Significance of the third hypervariable region of the antibody heavy (H) chain for antigen-specificity and expression of idiotypes during the thymus-dependent immune response

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To my parents

Summary

Antigen-induced lymphocytic (adaptive) immune responses rely on a complex contact- and cytokine-mediated interplay of different cells. Hapten-carrier complexes as antigens have been employed to elaborate many characteristic features of this process, *e.g.* action of antigen-presenting cells, specificity of T (TCR) and B (BCR) cell antigen receptors, collaboration of T and B lymphocytes, function of T cell subpopulations, generation and selection of the primary repertoire, immune maturation through somatic hypermutation, class switch recombination and isotype regulation, to name the most important ones. One of the best explored antibody responses is that of BALB/c mice to the hapten 2-phenyl-oxazolone (phOx) after coupling to the thymus-dependent (TD) carrier chicken serum albumin (CSA). The latter response is characterized by the dominant Ox1 idiotype (Id_{Ox1}) which is encoded by the canonical VH171/ Vκ072 gene combination and contains the typical amino acid sequence aspartic acid-arginine-glycine (DRG) in the middle of the third complementarity-determining region of the heavy chain (HCDR3). The affinity of Id_{Ox1} antibodies is 10-100-times higher than that of non-Id_{Ox1} antibodies and immune maturation is primarily based on the accumulation of somatic mutations in Id_{Ox1} antibodies. During memory responses, however, there is a repertoire shift to non-Id_{Ox1} antibodies encoded by alternative VH and/or VL genes. The propensity of non-Id_{Ox1} antibodies for somatic mutations is known.

According to the selection theory, many of these characteristics depend on the antigen-specificity of the antigen-binding site (paratope) of the BCR. In principle, two different processes participate in the generation of the BCR repertoire during the antigen-independent differentiation in the bone marrow, *i.e.* on the one hand, the recombination of the genomic variable (V), diversity-enhancing (D) and joining (J) genes and, on the other, a non-genetic enhancement of this genetic repertoire during V(D)J recombination by the action of three different enzyme systems. Exonucleases cut off terminal nucleotides, repair enzymes generate palindromic sequences (P nucleotides) and terminal deoxynucleotidyl-transferase (TdT) adds non-template nucleotides (N nucleotides) at random. In this way, an enormous non-genetic diversity is created at HCDR3 being large enough to allow efficient immune responses even when only a single VH and VL gene is available.

The experiments on the humoral anti-phOx response of DH mutant BALB/c mice, described in the present communication, were performed to elucidate how HCDR3-related non-genetic

and V gene-encoded genetic variability contribute to the thymus-independent type 2 (TI-2) and the TD response, primary TD repertoire, Id_{Ox1} dominance, class switch recombination, somatic hypermutation and immune maturation during memory TD responses. Two mutant BALB/c strains were available which harbored only one single DH gene in their genome instead of 13 DH genes in BALB/c wildtype (WT) mice. The DH gene segment of ΔD-iD mice codes for charged amino acids in HCDR3 while that of ΔD-DμFS mice codes for hydrophobic amino acids. The phOx-Ficoll-induced TI-2 and the phOx-CSA-induced TD immune response of both strains were compared with that of BALB/c WT mice and analyzed at the level of single antibody-forming plasma cells and serum anti-phOx antibody formation. The quality of the TD response was analyzed with the aid of hybridoma monoclonal antibodies generated after early and late primary, secondary and tertiary immunization.

The following results were obtained:

A Quantitative aspects of the humoral anti-phOx immune response in DH mutant mice

1.) The analysis with fluorescence-activated cell sorting (FACS) revealed that the numbers of total nucleated cells, lymphocytes as well as T and B cells in the bone marrow of both mutant strains were comparable to BALB/c WT mice.
2.) However, in the spleens of ΔD-iD and ΔD-DμFS mice, numbers of total mononuclear cells as well as both lymphocyte subpopulations were reduced to values of lower than 50 % of those found in WT mice.
3.) It is a striking result that the genetic alteration in both strains of mice also affects T cells although the genetic alteration is restricted to Ig genes and can thus not directly affect the germ line repertoire of TCR. In relation to and in accord with published data, this allows concluding that the T cell repertoire is indirectly controlled by B lymphocytes.
4.) The total immunoglobulin concentrations were reduced for IgM in ΔD-iD but not ΔD-DμFS mice while the IgG immunoglobulin class was clearly reduced in both strains.
5.) In contrast to these results, the background numbers of IgM but not IgG anti-phOx antibody-secreting cells were significantly *en*hanced in both DH mutant strains.
6.) The phOx-Ficoll-induced TI-2 response was comparable for the IgM and delayed for the IgG antibody response.
7.) The IgM production during the primary phOx-CSA-induced TD response was delayed and remained at lower maximal levels before falling back to similar low levels as in WT mice. IgG titers were severely suppressed during the entire primary response and remained at this lower level after secondary immunization.

