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A T Cell Receptor Locus Harbors a Malaria-Specific Immune Response Gene

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SUMMARY

Immune response (*Ir*) genes, originally proposed by Baruj Benacerraf to explain differential antigen-specific responses in animal models, have become synonymous with the major histocompatibility complex (MHC). We discovered a non-MHC-linked *Ir* gene in a T cell receptor (TCR) locus that was required for $CD8^+$ T cell responses to the *Plasmodium berghei* $GAP50_{40-48}$ epitope in mice expressing the MHC class I allele $H\text{-}2D^b$. $GAP50_{40-48}$-specific $CD8^+$ T cell responses emerged from a very large pool of naive $V\beta8.1^+$ precursors, which dictated susceptibility to cerebral malaria and conferred protection against recombinant *Listeria monocytogenes* infection. Structural analysis of a prototypical $V\beta8.1^+$ TCR-$H\text{-}2D^b$-$GAP50_{40-48}$ ternary complex revealed that germline-encoded complementarity-determining region 1β residues present exclusively in the Vβ8.1 segment mediated essential interactions with the $GAP50_{40-48}$ peptide. Collectively, these findings demonstrated that *Vβ8.1* functioned as an *Ir* gene that was indispensable for immune reactivity against the malaria $GAP50_{40-48}$ epitope.

INTRODUCTION

Genetic regulation of the immune system is a central determinant of health and disease. The observation that random-bred guinea pigs challenged with simple antigens segregate into responder and non-responder groups led to the notion of autosomal-dominant immune response (*Ir*) genes (reviewed in Benacerraf and Germain, 1978). *Ir* genes were first identified as major histocompatibility complex (MHC)-linked (McDevitt and Chinitz, 1969) and subsequently found to encode specific allotypes of antigen-presenting molecules, such as MHC class II (MHC II) (Benacerraf, 1974; Benacerraf and McDevitt, 1972). Although unidentified at the time, a determinative role was also suggested for the putative T cell receptor (TCR) (Benacerraf and Germain, 1978). MHC II-mediated antigen presentation is required to activate T helper cells and generate antibody responses to T-dependent antigens (Owens and Zeine, 1989). In addition, the host must assemble TCRs capable of engaging specific MHC-peptide (MHCp) complexes with sufficient avidity to trigger immune reactivity (Davis et al., 2003; van der Merwe and Dushek, 2011). Numerous studies of inbred animals have linked the absence of specific immune responses with a lack of appropriate MHC alleles (Marshak et al., 1977; Zinkernagel, 1978). In contrast, despite major advances in our understanding of antigen recognition over the last four decades, it remains unclear whether germline-encoded segments of the TCR can function as *Ir* genes.

$CD8^+$ T cells recognize MHC I-restricted peptides via heterodimeric TCRs (Davis and Bjorkman, 1988; Townsend et al., 1985). A vast number of different TCRs can be generated from a limited number of germline-encoded segments through the process of *V(D)J* gene recombination with junctional diversification and subsequent random pairing of the somatically rearranged TCRα and TCRβ chains (Cabaniols et al., 2001; Chothia et al., 1988; Davis and Bjorkman, 1988; Rossjohn et al., 2015; Turner et al., 2006). As a consequence, each individual harbors an extensive repertoire of naive $CD8^+$ T cells, which ensures broad recognition of a large number of foreign antigens presented by MHC I (Goldrath and Bevan, 1999). To meet the diversity criterion within space limitations, however, only a few naive precursors are specific for any given epitope (Blattman et al., 2002; Obar et al., 2008), and robust antigen-driven proliferation is required to establish effector and memory $CD8^+$ T cell populations (Busch et al., 1998; Goldrath and Bevan, 1999). It is estimated that most naive antigen-specific repertoires in mice do not contain more than 10–300 $CD8^+$ T cells (Obar et al., 2008).

Larger pre-immune repertoires comprising 1,000–1,500 naive $CD8^+$ T cells have been reported for the murine cytomegalovirus (MCMV) $M45_{985-993}$ and vaccinia virus (VacV) $B8R_{20-27}$ epitopes (Jenkins and Moon, 2012), but it remains unclear whether these specific precursor pools define the upper limits of antigen reactivity in the post-thymic landscape of clonotypically distributed TCRs.

Clonal selection ensures the recruitment of biologically and structurally optimal immune receptors from the naive repertoire (Malherbe et al., 2004; Price et al., 2005), frequently leading to biased TCR usage among memory $CD8^+$ T cell populations (Miles et al., 2011; Turner et al., 2006). In extreme cases, non-peptidic antigens restricted by non-classical MHC molecules elicit innate-like responses dominated by semi-invariant TCRs (Bendelac et al., 1997; Godfrey et al., 2015; Van Rhijn et al., 2015). Here, we found that a similar phenomenon can regulate conventional $CD8^+$ T cell immunity. We demonstrated that an epitope from the *Plasmodium berghei* (*P. berghei*) ANKA glideosome-associated protein ($GAP50_{40-48}$), a key pathogenic target in experimental cerebral malaria (ECM), was associated with the largest naive $CD8^+$ T cell repertoire yet described in laboratory mice (Jenkins and Moon, 2012). We also revealed the molecular properties of these $GAP50_{40-48}$-specific TCRs, demonstrating exclusive use of the Vβ8.1 segment underpinned by direct interactions with the malarial peptide. Mice lacking *Vβ8.1* did not respond to the $GAP50_{40-48}$ epitope after *P. berghei* infection and did not develop ECM. Moreover, the very large pool of naive precursors conferred enhanced control of primary infection with recombinant *Listeria monocytogenes* (*L. monocytogenes*) expressing the $GAP50_{40-48}$ epitope. Collectively, these findings extend the notion of *Ir* genes to incorporate germline-encoded components of antigen-specific TCRs.

RESULTS

$GAP50_{40-48}$-Specific $CD8^+$ T Cells Exhibit an Extreme TCR Bias

ECM in susceptible C57BL/6 (B6) mice infected with *P. berghei* ANKA (Engwerda et al., 2005) is a valuable model of severe malarial disease (Brewster et al., 1990). It is established that the development of ECM is critically dependent on pathogenic $CD8^+$ T cells (Amani et al., 2000; Haque et al., 2011; Yañez et al., 1996) expressing $V\beta8^+$ TCRs (Boubou et al., 1999; Mariotti-Ferrandiz et al., 2016), especially those specific for the $H\text{-}2D^b$-restricted $GAP50_{40-48}$ epitope (Howland et al., 2013). However, it is not known why $GAP50_{40-48}$-specific $CD8^+$ T cells are pathogenic in ECM. To address this issue, we set out to generate TCR retrogenic mice harboring monoclonal or oligoclonal $CD8^+$ T cell populations specific for individual epitopes derived from *P. berghei*, namely thrombospondin-related adhesion protein $(TRAP)_{130-138}$, sporozoite-specific protein 20 $(S20)_{318-326}$, and $GAP50_{40-48}$ (Holst et al., 2006; Hafalla et al., 2013; Howland et al., 2013). As a first step, we immunized three separate groups of B6 mice with peptide-pulsed dendritic cells (DCs) followed 7 days later by recombinant *L. monocytogenes* (LM) expressing the same epitope. This accelerated prime-boost approach (Badovinac et al., 2005) elicited large $CD8^+$ T cell responses specific for $TRAP_{130-138}$, $S20_{318-326}$, and $GAP50_{40-48}$ (Figure S1A). We then used the corresponding MHC I tetramers and a panel of anti-mouse Vβ antibodies to profile the constituent malaria-specific TCRs.

$CD8^+$ T cells specific for $TRAP_{130-138}$ and $S20_{318-326}$ expressed several different TCR Vβ segments with distinct preferences (Figures 1A and S1B). For example, almost 75% of the $S20_{318-326}$-specific repertoire was focused on Vβ8.1/8.2 and Vβ8.3, while the $TRAP_{130-138}$-specific repertoire was dominated by Vβ2 and Vβ7. In contrast, >99% of $GAP50_{40-48}$-specific $CD8^+$ T cells expressed Vβ8.1/8.2, which cannot be discriminated by antibody staining (Figures 1A and S1B). These segments comprised <15% of the corresponding $GAP50_{40-48}$ $tetramer^-$ repertoires in the same mice (Figure 1B). Moreover, an identical TCR bias was observed after inoculation of sporozoites by mosquito bite or immunization with radiation-attenuated sporozoites or DC-$GAP50_{40-48}$ alone (data not shown), and similar results were obtained with other strains of mice expressing the MHC I allele $H\text{-}2D^b$, including CB6F1 and BALB.b (Figure S1C). The $GAP50_{40-48}$ epitope therefore mobilized an almost exclusive repertoire of TCR Vβ-defined memory $CD8^+$ T cells.

