Published in final edited form as:
J Immunol. 2008 July 1; 181(1): 649–659.

Terminal Deoxynucleotidyl Transferase Establishes and Broadens Anti-Viral CD8+ T Cell Immunodominance Hierarchies

S.M. Mansour Haeryfar[1,2], **Heather D. Hickman**[1], **Kari R. Irvine**[1], **David C. Tscharke**[1,3], **Jack R. Bennink**[1], and **Jonathan W. Yewdell**[1,4]

1*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, 20892 USA*

2*Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada*

3*School of Biochemistry and Molecular Biology, Australian National University, Canberra ACT 0200, Australia*

Abstract

The action of terminal deoxynucleotidyl transferase (TdT) on mouse T cell receptor (TCR) genes accounts for ~ 90% of T cell repertoire diversity. We report that in TdT -/- mice, total T_{CD8+} responses to influenza and vaccinia viruses are reduced by ~ 30% relative to *wt* mice. We find that T_{CD8+} responses to 3 subdominant influenza virus determinants are reduced to background values in TdT -/- mice while responses to 3 immunodominant determinants undergo a major reshuffling. A similar reshuffling occurs in T_{CD8+} responses to immunodominant vaccinia virus determinants, and is clearly based on broad differences in TCR family usage and CDR3 length between *wt* and TdT -/- mice. These findings demonstrate that TdT plays a critical role in the magnitude and breadth of anti-viral T_{CD8+} responses toward individual determinants and suggests that germline TCR repertoire bias towards the most dominant determinants is a major factor in establishing immunodominance hierarchies.

Introduction

Infection of vertebrate cells with viruses with the largest genomes generates millions of distinct viral peptides that represent potential targets for $CD8^+$ T lymphocyte (T_{CD8+}) recognition. T_{CD8+} responses, however, are limited to a minute fraction of viral peptidomes. Even among the chosen few peptide determinants that elicit measurable T_{CD8+} responses, the magnitude of T_{CD8+} responses varies widely, creating a hierarchical pattern that is highly consistent between individuals sharing a given set of MHC class I alleles (1). Accordingly, immunodominant determinants provoke the most robust T_{CD8+} responses, while subdominant determinants elicit lower number of T_{CD8+}.

Immunodominance is a consistent feature of T_{CD8+} and T_{CD4+} responses to viruses and other intracellular pathogens, tumor cells, and transplanted tissues. In model mouse-virus systems

4 Correspondence should be addressed to Dr. Jonathan W. Yewdell, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Room 211, Building 4, 4 Center Drive, Bethesda, MD 20892-0440, or Dr. S.M. Mansour Haeryfar, Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario N6A 5C1, Canada. e-mail: jyewdell@nih.gov or mansour.haeryfar@schulich.uwo.ca.

Competing Interests Statement: The authors declare that they have no competing financial interests.

numerous factors are known to participate in shaping T_{CD8+} immunodominance hierarchies including:

1. Abundance and kinetics of viral gene product expression.
2. Proteolytic generation of peptides by proteasomes and other proteases.
3. Specificity of transporter associated with antigen (Ag) processing (TAP) for transporting cytosolic peptides into the endoplasmic reticulum (ER).
4. Affinity of peptide binding to class I allomorphs.
5. Ability of the T_{CD8+} repertoire to respond to a given peptide-class I complex based on precursor frequency and proliferative capacity.
6. Immunodomination, *i.e.* suppression of subdominant determinant T_{CD8+} by immunodominant determinant T_{CD8+} at the level of individual Ag-presenting cells (APCs).
7. Suppression of immunodominant determinant T_{CD8+} by naturally occurring $CD4^+CD25^+$ regulatory T cells (2).

In this study we have examined the shaping of the TCR repertoire, one of the most poorly defined factors in establishing immunodominance hierarchies. The TCR repertoire is thought to encompass approximately 10^7 and 10^8 distinct TCRs, respectively, in mice and humans. The diversity of TCRs is predominantly exhibited in their complementarity-determining regions (CDRs) that establish contact with cognate peptide-MHC complexes. CDR3 regions of antibodies and TCRs are unequivocally the most diverse structures known in biology.

TCR structural diversification is achieved by several mechanisms during the rearrangement of receptor genes and assembly of receptor chains in the thymus (reviewed in refs (3,4). Stochastic recombination of non-contiguous germline DNA segments known as variable (V), diversity (D), and joining (J) segments is initiated by recombination activating gene (RAG)-1 and RAG-2 proteins, giving rise to a multitude of V(D)J fusions. TCR diversity is further enhanced by variable removal of nucleotides from the exposed V, D, and J termini, and by addition of template-dependent or palindromic (P) as well as non-template-encoded (N) nucleotides at the V-D, D-J and V-J junctions. Finally, random pairing of TCR-α and -β chains followed by selection for functional TCRs introduces another layer of diversity to the TCR repertoire.

N-region diversity results from the transient expression during V(D)J recombination of terminal deoxynucleotidyl transferase (TdT). Mice with a targeted disruption of TdT (TdT -/- mice) demonstrate the nearly complete absence of N nucleotides in their B and T cells (5,6). TdT belongs to the pol × family of polymerases, a subgroup of an ancient nucleotidyltransferase superfamily defined by homologies within their nucleotide binding domains and active site motifs (Holm and Sander, 1995). TdT is unique among DNA polymerases in adding nucleotides to free 3′-OH ends of fragmented or nicked DNA in a template-independent fashion (7). TdT is expressed in human and mouse thymocytes 20 weeks post-gestation and 3-5 days after birth, respectively (8,9). In both cases, TdT expression temporally correlates with the onset of N-region diversity in immature thymocytes undergoing differentiation. Strikingly, TdT has been estimated to be responsible for at least 90% of diversity in αβ TCRs (10)).

Mice with a targeted deletion in TdT were generated by Mathis, Benoist and colleagues more than 15 years ago (6) and to date have demonstrated little in the way of disease susceptibility or alterations in their T_{CD4+} responses to soluble antigens. Gavin and Bevan reported that T_{CD8+} clones from TdT -/- mice were more generally cross-reactive between peptides present in a large synthetic library (11). But what is the effect of the knockout on the magnitude and breadth of T_{CD8+} responses to individual viral determinants? Here we provide the initial

description of the critical role that TdT plays in establishing the immunodominance hierarchy in T_{CD8+} anti-viral responses.

Materials and Methods

Mice

Adult sex- and age-matched mice were used in all experiments. TdT -/- mice on BALB/c background (H-2^d) and BALB/c mice expressing the Thy-1.1 congenic marker were generously provided by John Kearney (University of Alabama at Birmingham) and Ethan Shevach (NIAID, NIH), respectively. These mice were bred and housed together with *wt* BALB/c mice purchased from Taconic Farms in our animal care facility under specific, pathogen-free conditions.

Viruses and immunization

IAVs used in this study, namely IAV, IAV-SEQ12 and J-1 were grown in 10-day-old embryonated chicken eggs and used as infectious allantoic fluid. Mice received a single *i.p.* dose of IAV approximating 600 hemagglutinating units (HAUs). In memory T_{CD8+} responses, mice were primed with IAV and boosted a month later with IAV-SEQ12. In several experiments, we infected mice *i.p.* with 10^6 plaque forming units (PFUs) of rVV-ES-HA_{518}.

Peptides

All peptides used in this study (listed in Table I) were procured or synthesized, purified by high performance liquid chromatography (HPLC), and analyzed by mass spectrometry by or under the supervision of the Biologic Resource Branch, NIAID. In each case, substances with the predicted mass constituted >95% of the material analyzed. Stock solutions of peptides were prepared at 1 mM in dimethyl sulfoxide (DMSO) and stored at -30°C.

Antibodies

Anti-CD16/CD32 (clone 2.4G2, rat IgG_{2b}, Fc Block™), CyChrome- or Alexa Fluor® 647-conjugated anti-mouse CD8α (clone 53-6.7, rat IgG_{2a}), PE-conjugated anti-mouse Thy-1.2 (clone 53-2.1, rat IgG_{2a}) and FITC-conjugated anti-mouse IFN-γ (clone XMG1.2, rat IgG_1) mAbs were all from BD Biosciences.

ICS for IFN-γ

Erythrocyte-depleted splenocytes were prepared and PECs were collected via peritoneal lavage using sterile PBS, ICS was performed as described (12).

Cytotoxicity assays

CTL induction was assessed by conventional ^{51}Cr release assay. Erythrocyte-depleted splenocytes were prepared on d 7 post-infection and used as effector cells at indicated ratios against ^{51}Cr-labeled P815 target cells that were pre-sensitized with 100 nM IAV-derived peptides. Target cells were seeded at 10^4 cells/well together with effector splenocytes in 96-well U-bottom plates. The plates were spun at 400 × *g* for 5 min at the end of a 6-h incubation period at 37°C. A 100-μl aliquot of supernatant was then harvested from each well, and the ^{51}Cr content of the samples was determined by γ counting. Specific lysis of the target cells was determined using following formula: % specific lysis = [(ER - SR)/(TR - SR)] × 100, where ER (experimental release) is obtained from wells containing both effector and target cells, whereas SR (spontaneous release) and TR (total release) are determined from wells receiving only target cells plus medium or target cells plus a 1:7 dilution of 3.5% (w/v) cetrimide, respectively. *In vivo killing assay* was performed as described (13) with minor modifications. Syngeneic erythrocyte depleted splenocytes were split into three populations,

and were pulsed with either LCMV-NP_{118} at 1 μM and stained with 0.025 μM CFSE ($CFSE^{low}$) while $CFSE^{int}$ (0.2 μM CFSE) and $CFSE^{high}$ (1.6 μM CFSE) populations were pre-incubated with HA_{518} (1 μM) and NP_{147} (1 μM), respectively. After extensive washing, equal numbers of cells from each population were mixed, and each mouse (naive or previously infected with IAV) received a total of 3×10^7 target cells in 500 μl of PBS intravenously (*i.v.*). Two, 4 or 6 h later, spleens were harvested and analyzed by flow cytometry. Up to 1×10^4 $CFSE^{high}$ events were collected for each mouse for analysis and the percent specific killing was calculated as follows: 100 - [(% cognate peptide-pulsed in primed mouse/% control peptide-pulsed in primed mouse)/(% cognate peptide-pulsed in naive mouse/% control peptide pulsed in naive mouse)] × 100.