B VH/VL sequence analysis of monoclonal hybridoma antibodies from the different phases of the humoral anti-phOx immune response in DH mutant mice

Genetic analysis of the phOx-reactive repertoire of ΔD-DμFS mice

In the early primary response, the Id_{Ox1} gene combination is rarely used and not at all dominant. Antibodies with 5-100-times higher affinities than the normally dominant Id_{Ox1} occur. Usage of reading frame 1 (RF1), coding for hydrophobic amino acids, is counter selected during the generation of the primary repertoire of ΔD-DμFS mice. The length of the middle portion HCDR3 varied from 1-12 amino acids. This may suggest that it is not of prime importance for the phOx-specificity. The more D gene nucleotides were eliminated by exonucleases during VDJ recombination in the course of generation of the pre-immune repertoire, the shorter the HCDR3 length will be.

In contrast to wildtype mice, the late primary anti-phOx antibodies contain an astonishingly high number of 56% non-switched IgM antibodies. Since a dominant clonotype did also **not** develop at this time point, this process obviously depends on the available DH gene repertoire and can not or only very rarely be generated from the non-natural DH gene of ΔD-DμFS mice. An immune maturation is indicated by enhanced mutation rates in IgG antibodies, but the mutations do not accumulate in a particular clone. These antibodies preferably used germ line-related HCDR3 nucleotides sequences, but `tried to avoid´ RF1.

Antibodies obtained after secondary immunization showed again that class switch recombination is impaired in this mouse strain, that the phOx-specific B cell repertoire develops at random, that immune maturation occurs in individual, but not all ΔD-DμFS mice and that a few individual mice may even generate Id_{Ox1} antibodies. The HCDR3 sequence (CAR-DGG-AYW) of one these Id_{Ox1} antibodies probably derived from the canonical Id_{Ox1} sequence CAR-DRG-AYW. Hence, although ΔD-DμFS mice can generate the VH171/Vκ072 Id_{Ox1} gene combination, the Id_{Ox1}-specific DRG motive in the middle of HCDR3 can either not or only extremely rarely be generated.

An impaired class switch recombination is even observed after tertiary immunization since about one third of the established antibodies were of the IgM class. Id_{Ox1} antibodies did also not dominate the tertiary TD response. Instead, about one third of IgG antibodies were encoded by the VH/VL gene combination VH158/Vκ115 which has already been observed as a major clone in a memory response of BALB/c WT mice. During all phases (1° to 3°) of the TD anti-phOx response, ΔD-DμFS mice `try to avoid´ coding of HCDR3 in RF1 causing hydrophobic amino acids. Instead, RF2 usage and generation of new nucleotide sequences during recombination favor HCDR3 loops which are rich in glycine and tyrosine.

Genetic analysis of the phOx-reactive repertoire of ΔD- iD mice with a charged HCDR3

In the early primary response of ΔD-iD mice, the restriction to a single DH gene segment coding for charged amino acids changed the pattern of VH/VL combinations and led to the appearance of some hitherto not observed variable genes. Neither Id_{Ox1} nor other clonotypes dominated. In some IgM and IgG antibodies affinities were enhanced by factors of 5-10. The bias against RF1 usage is not as pronounced as in ΔD-DμFS mice. RF2 is not really dominant and in most of the antibodies the reading frame could not be assigned. These antibodies have shorter D-region lengths. B cells seem to escape the usage of charged HCDR3-loops by shifting the RF and an increased loss of nucleotides at the termini of DH gene, so that 53 % of the HCDR3 intervals contained no germ line-encoded DH nucleotides. Hydrophilic HCDR3 loops are strongly favored. Sequence comparisons showed that the Id_{Ox1} specificity relies not only on the DRG motive in the HCDR3 loop, but is also influenced in its 3-dimensional structure by other parts of the molecule.

The genetic repertoire of the late primary response of ΔD-iD mice is quite heterogeneous and there is again no dominant VH/VL gene combination. Id_{Ox1} antibodies were not observed. As in ΔD-DμFS mice, a high amount of IgM-secreting cells at this time point indicates impaired class switch recombination in ΔD-iD mice. These late IgM-secretors showed almost no somatic mutations. Hence, the restriction in ΔD-iD mice to a single DH gene coding for charged amino acids in HCDR3 drastically inhibits the early immune maturation. The bias against RF1 usage was not as pronounced as in ΔD-DμFS mice. RF2 and RF3 are least frequently used and antibodies with germ line non-related HCDR3 sequences are rather common. Non-assignable reading frames code for shorter D-region lengths. Late primary anti-phOx antibodies of ΔD-iD mice use mainly hydrophilic amino acids in HCDR3.

In the secondary response, it is again astonishing that an IgM antibody could be isolated already 3 days after secondary immunization with the TD antigen phOx-CSA. Enhanced mutations and an increased affinity of the IgG antibody indicated that secondary antibodies in ΔD-iD mice are derived from an immune maturated repertoire.