To gain a deeper understanding of this extreme bias, we sorted $GAP50_{40-48}$-specific $CD8^+$ T cells directly *ex vivo* from DC-LM-$GAP50_{40-48}$-immunized B6 mice and performed an unbiased molecular analysis of all expressed *TRA* and *TRB* gene rearrangements (Quigley et al., 2011). Sequence analysis showed that >98% of transcripts encoded Vβ8.1 (Figure 1C and Table S1). In contrast, a variety of *TRAV* gene segments were detected, indicating promiscuous pairing of diverse TCRα chains with a constrained repertoire of $V\beta8.1^+$ TCRβ chains (Figure 1D). Almost 40% of retrogenic T cells generated with a $GAP50_{40-48}$-specific TCRβ chain bound the $GAP50_{40-48}$ tetramer in naive mice, while only ~1% of retrogenic T cells generated with a $TRAP_{130-138}$-specific TCRβ chain bound the $TRAP_{130-138}$ tetramer (Figure S2). These data suggested that the Vβ8.1 segment was a major recognition element for $GAP50_{40-48}$-specific $CD8^+$ T cells.

Analysis of the third TCRβ chain complementarity-determining region (CDR3β) revealed additional features of the $GAP50_{40-48}$-specific repertoire (Figures S3A–S3C, Table S1). In particular, we identified a common CDR3β length (~60% of translated sequences incorporated 14 amino acids) (Figure S3A), an almost uniform bias toward *TRBD2* gene usage within a single reading frame (Figure S3B), and a strong preference for *TRBJ* gene segments from the Jβ2 cluster (Figure S3C). Moreover, the Vβ-Dβ junction lacked N additions, and a germline-encoded CDR3β motif (^{104}CASSDWG^{110}) was present in >95% of sequences (Table S1 and Figure 1E). These data demonstrated that a highly convergent pattern of gene rearrangements effectively "licensed" the Vβ8.1-driven immune response to the $GAP50_{40-48}$ epitope (Quigley et al., 2010).

The C-Terminal Region of the $GAP50_{40-48}$ Peptide Is a "Hotspot" for TCR Recognition

To understand the molecular basis of this extreme TCR bias, we first determined the structure of the $H\text{-}2D^b$-$GAP50_{40-48}$ complex at a resolution of 2.2 Å (Table S2). The bound peptide adopted a canonical extended conformation (Young et al., 1994), with four solvent-exposed amino acid residues (P4-L, where L is leucine; P6-A, where A is alanine; P7-K, where K is lysine; and P8-Y,

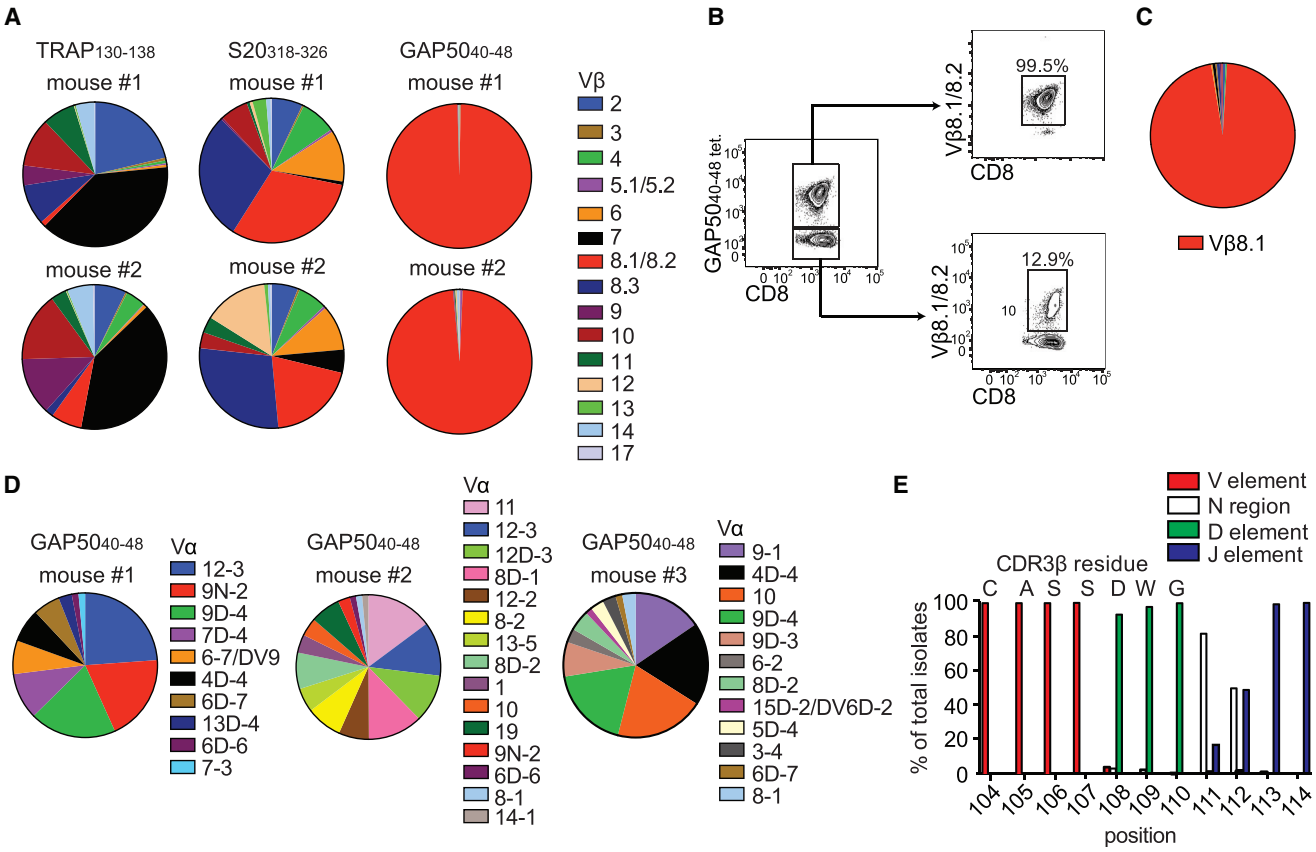


Figure 1. GAP50$_{40-48}$-Specific CD8$^+$ T Cells Express Biased TCRs

(A and B) Mice were primed with peptide-coated DCs and boosted on day 7 with recombinant *L. monocytogenes* expressing the same epitope.

(A) The TCR Vβ repertoire was assessed using splenocytes isolated from immunized mice by staining with the indicated tetramers and Vβ-specific antibodies. Results are shown for two mice for each antigen specificity. Data represent four independent experiments (n = 5 mice/group). Cumulative data are shown in Figure S1B.

(B) Extreme focusing of the GAP50$_{40-48}$-specific repertoire does not reflect the overall frequency of Vβ8.1/8.2$^+$ clonotypes in the CD8$^+$ T cell compartment. Data represent three independent experiments (n = 4–5 mice/group).

(C and D) Confirmation of TCR bias at the transcriptional level. Sequences were derived from GAP50$_{40-48}$-specific CD8$^+$ T cells isolated from the spleens of mice previously immunized with DC-LM-GAP50$_{40-48}$ (total = 316 molecular clones).

(C) Population-level analysis revealed an extreme bias toward Vβ8.1.

(D) TCR Vα sequence distributions from three different mice are depicted. No obvious bias was apparent in the Vα repertoire.

(E) Origin of CDR3β amino acids expressed relative to the total number of molecular Vβ8.1$^+$ clones (n = 5 mice). Vβ, red; Dβ, green; Jβ, purple; N segments, white. See also Figures S1–S3 and Table S1.

where Y is tyrosine) representing potential contacts for the TCR (Figure 2A).

To determine which GAP50$_{40-48}$ residues were important for TCR recognition, we replaced individual amino acids with alanine (Ala), except for the wild-type (WT) Ala residue at position 6 (P6), which was replaced with serine (Ser) (Figure 2B). We did not mutate the critical H-2D^b anchor residues at P5 or P9 (Valkenburg et al., 2013). The WT and mutant peptides were then used at saturating concentrations in intracellular cytokine staining assays to determine the impact of each residue on the functional reactivity of GAP50$_{40-48}$-specific CD8$^+$ T cells (Figures 2C and S4). Interferon-γ (IFN-γ) production relative to the WT peptide was unaffected by the substitutions at P1, P2, and P3 (Figure 2C). In contrast, the mutations at P4-L and P6-A diminished IFN-γ production, while the mutations at P7-K and P8-Y abolished IFN-γ production (Figure 2C). The C-terminal P7-K and P8-Y residues were therefore crucial for GAP50$_{40-48}$-specific CD8$^+$ T cell activation and represented "hotspots" for TCR engagement (Figure 2D).

