**Mutagenetix Phenotypic Mutation 'Carlsbad'

<table>
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<th><strong>Allele</strong></th>
<th><strong>Carlsbad</strong></th>
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<tr>
<td><strong>Base Change (assembly)</strong></td>
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<tr>
<td><strong>Gene</strong></td>
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<tr>
<td><strong>Gene Name</strong></td>
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<td>Beutler Lab</td>
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<td><strong>Phenotypic Category</strong></td>
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<tr>
<td><strong>Authors</strong></td>
<td>Ming Zeng, Xue Zhong, and Bruce Beutler</td>
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<td><strong>Illustrators</strong></td>
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Figure 1. Carlsbad mice exhibit increased CD4 to CD8 T cell ratios. Flow cytometric analysis of peripheral blood was utilized to determine T cell frequency. Normalized data are shown. Abbreviations: WT, wild-type; REF, homozygous reference mice; HET, heterozygous variant mice; VAR, homozygous variant mice. Mean (±) and standard deviation (±) are indicated.
Figure 2. *Carlsbad* mice exhibit reduced frequencies of CD8$^+$ T cells in CD3$^+$ T cells. Flow cytometric analysis of peripheral blood was utilized to determine T cell frequency. Normalized data are shown. Abbreviations: WT, wild-type; REF, homozygous reference mice; HET, heterozygous variant mice; VAR, homozygous variant mice. Mean (±) and standard deviation (±) are indicated.
Figure 3. *Carlsbad* mice exhibit increased frequencies of CD4$^+$ T cells in CD3$^+$ T cells. Flow cytometric analysis of peripheral blood was utilized to determine T cell frequency. Normalized data are shown. Abbreviations: WT, wild-type; REF, homozygous reference mice; HET, heterozygous variant mice; VAR, homozygous variant mice. Mean (\textit{l}) and standard deviation (\textit{?}) are indicated.
Figure 4. Homozygous *riogrande* mice exhibit increased T-dependent IgG responses to recombinant Semliki Forest virus (rSFV)-encoded β-galactosidase (rSFV-β-gal). IgG levels were determined by ELISA. Normalized data are shown. Abbreviations: WT, wild-type; REF, homozygous reference mice; HET, heterozygous variant mice; VAR, homozygous variant mice. Mean (±) and standard deviation (±) are indicated.

The *Carlsbad* phenotype was identified among G3 mice of the pedigree R2860, some of which showed an increase in the CD4⁺ to CD8⁺ T cell ratio (Figure 1) caused by a diminished frequency of CD8⁺ T cells in CD3⁺ T cells (Figure 2) coupled with an increase in the frequency of CD4⁺ T cells in CD3⁺ T cells (Figure 3), all in the peripheral blood. Also, the T-dependent antibody response to recombinant Semliki Forest virus (rSFV)-encoded β-galactosidase (rSFV-β-gal) was increased in the *Carlsbad* mice (Figure 4).

**Nature of Mutation**

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Whole exome HiSeq sequencing of the G1 grandsire identified 45 mutations. All of the above anomalies were linked by continuous variable mapping to a mutation in \(Cd8b1\): an A to G transition at base pair 71,334,101 (v38) on chromosome 6, or base pair 11,290 in the GenBank genomic region NC_000072 encoding \(Cd8b1\). The strongest association was found with an additive model of linkage to the normalized CD4:CD8 ratio, wherein four variant homozygotes and 15 heterozygotes departed phenotypically from five homozygous reference mice with a \(P\) value of \(1.124 \times 10^{-5}\) (Figure 5).

The mutation corresponds to residue 649 in the mRNA sequence NM_009858 within exon 5 of 6 total exons.

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The mutated nucleotide is indicated in red. The mutation results in an arginine (R) to glycine (G) substitution at position 202 (R202G) in the CD8? protein, and is strongly predicted by PolyPhen-2 to be damaging (score = 0.996) (1).

**Protein Prediction**

[Flash Content]

**Figure 6. Domain structure of CD8?**. The extracellular region of CD8? has an IgV domain. The Carlsbad mutation results in an arginine (R) to glycine (G) substitution at position 202 (R202G).
Figure 7. Crystal structure of the CD8?? Ig-like domains in complex with the extracellular region of H-2D<sup>d</sup> (yellow), ?2m (gray), and a bound 10 amino acid peptide (not shown). CD8? (orange) is proximal to the T cell membrane; CD8? (green) is distal. Model is based on PDB 3DMM, Wang et al. J. Immunol. 183, 2554-2564 (2009). Click on the 3D structure to view it rotate.