Adoptive transfer of TdT -/- T_{CD8+} into wt hosts

T_{CD8+} from pooled splenocytes of naïve TdT -/- mice were magnetically separated using an AutoMACS (Miltenyi Biotec). Anti-CD11c beads were simultaneously used during T_{CD8+} negative selection to eliminate splenic DCs. Cell preparations thus obtained were further enriched for T_{CD8+} using a FACSAria™ cell sorter (BD Biosciences) to achieve ~99.9% purity. Between $5\text{-}10 \times 10^6$ TdT -/- T_{CD8+} were then injected *i.v.* into BALB/c mice expressing the Thy-1.1 congenic marker one d prior to *i.p.* infection with IAV. IAV-specific responses of recipient ($Thy\text{-}1.1^+$ *wt*) and donor ($Thy\text{-}1.2^+$ TdT -/-) T_{CD8+} were evaluated by ICS seven d post-infection after live gating on $CD8^+$ events and using Thy-1.2 staining to distinguish donor cells from recipient cells.

Immunoscope Analysis of T_{CD8+}

BALB/c and TdT -/- mice were immunized i.p. with 1×10^6 pfu of VACV strain WR. Spleens and peritoneal exudate cells (PECs) were harvested 6 days following injection. CD8+ T cells were prepared from pooled spleens and PECs using a CD8+ isolation kit and autoMACs separator (Miltenyi Biotec, #130-090-859). The flow-through fraction containing CD8-enriched cells was collected, and resulted in >95% CD8+ expression in all populations. A fraction of cells was set aside for further CD8+ purification by flow sorting. The rest were stimulated in vitro for 3 h at 37°C with A52R peptide pulsed P815 cells. Stimulated CD8+ T cells were incubated for 20 minutes on ice to block the release of IFN-γ. Cells were resuspended 100μl of cytokine catch reagent consisting of an anti-CD45 antibody conjugated to an anti-IFN-γ antibody (Miltenyi) and incubated on ice for 5 minutes. 10 ml of warm DMEM were added and cells were incubated for another 45 minutes at 37°C under slow continuous rotation. This step is critical to reduce the amount of “trans” capture of IFN-γ. Cells were washed with cold buffer, incubated with Fc Block and labeled with anti-FITC CD8+ antibody (Pharmingen) and anti-APC IFN γ+ Microbeads (Miltenyi) for 15 minutes. CD8+, IFN-γ+ cells were sorted from IFN-γ - cells using a FACSVantage DIVA; BD Biosciences. Post sort analysis revealed that for *wt* and TdT -/- populations, respectively, 98% and 45% of cells were CD8+, IFN-γ+ (data not shown). This discrepancy is not related to differences in the singlet population sorted, which were highly similar. We believe instead, that it is due to increased losses post sorting in surface bound anti-IFN-γ antibody by the TdT -/- population, which remained 99% CD8+. We note that any putative differences in the purity of TdT -/- vs. *wt* cells would minimize, not accentuate, the differences we observe in the oligoclonal populations detected by CDR3 length analysis. Cells were snap frozen in Trizol (Invitrogen) and shipped to TcLand SA (Nantes, France) for Vβ typing and CDR3 length analysis as previously described (Sebille *et al.*, 2001). In brief, mRNA was reverse transcribed to cDNA that was then PCR amplified in 19 separate reactions using a Cβ primer and primers for each of 19 Vβ genes examined. Each amplification product was elongated using a dye labeled Cβ primer and its CDR3 length was then determined by sizing via arcrylamide–urea gel electrophoresis. Immunoscope software was used to display the CDR3 lengths of the amplified gene products. Real time PCR was used to determine the relative levels of each 19 Vβ genes transcripts. For each reaction,

hydroxyphosphoribosyltransferase (HPRT) transcripts were used as an internal control to correct for variability in RNA preparations and the conversion efficiency of the reverse transcription reaction.

Results

Immunodominance hierarchy of influenza A virus (IAV)-specific T_{CD8+} is altered in TdT -/- mice

Infection of BALB/c mice with the A/Puerto Rico/8/34 strain of IAV induces a well defined T_{CD8+} immunodominance hierarchy (14,15) that can be measured by intracellular cytokine staining (ICS) for interferon (IFN)-γ. Following intraperitoneal (i.p.) infection, responses to individual determinants can be quantitated both locally in peritoneal exudate cells (PECs) and systemically in spleens. The typical immunodominance hierarchy is seen in Fig. 1 where NP_{147} is clearly the immunodominant determinant among the 7 defined IAV determinants (Table I) tested for their recognition by splenic or peritoneal T_{CD8+}. Overall anti-IAV responses can be measured by the activation of T_{CD8+} by IAV-infected P815 cells. This clearly represents only a fraction of the total response since the numbers of responding cells are less than the sum of the responses to the defined individual determinants (and other major determinants are recognized by BALB/c mice, unpublished findings). Still, this provides a measure of the magnitude of the anti-IAV T_{CD8+} response.

In the same experiment, we measured T_{CD8+} responses in IAV-infected TdT -/- mice. We consistently observed that although the cellularity of TdT -/- spleens was reduced by ~20% relative to *wt* spleens, this was usually compensated by a 10-20% increase in the fraction of T_{CD8+}, so that the % of responding T_{CD8+} in -/- *vs. wt* mice generally corresponds well to the absolute numbers of responding cells. Overall IAV-specific splenic and peritoneal T_{CD8+} responses were reduced by ~30% relative to *wt* mice. Remarkably, the immunodominance hierarchy was greatly altered in TdT -/- mice: there was a reduction in T_{CD8+} specific for the immunodominant determinant, NP_{147}, with a concomitant increase in HA_{518}-specific T_{CD8+}, which now assumed immunodominant status. The rank promotion of HA_{518} was highly consistent and observed in 10 independent experiments each typically consisting of 3-4 mice/group. Further, we also consistently observed a narrowing of the response, with decreases to background levels in responses to NP_{39}, NP_{218}, and HA_{462}.

IFN-γ secretion is but one of multiple T_{CD8+} functions. To broaden these findings, we measured the *in vitro* and *in vivo* cytotoxic functions of IAV-specific T_{CD8+} in *wt* and TdT -/- mice. This confirmed that splenic T_{CD8+} from *wt* and TdT -/- mice demonstrated a similar reversal in cytotoxic activity against target cells pulsed with NP_{147} or HA_{518} (Fig 2a). To extend this finding *in vivo*, we injected mice infected 7 d earlier with a mixture of three target cell populations (BALB/c splenocytes pulsed with synthetic lymphocytic choriomeningitis virus (LCMV) NP_{118} [negative control], NP_{147} or HA_{518}) that were distinguished by levels of labeling carboxyfluorescein diacetate succinimidyl ester (CFSE) (13). While most NP_{147}-pulsed target cells were cleared from *wt* spleens 4 h after *i.v.* injection, only a small fraction of HA_{518}-pulsed target cells were cleared (Fig 2b). By contrast, the opposite pattern was observed in TdT -/- mice. We observed a similar hierarchical switch when spleens were harvested 2 or 6 h after target cell injection (data not shown). Similar numbers and ratio of target cells were recovered from naïve TdT -/- *vs. wt* mice, indicating that the results are unlikely to be attributed to differences in target cell homing patterns in *wt vs.* TdT -/- mice (data not shown).

These data collectively demonstrate that TdT -/- mice exhibit a marked change in the breadth and composition of the T_{CD8+} immunodominance hierarchy elicited by IAV infection as measured either by IFN-γ secretion or lytic activities.

T_{CD8+} hierarchical switch in TdT -/- mice is not due to altered Ag presentation but intrinsic to TdT -/- T_{CD8+} repertoire

We examined the potential contribution of altered Ag presentation in TdT -/- mice to the phenomena described above. We employed three approaches to address this issue. First, we generated bone marrow-derived dendritic cells (BMDCs) from *wt* and TdT -/- mice. IAV-infected BMDCs or P815 cells were tested for their ability to activate splenic and peritoneal primary (d 7) T_{CD8+} obtained from IAV-infected *wt* mice as determined by ICS. BMDCs from *wt* and TdT -/- mice were equally potent stimulators on a per cell basis under conditions activation is limited by numbers of APCs (data not shown).

Next, we examined HA_{518}-specific T_{CD8+} responses following infection of mice with a recombinant vaccinia virus (rVV) that expresses HA_{518} as an ER-targeted minigene product (rVV-ES-HA_{518}) (Fig. 3, right side). We previously provided evidence that this virus generates super-normal levels of HA_{518}-K^d complexes on infected cells (16). If enhanced presentation of HA_{518} contributes to the enhanced immunodominance status of HA_{518} in TdT -/- mice, then saturating HA_{518} presentation should equalize responses between *wt* and TdT -/- mice. ollowing 7-d infection, VV-infected TdT -/- mice maintained enhanced primary splenic and peritoneal T_{CD8+} responses to HA_{518}.

Finally, we adoptively transferred highly purified T_{CD8+} from TdT -/- mice (which express the Thy 1.2 allele) into *wt* BALB/c mice expressing the Thy-1.1 allele (Fig 4a). Using a fluorochrome-labeled monoclonal antibody (mAb) specific for Thy 1.2, we could thereby distinguish donor *vs.* recipient T_{CD8+} responses following IAV infection. This revealed a clear dichotomy between responses of *wt* and TdT -/- T_{CD8+} in a single environment: *wt* cells responded more vigorously to NP_{147} while donor cells preferred HA_{518}.

These experiments clearly indicate that the reversal in the immunodominance hierarchy between NP_{147} and HA_{518} is due largely, if not exclusively, to properties of the TdT-deficient T_{CD8+} repertoire.

Immunodomination is not required for the ascension of HA_{518}-specific T_{CD8+} in TdT -/- mice

We next examined the role of immunodomination mediated by HA_{518}-specific T_{CD8+} in the decreased response to NP_{147} in TdT -/- mice. Immunodomination is experimentally defined as augmented responses to subdominant determinants when responses to immunodominant determinants are diminished by removing or altering the determinant *per se*, the restricting MHC class I allomorph, or immunodominant determinant-specific T_{CD8+}. We compared the responses of *wt* and TdT -/- to IAV vs. a recombinant virus (J-1) that possesses 7 genes from IAV with an HA gene derived from the A/Hong Kong/68 IAV strain (HK). The HK HA does not cross-react with HA_{518}-specific T_{CD8+}, and in fact, does not appear to induce a T_{CD8+} response in BALB/c mice (17).