GAP50$_{40-48}$ Peptide "Hotspots" Interact Exclusively with the TCRβ Chain

Next, we determined the structure of a prototypical Vβ8.1$^+$ TCR, derived from the GAP50$_{40-48}$-specific CD8$^+$ T cell clone NB1 (Table S3), in complex with H-2D^b-GAP50$_{40-48}$ (Figure 3A, Table S2). The NB1 TCR docked centrally atop the H-2D^b cleft, with an angle of 43° and a buried surface area (BSA) of approximately 2,200 Å^2, values that fall within the range previously determined for TCR-MHCp complexes (Figure 3B; Rossjohn et al., 2015). All six CDR loops were involved to a varying extent in the interaction with H-2D^b-GAP50$_{40-48}$ (Figure 3C). Namely, the CDR3α and CDR3β loops contributed 24% and 20% of the

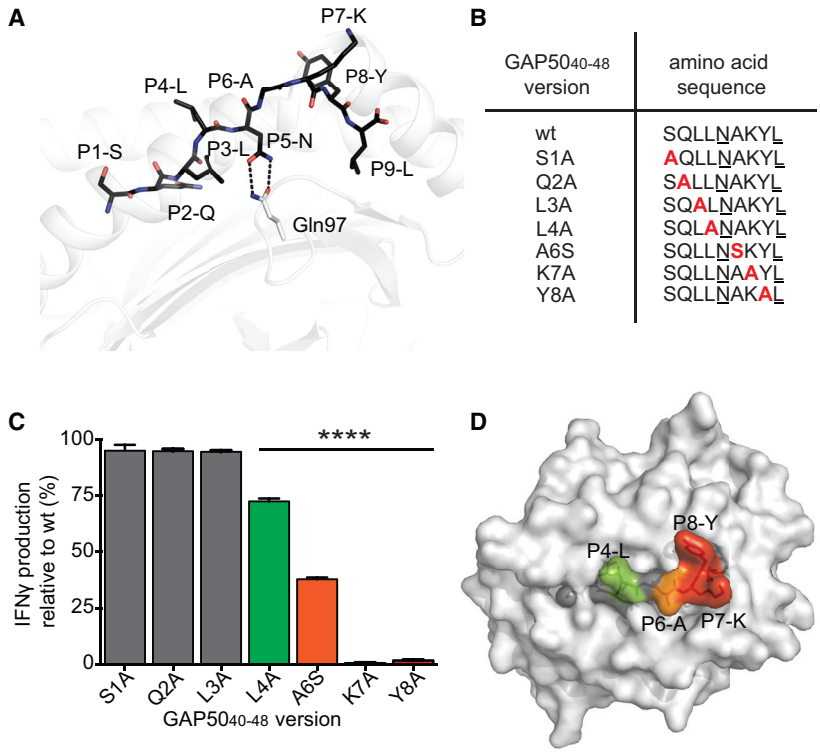


Figure 2. TCR Interactions Focus on the C-Terminal Region of the GAP50$_{40-48}$ Peptide

(A) The structure of the GAP50$_{40-48}$ peptide in complex with H-2D^b. The GAP50$_{40-48}$ peptide is represented as black sticks in the antigen-binding cleft of H-2D^b (white cartoon). The P2-Q and P9-L anchor residues are buried within the antigen-binding cleft, and P5-N acts as a secondary anchor residue forming hydrogen bonds (black dashes) with Gln97 (white stick) at the base of the antigen-binding cleft of H-2D^b (white cartoon).

(B) A panel of peptide mutants was generated by replacing amino acids with Ala or Ser at selected positions (red). Anchor residues are underlined.

(C) Splenocytes isolated from GAP50$_{40-48}$-immunized mice were restimulated for 5 hr *in vitro* using WT or mutant peptides. The production of IFN-γ induced by stimulation with the different mutant peptides is expressed relative to the production of IFN-γ induced by stimulation with the WT peptide. Data represent two independent experiments performed in triplicate. Bars represent mean ± SD. Significance was assessed using a one-way ANOVA (****$p < 0.0001$).

(D) The effect of peptide substitutions on recognition of the H-2D^b-GAP50$_{40-48}$ complex by specific CD8$^+$ T cell clones. The surface of H-2D^b is shown in white. Peptide residues that were not mutated or did not affect IFN-γ production are shown in gray. Green represents up to a 25% decrease in T cell activation; orange represents up to a 70% decrease in T cell activation; red represents a total abrogation of T cell activation.

See also Figure S4 and Table S2.

BSA, respectively, while the CDR1α and CDR2α loops each contributed ~17% of the BSA (Figure 3C). Somewhat unexpectedly given the extreme TCR Vβ bias, the germline-encoded CDR1β and CDR2β loops each contributed only ~10% of the BSA. Thus, the cumulative β chain contribution to the overall BSA was substantially smaller (39%) relative to the cumulative α chain contribution (61%) (Figure 3C). However, >95% of TCR-GAP50$_{40-48}$ peptide interactions were mediated by the TCRβ chain (Figure 3D, Table S4), while the TCRα chain primarily contacted the MHC molecule (74% of the BSA) (Figure 3E, Table S4).

The CDR1α loop stretched between the peptide-binding α helices of H-2D^b, with tyrosine (Tyr) 28α playing a principal role by contacting glutamic acid (Glu) 163, Glu166, and tryptophan (Trp) 167 at the N-terminal end of the cleft (Figure 3F, Table S4). The CDR2α loop sat above the α2 helix, with arginine (Arg) 57α lying flat above residues Glu154 and Ala158 (Figure 3G). The CDR3α loop contacted a large stretch of the α1 helix (spanning residues 58–72), with hydrophobic and hydrogen bond contacts exclusively mediated by germline-encoded residues from the Jα segment (^{108}YAQ^{110}, where Q is glutamine) (Figure 3H). The NB1 TCRα chain engaged the H-2D^b molecule with a large footprint over the N-terminal region of the antigen-binding cleft (Figure 3B). Mutational analyses of H-2D^b binding performed using three distinct GAP50$_{40-48}$-specific CD8$^+$ T cell clones further indicated that different Vβ8.1$^+$ TCRs used an identical MHC I docking strategy (Figure S5, Table S5).

Interactions between the NB1 TCRα chain and the GAP50$_{40-48}$ peptide were limited to two van der Waals interactions between the CDR3α loop and P4-L (Table S4). In contrast, the NB1 TCR Vβ8.1 region dominated contacts with the bound epitope (44 of 46 contacts) (Table S4). Moreover, all three CDRβ loops contacted the C terminus of the peptide (P6–8) (Figures 3I–3K), encompassing the previously identified "hotspots" for CD8$^+$ T cell recognition (Figure 2D). The CDR1β loop interacted with both P7-K and P8-Y via its ^{28}N-DY^{31} motif (where N is asparagine and D is aspartic acid). More specifically, P7-K and P8-Y formed a notch in which aspartic acid (Asp) 30β was inserted as a peg, forming a salt bridge with P7-K (Figure 3J). In addition, asparagine (Asn) 28β hydrogen bonded via its main chain with P7-K, while the aromatic group of Tyr31β sat above P8-Y (Figure 3J). Contacts were also established between P8-Y and the CDR2β loop via Tyr57. Accordingly, P8-Y was closely sequestered upon TCR binding (Figure 3J). The conserved CDR3β motif ^{108}DW^{109} (where W is tryptophan), which was most frequently germline-encoded within the *D* gene (Figure 1E), interacted with P6-A and P7-K (Figure 3K). The large Trp109β side chain lay flat on top of P6-A, acting as a lid covering the central part of the epitope, while Asp108β and P7-K formed a salt bridge (Figure 3K).