In the tertiary response of ΔD-iD mice, a high percentage of naïve, non-mutated and non-maturated IgM antibodies were obtained which thus resemble IgM antibodies from the primary response. In contrast to anti-phOx antibodies from earlier stages of TD immune responses, tertiary antibodies contained again VH171/Vκ072-encoded IgM and IgG Id_{Ox1} antibodies. The IgG Id_{Ox1} antibody contained somatic mutations and was affinity-maturated. However, the Id_{Ox1} clonotype was not dominant and there was ***no*** other dominant clone. Usage of RF1

in HCDR3 was suppressed, but not avoided. RF2 and RF3 were not used and in a rather high proportion of antibodies, HCDR3 reading frames could not be assigned. Tertiary antibodies as those from earlier phases of the response reveal a selection of hydrophilic HCDR3-loops. The obtained sequences confirmed that the original Id_{Ox1} motive DRG is completely associated with the VHOx1gene VH171.

Hence, the findings in the anti-phOx response of the 2 DH mutant mouse strains ΔD-DμFS and ΔD-iD mice allow the following general conclusions:

1.) Under the conditions of a single artificial DH gene, the enzymatically created extraordinary variability at the third hypervariable region becomes visible. The data confirm previous findings that neutral amino acids like tyrosine and glycine are preferred in HCDR3.

2.) Since the T cell-mediated class switch-recombination as well as the expression of dominant B cell clonotypes obviously depends on the available DH genes it can be concluded that both processes are not only antigen-driven. This supports the further argument that T cells are involved in the selection of particular B cell clones and that this selection depends in a hitherto unknown way on particular HCDR3 sequences.

3.) A comparison with a large panel of anti-phOx antibodies from BALB/c WT mice (44 VH and 40 VL genes) revealed that DH mutant mice made use of 28 more VH and 12 more VL genes.

Further work in ΔD-DμFS and ΔD-iD DH mutant mice, in comparison to BALB/c WT mice, must elucidate the precise T cell involvement in regulation of class switch recombination and HCDR3-dependent selection of IgG-secreting B cell clones.

Zusammenfassung

Antigen-induzierte lymphozytäre (adaptive) Immunantworten beruhen auf komplexen kontakt- und zytokin-vermittelten Wechselwirkungen verschiedener Zellen. Vielfach wurden Hapten-Träger-Komplexe als Antigene eingesetzt, die verschiedenen charakteristischen Funktionen der einzelnen Komponenten dieses Prozesses zu entschlüsseln, z. B. die Wirkung der antigen-präsentierenden Zellen, Spezifität der T- (TCR) und B (BCR) Zell Antigenrezeptoren, die Kooperation von T- und B-Lymphozyten, die Funktion der Subpopulationen der T-Zellen, die Entstehung und Selektion des primären Repertoires, die Immunreifung durch somatische Hypermutationen, den Klassenwechsel und die Isotypregulation, um die wichtigsten zu nennen. Eine der am besten erforschten Antikörper-Antworten ist die in BALB/c-Mäusen gegen das Hapten 2-phenyl-oxazolone (phOx) nach Kopplung an das thymus-abhängige (TD) Trägerprotein Hühnerserum-Albumin (CSA). Die Immunantwort gegen phOx-CSA ist durch den dominierenden Ox1 Idiotype (Id_{Ox1}) gekennzeichnet, der von der kanonischen VH171/Vκ072 Genkombination kodiert wird und die typische Aminosäure-Sequenz Asparaginsäure-Arginin-Glycin (DRG) in der Mitte der dritten hypervariablen Region der schweren Kette (HCDR3) enthält. Die Affinität der Id_{Ox1}-Antikörper ist 10-100-fach höher als die der Non-Id_{Ox1}-Antikörper und die Immunreifung beruht in erster Linie auf der Anhäufung von somatischen Mutationen in Id_{Ox1}-Antikörpern. Während der Gedächtnisantwort gibt es jedoch eine Repertoireverschiebung zu Non-Id_{Ox1}-Antikörpern, die durch alternative VH- und/oder VL-Gene kodiert sind. Die Neigung von Non-Id_{Ox1}-Antikörpern für die somatischen Mutationen ist bekannt.

Nach der Selektionstheorie hängen viele dieser Eigenschaften von der Antigen-Spezifität der Antigen-Bindungsstelle (Paratop) des BCR ab. Grundsätzlich sind zwei verschiedene Prozesse an der Entstehung des Antiköprper-Repertoires während der antigen-unabhängigen Differenzierung im Knochenmark beteiligt, nämlich einmal die Rekombination der genomischen variablen (V), die Diversität erhöhenden (D) und der verbindenden (J) Gene und zum anderen eine nicht-genetische Erweiterung dieses genetischen Repertoires während V(D)J-Rekombination durch die Wirkung von drei verschiedenen Enzym-Systemen. Exonucleasen schneiden terminale Nukleotide ab, Reparatur-Enzyme generieren palindromische Sequenzen (P-Nukleotide) und die terminale Deoxynukleotidyl-Transferase (TDT) fügt zufällig nicht-kodierte Nukleotide (N-Nukleotide) hinzu. Auf diese Weise wird in der HCDR3 eine enorme nicht-genetische Diversität geschaffen, die ausreicht, eine effiziente Immunantwort zu ermöglichen, selbst wenn nur ein einziges VH- und VL-Gen vorhanden sind.