Following infection with J-1 virus, TdT -/- mice demonstrate a decreased response to NP_{147} relative to the response of *wt* mice (Fig. 4b) (the alteration in the magnitude of the response is most likely due to differences in viral tropism and replication rather than to alterations in presentation of the viral peptide determinants). This decrease of a nearly identical magnitude (in relative terms) to the difference observed to IAV in the same experiment supports the conclusion that enhanced immunodominance status of these clones in TdT -/- mice is not due to immunodomination mediated by HA_{518} T_{CD8+}.

TCR avidity of HA_{518}-specific T_{CD8+} is higher in TdT-deficient mice than in wt controls

There is evidence that T_{CD8+} with increased sensitivity for cognate peptide-class I complexes exhibit a proliferative edge *in vivo* (18). We therefore measured the ability of NP_{147}- and

HA_{518}-specific T_{CD8+} to respond to APCs incubated with decreasing concentrations of cognate synthetic peptide determinants (Fig. 5). Day 7 splenic T_{CD8+} obtained from *wt vs.* TdT -/- mice specific for NP_{147} demonstrated highly similar sensitivity (half-maximal activation at ~ 10^{-11}M) Critically, however, HA_{518}-specific T_{CD8+} derived from TdT -/- mice demonstrated approximately 10-fold higher sensitivity to peptide than corresponding T_{CD8+} from *wt* mice. A similar trend was observed in an independent experiment involving 4 mice per group that used APCs pulsed with peptide and washed prior to incubation with T_{CD8+}.

These findings are consistent with the idea that the lack of TdT results in the generation of a TCR repertoire with greater sensitivity for detecting HA_{518}-K^d complexes, most likely due to an intrinsically higher affinity of their TCR for the complexes. These data also indicate that TdT is not required to generate NP_{147}-specific T_{CD8+} with high functional avidity for cognate peptide class I complexes.

Hierarchy shuffling in secondary anti-IAV responses of TdT -/- mice

To characterize immunodominance hierarchies of memory T_{CD8+} we needed to boost with an IAV that escapes neutralization by Abs induced by the priming virus. We could neatly do this by boosting with an IAV mutant selected by sequential neutralization with a panel of 12 HA-specific mAbs (19). This mutant has accumulated mutations in the globular domain but retains HA structure and possesses an identical sequence in and around the HA_{518} sequence. Heterologous prime-boosting with IAV and IAV-SEQ12 indeed resulted in greatly enhanced secondary T_{CD8+} responses relative to homologous prime-boosting with IAV (data not shown).

Primary infection of mice with IAV-SEQ12 recapitulated the differential hierarchy between *wt* and TdT -/- mice shown in Fig. 1 (data not shown), indicating the T_{CD8+} determinants are presented similarly by the two viruses *in vivo*. We next boosted IAV primed *wt* and TdT -/- mice with IAV-SEQ12 (Fig. 6). As in primary responses, the dominance pattern of T_{CD8+} in TdT -/- mice was changed in favor of HA_{518} and in a very similar fashion to what is observed in primary responses. Equally notable is the severe narrowing of the T_{CD8+} response and the decreased overall response to IAV. Despite the prime-boost regimen, we failed to detect responses to three subdominant determinants, NP_{39}, NP_{218}, and HA_{462}. Reciprocal priming with IAV-SEQ12 and boosting with IAV gave similar results (data not shown).

These findings demonstrate that the alterations in the ID hierarchy of TdT -/- mice extends to secondary responses to IAV, and underscores the marked narrowing of the response as well as its decreased magnitude.

TdT -/- mice exhibit a shuffled VV-specific T_{CD8+} immunodominance hierarchy

We generalized these findings to anti-VV responses by measuring the T_{CD8+} responses to three H-2^d-restricted determinants (20) (Fig 3A: these data were obtained in the same experiment that examined responses to the HA_{518} minigene product). Infection of *wt* BALB/c mice with VV leads to a highly robust T_{CD8+} response to $F2L_{23}$, $A52R_{75}$ and $E3L_{140}$ in order of hierarchical dominance. Remarkably, TdT -/- mice demonstrate alterations in their immunodominance hierarchy similar to that observed in IAV responses. Reponses to the immunodominant determinant $F2L_{23}$ were decreased 2-3 fold while responses to the subdominant determinant $A52R_{75}$ were increased by ~ 2-fold, placing it atop the ID hierarchy.

TdT -/- mice generate a highly diverse response to viral antigens

To gain molecular insight into the effect of TdT on the anti-viral T_{CD8+} response, we examined TCR Vβ gene usage (19 of 27 mouse Vβ genes analyzed) and CDR3 length by Immunoscope analysis of mRNA (21) isolated from purified T_{CD8+} from naïve and d6 primary VV-infected mice (Fig 7). CDR3 is the sole variable region of each α and β TCR gene product, and it typically

forms the most intimate contacts with MHC bound peptides in TCR-MHC structures determined by X-ray crystallography (22). Hence, CDR3 plays the most critical role in T cell specificity of cognate peptides.

The validity of our Immunoscope analysis is supported by: 1) the paucity of T_{CD8+} expressing Vβ3, Vβ5 and Vβ12; this is expected due to the deletion of clones bearing these TCRs due their interaction with endogenous retroviral elements in BALB/c mice (23) and 2) the distribution of Vβ chains, which is consistent with previous reports of the naïve BALB/c T cell repertoire, as is the Gaussian distribution of CDR3 segment length centered around 7 amino acids (24). Surprisingly, while *TdT* deletion had the anticipated effect of shortening the average length of CDR3 sequences in each Vβ family by 1-2 amino acids, it had only minor effects on Vβ chain representation in the naïve repertoire, which was nearly identical to *wt* mice. This indicates that Vβ usage occurs independently of TdT expression, which is consistent with the conservation between Vβ usage in neonatal and adult T cell repertoires (25).

Antigen driven clonal expansions can frequently be observed as perturbations in the Gaussian CDR3 distribution as a relative increase in the fraction of TCRs with a given CDR length. These can be observed even in a total T_{CD8+} population if the overall response is of sufficient magnitude (24) (which is why we studied T_{CD8+} induced by VV instead of IAV, since the response is of much greater magnitude). On day 6 of a primary VV infection major expansion of individual CDR3 populations are indicated in Fig 7 by a red arrowhead. It can be seen that 11 such populations can be identified by eye in the *wt* anti-VV response. A disproportionate number of these responses occur in minor populations (Vβ3, 5, 12, 18, & 20). This is to be expected since these populations should have less diversity (based on their selection of avoiding super antigen deletion), which should facilitate detection of oligoclonal expansions. Expansions are also detected in Vβ1 and Vβ7 populations, however, which together constitute 20% of the total T_{CD8+} response. Notably, by the criteria of detectable CDR3 population expansions, the polyclonal anti-VV response of TdT -/- mice is broader than *wt* mice, with 17 clearly perturbed populations with the increase due to more perturbations in the predominant Vβ families (i.e. the families expressed by the highest % of T_{CD8+}) (Fig 7).

This analysis of total T_{CD8+} populations provides a rough idea of clonal diversity, as it is obviously limited by the presence of the majority of T_{CD8+} that do not respond to VV. To get a better idea of the effect of TdT in shaping the anti-viral repertoire, and particularly the phenomenon of the promotion of subdominant determinant to immunodominant status, we analyzed T_{CD8+} that were sorted *ex vivo* on the basis of IFN-γ secretion upon activation by $A52R_{75\text{-}83}$ peptide coated APCs. In this case, nearly every Vβ population in both *wt* and TdT -/- mice exhibited oligoclonal expansion, demonstrating the validity of the Immunoscope analysis and the effectiveness of purifying $A52R_{75}$-specific T_{CD8+}. These data are all the more remarkable since the analysis was performed on T_{CD8+} pooled from many individual animals (10 *wt* and 6 TdT -/- mice were used). Nearly all of the specifically expanded populations in TdT -/- polyclonal anti-VV population were also present in the $A52R_{75}$-specific population, while only a few of expanded populations in *wt* mice were similarly matched. This is completely consistent with the immunodominance status of $A52R_{75}$-specific T_{CD8+} in TdT -/- vs. *wt* mice.

The most important finding in this experiment is that $A52R_{75}$-specific responses of *wt* and TdT use a similarly wide, but clearly distinct range of Vβ chains. Notably, the CDR3 regions of responding T_{CD8+} in TdT -/- mice are shorter by an average of 1.25 amino acids (5.1 vs. 6.35), and are more uniform in length as reflected by a narrower standard deviation (TdT -SD=1.1 (n= 20) vs. *wt* SD= 1.9 (n=21)). These data indicate that the greater immunogenicity of $A52R_{75}$ in TdT -/- mice is not due to a “jackpot” scenario of expansion of a single closely related family of clones. Rather, it reflects the activation of a wide array of clones scattered

among virtually all of the Vβ families examined. Notably, most of responding TdT clones use shorter CDR3 segments than their counterparts in the corresponding *wt* Vβ families.

Discussion

The quality, quantity, and kinetics of Ag presentation have been the focus of most mechanistic investigations into immunodominance in T_{CD8+} responses to intracellular pathogens. This is but half-of the equation, however, as much remains to be learned how determinant-specific T_{CD8+} clonal variation affects immunodominance. Variation in T_{CD8+} precursor frequencies and doubling times would obviously be intrinsic contributors to the immunodominance hierarchy. Clonal differences in the numbers of complexes or degree of co-stimulation needed for T_{CD8+} activation must also contribute to immunodominance, though more subtly, as they would operate in conjunction with variables in Ag presentation. Clonal differences in the abilities of T_{CD8+} to modulate the behavior of APCs and other T cells would also influence immunodominance.

The TdT gene is highly conserved among vertebrates. The sole defined function of TdT is to generate lymphocyte Ag receptor diversity. It might be anticipated that TdT -/- mice would demonstrate easily detected differences in their immunity to pathogens. Strikingly, TdT -/- mice do not exhibit increased susceptibility to pathogens that commonly cause morbidity and/or mortality in immunodeficient animals in conventional animal care facilities (6). TdT -/- mice survived an outbreak of Sendai virus that annihilated immunocompromised strains in same colony. TdT -/- mice also cope well with LCMV, a natural mouse pathogen. Gilfillan *et al* (26) further showed that *wt* and TdT -/- mice are similar in T cell proliferation and Ab responses to soluble protein immunogens and CTL responses to LCMV, VV, or vesicular stomatitis virus infections. No consistent differences were noted in T_{CD4+} immunodominance hierarchies to soluble Ages. Anti-viral T_{CD8+} responses in this work were measured by ^{51}Cr release assay using virus infected cells, which provides a semi-quantitative measure for enumerating T_{CD8+} responses since it is based not only on numbers of responding T_{CD8+} but also their capacity to lyse multiple target cells during the course of the assay. Responses to individual defined determinants were not measured, so the role of TdT in generating the immunodominance hierarchy was not explored.