TCRβ chain interactions with the H-2D^b molecule were modest in comparison to the TCRα chain (Table S4). The CDR1β loop contacted only glutamine (Gln) 72 via Tyr31β (Figures 3I and 3J). The side chains of valine (Val) 58β from the CDR2β loop abutted Val76 from H-2D^b, establishing an interaction

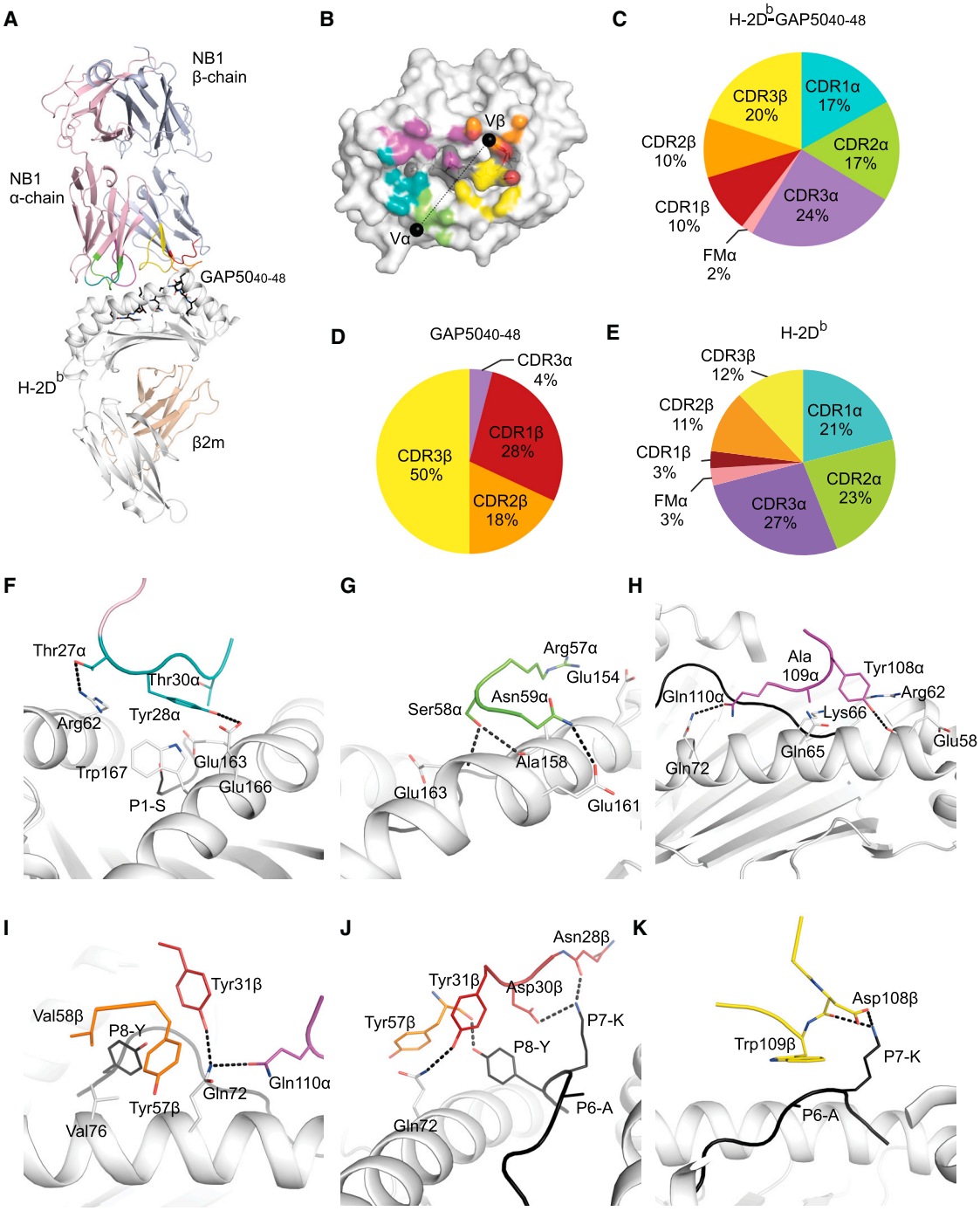


Figure 3. GAP50$_{40-48}$-Specific TCR Bias Is Peptide Driven

(A) The crystal structure of the NB1 TCR (α chain, pink cartoon; β chain, purple cartoon) bound to the GAP50$_{40-48}$ peptide (black sticks) presented by H-2D^b (heavy chain, white cartoon; β2 m, wheat cartoon).

(B) The CDR loop contribution to the BSA is represented as teal (CDR1α), green (CDR2α), purple (CDR3α), red (CDR1β), orange (CDR2β), or yellow (CDR3β). Black spheres represent the center of mass for the Vα and Vβ.

(C–E) The contribution (%) of the CDR loops to the interaction with H-2D^b-GAP50$_{40-48}$ (C), GAP50$_{40-48}$ (D), and H-2D^b (E).

(F–K) Contact residues between H-2D^b-GAP50$_{40-48}$ and CDR1α (F), CDR2α (G), CDR3α (H), CDR1β (I, J), CDR2β (J), and CDR3β (K). The CDR color scheme is maintained. Residues that make contacts with the GAP50$_{40-48}$ peptide or H-2D^b (white) are depicted as black and white sticks, respectively. The H-2D^b molecule is represented as white cartoon, and hydrogen bonds are shown as black dashes.

See also Figure S5 and Tables S2–S5.

network extended by Tyr57β-mediated contacts with Gln72, Arg75, and Val76. The conserved CDR3β ^{108}DW^{109} motif from the *TRBD2* gene segment formed interactions with the hinge region of the α2 helix, whereby Trp109β nestled between histidine (His) 155 and Ser150. Thus, germline-encoded TCRβ chain residues played a minor role in contacting the H-2D^b molecule.

The observation that the prototypical NB1 TCR interacted with GAP50$_{40-48}$ extensively via its β chain suggested that the peptide itself drove the extreme bias toward Vβ8.1. This notion contrasts with prevailing dogma, which asserts that germline bias arises as a consequence of allotype-specific MHC reactivity (Garcia et al., 2009).

CDR1 Residues Unique to Vβ8.1 Are Critical for GAP50$_{40-48}$ Peptide Recognition

These structural insights allowed us to identify candidate factors underlying the exclusive recruitment of Vβ8.1$^+$ TCR clonotypes into the immune repertoire. To determine the precise amino acid sequences involved in this process, we initially focused on the germline-encoded CDR1β and CDR2β loops. Sequence alignments revealed that the CDR1β loops encoded by six *TRBV* genes shared a common Asp30β, while the Asp30β-Tyr31β motif was unique to Vβ8.1. In the other five *TRBV* genes, Tyr31β was replaced by a smaller threonine (Thr)/Ser residue (Table S6A). Three *TRBV* genes shared a common Val58β residue in the encoded CDR2β loop, but only Vβ8.1 incorporated the additional Tyr57β required to sequester P8-Y (Table S6B).

Although the conserved CDR3β region interacted with both the peptide and H-2D^b, we wanted to determine whether the germline-encoded Vβ8.1 residues were essential for TCR-mediated recognition of H-2D^b-GAP50$_{40-48}$. We therefore conducted affinity measurement studies using targeted mutants of the NB1 TCR. Ala mutagenesis was performed on residues Asp30β and Tyr31β in the CDR1β loop and residues Tyr57β and Val58β in the CDR2β loop (Figures 4A and 4B). In addition, Tyr31β was mutated to Thr to mimic the CDR1β loop encoded by the five other *TRBV* genes that shared Asp30β (Figures 4A and 4B). Affinity values for each TCR mutant, measured by surface plasmon resonance (SPR), were compared with the affinity of the WT TCR. The results showed that mutations in the CDR2β loop exerted a moderate effect, decreasing binding affinity by 3-fold compared with the NB1 TCR (Figures 4A and 4B). In contrast, mutation of Tyr31β to either Ala or Thr in the CDR1β loop substantially reduced binding to H-2D^b-GAP50$_{40-48}$, resulting in Kd$_{eq}$ values >200 μM (Figures 4A and 4B). As shown in Figure 3I and Table S4, Tyr31β contacted both H-2D^b and P8-Y. Additionally, mutation of Asp30β to Ala abolished binding to H-2D^b-GAP50$_{40-48}$ (Figures 4A and 4B). The Asp30β residue contacted the peptide alone via a salt bridge with P7-K, one of the two defined "hotspot" residues for TCR recognition (Figure 3J and Table S4).

The unique germline-encoded CDR1β ^{30}DY^{31} motif within the Vβ8.1 segment therefore underpinned both the extreme TCR bias and the residue-specific patterns of epitope recognition that characterized the CD8$^+$ T cell response to H-2D^b-GAP50$_{40-48}$.