In der vorliegenden Arbeit werden Experimente zur humoralen Anti-phOx-Immunantwort von zwei transgenen BALB/c-Mäusestämmen beschrieben, welche jeweils nur ein DH–Gen enthalten. Ziel dieser Untersuchungen war es zu bestimmen, welchen Anteil die in HCDR3 beobachtete nicht-genetische und die auf VH/VL-Genen beruhende genetische Variabilität haben: an der thymus-unabhängigen Typ 2 (TI-2) und der thymus-abhängigen Immunantwort, dem primären TD-Repertoire, der Id_{Ox1}-Dominanz, dem Klassenwechsel, der somatischen Hypermutation und der Immunreifung während der TD Gedächtnisantwort. Zwei Mutanten des BALB/c–Mäusestammes standen zur Verfügung, die statt der 13 DH-Gene in BALB/c Wildtyp-Mäusen nur ein einziges DH-Gen in ihrem Genom tragen. Das DH-Gensegment der ΔD-iD Mäuse kodiert für geladene Aminosäuren in der HCDR3, während das DH-Gen der ΔD-DμFS Mäuse für hydrophobe Aminosäuren kodiert. Die phOx-Ficoll-induzierte TI-2 und die phOx-CSA- induzierte TD-Immunantwort der beiden Stämme wurden auf der Ebene der einzelnen antikörper-bildenden Zellen und der Serum-anti-phOx Antikörperbildung analysiert und mit denen in BALB/c Wildtyp-Mäusen verglichen. Die Qualität der TD Immunantwort wurde mit Hilfe monoklonaler Antikörper, welche aus Hybridomen gewonnen wurden, analysiert, die nach früher und später primärer, sekundärer und tertiärer Immunisierung durch Zellfusion hergestellt wurden.

Die folgenden Ergebnisse wurden erhalten:

A <u>Quantitative Aspekte der humoralen Anti-phOx Immunantwort in DH –Mutanten-Mäusen</u>

1). Die Analyse mittels Durchflusszytometrie (FACS) ergab, dass die Gesamtzahl der kernhaltigen Zellen, Lymphozyten sowie T- und B-Zellen, im Knochenmark der beiden Mutantenstämme vergleichbar war mit denen in BALB/c Wildtyp-Mäusen.

2.) Allerdings war die Gesamtzahl der mononukleären Zellen, ebenso wie der beider Lymphozyten-Subpopulationen in der Milz von ΔD-iD und ΔD-DμFS Mäusen auf Werte von weniger als 50 % der Wildtyp-Mäuse reduziert.

3.) Bemerkenswerterweise wirkte sich die genetische Veränderung in beiden Mäusestämmen auch auf T-Zellen aus, obwohl die genetische Veränderung auf die Immunglobulin-Gene beschränkt ist und deshalb keine direkte Auswirkung auf das TCR-Keimbahn-Repertoire

haben kann. Das Ergebnis in Zusammenhang mit den publizierten Daten erlaubt die Schlussfolgerung, dass die B-Lymphozyten das T-Zell-Repertoire indirekt kontrollieren.

4.) Die IgM-Immunglobulin-Konzentrationen waren im Serum von ΔD-iD, nicht aber in ΔD DμFS-Mäusen verringert, während die der IgG-Immunglobulin-Klasse deutlich in beiden Stämmen reduziert war.

5.) Im Gegensatz dazu war die Zahl der `*background´* IgM-anti-phOx Antikörper-sezernierende Zellen in beiden DH Mutantenstämmen deutlich erhöht, nicht aber der IgG-sezernierenden Zellen.

6.) Verglichen mit BALB/c-Wildtypmäusen war die phOx-Ficoll-induzierte TI-2 Antwort vergleichbar für IgM, während die für IgG sich verzögert entwickelte.

7.) Die IgM-Produktion während der primären phOx-CSA- induzierten TD-Antwort verlief verzögert und blieb auf niedrigeren Maximalwerten, bevor sie auf ein ähnliches Niveau wie bei Wildtyp-Mäusen zurückfiel. IgG-Titer waren während der gesamten primären Antwort stark supprimiert und verblieben auf diesem niedrigen Niveau auch nach sekundärer Immunisierung.