CD8? (also known as Lyt-3) is a 213-amino acid single pass type I transmembrane glycoprotein of the immunoglobulin (Ig) superfamily (Figure 6) (2). CD8? heterodimerizes with CD8? (also known as Lyt-2; see the record for alfalfa) to form CD8??, which functions as a coreceptor for the T cell receptor (TCR) [reviewed in (3)]. CD8? requires an association with CD8? to translocate to the cell surface; unassociated CD8? is retained in the endoplasmic reticulum and degraded (4-6). Human CD8?, but not mouse CD8?, can form a CD8?? homodimer (7). The lack of CD8?? homodimers in the mouse is attributed to the Ig domain found in mouse, but not human, CD8?. The CD8?? could not bind MHC class I.
intracellular domain of CD8? promotes the association with Lck and LAT, two proteins required for TCR signal transduction, as well as (9). The intracellular and extracellular domains of CD8? can both independently promote CD8+ T cell development, but both domains together is most efficient (8 ;9).

Connecting the Ig-like domain to the transmembrane domain is the stalk region of CD8? . The stalk region of CD8? is essential for selection of CD8+ class I MHC-restricted T cells as well as the response of peripheral T cells; the stalk region is not required for expression of CD8? on the cell surface (10). The stalk region enhances the coreceptor function of CD8, making the CD8?? heterodimer a better coreceptor than CD8?? (11). The stalk region is heavily O-glycosylated. O-glycosylation of CD8? is developmentally regulated such that immature double positive thymocytes display lower levels than mature thymocytes (12-15). This relative difference influences thymocyte selection in that decreased levels of CD8? O-glycosylation in immature thymocytes correlate with increased affinity for pMHC I; the enhanced CD8-pMHC I binding promotes elimination of autoreactive T cells in the thymus (12;15). In contrast, higher levels of O-glycosylation in mature T cells reduce CD8 affinity for pMHC I, thereby requiring stronger TCR-pMHC I binding for T cell activation. The structural mechanism by which O-glycosylation alters CD8 affinity for pMHC I is unknown (16); in published 3D crystal structures the stalk region has been disordered. O-glycosylation has been reported to increase the rigidity of polypeptides, as observed in mucins, and may limit the mobility of the Ig-like domains of CD8?? and CD8?? [reviewed in (3)]. O-glycosylation of CD8? does not appear to be developmentally regulated (14).

CD8? and CD8? share approximately 20% homology within the Ig domains. The crystal structure of the Ig-like domains of mouse CD8?? [Figure 7; PDB:2ATP; (17)] resembles the variable domain fragment (Fv) of an antibody, with each chain adopting a V-set type Ig fold (17;18); this structure is similar to that of the Ig-like domains in the CD8?? dimer (19;20). The two chains of both dimers have similar conformations, including a disulfide bond between the B and F ?-strands, and a tryptophan on the C strand.