The Naive GAP50$_{40-48}$-Specific CD8$^+$ T Cell Repertoire Is Extraordinarily Large

Extreme TCR focusing described in the literature appears to result primarily from the selection of high-affinity clonotypes during repeated or persistent infection (Busch and Pamer, 1999; Malherbe et al., 2004; Price et al., 2005; Savage et al., 1999), but recombinatorial bias and structural constraints dictate the available pre-immune repertoire (Miles et al., 2011; Neller et al., 2015; Turner et al., 2006). We therefore adapted a tetramer-based enrichment protocol (Moon et al., 2007) to enumerate the naive repertoires specific for the *P. berghei* epitopes TRAP$_{130-138}$, S20$_{318-326}$, and GAP50$_{40-48}$. Parallel experiments were performed with tetramers representing epitopes derived from ovalbumin (H-2K^b-OVA$_{257-264}$) and lymphocytic choriomeningitis virus glycoprotein (H-2D^b-LCMV GP$_{33-41}$) to allow comparison with previously reported evaluations of the naive antigen-specific T cell repertoire (Jenkins and Moon, 2012).

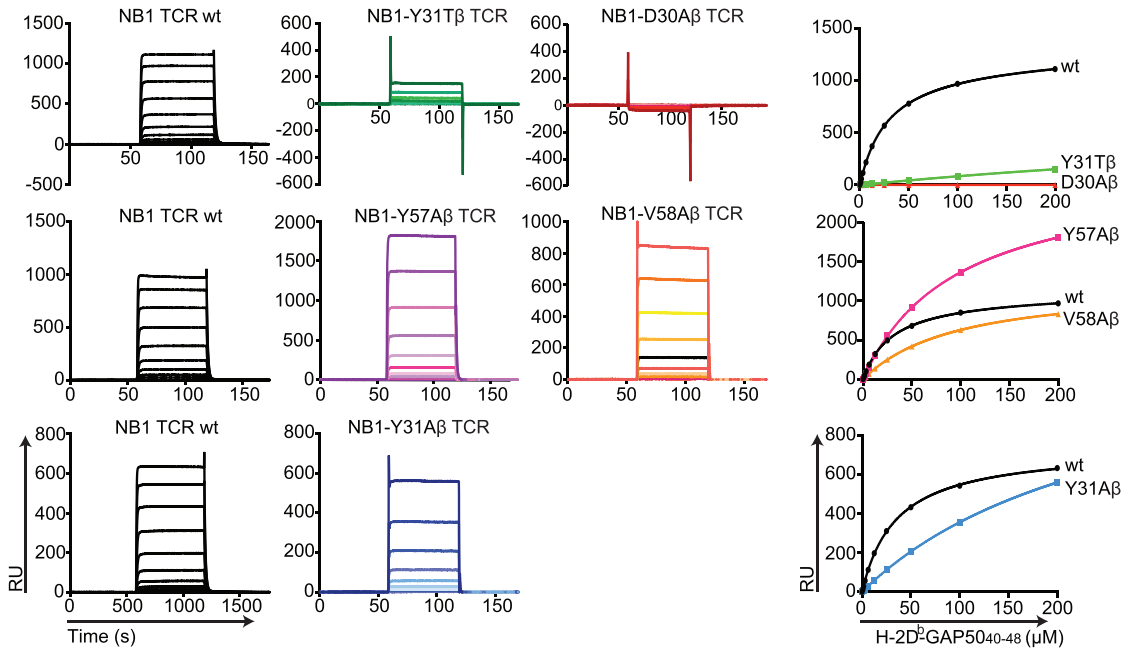
Precursor numbers specific for OVA$_{257-264}$ and GP$_{33-41}$ were consistent with prior reports (183 ± 33 and 358 ± 40 cells, respectively) (Figure 5A; Jenkins and Moon, 2012). The naive S20$_{318-326}$-specific repertoire was relatively small (~79 ± 19 cells), while fewer than 10 cells were counted in the TRAP$_{130-138}$-specific repertoire (Figure 5A). In contrast, as described recently (Gordon et al., 2015), the GAP50$_{40-48}$-specific naive repertoire was extraordinarily large (2,935 ± 305 cells; Figure 5A). The upper limit for a naive antigen-specific CD8$^+$ T cell repertoire in mice was previously established at 1,200–1,500 cells for the MCMV M45$_{985-993}$ and VacV B8R$_{20-27}$ epitopes (Jenkins and Moon, 2012). The GAP50$_{40-48}$-specific repertoire therefore constituted the largest naive antigen-specific CD8$^+$ T cell pool yet described (Figure 5B). Moreover, >99% of naive GAP50$_{40-48}$-specific CD8$^+$ T cells expressed Vβ8.1 (Figure 5A). These findings indicated that the extreme TCR Vβ bias observed in the memory CD8$^+$ T cell pool did not evolve as a consequence of repertoire focusing in response to antigenic challenge, but instead reflected intrinsic recognition of the GAP50$_{40-48}$ epitope characterized by absolute dependence on the interaction with Vβ8.1.

Phenotypically, the GAP50$_{40-48}$-specific precursors resembled classical naive CD8$^+$ T cells, comprised of a large CD44^{lo} population (>90%) and a small CD44^{hi} "virtual memory" population (<10%) akin to OT-I and P14 TCR-Tg cells from naive mice (Figure 5C; Akue et al., 2012). Expression of the CD5 surface protein, which acts as a surrogate marker for the strength of TCR activation induced by self-derived MHCp complexes during thymic selection and frequently correlates with T cell avidity for antigen (Fulton et al., 2015; Mandl et al., 2013), was uniformly higher on naive GAP50$_{40-48}$-specific CD8$^+$ T cells compared with the majority of non-GAP50$_{40-48}$-specific naive CD8$^+$ T cells in the same host and mirrored levels expressed by OT-I cells, which bear high-affinity TCRs (Figure 5D; Kedl et al., 2000). These data suggested that the extremely large pool of Vβ8.1$^+$ precursors specific for the GAP50$_{40-48}$ epitope arose as a consequence of strong thymic selection.

Absence of Vβ8.1 Compromises the GAP50$_{40-48}$-Specific CD8$^+$ T Cell Response

The results of our structural and mutagenesis studies indicated that immune responses to GAP50$_{40-48}$ required the presence of Vβ8.1. To test this hypothesis, we studied the generation of GAP50$_{40-48}$-specific CD8$^+$ T cell responses in C57/L (H-2^b)

A



B

NB1 TCR	Kdeq (μM)
wt	19.0 ± 0.3
NB1 TCR mutants	**Kdeq (μM)**
Tyr31Alaβ	> 200
Tyr31Thrβ	> 200
Asp30Alaβ	NB
Tyr57Alaβ	59.0 ± 0.9
Val58Alaβ	59.7 ± 1.2

Figure 4. Binding Affinities of NB1 TCR Mutants for H-2D^b-GAP50$_{40-48}$

(A) Surface plasmon resonance (SPR) analysis of NB1 WT (black) and the indicated mutant (colored) TCRs across a range of H-2D^b-GAP50$_{40-48}$ concentrations up to a maximum of 200 μM. Representative SPR binding curves are shown for the NB1 WT TCR (black) and NB1 mutant TCRs with the following β chain substitutions: Y31A (green), D30A (red), Y57A (pink), V58A (orange), and Y31A (blue). Each NB1 mutant TCR was tested in parallel with the NB1 WT TCR.

(B) Summary table representing equilibrium binding affinities of NB1 TCR mutants for H-2D^b-GAP50$_{40-48}$. NB, no binding; RU, response units. Data represent two independent experiments performed in duplicate (mean ± SEM).

See also Tables S6A and S6B.

mice, which lack Vβ8 (Behlke et al., 1986). For this purpose, C57/L mice and control B6 mice were exposed to infection with 10^3 *P. berghei* ANKA sporozoites, a challenge that induces ECM in susceptible mouse strains.