B VH/VL Sequenzanalyse von monoklonalen Hybridom-Antikörpern aus den verschiedenen Phasen der humoralen Anti-phOx Immunantwort in DH Mutante Mäusen

Genetische Analyse des phOx-reaktiven Repertoires in ΔD-DμFS Mäusen

In der frühen primären Immunantwort der ΔD-DμFS-Mäuse (RF1 kodiert für hydrophobe HCDR3 Aminosäuren) wurde die Id_{Ox1} Genkombination VH171/Vκ072 selten benutzt und dominierte in keiner Weise. Antikörper traten auf, die 5–100-fach höhere Affinität als die normalerweise dominierenden Id_{Ox1}-Antikörper besaßen. Die Verwendung des RF1 war im primären Repertoire in ΔD DμFS-Mäusen stark eingeschränkt. Die Länge des mittleren Teils des HCDR3 variierte von 1-12 Aminosäuren. Dies könnte darauf hindeuten, dass HCDR3 nicht von zentraler Bedeutung für die phOx-Spezifität ist. Je mehr Nukleotide vom DH-Gen durch Exonukleasen während der Entwicklung des Prä-Immunrepertoires durch VDJ Rekombination eliminiert werden, desto mehr verkürzt sich die Länge der HCDR3.

Im Gegensatz zu Wildtyp-Mäusen enthielten die späten primären Anti-phOx Antikörper einen erstaunlich hohen Anteil von 56 % IgM-Antikörpern. Da sich auch zu diesem Zeitpunkt kein dominanter Klon entwickelt hatte, folgt, dass eine solche Auswahl offensichtlich von dem zur Verfügung stehenden DH-Gen-Repertoire abhängt. Der Id_{Ox1}-Klon kann entweder gar nicht oder nur sehr selten aus dem artifiziellen DH-Gen der ΔD-DμFS-Mäuse generiert werden. Dass eine Immunreifung stattgefunden hatte, wird durch eine erhöhte Anzahl von Mutationen

in IgG-Antikörpern angezeigt, aber die Mutationen sind nicht in einem bestimmten Klon akkumuliert. Die HCDR3-Nukleotidsequenzen basieren bevorzugt auf dem DH-Keimbahngen, RF1 ist aber unterrepräsentiert.

Antikörper, die nach sekundärer Immunisierung erhalten wurden, zeigten, dass die erneute Rekombination beim Klassenwechsel in diesen Mäusen beeinträchtigt ist, sich das phOx-spezifische B-Zell-Repertoire zufällig entwickelte, die Immunreifung in einzelnen aber nicht in allen ΔD-DμFS Mäuse ablief und einzelne Mäuse sogar Id_{Ox1}-Antikörper mit der VH171/Vκ–072 Gen-Kombination bilden können. Die HCDR3-Sequenz (CAR-DGG-AYW) eines dieser Id_{Ox1}-Antikörper leitet sich wahrscheinlich von der kanonischen Id_{Ox1}-Sequenz CAR-DRG-AYW ab. Das Id_{Ox1} spezifische DRG-Motiv in der Mitte der HCDR3 kann aber entweder gar nicht oder nur äußerst selten gebildet werden.

Eine Beeinträchtigung des Klassenwechsels war sogar nach tertiärer Immunisierung zu beobachten, denn etwa ein Drittel der etablierten Antikörper gehörte zur IgM-Klasse. Id_{Ox1}-Antikörper dominierten auch nicht in der tertiären TD Immunantwort. Stattdessen wurde über ein Drittel der IgG-Antikörper von der VH/VL-Gen Kombination VH158/Vκ115 kodiert, welche bereits einmal als ein dominanter Klon während der Gedächtnisantwort in BALB/c Wildtyp-Mäusen beobachtet worden war. In allen Phasen der primären bis tertiären Anti-phOx TD-Immunantwort in ΔD-DμFS-Mäusen war die Kodierung der HCDR3 in RF1, welche hydrophobe Aminosäuren bedingt, unterrepräsentiert. Stattdessen führten die Verwendung des RF2 und die Bildung von keimbahn-unabhängigen Nukleotidsequenzen zu HCDR3-Schleifen, die reich an Glycin und Tyrosin sind.

Genetische Analyse des phOx-reaktiven Repertoires der ΔD-iD-Mäuse

In den frühen primären Antwort der ΔD-iD-Mäuse, deren in RF1 translatiertes DH-Gen für geladene HCDR3-Aminosäuren kodiert, war das Muster der VH/VL-Kombinationen geändert, und es wurden bisher nicht beobachtete V-Gene benutzt. Es dominierten aber weder Id_{Ox1}–Antikörper noch andere Klonotypen. Einige IgM- und IgG-Antikörper hatten 5-10-fach höhere Affinitäten als die normalerweise höchst-affinen Id_{Ox1}-Antikörper. Die Tendenz gegen eine Verwendung des RF1 war nicht so ausgeprägt wie in ΔD-DμFS-Mäusen. Allerdings war auch RF2 nicht wirklich dominant, und in den meisten Antikörpern konnte der Leserahmen nicht zugeordnet werden. Die D-Regionen dieser Antikörper waren kürzer als es sonst beobachtet wird. Die B-Zellen scheinen die Verwendung von geladenen HCDR3-Schleifen einmal durch eine Verlagerung des Leserasters und zum anderen durch einen erhöhten Verlust von Nukleotiden an beiden Enden des DH-Gens zu vermeiden, so dass etwa 53 % der HCDR3-

