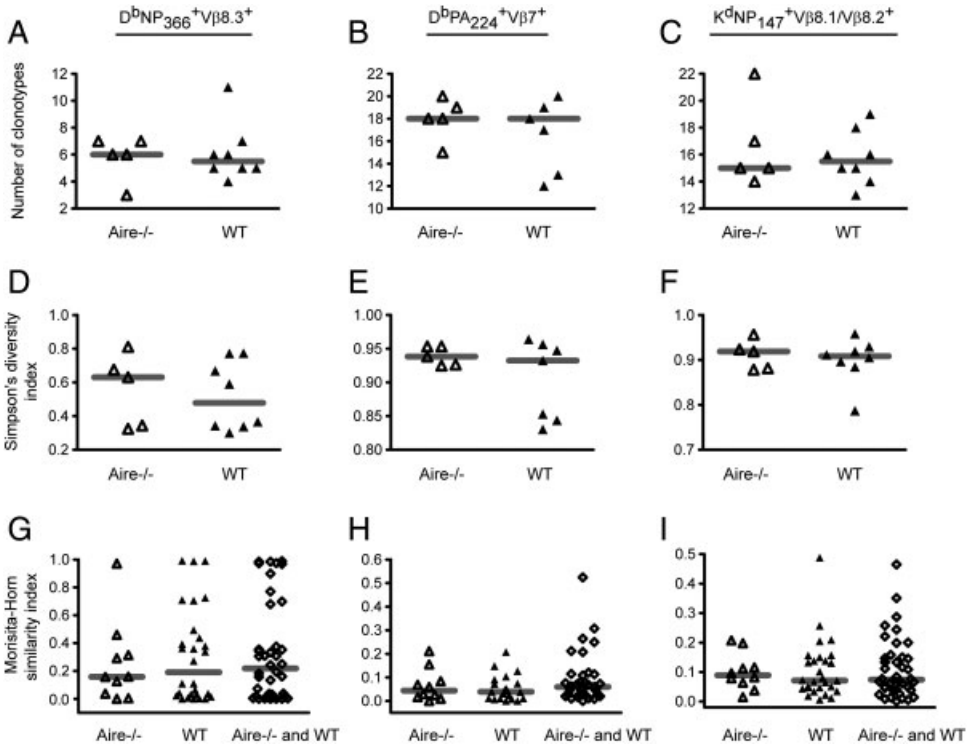

Figure 5. Comparison of the TCRβ repertoires between the Aire$^{-/-}$ and wt mice in the $D^bNP_{366}{}^+V\beta8.3^+$, $D^bPA_{224}{}^+V\beta7^+$ and $K^dNP_{147}{}^+V\beta8.1/V\beta8.2^+$ $CD8^+$ T-cell responses. The measures of TCRβ repertoire diversity include the number of different TCRβ clonotypes (A–C) and the Simpson's diversity index (D–F). The Morisita-Horn similarity index was used to determine the similarity between all pairs of Aire$^{-/-}$ mice, all pairs of wt mice, and all wt mice paired with all Aire$^{-/-}$ mice (Aire$^{-/-}$ and wt) (G–I). The Simpson's diversity and Morisita-Horn similarity index account for both the number of different TCRβ clonotype and the number of copies of each clonotype (*i.e.* the clonal dominance hierarchy). The diversity and similarity measures have been estimated for all samples having an equal sample size of 37 TCRβ sequences. The median values for each group of mice are shown as a horizontal line. The TCRβ diversity measures were compared between the Aire$^{-/-}$ and wt mice using a Mann–Whitney test and no significant differences in TCRβ diversity were found. There were no significant differences in TCRβ repertoire similarity between the three groups of pairings of mice (as determined by a Kruskal–Wallis test), indicating that the similarity in these epitope-specific TCRβ repertoires between Aire$^{-/-}$ and wt mice was not significantly less than observed between Aire$^{-/-}$ or between wt mice.

similar to the TCRβ repertoires between Aire$^{-/-}$ mice and between wt mice (Fig. 5H).