Here, we provide the initial evidence for the importance of TdT in anti-pathogen immunity. We show that TdT has a major influence in establishing the immunodominance hierarchy to IAV and VV, two radically different viruses in terms of complexity, structure, genome, replication strategy and host cell range. Detailed study of the IAV response conclusively demonstrates that these differences are directly related to intrinsic differences between the T_{CD8+} compartment of *wt* and TdT -/- mice and are not due to alterations in Ag presentation.

We find that TdT plays a major role in setting the hierarchy among the most dominant determinants in both IAV and VV infection. In IAV infections, it appears that increased sensitivity of T_{CD8+} activation explains the enhanced status of HA_{518}-specific clones. Assuming that this is due to increased TCR affinity for the HA_{518}-K^d complex (which remains to be established experimentally), this would mean that TdT expression results in the loss of a fraction of high affinity clones for some determinants. The implication is that shorter CDR3 regions enable higher affinity TCRs for a subset of determinants. The key word here is subset, since the cost of better recognition appears to be diminished recognition of a larger subset determinants that for IAV includes NP_{147} and all of the subdominant determinants.

Gavin and Bevan (11) reported that highest affinity T_{CD8+} clones from TdT -/- mice were more cross-reactive for peptides present in a large synthetic library. The authors speculated that this was based on higher affinity docking with class I alpha-helices and further, that such clones

might provide protective immunity against multiple pathogens at the expense of increased susceptibility to autoimmune abnormalities. To date, however, it appears that the opposite is true. TdT -/- mice are reported to be protected against autoimmune diabetes in the NOD model (27) and glomerulonephritis in lupus-prone MRL-*Fas*^{lpr} mice (28) and (NZB × NZW) F1 mice (29). A second school of thought advocates a link between higher numbers of TCR specificities (rather than cross-reactivity) and autoimmune process (30). The delay in TdT expression in ontogeny may therefore allow peripheral tolerance mechanisms to develop prior to the acquisition of a highly diverse repertoire with increased self-recognition.

Turner, Doherty and colleagues have recently explored the relationship between peptide class I complex structure and the mouse TCR repertoire (31,32). They found a correlation between the dominant use of public TCR specificities (*i.e.* sequences closely related to germline sequences shared between all responding individual inbred mice) and the lack of prominent peptide side chain structures protruding from the class I groove. Based on Mitaksov & Fremont's recent x-ray crystallographic structural determination of NP_{147}-K^d complexes (33), NP_{147} qualifies as a "structure rich" peptide (with Thr, Arg, and Arg oriented to the TCR at positions P1, P4 and P6, respectively), while HA_{518} is more "vanilla" (Ile, Ala, Ala) (Table I). The decreased recognition of NP_{147} and increased recognition of HA_{518} by TdT -/- T_{CD8+} is consistent with the Turner-Doherty model. On the other hand, TdT -/- T_{CD8+} also demonstrate enhanced recognition of the structure rich K^d-restricted $A52R_{75}$ VV determinant. This suggests either that the correlation between peptide structure and public TCR usage is not universal, or that TdT activity is necessary to generate the core public specificities that demonstrate the proposed correlation.

By analyzing Vβ gene expression and CDR3 length we found that TdT is not required to mount a diverse response to the VV $A52R_{75}$ determinant, as the response includes T_{CD8+} expressing each of the 19 Vβ families we examined. Since we sorted $A52R_{75}$-specific T_{CD8+} on basis of antigen induced IFN-γ secretion, it appears that each of the Vβ families contributes functionally relevant T_{CD8+} that recognize this one determinant. CDR3 length analysis clearly indicates that in most of these families the dominant clones are shorter by a single amino acid relative to the TCRs from *wt* mice. Thus, for some determinants, TdT mediated insertion of amino acids in a wide variety of Vβ chains generally results in a less fit TCR by the criteria of the magnitude of response. On the other hand, our finding that overall anti-viral responses are decreased and narrowed in TdT -/- mice demonstrates that the negative effect of TdT on responses to some individual determinants (like $A52R_{75}$ and HA_{518}) is outweighed by its effects on TCRs that recognize the majority of immunogenic viral determinants.

The role of TdT in shaping anti-viral determinant responses was first explored by Fazilleau *et al* (34) who used TdT -/- C57Bl/6 mice to study T_{CD8+} responses to a hepatitis B virus T_{CD4+} determinant and a IAV T_{CD8+} determinant (NP_{366}) introduced as immunogens as, respectively, a synthetic peptide or a NP-expressing plasmid. In contrast to our findings, the overall response to these determinants was extremely narrow in both *wt* and TdT -/- mice, being limited to a single predominant Vβ receptor (Vβ8.3 in the case of NP_{366}). This may have resulted from the *in vitro* antigen driven expansion of responding T cells, from DNA vaccination, or from the use of tetramers to isolate T_{CD8+}, since NP_{366} specific T_{CD8+} induced by IAV infection have been reported to comprise at least 11 different Vβ gene products (35). Indeed, using mAbs specific for 15 individual Vβ gene products, we reported that in the BALB/c response to 5 different IAV determinants, multiple Vβ families (typically 9) contributed to the response to each determinant (Chen et al., 2000).

Using D^b-NP_{366} tetramer off-rate as a measure of TCR affinity, Fazilleau *et al.* found that TdT -/- and *wt* T_{CD8+} have a similar affinity for D^b-NP_{366} complexes. This is consistent with our findings regarding similar sensitivity of *wt* and TdT -/- T_{CD8+} for NP_{147}-K^d complexes.

Performing detailed molecular analysis of V chain expression, Fazilleau *et al.* reported that TdT expression had minor effect on the Jβ gene usage and CDR3 sequences, exerting larger effects on Jα usage and CDR sequences. We extend these findings by showing that for $A52R_{75}$-specific T_{CD8+}, TdT expression has protean effects on Vβ chain usage itself. This suggests that in broadening the overall T_{CD8+} repertoire, TdT creates holes in the repertoire among a wide variety of CDR3 regions that modifies the ability of Vβ family members to recognize certain antigenic peptides. Presumably, in the case of HA_{518} and $A52R_{75}$, the TdT-dependent holes cannot be completely compensated by recruiting other Vβ family members, thus resulting in a diminished response to these determinants in *wt* mice.

The positive effect of TdT expression on the overall magnitude and breadth of anti-viral T_{CD8+} is probably our most important finding. The decreased magnitude of the T_{CD8+} response in TdT -/- mice may compromise their capacity to eradicate viruses (and other intracellular pathogens) under circumstances where the immune system is just able to cope with the infectious burden. Further, the narrowing of the response to fewer viral determinants should favor the generation of viral escape mutants, and concomitantly decrease the functional diversity of the response, which has been linked to T_{CD8+} specificity (36-38). Together, these factors likely contribute to the evolution and maintenance of the TdT in vertebrate genomes by providing positive selective pressure based on resistance to infection.

Acknowledgements

Deborah Tokarchick and Dr. James Gibbs provided outstanding technical support. We are indebted to John Kearney for providing TdT -/- mice.

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH.

References

1. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol 1999;17:51–88. [PubMed: 10358753]
2. Haeryfar SM, DiPaolo RJ, Tscharke DC, Bennink JR, Yewdell JW. Regulatory T cells suppress CD8 + T cell responses induced by direct priming and cross-priming and moderate immunodominance disparities 2. J Immunol 2005;174:3344–3351. [PubMed: 15749866]
3. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395–402. [PubMed: 3043226]
4. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat Rev Immunol 2004;4:123–132. [PubMed: 15040585]
5. Komori T, Okada A, Stewart V, Alt FW. Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. Science 1993;261:1171–1175. [PubMed: 8356451]
6. Gilfillan S, Dierich A, Lemeur M, Benoist C, Mathis D. Mice lacking TdT: mature animals with an immature lymphocyte repertoire. Science 1993;261:1175–1178. [PubMed: 8356452]
7. Landreth KS, McCoy K, Clagett J, Bollum FJ, Rosse C. Deficiency in cells expressing terminal transferase in autoimmune (motheaten) mice. Nature 1981;290:409–411. [PubMed: 7219527]
8. Bonati A, Zanelli P, Savi M, Neri TM. TCR-beta chain gene rearrangement and expression in human T-cell development and in leukemia. Leukemia 1994;8:918–923. [PubMed: 8207984]
9. Bogue M, Gilfillan S, Benoist C, Mathis D. Regulation of N-region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny. Proc Natl Acad Sci U S A 1992;89:11011–11015. [PubMed: 1438306]
10. Cabaniols JP, Fazilleau N, Casrouge A, Kourilsky P, Kanellopoulos JM. Most alpha/beta T cell receptor diversity is due to terminal deoxynucleotidyl transferase. J Exp Med 2001;194:1385–1390. [PubMed: 11696602]