Segmente keine keimbahn-kodierten Nukleotide enthalten. Dagegen sind hydrophile HCDR3-Schleifen stark begünstigt. Sequenzvergleiche zeigten, dass die Id_{Ox1}-Spezifität nicht nur durch das DRG-Motiv in der HCDR3-Schleife bedingt ist, sondern auch durch die 3-dimensionale Struktur anderer Teile des Moleküls beeinflusst wird.

Das genetische Repertoire der <u>späteren primären Antwort</u> der ΔD-iD-Mäuse ist sehr heterogen, und es zeigt wieder keine dominierende VH/VL Gen-Kombination. Id_{Ox1}-Antikörper wurden nicht beobachtet. Wie in ΔD-DμFS-Mäusen zeigte der hohe Anteil an IgM-sezernierenden Zellen zu diesem Zeitpunkt, daß der Klassenwechsel auch in ΔD-iD-Mäusen beeinträchtigt ist. Die fast vollständig fehlenden somatischen Mutationen in diesen späten IgM-Antikörpern zeigen, daß die Beschränkung in ΔD-iD-Mäusen auf ein einziges DH-Gen, welches für geladene HCDR3-Aminosäuren kodiert, auch die frühe Immunreifung hemmt. Die Nicht-Verwendung des RF1 war nicht so ausgeprägt wie in ΔD DμFS-Mäusen. RF2 und RF3 wurden am wenigsten verwendet, und Antikörper mit keimbahn-unabhängigen HCDR3-Sequenzen waren am häufigsten. Letztere Antikörper besaßen wiederum kürzere D-Regionen, und die späten primären Anti-phOx-Antikörper der ΔD-iD-Mäuse besaßen hauptsächlich hydrophile HCDR3-Aminosäuren.

In der <u>sekundären Antwort</u> erstaunte es wieder, dass ein IgM-Antikörper bereits 3 Tage nach Immunisierung mit dem TD-Antigen phOx-CSA isoliert werden konnte. Vermehrte Mutationen und eine erhöhte Affinität des IgG-Antikörpers weisen darauf hin, dass sekundäre Antikörper in ΔD-iD-Mäusen sich aus einem immungereiften Repertoire entwickeln.

In der <u>tertiären Antwort</u> der ΔD-iD-Mäuse wurde ebenfalls ein hoher Prozentsatz an naiven, unmutierten und ungereiften IgM-Antikörpern erhalten, die damit IgM-Antikörpern der primären Immunantwort ähneln. Im Gegensatz zu Anti-phOx-Antikörper aus früheren Phasen der TD-Immunantwort, waren unter den tertiären Antikörpern wieder VH171/Vκ072-kodierte IgM und IgG Id_{Ox1}-Antikörper zu finden. Der IgG Id_{Ox1}-Antikörper enthielt somatische Mutationen und war immungereift. Allerdings waren Id_{Ox1}-Antikörper nicht dominant, und es gab auch ***keine*** anderen dominanten Klone. Die Verwendung des RF1 in HCDR3 war zwar stark vermindert, aber nicht völlig unterdrückt. RF2 und RF3 wurden nicht benutzt, und in einem recht hohen Anteil von Antikörpern konnte das verwendete HCDR3-Leseraster nicht zugeordnet werden. Wie es auch schon in den frühen Phasen der TD-Immunantwort beobachtet worden war, zeigten die tertiären Antikörper eine Tendenz zu hydrophilen HCDR3-Schleifen. Die erhaltenen Sequenzen bestätigten, dass das ursprüngliche Id_{Ox1}-Motiv DRG vollständig mit der VHOx1gene VH171 assoziiert ist.

Die Beobachtungen zur Anti-phOx-Immunantwort in den beiden DH-transgenen Mäusestämmen ΔD-DμFS und ΔD-iD erlauben die folgenden allgemeinen Schlussfolgerungen:

1.) Unter den Bedingungen eines einzigen künstlichen DH-Genes wurde die außergewöhnliche Variabilität in der dritten hypervariablen Region der schweren Immunglobulinkette, welche durch die Wirkung von 3 Enzymsystemen bedingt ist, sichtbar. Die Daten bestätigen frühere Ergebnisse, dass neutrale Aminosäuren wie Tyrosin und Glycin in HCDR3 bevorzugt werden.