These results suggest that the absence of Aire does not significantly affect the composition or diversity of the mature $V\beta8.3^+CD8^+$ and $V\beta7^+CD8^+$ TCRβ repertoires responding to the D^bNP_{366} and D^bPA_{224} influenza A virus epitopes. In particular, the absence of Aire does not contribute to a broadening of the $D^bNP_{366}{}^+V\beta8.3^+CD8^+$-specific repertoire, suggesting that the narrow composition seen in wt animals is not a result of deletional tolerance of Aire-derived peptides. Similarly, the $D^bPA_{224}{}^+V\beta7^+CD8^+$ TCR repertoire appears unchanged in the absence of Aire.

TCR Vβ repertoire of $K^dNP_{147}{}^+CD8^+$ T-cell responses in Aire$^{-/-}$ BALB/c mice

Having shown that the TCRβ repertoire of influenza-specific immunodominant $CD8^+$ T-cell responses in B6 mice are not under the restriction of Aire control, we needed to determine whether these findings would hold true for other influenza-specific TCR repertoires, and mouse strains. To answer this question, we investigated influenza-specific TCR repertoires in Aire$^{-/-}$ mice on a BALB/c background. We firstly needed to characterize the nature and clonotype composition of previously undescribed TCR repertoire for immunodominant influenza-specific $K^dNP_{147}{}^+CD8^+$ T cells in wt BALB/c mice. Since $K^dNP_{147}{}^+CD8^+$ T cells had a strong Vβ8.1/8.2 bias, with $V\beta8.1/8.2^+$ cells comprising $39.5\pm4.2\%$ of $CD8^+$ T cells in the spleen and $48.9\pm10.1\%$ of $CD8^+$ T cells in BAL (Fig. 3C), we designed specific primers amplifying the Vβ8.1/8.2 region. Our single cell RT-PCR analysis of $K^dNP_{147}{}^+V\beta8.1^+CD8^+$ and $K^dNP_{147}{}^+V\beta8.2^+CD8^+$ T cells following HK-x31 infection in wt BALB/c mice (Table 1) showed diverse repertoires with predominant CDR3β loop lengths of 8, 9 or 10 aa for both $K^dNP_{147}{}^+V\beta8.1^+CD8^+$ T cells and $K^dNP_{147}{}^+V\beta8.2^+CD8^+$ T-cell sets (Supporting Information Table 2, Fig. 4A). Specific clonotypes utilized diverse Jβ profiles, such as Jβ1.5, Jβ2.1 and Jβ2.4 for $K^dNP_{147}{}^+V\beta8.1^+CD8^+$ T cells and Jβ1.2, Jβ2.1 and Jβ2.6 for $K^dNP_{147}{}^+V\beta8.2^+CD8^+$ sets (Supporting Information Table 2, Fig. 4B). Across the pooled repertoires of all mice, ~11% of clonotypes were present in more than one mouse and one clonotype, GDATNSDY, using the Vβ8.2 and Jβ1.2 genes, was found in all eight mice (Supporting Information Table 2). Thus, it appears that the TCRβ repertoire

of $K^dNP_{147}^+$ Vβ8.1/8.2$^+$ CD8$^+$ T cells is relatively diverse, similar to the $D^bPA_{224}^+$Vβ7$^+$CD8$^+$ TCRβ repertoire, rather than restricted like the $D^bNP_{366}^+$Vβ8.3$^+$ CD8$^+$ T-cell repertoire.

Clonal analysis of $K^dNP_{147}^+$Vβ8.1/8.2$^+$CD8$^+$ T cells in Aire$^{-/-}$ BALB/c mice confirmed results obtained in wt BALB/c mice. Similarly, the TCRβ repertoire for $K^dNP_{147}^+$Vβ8.1/8.2$^+$CD8$^+$ T cells in Aire$^{-/-}$ mice was diverse (Table 1, Supporting Information Table 3). $K^dNP_{147}^+$Vβ8.1/8.2$^+$CD8$^+$clonotypes in Aire$^{-/-}$ mice also utilized diverse Jβ profiles, with a bias toward Jβ1.2 and Jβ2.6, and CDR3β lengths of 8, 9 and 10 aa (Fig. 4). Fourteen out of 88 TCRβs (15.9%) were found in two or more Aire$^{-/-}$ mice (compared with 11.3% for wt mice) and the clonotype GDATNSDY, using the Vβ8.2 and Jβ1.2 genes, which was present in all wt mice, was also was detected in all five Aire$^{-/-}$ mice (Supporting Information Table 3).