In contrast to B6 mice, which generated GAP50$_{40-48}$-specific responses comprising 45% of the total CD8$^+$ T cell pool in peripheral blood and >10% of the total CD8$^+$ T cell pool in the brain by day 7 after infection, the GAP50$_{40-48}$-specific response in C57/L mice was undetectable in both compartments (Figures 6A and 6B). This finding strongly suggested that the GAP50$_{40-48}$-specific CD8$^+$ T cell response was critically dependent on Vβ8.1. In addition, B6 x C57/L F1 mice mounted substantial Vβ8.1$^+$ GAP50$_{40-48}$-specific CD8$^+$ T cell responses after infection with LM-GAP50$_{40-48}$ or *P. berghei* ANKA (data not shown), excluding negative selection of GAP50$_{40-48}$-specific naive precursors in C57/L mice. However, C57/L mice lack other *Vβ* genes (*Vβ5*, *Vβ8*, *Vβ9*, *Vβ11*, and *Vβ12*) (Behlke et al., 1986), which could potentially compromise the ability to mount alternative responses to GAP50$_{40-48}$. Countering this argument, the magnitude of the total *P. berghei*-specific CD8$^+$ T cell response, as detected by the frequency of CD8$^+$ T cells expressing surrogate activation markers (CD11a^{hi}CD8^{lo}) (Rai et al., 2009) at day 7 after infection, was essentially identical in B6 and C57/L mice (Figure 6C). Moreover, C57/L mice did not develop ECM. Instead, these mice survived beyond day 9 after infection and succumbed to high-level parasitemia after ~3 weeks (Figure 6D). Thus, *Vβ8.1* was

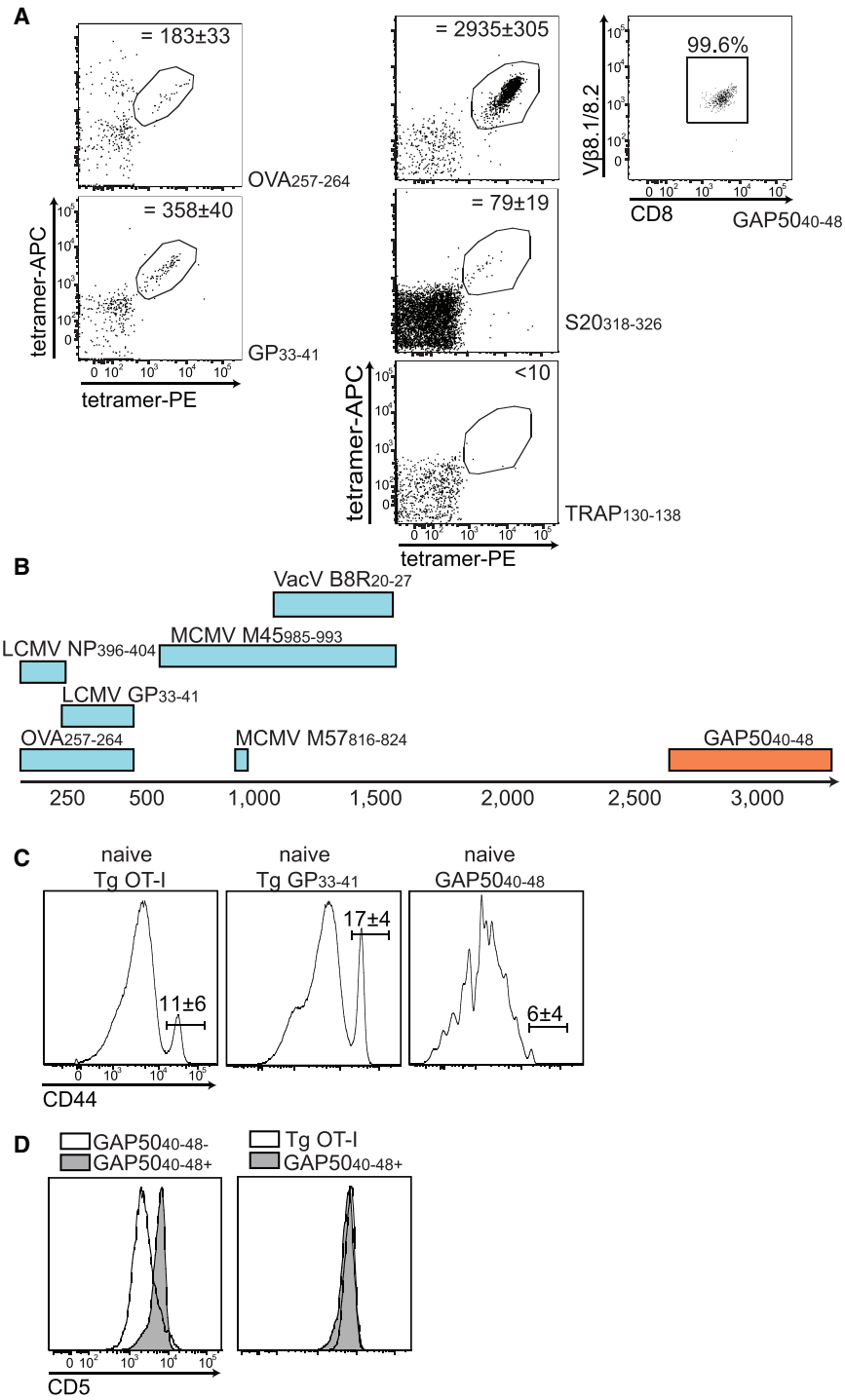


Figure 5. The Naive $GAP50_{40-48}$-Specific $CD8^+$ T Cell Repertoire Is Extremely Large

(A) $CD8^+$ T cells specific for $OVA_{257-264}$, GP_{33-41}, $GAP50_{40-48}$, $S20_{318-326}$, and $TRAP_{130-138}$ were enriched from the spleens and macroscopic lymph nodes of naive C57BL/6 mice and used to calculate the final numbers of $tetramer^+$ cells indicated in each plot. Target cells were identified as $CD8^+CD90.2^+CD11b^-CD11c^-B220^-$ $tetramer(APC)^+$ $tetramer(PE)^+$. Depicted numbers of naive precursors were calculated as mean ± SD from 11 mice for the $GAP50_{40-48}$-specific repertoire. Right panel: Vβ8.1/8.2 expression on naive $GAP50_{40-48}$-specific $CD8^+$ T cells.

(B) Sizes of the naive repertoires specific for various $CD8^+$ T cell epitopes. The number of naive $GAP50_{40-48}$-specific precursors is represented as a range from 2,700–3,250 cells (total n = 11 mice).

(C and D) Phenotypic characterization of naive $GAP50_{40-48}$-specific $CD8^+$ T cells.

(C) Expression of CD44 on naive $GAP50_{40-48}$-specific $CD8^+$ T cells was compared with $CD8^+$ T cells obtained from naive Tg OT-I and P14 mice.

(D) Expression of CD5 on naive $GAP50_{40-48}$-specific $CD8^+$ T cells was compared with total naive, non-$GAP50_{40-48}$-specific $CD8^+$ T cells (left) and with naive Tg OT-I cells (right). Data represent two independent experiments (n = 4 mice/group). Bars depict mean ± SD.

necessary for the generation of detectable $GAP50_{40-48}$-specific $CD8^+$ T cell responses and the development of ECM.

$GAP50_{40-48}$-Specific $CD8^+$ T Cells Are Pathogenic in ECM

A recent publication suggested that $GAP50_{40-48}$-specific $CD8^+$ T cells play a key role in the pathogenesis of ECM in susceptible B6 mice (Howland et al., 2013). Mechanistically, accumulation of $GAP50_{40-48}$-specific $CD8^+$ T cells in the brain could lead to eventual rupture of the blood-brain barrier, which triggers well-recognized neurological symptoms leading to rapid death (7–9 days after infection) (Howland et al., 2013). In contrast to susceptible B6 mice, which all succumbed to ECM by day 7 after infection with *P. berghei* ANKA-parasitized red blood cells (pRBCs), only 20% of CB6F1 mice succumbed to ECM (Figure 6E). We therefore considered the possibility that a strain-specific difference in the size of the naive $GAP50_{40-48}$-specific $CD8^+$ T cell repertoire might underlie these differential outcomes. Tetramer pull-down of naive $GAP50_{40-48}$-specific $CD8^+$ T cells revealed that CB6F1 mice harbored ~3-fold fewer precursors than B6 mice (Figure 6F). Moreover, this smaller naive pool resulted in a smaller $GAP50_{40-48}$-specific $CD8^+$ T cell response in CB6F1 mice compared with B6 mice at day 6 after infection with *P. berghei* ANKA pRBCs (Figure 6G). These results suggested that a $GAP50_{40-48}$-specific $CD8^+$ T cell response threshold might be required for the induction of ECM. To confirm a threshold-dependent pathogenic role for $GAP50_{40-48}$-specific $CD8^+$ T cells in ECM, we generated a large $GAP50_{40-48}$-specific $CD8^+$ T cell response in CB6F1 or B6 mice prior to infection with *P. berghei* ANKA. We observed no meaningful difference in the