2.) Da sowohl die durch T-Zellen vermittelte erneute Genrekombination des Klassenwechsels als auch die Entwicklung eines dominanten B-Zellklons offensichtlich von den verfügbaren DH-Genen abhängt, kann man schließen, dass beide Prozesse nicht nur durch eine durch das Antigen vermittelten Selektion angetrieben werden. Dies unterstützt das weitere Argument, dass T-Zellen an der Auswahl von bestimmten B-Zell-Klonen beteiligt sind und dass diese Auswahl in einer bisher unbekannten Art und Weise von bestimmten HCDR3-Sequenzen abhängig ist.

3.) Ein Vergleich mit einer großen Gruppe der Anti-phOx-Antikörpern von BALB/c Wildtypmäusen (44 VH- und 40 VL-Gene) ergab, dass in den DH–transgenen Mäusen weitere 28 VH- und 12 VL-Gene benutzt werden.

Es sind weitere Arbeiten in ΔD-iD und ΔD-DμFS DH-transgenen Mäusen und der Vergleich mit BALB/c Wildtypmäusen erforderlich, die genaue Beteiligung der T-Zellen an der Regulation des Klassenwechsels und der HCDR3-abhängigen Selektion der IgG-sezierenden B-Zellklone aufzuklären.

Table of contents

1 Introduction

The immune system fulfills two major tasks, namely protection against foreign harmful invaders on the one hand and preservation of the inner integrity of the organism on the other. A variety of mechanisms enables the organism to fight infections by identifying and killing microbial pathogens which range from viruses to parasitic worms. A prerequisite for this action is that the immune system has to distinguish them from the organism's normal cells and tissues. Hence, the immune system has to and can distinguish `*Self*´ from `*Non-Self*´. This ability not only allows the elimination of potentially harmful `*Non-Self*´ molecules and microbes, but also to recognize and destroy abnormal cells that derive from host tissues by malignant transformation.

To survive a pathogenic challenge, several mechanisms have evolved that recognize and neutralize pathogens. Even unicellular organisms have rich arsenals of mechanisms for protection, such as restriction endonucleases [1]. Other basic immune mechanisms evolved in ancient eukaryotes and remain in their modern descendants, such as plants, fish, reptiles, insects, birds and mammals. These mechanisms include various antimicrobial peptides such as defensins [2], pattern recognition receptors, and the complement system. More sophisticated mechanisms, however, developed relatively recently with the evolution of vertebrates [3]. The immune systems of vertebrates enclose innate and adaptive immunity and consist of many types of proteins, cells, tissues, and organs which interact in an elaborate and dynamic network.

1.1 Innate and adaptive immunity

The mammalian body is susceptible to infection by many pathogens which, after contacting the host, establish a focus of infection. Since the pathogens differ greatly in their surface proteins and mechanisms of pathogenesis, hosts developed two defense systems, namely innate and adaptive immune responses. Both responses are able to distinguish *Self* from *Non-Self* but with different degree of specificity. Also the mechanisms of antigen recognition by both innate and adaptive immunity are very diverse. Cooperation between both types of immune responses is required to achieve optimal responses against pathogens.

Innate immunity represents the first line of host defense through effector mechanisms that act immediately on contact with the pathogen at any time. Innate immunity is relatively non-specific for a particular pathogen. Anatomic barriers like skin and physiologic conditions like low pH values of the stomach inhibit the entrance of pathogens and protect against viral and

bacterial colonization. Bacteria, viruses, and parasites that overcome this barrier are faced immediately with tissue macrophages equipped with surface receptors (*e.g.* Toll-like receptor) that recognize a limited number of molecular patterns common to a wide variety of pathogens [4]. This, in turn, leads to an inflammatory response, which causes the accumulation of phagocytic monocytes, neutrophils and macrophages at the site of infection [5].

Since the mechanisms of innate immune responses do not develop a memory, they are incapable to improve their ability to resist a subsequent challenge with either the same or a different pathogen. These mechanisms prevent the vast majority of infections from becoming established or to develop further to a state of disease. However, innate responses often fail to clear the infection. In that case, dendritic cells, macrophages and other antigen presenting cells (APC), activated in the early innate immune response, help to initiate the development of adaptive immune responses [6, 7].

Adaptive immune responses are carried out by B and T lymphocytes which can, in principle, interact with *Non-Self* as well as *Self* molecules with membrane-bound antigen-receptors, *i.e.* the B cell antigen-receptor (BCR) and the T cell antigen-receptor (TCR). Antigens are recognized by BCR and TCR in two fundamentally different ways. BCR is a membrane-bound immunoglobulin = antibody (see below) which can directly interact with the antigenic molecule. The TCR is composed of 2 polypeptide chains, being either α plus β or γ plus δ. This allows the classification of two large subpopulations of T cells. While $\gamma\delta$ T cells are able to recognize antigens directly (like B cells), $\alpha\beta$ T cells can only do so when peptide fragments of protein antigens are bound to major histocompatibility (MHC) molecules. Protein antigens can be taken up and degraded to peptide fragments by antigen-presenting cells (APC) of the innate immune system as well as B lymphocytes. Hence, $\alpha\beta$ T cells recognize inner peptides of proteins while B cells recognize the surface of antigens. Since lymphocytes express a huge repertoire of antigen-receptors, adaptive responses to pathogens are highly specific for particular molecules of such microbes and this interaction can provide long-lasting memory cell-mediated protection. Thus, a second attack with the same pathogen induces an adaptive response that develops much faster and stronger than the first.