We compared the $K^dNP_{147}^+$Vβ8.1$^+$/Vβ8.2$^+$CD8$^+$ TCRβ repertoire diversity between Aire$^{-/-}$ and wt mice and found no significant differences (Fig. 5C and F). There was also no significant difference between the similarity of the $K^dNP_{147}^+$ Vβ8.1$^+$/Vβ8.2$^+$CD8$^+$ TCRβ repertoires between pairs of Aire$^{-/-}$ and wt mice and pairs of Aire$^{-/-}$ or pairs of wt mice (Fig. 5I), further indicating the likeness of the $K^dNP_{147}^+$Vβ8.1$^+$/Vβ8.2$^+$ CD8$^+$ repertoires between Aire$^{-/-}$ and wt mice.

Taken together, our study shows that TCRβ repertoires of the three immunodominant influenza-specific CD8$^+$ T-cell responses in B6 and BALB/c mice are not under the control of Aire. The nature of the TCRβ clonotypes (Fig. 4, Table 2, Supporting Information Tables 1 and 3), number of different clonotypes (Table 1, Fig. 5A–C) and clonal diversity (Fig. 5D–F) was comparable for wt and Aire$^{-/-}$ mice for the $D^bNP_{366}^+$Vβ8.3$^+$ CD8$^+$, $D^bPA_{224}^+$Vβ7$^+$CD8$^+$ and $K^dNP_{147}^+$Vβ8.1/8.2$^+$CD8$^+$ T-cell responses.

Discussion

The acquisition of self-tolerance through thymic selection is essential for avoiding autoimmunity. The Aire molecule plays a role in promoting the thymic expression of tissue-specific Ag and thus allows tolerance to a wider range of self-Ag, and autoimmunity is seen in the absence of Aire [3–6]. However, it remained unclear whether Aire-deficiency can also affect the selection of Ag-specific T-cell repertoire. Here we focus on three narrow portions of the CD8$^+$ T-cell repertoire responding to influenza virus. We find no detectable difference in the T-cell clonotypes responding to infection in wt compared to Aire$^{-/-}$ mice, demonstrating that there are no major perturbations of these immune responses in the absence of Aire. This does not indicate that the naive T-cell repertoires are identical in wt and Aire-deficient mice, as the development of autoimmunity suggests the presence of self-reactive clones in the latter.

Four Aire-deficient mouse models have been reported [3, 14, 16, 34, 35]. The similar phenotype shared by these models is less severe than that of APECED patients [16, 35–37]. Although some disturbance of the medullary epithelial compartment is observed in our Aire-deficient mice, their T-cell compartment appeared relatively normal in the thymus and periphery at the phenotypic level. An increase in the number of activated T cells was evident, and autoantibodies against several organs were detected. At the histological level, lymphocytic infiltration of eyes and salivary glands indicated the development of autoimmunity, though symptoms were mild and the quality of life for our Aire-deficient mice appeared equivalent to wt littermates. Vβ gene usage and CDR3β length analysis of naive CD4$^+$ and CD8$^+$ T cells suggested that each Aire-deficient mouse develops its own polyclonal autoimmune repertoire [16].

Previous work suggested either minimal [16] or significant [15] differences in TCRβ repertoires between the wt and Aire-deficient mice when naive CD4$^+$ T cells [16] or bulk spleen samples [15] were analyzed. The earlier study [15] found differences in the spectratypes for 3 of 24 Vβ families. However, the relative contributions of these Vβ families to the total TCRβ repertoire are not discussed. The non-Gaussian nature of the spectratypes for Vβ19 and Vβ20 for both the wt and Aire-deficient mice suggests that there may have been few cells using these Vβ families. This may account for the variation between wt and Aire-deficient mice observed for these Vβ families. In a more recent study, minimal differences between wt and Aire-deficient mice in the naive CD4$^+$ TCRβ repertoires (as determined by Vβ usage and CDR3β lengths) were observed [16]. In the present study, we examined TCRβ repertoires of Ag-specific CD8$^+$ T cells in Aire$^{-/-}$ mice following influenza A virus infection. The absence of any significant difference between the repertoires of Aire$^+$ and Aire$^{-/-}$ mice suggests that the absence of Aire does not impose significant changes in the naive repertoire available to respond to influenza infection. However, further analysis of other Ag-specific TCR repertoires needs to be performed in order to confirm our observations obtained in the context of influenza infection. Given the prevalence of candidiasis in APECED patients, it would be of interest to examine repertoires of T cells reacting towards *Candida albicans*-derived epitopes.