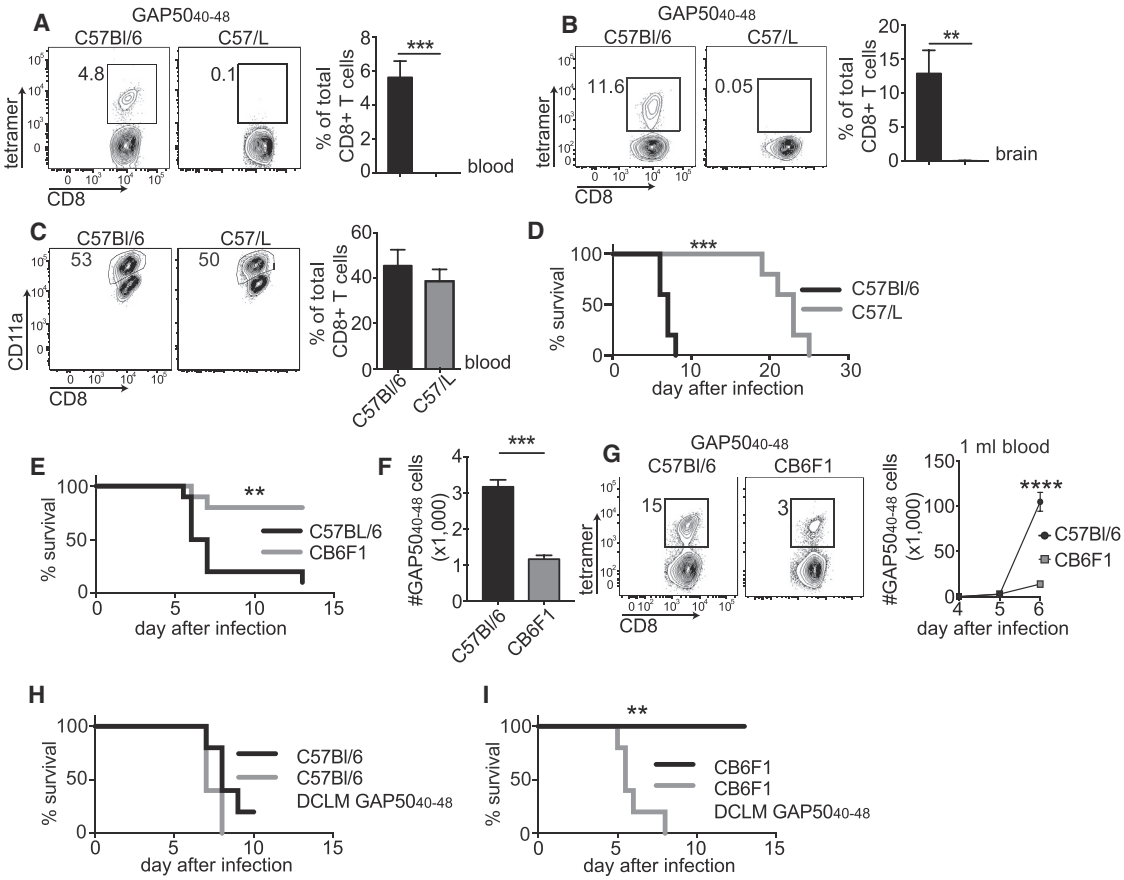


Figure 6. Vβ8.1 Is Required for the Generation of GAP50$_{40-48}$-Specific CD8$^+$ T Cells and the Development of ECM

(A and B) C57BL/6 and C57/L mice were infected with 10^3 *P. berghei* ANKA sporozoites. H-2D^b-GAP50$_{40-48}$ tetramer$^+$ cells were quantified in the blood (A) and brains (B) at day 7 post-infection (representative plots: left; summary graph: right).

(C) Magnitude of the activated CD8$^+$ T cell response at day 7 post-infection, expressed as percent CD11a^{hi}CD8^{lo} of total CD8$^+$ T cells (representative plots: left; summary graph: right). Naive control values were subtracted from individual values. Data represent two independent experiments (n = 3 mice/group). Bars depict mean ± SD. Significance was assessed using an unpaired, two-tailed t test (**p < 0.01, ***p < 0.001).

(D) Survival curves for C57BL/6 mice and C57/L mice after infection with *P. berghei* ANKA. Data represent three independent experiments (n = 5 mice/group). Significance was assessed using the Mantel-Cox log rank test (***p = 0.001).

(E) C57BL/6 and CB6F1 mice were infected with 10^6 *P. berghei*-parasitized red blood cells (pRBCs). Mice were monitored for the development of ECM symptoms and scored for survival over a period of 2 weeks. Data represent three independent experiments (n = 5 mice/group). Significance was assessed using the Mantel-Cox log rank test (**p = 0.0025).

(F) GAP50$_{40-48}$-specific CD8$^+$ T cells were tetramer-enriched from naive C57BL/6 and CB6F1 mice and quantified to estimate repertoire size. Cumulative results are shown from two independent experiments (total n = 4 mice/group). Bars depict mean ± SEM. Significance was assessed using an unpaired, two-tailed t test (***p < 0.001).

(G) C57BL/6 and CB6F1 mice were infected with 10^6 pRBCs. The GAP50$_{40-48}$-specific CD8$^+$ T cell response was followed in the blood of infected mice using H-2D^b-GAP50$_{40-48}$ tetramers. All C57BL/6 mice succumbed to ECM by days 6 and 7, while all CB6F1 mice survived. Data represent two independent experiments (n = 5 mice/group). Bars depict mean ± SD. Significance was assessed using an unpaired, two-tailed t test (****p < 0.0001).

(H and I) C57BL/6 and CB6F1 mice were injected i.v. with 5 × 10^5 GAP50$_{40-48}$ peptide-coated DCs and then infected 7 days later with recombinant attenuated LM-GAP50$_{40-48}$. At a memory time point after LM infection, DC-LM-GAP50$_{40-48}$-immunized or non-immunized mice were infected with 10^6 pRBCs. Survival curves are shown for C57BL/6 mice (H) and CB6F1 mice (I). Data represent two independent experiments (n = 5 mice/group). Significance was assessed using the Mantel-Cox log rank test (p = 0.0993 in H; **p = 0.0018 in I).

onset of ECM symptoms or the mortality rate between previously immunized and non-immunized B6 mice, demonstrating that sufficient numbers of GAP50$_{40-48}$-specific effector CD8$^+$ T cells can be generated from the large pool of naive precursors in this susceptible strain (Figure 6H). In contrast, prior immunization against GAP50$_{40-48}$ was required to render all CB6F1 mice susceptible to ECM after *P. berghei* ANKA infection (Figure 6I). Collectively, these findings demonstrated that a numerical threshold delimited the pathogenicity of GAP50$_{40-48}$-specific CD8$^+$ T cells in the etiology of ECM.

GAP50$_{40-48}$-Specific CD8$^+$ T Cells Protect against LM-GAP50$_{40-48}$

Although pathogenic in the context of ECM, we hypothesized that the rapid generation of a substantial GAP50$_{40-48}$-specific response from the large number of naive precursors may protect

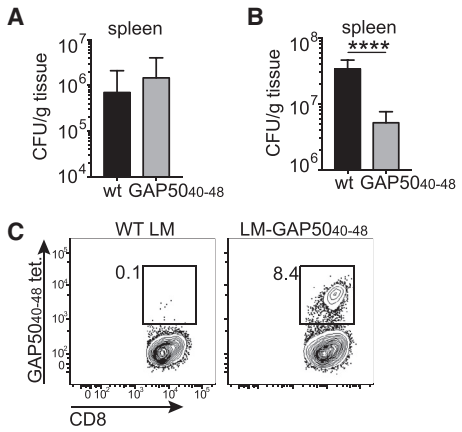


Figure 7. Large Numbers of GAP50$_{40-48}$-Specific CD8$^+$ T Cells Control Primary *L. monocytogenes* Infection

(A) BALB/c mice were infected i.v. with 5 × 10^3 CFU of WT LM or recombinant LM-GAP50$_{40-48}$. Bacterial burden was measured in the spleen on day 5. Data represent two independent experiments (n = 4 mice/group). Bars depict mean ± SD.

(B) C57BL/6 mice were infected i.v. with 10^4 CFU of WT LM or recombinant LM-GAP50$_{40-48}$. Bacterial burden was measured in the spleen on day 5. Cumulative results are shown from three independent experiments (total n = 14–15 mice/group). Bars depict mean ± SEM. Significance was assessed using an unpaired, two-tailed t test (****p < 0.0001).