11. Gavin MA, Bevan MJ. Increased peptide promiscuity provides a rationale for the lack of N regions in the neonatal T cell repertoire. Immunity 1995;3:793–800. [PubMed: 8777724]
12. Chen W, Yewdell JW, Levine RL, Bennink JR. Modification of cysteine residues in vitro and in vivo affects the immunogenicity and antigenicity of major histocompatibility complex class I-restricted viral determinants. J Exp Med 1999;189:1757–1764. [PubMed: 10359579]
13. Coles RM, Mueller SN, Heath WR, Carbone FR, Brooks AG. Progression of armed CTL from draining lymph node to spleen shortly after localized infection with herpes simplex virus 1 3. J Immunol 2002;168:834–838. [PubMed: 11777979]
14. Chen W, Anton LC, Bennink JR, Yewdell JW. Dissecting the multifactorial causes of immunodominance in class I- restricted T cell responses to viruses. Immunity 2000;12:83–93. [PubMed: 10661408]
15. Chen W, Bennink JR, Morton PA, Yewdell JW. Mice deficient in perforin, CD4+ T cells, or CD28-mediated signaling maintain the typical immunodominance hierarchies of CD8+ T-cell responses to influenza virus. J Virol 2002;76:10332–10337. [PubMed: 12239309]
16. Deng Y, Yewdell JW, Eisenlohr LC, Bennink JR. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. J Immunol 1997;158:1507–1515. [PubMed: 9029084]
17. Bennink JR, Yewdell JW, Smith GL, Moss B. Recognition of cloned influenza virus hemagglutinin gene products by cytotoxic T lymphocytes. J Virol 1986;57:786–791. [PubMed: 3485199]
18. Kedl RM, Kappler JW, Marrack P. Epitope dominance, competition and T cell affinity maturation. Curr Opin Immunol 2003;15:120–127. [PubMed: 12495743]
19. Mozdzanowska K, Feng J, Eid M, Kragol G, Cudic M, Otvos L Jr, Gerhard W. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. Vaccine 2003;21:2616–2626. [PubMed: 12744898]
20. Tscharke DC, Woo WP, Sakala IG, Sidney J, Sette A, Moss DJ, Bennink JR, Karupiah G, Yewdell JW. Poxvirus CD8+ T-cell determinants and cross-reactivity in BALB/c mice. J Virol 2006;80:6318–6323. [PubMed: 16775319]
21. Sebille F, Gagne K, Guillet M, Degauque N, Pallier A, Brouard S, Vanhove B, Delsuc MA, Soulillou JP. Direct recognition of foreign MHC determinants by naive T cells mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution. J Immunol 2001;167:3082–3088. [PubMed: 11544292]
22. Garcia KC, Teyton L, Wilson IA. Structural basis of T cell recognition. Annu Rev Immunol 1999;17:369–397. [PubMed: 10358763]
23. Hodes RJ, Abe R. T cell recognition of Mls-like superantigens: analysis of TCR requirements, superantigenic ligands, and signal transduction. Semin Immunol 1992;4:319–327. [PubMed: 1336682]
24. Sourdive DJ, Murali-Krishna K, Altman JD, Zajac AJ, Whitmire JK, Pannetier C, Kourilsky P, Evavold B, Sette A, Ahmed R. Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection. J Exp Med 1998;188:71–82. [PubMed: 9653085]
25. Gonzalez-Quintial R, Baccala R, Balderas RS, Theofilopoulos AN. V beta gene repertoire in the aging mouse: a developmental perspective. Int Rev Immunol 1995;12:27–40. [PubMed: 7595012]
26. Gilfillan S, Bachmann M, Trembleau S, Adorini L, Kalinke U, Zinkernagel R, Benoist C, Mathis D. Efficient immune responses in mice lacking N-region diversity. Eur J Immunol 1995;25:3115–3122. [PubMed: 7489751]
27. Gilfillan S, Benoist C, Mathis D. Mice lacking terminal deoxynucleotidyl transferase: adult mice with a fetal antigen receptor repertoire. Immunol Rev 1995;148:201–219. [PubMed: 8825288]
28. Robey IF, Peterson M, Horwitz MS, Kono DH, Stratmann T, Theofilopoulos AN, Sarvetnick N, Teyton L, Feeney AJ. Terminal deoxynucleotidyltransferase deficiency decreases autoimmune disease in diabetes-prone nonobese diabetic mice and lupus-prone MRL-Fas(lpr) mice. J Immunol 2004;172:4624–4629. [PubMed: 15034081]
29. Conde C, Weller S, Gilfillan S, Marcellin L, Martin T, Pasquali JL. Terminal deoxynucleotidyl transferase deficiency reduces the incidence of autoimmune nephritis in (New Zealand Black × New Zealand White)F1 mice. J Immunol 1998;161:7023–7030. [PubMed: 9862739]

30. Benedict CL, Gilfillan S, Thai TH, Kearney JF. Terminal deoxynucleotidyl transferase and repertoire development. Immunol Rev 2000;175:150–157. [PubMed: 10933600]

31. Turner SJ, Kedzierska K, Komodromou H, La Gruta NL, Dunstone MA, Webb AI, Webby R, Walden H, Xie W, McCluskey J, Purcell AW, Rossjohn J, Doherty PC. Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations. Nat Immunol 2005;6:382–389. [PubMed: 15735650]

32. Turner SJ, Kedzierska K, La Gruta NL, Webby R, Doherty PC. Characterization of CD8+ T cell repertoire diversity and persistence in the influenza A virus model of localized, transient infection. Semin Immunol 2004;16:179–184. [PubMed: 15130502]

33. Mitaksov V, Fremont DH. Structural Definition of the H-2Kd Peptide-binding Motif. J Biol Chem 2006;281:10618–10625. [PubMed: 16473882]

34. Fazilleau N, Cabaniols JP, Lemaitre F, Motta I, Kourilsky P, Kanellopoulos JM. Valpha and Vbeta public repertoires are highly conserved in terminal deoxynucleotidyl transferase-deficient mice. J Immunol 2005;174:345–355. [PubMed: 15611258]

35. Deckhut AM, Allan W, McMickle A, Eichelberger M, Blackman MA, Doherty PC, Woodland DL. Prominent usage of Vá8.3 T cells in the H-2D b -restricted response to an influenza A virus nucleoprotein epitope. J Immunol 1993;151:2658–2666. [PubMed: 7689611]

36. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, Chetty S, Rathnavalu P, Moore C, Pfafferott KJ, Hilton L, Zimbwa P, Moore S, Allen T, Brander C, Addo MM, Altfeld M, James I, Mallal S, Bunce M, Barber LD, Szinger J, Day C, Klenerman P, Mullins J, Korber B, Coovadia HM, Walker BD, Goulder PJ. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature 2004;432:769–775. [PubMed: 15592417]

37. Crowe SR, Miller SC, Shenyo RM, Woodland DL. Vaccination with an acidic polymerase epitope of influenza virus elicits a potent antiviral T cell response but delayed clearance of an influenza virus challenge. J Immunol 2005;174:696–701. [PubMed: 15634888]

38. Yant LJ, Friedrich TC, Johnson RC, May GE, Maness NJ, Enz AM, Lifson JD, O'Connor DH, Carrington M, Watkins DI. The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol 2006;80:5074–5077. [PubMed: 16641299]

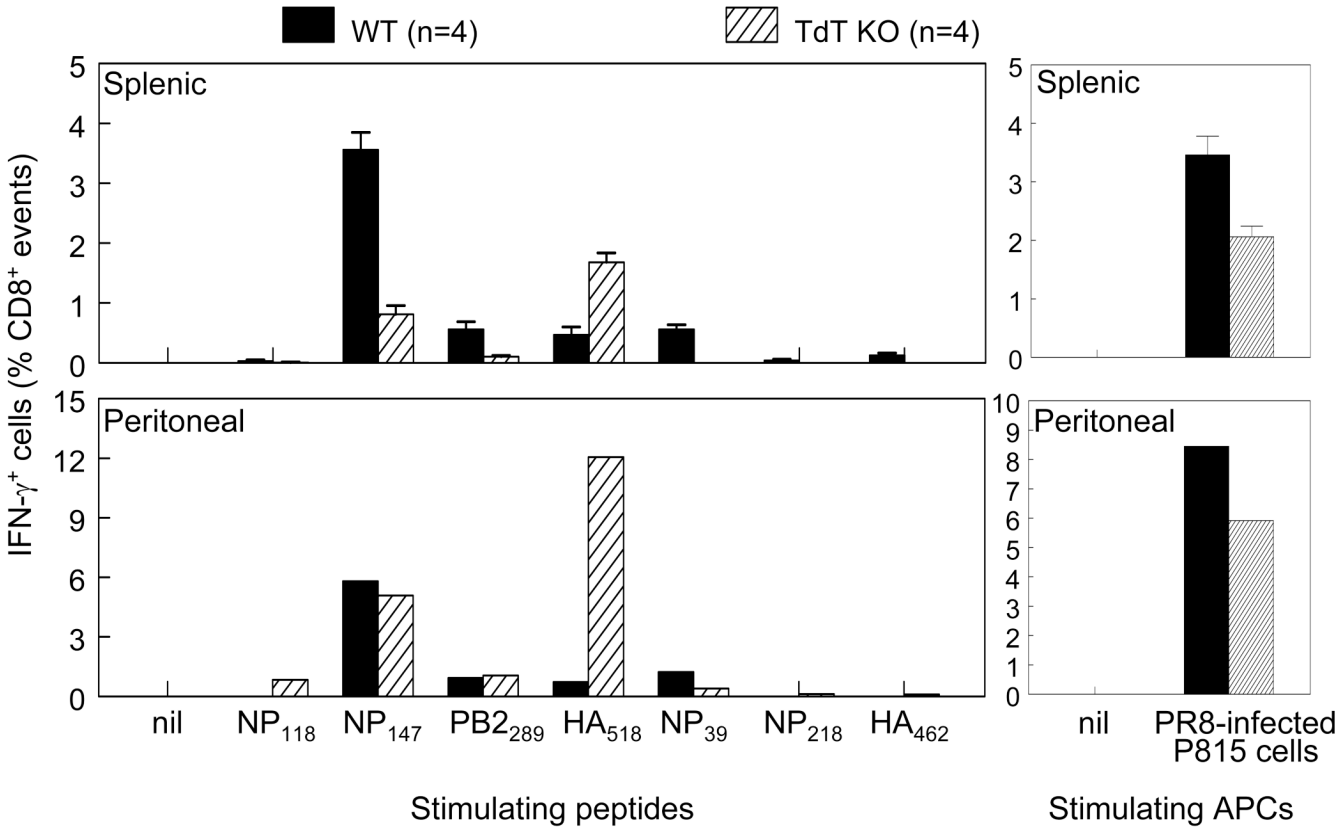

Figure 1. Distinct IAV-specific T_{CD8+} hierarchy in TdT -/- mice

wt and TdT-deficient mice were injected *i.p.* with IAV. Seven days later, splenocytes and PECs were examined *ex vivo* for IFN-γ accumulation following restimulation with indicated peptides (left panels) or IAV-infected P815 cells (right panels) used at 4×10^5 cells/well. NP_{118} is an H-2^d- binding immunodominant peptide of LCMV that was used as a negative control. Values are subtracted from background obtained from wells receiving no peptides and are expressed as mean ± SEM of four individual mice/group for splenic responses. For peritoneal responses, PECs were pooled and analyzed simultaneously. Data are representative of more than 10 independent experiments yielding similar results.