Taken together, our study used three immunodominant influenza-specific CD8$^+$ T-cell responses characterized by TCR repertoires of distinct natures (public and restricted *versus* private and diverse) in two different mouse models (B6 and BALB/c mice) to provide, to our knowledge, the first evidence that Ag-specific TCR repertoires are unperturbed in the absence of Aire. Our findings have important implications for our understanding of normal TCR selection, and the role of Aire-dependent peptides in the development of the natural and autoantigen-specific repertoire.

Materials and methods

Mice and virus infections

C57Bl/6J (H-2^b) and BALB/c mice (both wt and Aire$^{-/-}$) were bred in the specific pathogen-free breeding facility at the Walter and Eliza Hall Institute (Parkville, Vic, Australia) and maintained

under conventional conditions. Wt and Aire$^{-/-}$) mice were bred and housed as littermates. C57BL/6 mice deficient for Aire gene were generated *via* homologous recombination of targeting vectors in mouse C57BL/6 ES cells. Insertion of the Aire targeting vector disrupted exon 8 and brought the LacZ reporter gene under the control of the endogenous Aire promoter, creating an Aire-LacZ fusion (17). The Aire knockout mutation was back-crossed onto BALB/cJ (BALB/c), for ten generations and intercrossed once for analysis. Naive mice of 8–10 wk of age were anesthetized by isofluorane inhalation and infected i.n. with 10^4 pfu of the HKx31 (H3N2) influenza A virus in 30 μL of PBS. Virus stocks were grown in the allantoic cavity of 10-day (d) embryonated hen's eggs and quantified as pfu on monolayers of Madin Darby canine kidney cells. All animal experiments followed the guidelines set by the NHMRC Code of Practice for the Care and Use of Animals for Scientific Purposes and have been approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

Tissue sampling and cell preparation

Spleen and BAL samples were recovered from mice at the acute phase of the primary infection (d10). BAL samples were incubated on plastic petri dishes for 1 h at 37°C to remove macrophages. Spleens were disrupted and enriched for CD8$^+$ T cells using goat anti-mouse IgG and IgM Ab (Jackson ImmunoResearch Labs, West Grove, PA, USA). Cells were washed and resuspended either in FACS buffer (1% BSA/0.2% NaN_3 in PBS) for flow cytometric analysis or sort buffer (0.1% BSA in PBS) for single-cell sorting.

Tetramer staining and tetramer dissociation assay

CD8$^+$ T-cell-enriched lymphocytes from spleen were stained with D^bNP_{366} or D^bPA_{224} tetramers conjugated to Streptavidin-PE (Molecular Probes, Eugene, OR, USA) for 60 min at room temperature. Cells were washed twice in FACS buffer (10% BSA/0.02% NaAz in PBS). As a measure of TCR avidity, spleen samples were used in tetramer dissociation assay [25]. After cells were stained with tetramers, they were washed and incubated in the presence of anti-H2D^b Ab (28-14-8, BD Biosciences Pharmingen) at 5 μg/mL at 37°C to prevent tetramer rebinding. At specific times, cells were removed into FACS buffer and placed on ice, stained with anti-CD8 mAb conjugated to FITC (BD Biosciences Pharmingen) for 30min on ice, washed twice and analyzed by flow cytometry. Loss of tetramer$^+$CD8$^+$ T cells at particular time-points was calculated in comparison to tetramer staining at $t = 0$ min.

Vβ analysis of influenza-specific CD8$^+$ T cells

Spleen cells were enriched for CD8$^+$ cells and stained with D^bNP_{366}-PE, D^bPA_{224}-PE or K^dNP_{147}-PE tetramers for 60min at room temperature. After two washes, cells were stained for surface CD8-APC and the panel anti-mouse Vβ-FITC Ab for 30 min on ice. Lymphocytes were washed twice and analyzed by flow cytometry.