(C) Representative flow cytometry plots showing H-2D^b-GAP50$_{40-48}$ tetramer staining of splenocytes isolated from infected C57BL/6 mice at day 5 post-infection.

against infection controlled primarily by CD8$^+$ T cells. To address this possibility, we determined whether B6 mice could better control primary infection with virulent LM-GAP50$_{40-48}$ compared with virulent WT LM.

To ensure comparability, we first established that the two different strains of LM exhibited similar virulence in the absence of a GAP50$_{40-48}$-specific CD8$^+$ T cell response. For this purpose, we infected BALB/c mice (H-2^d), which were non-responsive to the H-2D^b-restricted GAP50$_{40-48}$ epitope (Figure 7A). Bacterial burden assessed in the spleens of infected mice 5 days after infection with 5 × 10^3 colony-forming units (CFU) of each strain was similar between the two groups, suggesting equivalent virulence *in vivo* (Figure 7B). In contrast, we measured ~10-fold fewer bacteria in the spleens of LM-GAP50$_{40-48}$-infected B6 mice compared with WT LM-infected B6 mice (Figures 7C and 7D). This superior control of bacterial infection in LM-GAP50$_{40-48}$-infected B6 mice was accompanied by a large GAP50$_{40-48}$-specific response, comprising >8% of the total CD8$^+$ T cell pool in the spleen (Figure 7E). The expansion of GAP50$_{40-48}$-specific effector cells from a large pool of naive precursors therefore conferred protection in the context of an infection controlled by CD8$^+$ T cells.

DISCUSSION

Conceptual frameworks developed in the 1970s, prior to a detailed understanding of the molecular interactions that govern T cell antigen recognition, established the notion of genetically controlled immune responsiveness. Overwhelming experimental data have since accumulated to validate one original proposition that MHC-linked *Ir* genes dictate the presentation of specific antigens in immunogenic form (Benacerraf and Germain, 1978). There is also some evidence to support the idea that germline-encoded components of the TCR interact preferentially with defined MHC allotypes (Garcia et al., 2009; Scott-Browne et al., 2009), building on the earlier theoretical work of Niels Jerne (Jerne, 1971). However, it has not been shown previously that heritable elements of an antigen receptor can license immune reactivity, a scenario postulated almost four decades ago as an alternative model to explain the *Ir* gene phenomenon (Benacerraf and Germain, 1978). In this study, we demonstrated that CD8$^+$ T cell responses specific for the murine malaria epitope GAP50$_{40-48}$ were entirely dependent on amino acid residues unique to the TCR Vβ8.1 segment, thereby providing insights into the genetic mechanisms that control adaptive immunity.

As a consequence of structural constraints focused primarily on the CDR1β loop, both naive and antigen-expanded H-2D^b-restricted GAP50$_{40-48}$-specific CD8$^+$ T cells almost invariably express Vβ8.1$^+$ TCRs. Similarly extreme biases are typically associated with innate-like responses to non-peptidic antigens presented by non-classical MHC molecules, although immune reactivity among these unconventional T cell subsets is generally endowed by a conserved TCRα chain (Van Rhijn et al., 2015). Narrow TCR repertoires specific for peptide epitopes have previously been linked with low numbers of naive precursors (Moon et al., 2007) and clonal selection during the genesis of memory populations, especially in the presence of constant or repetitive antigen stimulation (Busch and Pamer, 1999; Price et al., 2005; Savage et al., 1999). Paradoxically, the pre-immune GAP50$_{40-48}$-specific repertoire was the largest yet described in mice, numbering ~3,000 cells in a naive B6 host. Moreover, this substantial precursor pool did not seem to arise as a consequence of expansion into the virtual memory subset (Haluszczak et al., 2009), as >90% of naive GAP50$_{40-48}$-specific CD8$^+$ T cells expressed low levels of CD44 (Akue et al., 2012). Although the self-derived peptides associated with positive selection of these antigen-specific precursors remain unknown, the uniformly high expression of CD5 by naive GAP50$_{40-48}$-specific CD8$^+$ T cells suggested the existence of strong TCR-mediated interactions in the thymus (Mandl et al., 2013). It is also notable that *Vβ8* gene segments are highly represented in laboratory mouse strains (Wilson et al., 2001). High numbers of naive GAP50$_{40-48}$-specific CD8$^+$ T cells therefore most likely emerged as a consequence of both permissive thymic selection and the frequent generation of Vβ8.1$^+$ transcripts during the process of somatic recombination. In addition, the presence of a highly conserved Vβ-Dβ junction lacking N additions suggested that convergent gene rearrangements, which occur more commonly on a probabilistic basis (Quigley et al., 2010), effectively licensed the extreme penetrance of the *Vβ8.1* gene-encoded phenotype.

So what are the immunological consequences of such a large pool of antigen-specific precursors in the naive CD8$^+$ T cell compartment? Earlier work indicated that CD8$^+$ T cells expressing Vβ8.1/Vβ8.2$^+$ TCRs are highly prevalent during malaria infection and contribute to the pathogenesis of ECM (Mariotti-Ferrandiz et al., 2016). In addition, it had been shown previously that high-dose tolerization with the GAP50$_{40-48}$ peptide abrogates disease susceptibility in B6 mice (Howland et al., 2013). These observations can be explained by our finding that an

extremely large pool of Vβ8.1$^+$ antigen-specific naive precursors (~3,000 cells) underpinned the immunodominant CD8$^+$ T cell response to GAP50$_{40-48}$ and the susceptibility of B6 mice to ECM. In contrast, CB6F1 mice harbored a diminished pool of antigen-specific naive precursors (~1,000 cells), leading to smaller GAP50$_{40-48}$-specific CD8$^+$ T cell responses and relative resistance to ECM. This causal association was confirmed by the observation that increasing the number of GAP50$_{40-48}$-specific CD8$^+$ T cells in CB6F1 mice prior to infection rendered them susceptible to ECM. It remains unclear whether similar germline biases can dictate immune responses to malarial antigens in humans, but our data nonetheless provided formal proof of concept that genetic associations with disease outcome can be extended to loci encoding components of the TCR. Moreover, the peak incidence of severe anemia during malaria infection occurs in children under 2 years of age, while cerebral malaria occurs most commonly in children aged 3–5 years who have prior exposure to infection (Struik and Riley, 2004). These epidemiological patterns are consistent with the possibility that larger pre-infection repertoires of malarial antigen-specific T cells predispose to the development of cerebral malaria.

GAP50$_{40-48}$-specific memory CD8$^+$ T cells are not protective during the liver stage of murine malaria (Doll et al., 2016; Horne-Debets et al., 2016; van der Heyde et al., 1993; Vinetz et al., 1990), and the capacity of CD8$^+$ T cells to protect during blood-stage malaria remains controversial (Horne-Debets et al., 2016; Imai et al., 2010; van der Heyde et al., 1993; Vinetz et al., 1990). Thus, the pathogenic effects of GAP50$_{40-48}$-specific CD8$^+$ T cells in ECM most likely resulted from a large population of precursors, which expanded rapidly after infection to generate substantial numbers of effector cells, leading to sustained immune activation without elimination of the pathogen. In contrast, rapid expansion from the same population of precursors enhanced immune control of primary infection with *L. monocytogenes* expressing the GAP50$_{40-48}$ epitope. An unusually large naive CD8$^+$ T cell pool can therefore be pathogenic or protective, depending on the nature of the infectious challenge.

In conclusion, we have provided direct evidence that a germline-encoded TCR segment can determine immune responsiveness to an exogenous peptide antigen, thereby extending the concept of *Ir* genes beyond the MHC. The unique features associated with this phenomenon may allow novel interventions to improve vaccine efficacy and limit immune pathology in humans, pending further studies to identify similar genetic associations between disease outcome and heritable components of the TCR.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and six tables and can be found with this article online at https://doi.org/10.1016/j.immuni.2017.10.013.

AUTHOR CONTRIBUTIONS

N.V.B.B., S.G., K.L., T.M.J., L.P., S.L.U., C.F., D.A.P., J.R., and J.T.H. designed the study. N.V.B.B., S.G., K.L., T.M.J., L.P., S.L.U., K.L.M., and C.F. conducted experiments. N.V.B.B., S.G., K.L., T.M.J., L.P., S.L.U., K.L.M., C.F., D.A.P., J.R., and J.T.H. analyzed data and interpreted results. N.V.B.B. and S.G. drafted the manuscript. N.V.B.B., S.G., K.L., T.M.J., L.P., S.L.U., C.F., D.A.P., J.R., and J.T.H. edited the manuscript.

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