Palmitoylation of CD8? promotes the segregation of CD8 into lipid rafts, a critical step in facilitating the function of CD8 as a coreceptor with the TCR (21). Furthermore, CD8? directly associates with CD3? to facilitate the trafficking of the TCR into lipid rafts (22).
recognition of antigen-MHC complexes on the surface of antigen presenting cells. Expression of CD4 versus CD8 by a given T cell restricts TCR recognition of peptides to those bound to either MHCII or MHCI, respectively. CD4 and CD8 contact a conserved membrane-proximal region of their MHC target to enforce MHC class restriction. The molecular details of the interaction between CD8 with the peptide-MHCI complex (pMHCI) have been investigated by X-ray crystallography (18;20;23). The 3D structures revealed that the extracellular Ig-like domain of both the CD8 and the CD8 dimers contact the \(\alpha\)3 domain of the MHC I heavy chain, whereas CD8 additionally contacts the \(\alpha\)2 domain and \(\beta\)-2-microglobulin. Three complementarity-determining regions (CDRs), consisting of the BC loop (CDR1), the \(\alpha\)’ \(\alpha\)’’ loop (CDR2), and the FG loop (CDR3), are involved in binding to the \(\alpha\)3 domain of pMHCI. Residues in the CDR1 (Lys23), CDR2 (Lys55), and CDR3 (Val99, Ser101, and Lys103) loops of CD8 decreased MHC I binding and T cell activation (24). The CD8 subunit of CD8 is positioned next to the T cell membrane, while the CD8 subunit is distal from the T cell and close to the C-terminus of the MHCI \(\alpha\)3 domain. Differences exist between the CD8/pMHCI interface and the CD8/pMHCI interface despite the fact that overall conformations are quite similar. Importantly, nearly all MHCI residues making key contacts with CD8 and CD8 are non-polymorphic; thus CD8 and CD8 bind to different MHCI alleles with only subtly different affinities (25;26). However, reports suggest that CD8 is a more effective coreceptor than CD8 in enhancing the range and sensitivity to peptide antigens and alloantigens recognized by TCRs (27-30). The stalk or intracellular region of CD8 may facilitate a configuration that promotes this augmented function (30-33).

Human CD8B has several alternatively spliced variants that encode different cytoplasmic tails (M-1, M-2, M-3, M-4); mouse Cd8b1 does not encode multiple transcripts (34). In total CD8+ T cells, the expression level of the M-1 variant was the highest and the M-3 variant was the lowest. The M-4 isoform was the highest expressed variant in effector memory CD8+ T cells. After CD8+ T cell stimulation, the expression level of the M-2 variant increased 10 to 20 fold relative to resting cells in contrast to the other isoforms.

The mutation results in an arginine (R) to glycine (G) substitution at position 202 (R202G); residue 202 is within the cytoplasmic domain.
CD8 is expressed primarily on TCR+ thymocytes and peripheral cytotoxic T cells (35,36); the cell surface expression of CD8 requires association with CD8 (37,38).

**Background**

Figure 8. TCR signaling pathway. Together with CD4 or CD8 (depicted here), TCRs are responsible for the recognition of major...
histocompatibility complex (MHC) class II and I, respectively, as well as other antigens found on the surface of antigen presenting cells (APCs). Binding of these ligands to the TCR, and CD4/8 engagement of MHC, initiates signaling and T cell activation. The TCR is composed of two separate peptide chains (TCRα/β), and is complexed with a CD3 heterodimer (CD3γ/δ or CD3ε/ζ) and a ζ homodimer. One of the first steps in TCR signaling is the recruitment of the tyrosine kinases Lck and Fyn to the receptor complex. Lck and Fyn are regulated by the phosphorylation of two key tyrosine residues, an activating tyrosine located in the activation loop, and an inhibitory tyrosine located in the C-terminal tail. CD45 dephosphorylates the C-terminal inhibitory tyrosine, thereby promoting the activation of Lck and Fyn. Once activated, they phosphorylate ITAMS present on the CD3 and ζ chains. Phosphorylation of the ITAM motifs results in recruitment of ZAP-70 and Syk, which trans- and auto-phosphorylate to form binding sites for SH2 domain- and protein tyrosine binding domain-containing proteins. The Syk family kinases phosphorylate LAT and SLP-76. LAT binds to the adaptor proteins growth factor receptor-bound 2(Grb2), Src homologous and collagen (Shc) and GRB2-related adaptor downstream of Shc (Gads), as well as phosphatidylinositol 3-kinase (PI3K) and PLC-γ 1. SLP-76 is then recruited to the complex via Gads and binds the guanine nucleotide exchange factor Vav1, Nck (non-catalytic region of tyrosine kinase adaptor protein), IL-2-induced tyrosine kinase (Itk), PLC-γ 1, adhesion and degranulation-promoting adaptor protein (ADAP), and hematopoietic progenitor kinase 1 (HPK1). This proximal signaling complex is required for PLC-γ 1-dependent pathways including calcium (Ca^{2+}) mobilization and diacylglycerol (DAG)-induced responses, cytoskeleton rearrangements, and integrin activation pathways. Activated PLC-γ 1 hydrolyzes the membrane lipid phosphatidylinositol-3,4-diphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and DAG resulting in Ca^{2+}-dependent signal transduction including activation of nuclear factor of activated T cells (NF-AT), and activation of protein kinase C? and Ras, respectively. PKC? regulates nuclear factor-κB (NF-κB) activation via the trimolecular complex composed of Bcl10,
mucosa-associated lymphoid tissue translocation gene 1 (MALT1), and caspase recruitment domain family, member 11 (CARMA1). Ras initiates a mitogen-associated protein kinase (MAPK) phosphorylation cascade culminating in the activation of various transcription factors. The image is interactive; click to view mutations associated with the TCR signaling pathway.