Peptide stimulation and intracellular cytokine staining

T-cell populations from the spleen and BAL were stimulated with NP_{366} or PA_{224} peptides (Auspep) for 5 h in 200 μL complete-RPMI medium containing 1 μg/mL Golgi-Plug (BD Biosciences Pharmingen). Cells were washed in FACS buffer and stained with a PerCP-Cy5.5 conjugated mAb to CD8 for 30 min on ice. After two washes, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit. Cells were stained with mAb to IFN-γ (FITC), TNF-α (APC) and IL-2 (PE) (BD Biosciences Pharmingen) for 30 min on ice, washed and analyzed by flow cytometry.

Single-cell RT-PCR and sequencing

CD8$^+$ T cell-enriched lymphocyte populations were stained either with D^bNP_{366}-PE, D^bPA_{224}-PE or K^dNP_{147}-PE for 60 min at room temperature, followed by two washes in sort buffer. Cells were then stained with anti-CD8-APC and anti-Vβ8.3-, anti-Vβ7-FITC or anti-Vβ8.1/8.2-FITC Ab for $D^bNP_{366}^+CD8^+$, $D^bPA_{224}^+CD8^+$ or $K^dNP_{147}^+CD8^+$ T cells, respectively (BD Pharmingen, San Diego, CA, USA). After two washes, cells were resuspended in 500 μL sort buffer and transferred to polypropylene FACS tubes (BD Labware, Franklin Lakes, NJ, USA) for single-cell sorting. Lymphocytes were isolated using a FACSAria sorter (Cytomation, Fort Collins, CO, USA). Single $D^bNP_{336}^+V\beta8.3^+CD8^+$, $D^bPA_{224}^+V\beta7^+CD8^+$ or $K^dNP_{147}^+V\beta8.1/8.2^+$ CD8$^+$ T cells were sorted directly into a 96-well PCR plate (Eppendorf, Hamburg, Germany) containing 5 μL of cDNA reaction mix [22]. Negative controls were interspersed between the samples (1 in 10), and 80 cells were sorted *per* plate. After sorting, plates were incubated at 37°C for 90 min for cDNA synthesis, followed by 5 min at 95°C. The Vβ8.3 [22] and Vβ7 [24] transcripts were amplified and sequenced. Vβ8.1/8.2 transcripts were amplified using an external sense Vβ8.1/8.2 primer TGGCAGTAACAGGAGGAAAG and an internal sense Vβ8.1/8.2 primer TACAAGGCCTCCAGACCAAGCCAA.

Statistical analysis

The number of unique TCRβ amino acid clonotypes and Simpson's diversity index [38] were used as measures of TCR repertoire diversity. The Morisita-Horn similarity index [39] was used to measure the similarity between TCR repertoires. The Simpson's diversity and Morisita-Horn similarity indices account for both the variety of distinct clonotypes (defined by the Vβ and Jβ gene usage and the CDR3β amino acid sequence) and the clone size (number of copies) of each clonotype involved in the epitope-specific response within each mouse. The Simpson's diversity and Morisita-Horn similarity indices range in value from 0 (minimal diversity/similarity) to 1 (maximal diversity/similarity). The diversity and similarity measures were calculated

in conjunction with a randomization procedure to correct for the differences in sample sizes between mice [38, 39], and were estimated as if 37 TCRβ sequences had been obtained for each sample. A Mann–Whitney test was used to compare TCRβ repertoire diversity between the wt and Aire$^{-/-}$ mice. A Kruskal–Wallis test was used to compare the similarity of epitope-specific TCRβ repertoires between all pairs of wt mice, all pairs of Aire$^{-/-}$ mice, and all wt mice paired with all Aire$^{-/-}$ mice. The calculations of the diversity and similarity measures and the randomization techniques were performed using Matlab (The Mathworks, Natick, MA, USA). All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

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Abbreviations: **BAL:** bronchoalveolar lavage · **H:** viral hemagglutinin molecule · **N:** viral neuraminidase · **NP:** influenza nucleoprotein · **PA:** influenza acid polymerase · **pMHC:** peptide-MHC · **TRA:** tissue-restricted antigens

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