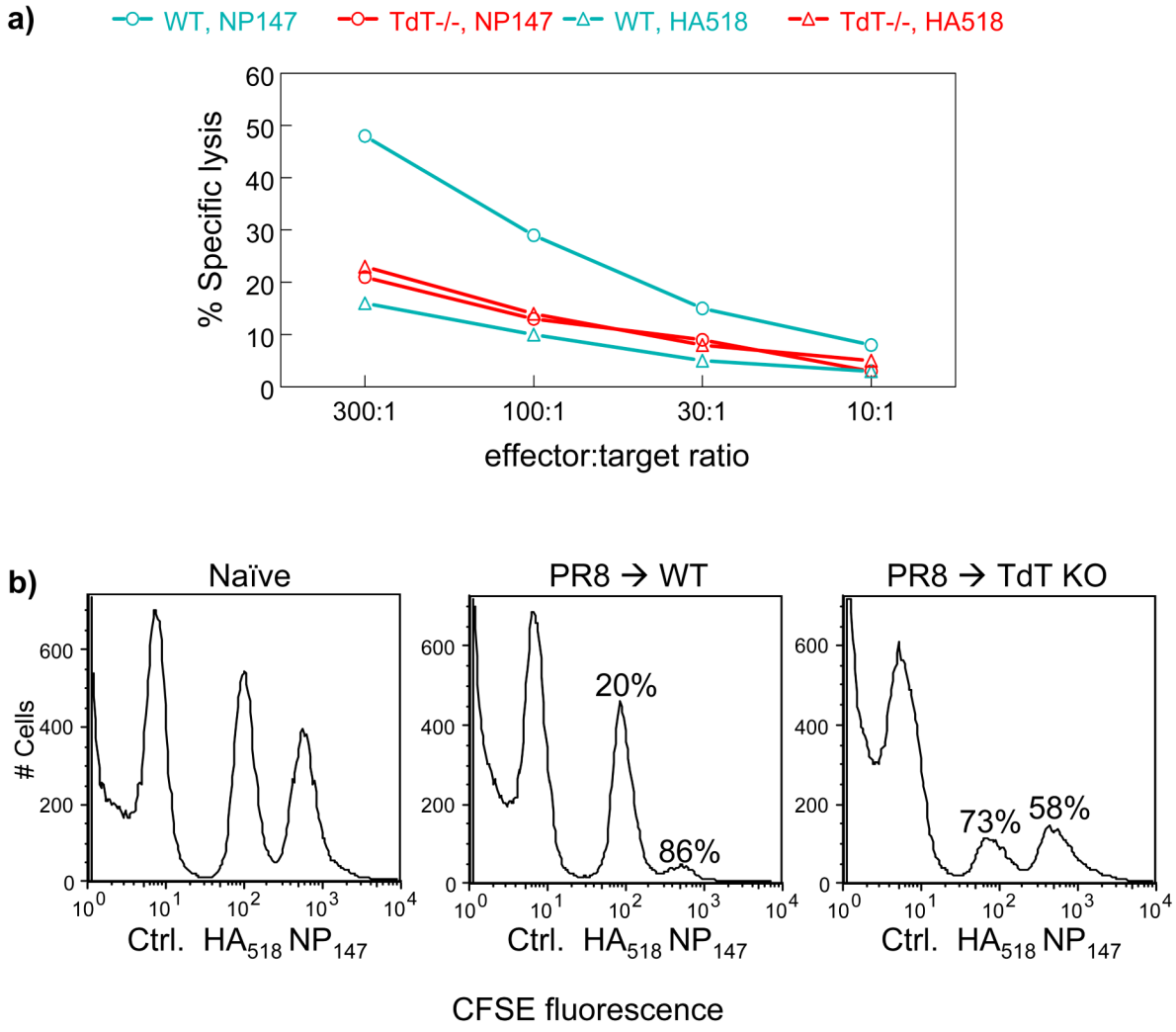

Figure 2. Altered immunodominance hierarchy of anti-IAV T_{CD8+} is also manifest at the level CTL effector function both ex vivo and in vivo

a) Three mice/group were infected *i.p.* with IAV. Seven days later, *wt* and TdT-deficient splenocytes from each group were pooled and used at indicated effector:target ratios against P815 cells sensitized with either NP_{147} or HA_{518} in a 6-h ^{51}Cr release assay as indidated. Data are from one experiment, another independent experiment gave similar results. Background lysis obtained from wells containing non-sensitized P815 or P815 cells pulsed with NP_{118} was always between 1-4% at the highest effector:target ratio used. Spontaneous release of P815 target cells was always <10%. Each data point represents the mean of triplicate samples. b) Target splenocytes were pulsed with Np_{118} (control) peptide, HA_{518} or NP_{147}, and stained with CFSE at 0.025, 0.2 and 1.6 μM, respectively. These cells were washed thoroughly, mixed in equal numbers and injected into tail veins of *wt* or TdT -/- mice. Mice were either uninfected or infected with PR8 7d previously. Two hours after injection, splenocytes from each mouse were harvested and analyzed by flow Cytometry using differential CFSE fluorescence to distinguish CFSE target cells. The percent specific killing of each target cell population was calculated as described in the *Material and Methods* section, and are shown in the Figure. Similar results were obtained in two additional experiments.

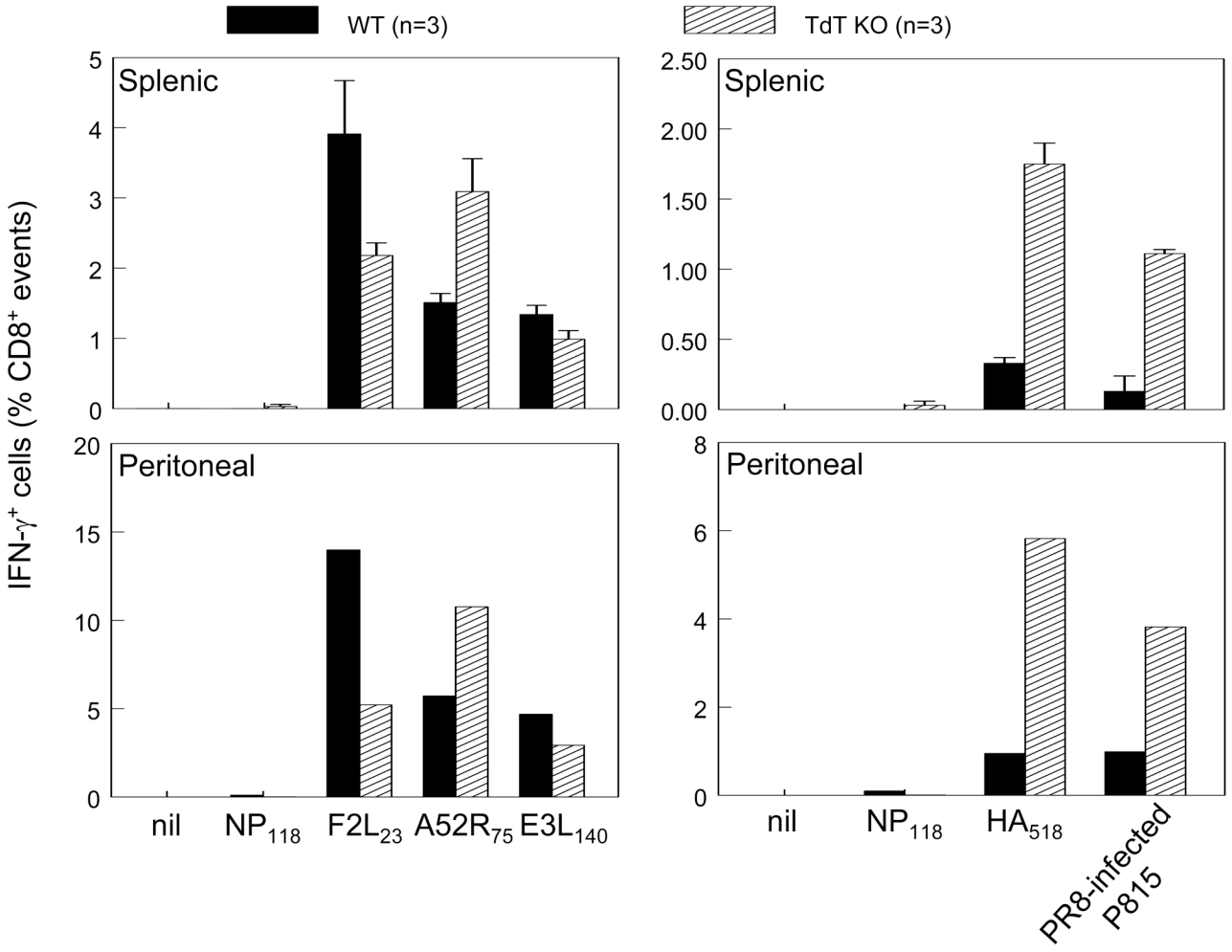

Figure 3. Effect of TdT on response to VV-encoded antigens

Three mice/group were injected with rVV-ES-HA_{518} *i.p.* Seven days later, individual spleens and pooled PECs were examined for the presence of T_{CD8+} responding to HA_{518} or IAV-infected P815 cells (right panels). At the same time, T_{CD8+} responses to three H-2^d-resricted peptides of VV were evaluated in *wt* and TdT -/- mice (left panels). Nearly identical results were obtained in an additional experiments also consisting of 3 mice/group.