T cells become activated when the TCR engages a peptide antigen in complex with an MHC molecule on the surface of a target or antigen-presenting cell. However, TCR-pMHC interaction is not by itself sufficient to induce an effective T cell response. Rather, engagement of pMHC by the TCR and either the CD4 or CD8 coreceptor is necessary for optimal T cell stimulation \((39;40)\). For cytotoxic T cells, the interaction of CD8 with MCHI enhances the antigen sensitivity and response of the T cells to pMHC ligands \((41)\). CD8 contacts invariant sites on the membrane-proximal region of the MHCI molecule in the context of the composite pMHCI complex. These sites are distinct and physically distant from the sites bound by the TCR, permitting both coreceptor and TCR to bind the same pMHCI complex \((20)\). Absent CD8-pMHCI interaction, MHC class I-restricted immune responses including cytokine production and cytotoxic effector function are impaired \((39;40)\). However, high-affinity pMHC/TCR interactions can in some cases compensate for a lack of CD8 engagement in terms of Lck activation, T cell proliferation, and cytotoxic T lymphocyte (CTL) activity \((42)\). Discussed here is the TCR coreceptor function of the CD8**h** heterodimer.

For an in-depth discussion on the function of the CD8**h** heterodimer, please see the record for alfalfa.

Rather than physically promoting initial TCR-pMHCI binding, the major function of CD8**h** is to recruit the kinase Lck to the TCR-MHC interaction site where it can phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of associated CD3 proteins (see tumormouse, a mutation of Cd3e, and allia, a mutation of Cd247 \((43;44)\)). Their phosphorylation recruits and activates other proteins including ZAP-70 (?-chain-associated protein of 70 kDa; see murdock), SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), and LAT (linker for activation of T cells) necessary for signaling leading to T cell activation, proliferation, and differentiation (Figure 8; also see iconoclast for a description of TCR signaling). The CD8-pMHCI interaction synergistically stabilizes TCR-pMHCI interactions \((45)\). However, it has also been reported that CD8**h** can play a critical role in promoting TCR-pMHCI binding, particularly for multimeric low-affinity pMHCI ligands \((46;47)\).
Putative Mechanism

Figure 9. Thymocyte differentiation. CD4+ and CD8+ T cells expressing ?? TCRs begin their development as double negative CD4−CD8− precursor thymocytes in the capsule of the thymus. After TCR rearrangement, progression to the double positive CD4+CD8+ stage, and TCR rearrangement (in the
cortex of the thymus), the yet immature TCR??+CD4+CD8+ thymocytes are then subject to positive or negative selection to generate mature CD4+ helper T cells and CD8+ cytotoxic T cells in the medulla of the thymus.

TCR signaling is necessary for both adaptive immune responses and for thymocyte differentiation (Figure 9). CD8?? is required for both positive and negative selection of double positive cells in the thymus (48-50). Cd8b1-deficient mice exhibited a five-fold reduction in the numbers of CD8+ T cells in the thymus and the periphery; the CD8+ T cells that remained had normal cytotoxic T lymphocyte activity (48-51). CD8? expression on thymocytes and peripheral T cells was reduced compared to that in wild-type mice (51). The population size and CD8? expression was normal in intraepithelial lymphocytes from the Cd8b1-deficient mice due to the predominant expression of CD8?? homodimers on these cells (51). Peripheral T cells from the Cd8b1-deficient mice did not exhibit defective cytotoxic activity against lymphocytic choriomeningitis virus or vesicular stomatitis virus (51-53). The phenotype of the Carlsbad mice indicates loss of CD8? function.

References
Mouse CD8 Beta, can be Expressed in the Absence of CD8 Alpha as a Beta Beta Homodimer. *J Immunol.* 164, 833-838.


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