Figure 4a

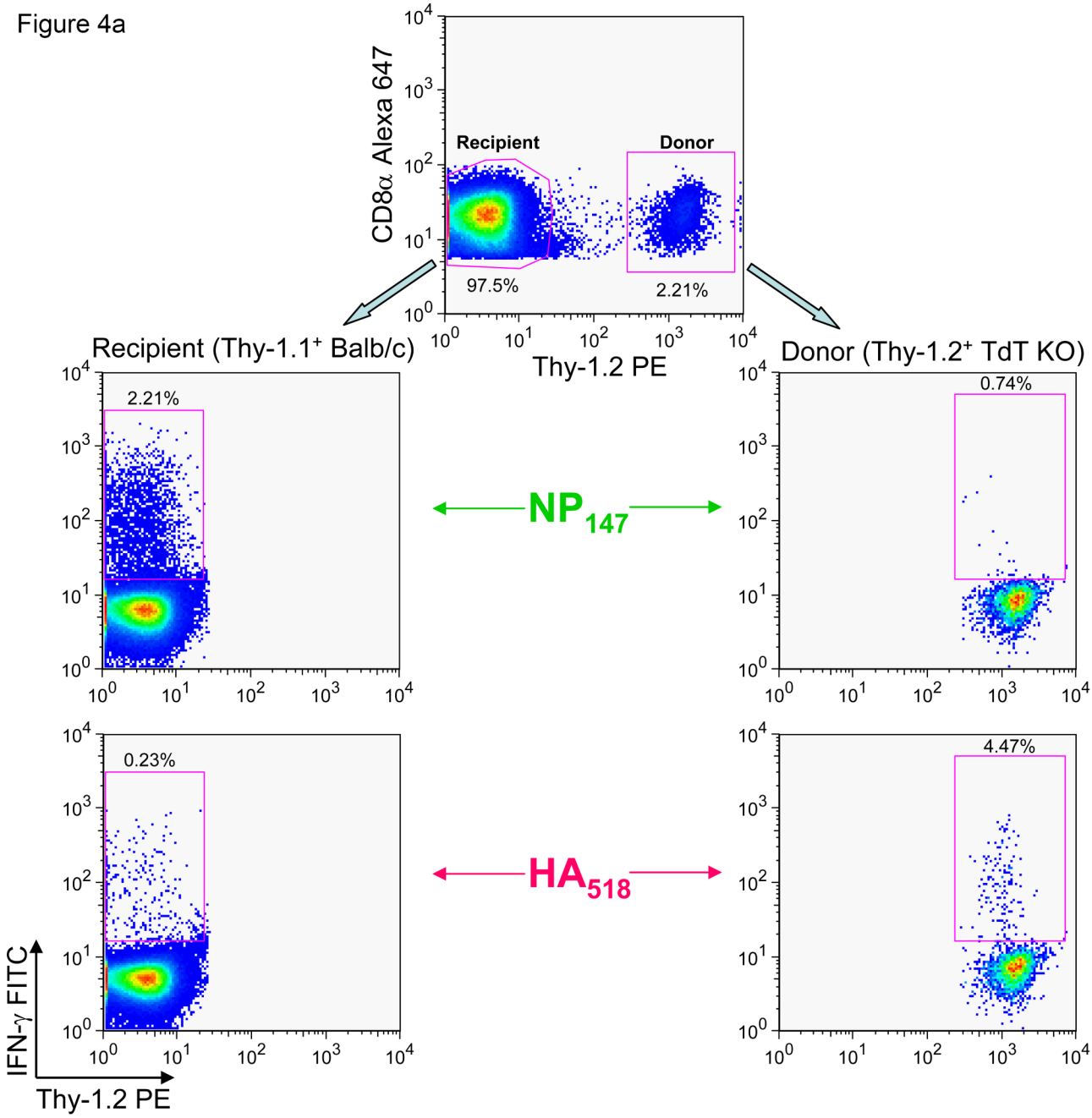

Figure 4b

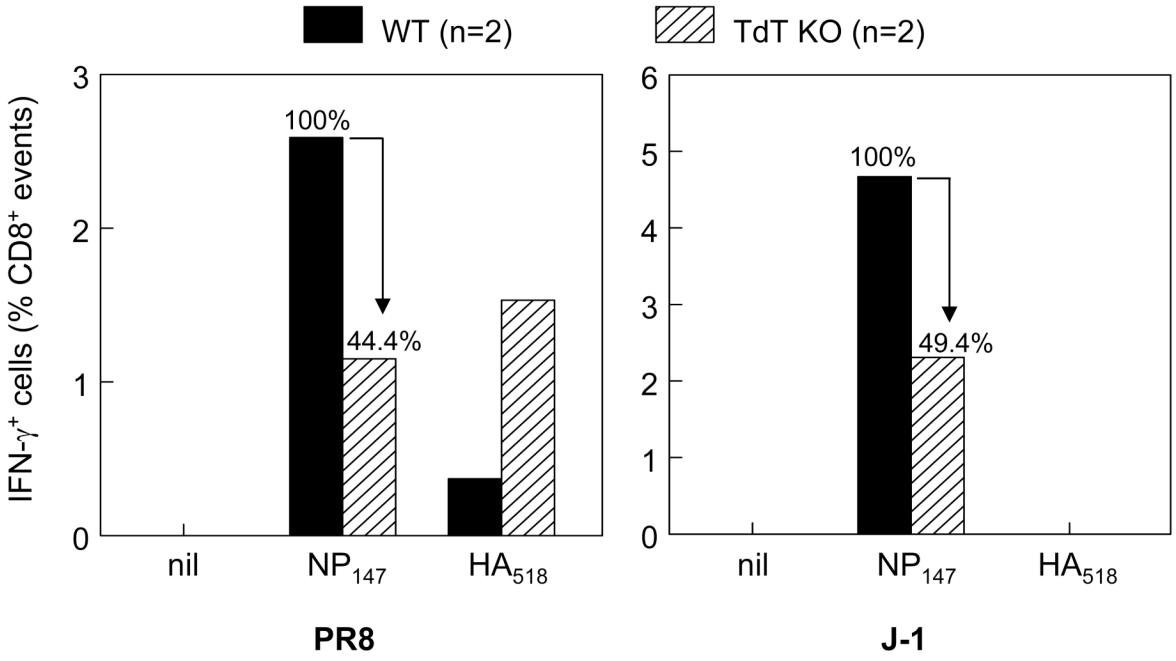

Figure 4. Mechanism of Altered Immunodominance Status of HA_{518}

A. Approximately 10 million highly purified T_{CD8+} from pooled splenocytes of naïve TdT -/- mice were injected *i.v.* into BALB/c mice expressing the Thy-1.1 congenic marker one day prior to *i.p.* infection with IAV. NP_{147}- and HA_{518}-specific responses of recipient (Thy-1.1^+ *wt*) and donor (Thy-1.2^+ Tk) T_{CD8+} were evaluated by ICS seven days post-infection after live gating on $CD8^+$ events and using Thy-1.2 staining to distinguish donor cells from recipient cells. The frequencies of determinant specific T_{CD8+} are shown from one representative mouse. This experiment was performed twice, each involving multiple mice, and similar results were obtained for each individual mouse. B. *wt* and TdT -/- mice were immunized with IAV or the J-1 reassortant virus lacking IAV HA. NP_{147}- and HA_{518}-specific T_{CD8+} responses were measured by ICS seven days later. NP_{147}-direced responses were similarly decreased following infection with IAV or J-1 (55.6% and 50.6% reduction, respectively).

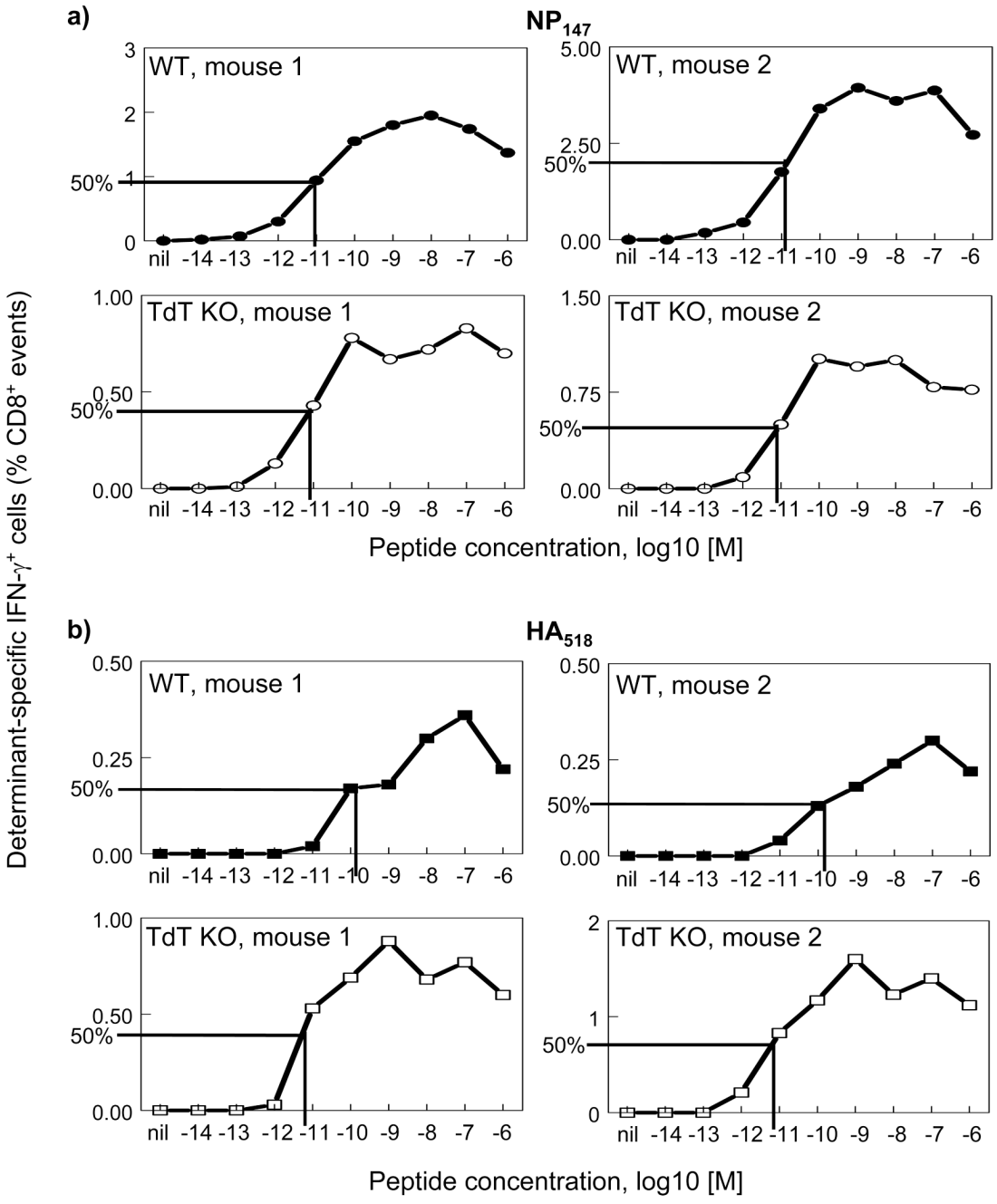

Figure 5. Antigen sensitivity of T_{CD8+} from wt and TdT -/- mice

Two *wt* and two TdT -/- mice were infected *i.p.* with IAV. Seven days later, splenic T_{CD8+} were re-stimulated with indicated concentrations of NP_{147} or HA_{518} and IFN-γ production by these cells was assessed by ICS. The concentration at which a half maximal response was achieved is demonstrated. These results were repeated in an additional experiment looking at T_{CD8+} responses of 4 mice pergroup.

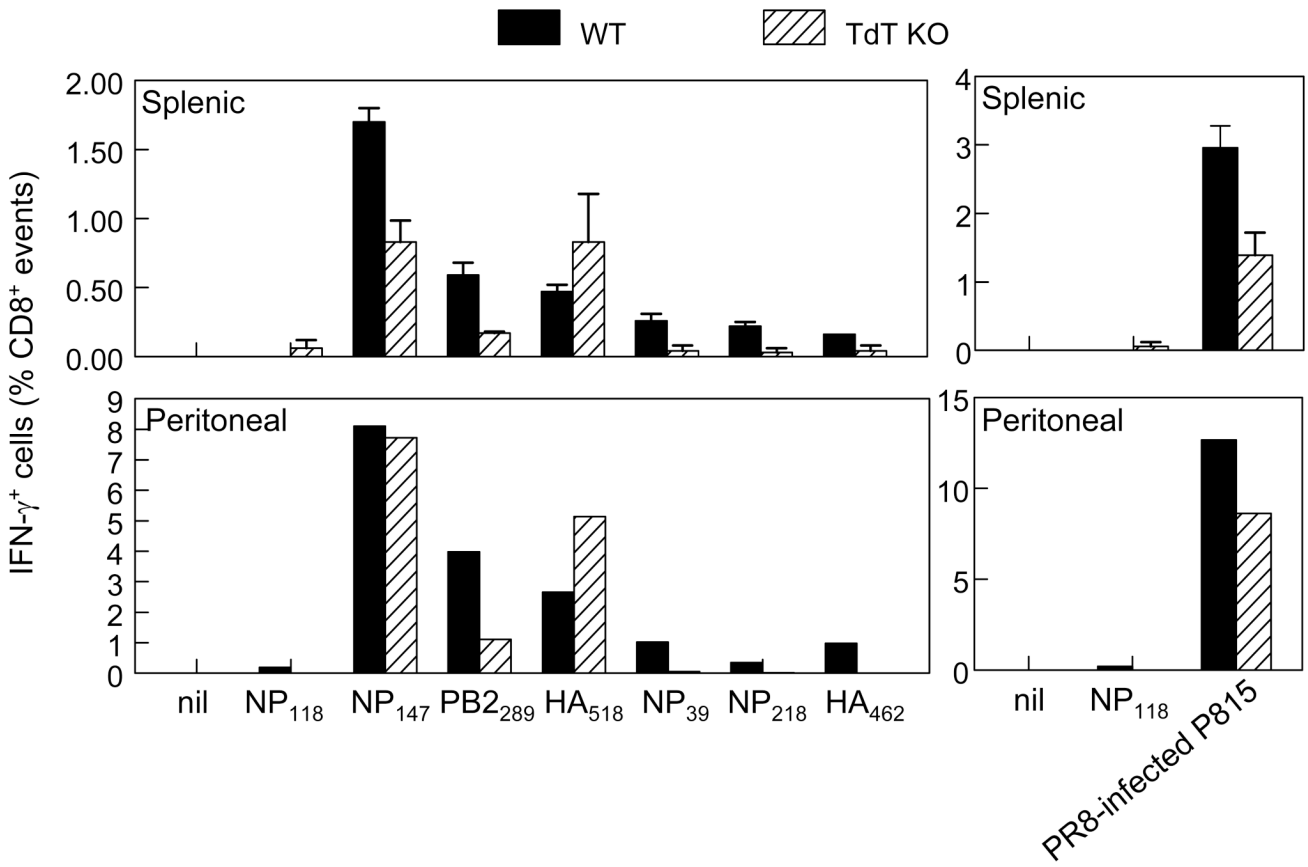

Figure 6. TdT shuffles and narrows the immunodominance hierarchy in memory T_{CD8+} responses

Mice were primed with IAV and boosted a month later with IAV-SEQ12. Seven days after the boost, splenic and peritoneal T_{CD8+} responses of *wt* and TdT-deficient mice were examined against IAV-derived antigenic peptides (left panels) as well as IAV-infected P815 cells (right panels). Error bars for splenic responses represent SEM among 3 mice per group. The same trend was found in 3 similar experiments.

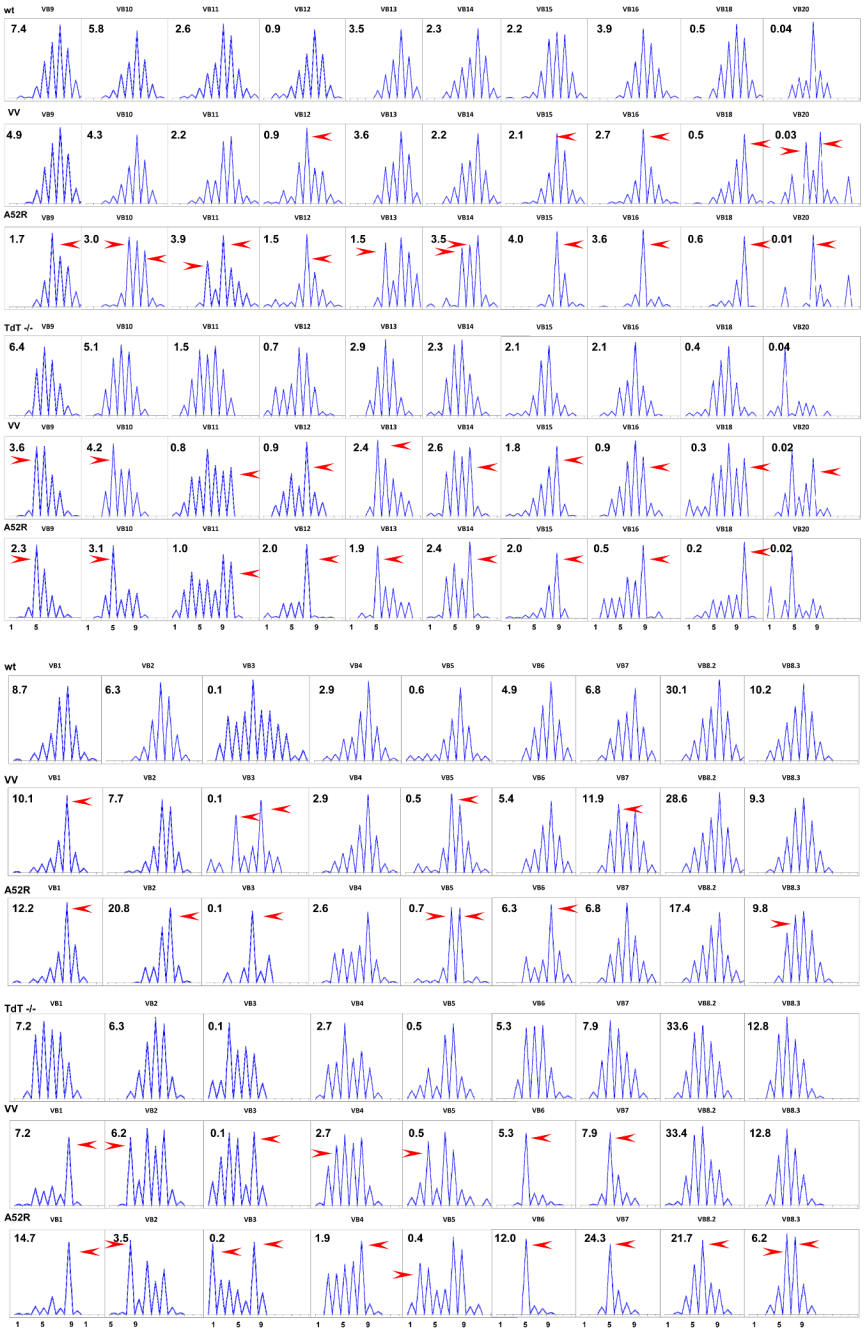

Figure 7. Vβ repertoire analysis of T_{CD8+}

T_{CD8+} were purified from splenocytes pooled from six naïve or ten TdT -/- mice immunized 6 d previously with VV via i.p. injection, or from two *wt* or one TdT -/- naïve mice. RNA prepared from purified T_{CD8+} was analyzed for Vβ usage and CDR3 length using Immunoscope technology. The % of total T_{CD8+} expressing the indicated Vβ chain is given in the upper left corner of each histogram. The distribution of CDR3 lengths is shown by the peak heights plotted against CDR3 length on the X-axis (each histogram starts with CDR3 = 1 amino acid and extends to the longest CDR3 segment detect). Arrows depict populations demonstrating significant expansion relative to naïve mice.

Table 1

Synthetic peptides used in this study. Predicted TCR contact residues for K^d (Mitaksov and Fremont, 2006) restricted are shown in red.

Virus	Determinant	Designation	Sequence	Restricting MHC	Reference
IAV	NP_{147}	NP_{147}	TYQRTRALV	K^d	(Sherman et al., 1992)
IAV	$PB2_{289-297}$	$PB2_{289}$	IGGIRMVDI	D^d	(Chen et al., 2002)
IAV	HA_{518}	HA_{518}	IYSTVASSL	K^d	(Sweetser et al., 1989)
IAV	NP_{39-47}	NP_{39}	FYIQMCTEL	K^d	(Deng et al., 1997)
IAV	$NP_{218-226}$	NP_{218}	TAYERMCNIL	K^d	(Deng et al., 1997)
IAV	$HA_{462-470}$	HA_{462}	LYEKVKSQL	K^d	(Deng et al., 1997)
VV	$F2L_{23-31}$	$F2L_{23}$	SPYAAGYDL	L^d	(Tscharke et al., 2006)
VV	$A52R_{75-83}$	$A52R_{75}$	KYGRLFNEI	K^d	(Tscharke et al., 2006)
VV	$E3L_{140-148}$	$E3L_{140}$	VGPSNSPTF	D^d	(Tscharke et al., 2006)
LCMV	$NP_{118-126}$	NP_{118}	RPQASGVYM	L^d	(Whitton et al., 1989)