Like innate immunity, adaptive immunity has the ability to distinguish between *Self* from *Non-Self* and responds, under normal physiological conditions, only to *Non-Self* molecules. However, occasionally, the system fails to make this distinction and reacts destructively

against the host's own molecules, giving rise to autoimmune diseases. In addition, many foreign molecules entering the body are harmless *per se*, but can activate pathogenic immune reactions under special conditions. Allergic conditions such as hay fever and asthma are examples of these deleterious adaptive immune responses against normally harmless foreign molecules.

Another distinction concerning adaptive immune responses carried out by B and T lymphocytes is that they mediate two broad classes of immune responses, humoral and cell-mediated responses, respectively. In the humoral response, antigen-activation of B cells induces their differentiation into plasma cells which secrete huge amounts of antibodies which circulate with the bloodstream. The protective function of these antibodies can be ascribed to different mechanisms. a) The binding of antibodies can inactivate viruses and microbial toxins by blocking their ability to bind to receptors on host cells. b) The formation of antigen-antibody complexes (immune complexes – IC) can activate secondary effector functions, *i.e.* activation of the complement system or marking invading pathogens for destruction, mainly by making it easier for phagocytic cells of the innate immune system to ingest them via cell membrane receptors for the constant Fc part of the antibody (Fc receptors = FcR) or complement receptors like those for C3b. c) Moreover, the formation of IC may induce further activation of T lymphocytes which supports the differentiation into memory T as well as B cells.

Cell-mediated or cellular immunity is mediated by cytotoxic T lymphocytes (CTL) which are able to kill virus-infected and tumor cells [8, 9]. Moreover, B cell responses to protein antigens need the help from T cells. Thus, proteins are so-called thymus-dependent (TD) antigens which induce $CD4^+$ T helper (T_H) cells. Their interaction with B cells depends on recognition of an antigenic peptide presented by MHC class II molecules on the membrane of the B cell. Helper T cells can further be subdivided into T_H1 cells secreting as major cytokines interleukin 2 (IL-2) and interferon γ (IFN-γ) and T_H2 cells secreting cytokines like IL-4, -5, -10 and -13 [10]. This cytokine network decides, for instance, about class switch recombination from primarily activated IgM-producing plasma cells to different IgG subclasses, mutual inhibition of T_H cell subpopulations and regulatory feedback to antigen-presenting cells. This complex cellular interplay is, in addition, regulated by $CD8^+$ suppressor / regulatory T cells [11].

1.2 Structure of B cell antigen receptor

The B cell antigen receptor, immunoglobulin or membrane-bound antibody, is a heterodimeric protein composed of two heavy (H) chains which are connected via disulfide bonds and two light (L) chains each of which is likewise bound via disulfide bridges to one of the H chains. The prototypic Y-shaped structure of the secreted form of an antibody is depicted Fig. 1. Thus, the transmembrane domains are omitted. Both chains are composed of one or more constant regions (or domains) that define their class- and subclass-dependent effector functions and one variable region that determines antigen specificity and affinity [12]. Immunoglobulins exist in several isotypic variants, the antibody classes IgM, IgD, IgG, IgA and IgE which are characterized by the constant parts of the heavy chains. They are designated μ for IgM, δ for IgD, γ for IgG, α for IgA and ε for IgE antibodies. In addition, IgG can be subdivided in 4 subclasses, namely IgG1-4 in humans and IgG1, 2a, 2b and 3 in mice. IgA appears in 2 subclasses, IgA1 and 2. There are 2 types of L chains named κ (kappa) and λ (lambda).

Each of the variable domains of the heavy chain (VH) and the light chain (VL) contain 3 hypervariable (hv) or complementarity-determining regions (CDR). Since the immunoglobulin domains are folded in a secondary structure with 2 ß-folds, the CDRs of each chain point into one direction. Together, they form the antigen-binding site = paratope which recognizes and binds the antigenic determinant = epitope. The location of the paratope-forming 3 VL and 3 VH CDRs are depicted in Fig. 2. The third CDR of the heavy chain (HCDR3) is especially important for a specific binding of the antigen. Together with the LCDR3, HCDR3 forms the center of the paratope. The immune system is able to create an extraordinary variability at HCDR3 (see below). Whereas variable domains allow specific recognition of the antigen, constant domains define the effector functions [12]. The C domains are directly germ line encoded, whereas V domains are created *de novo* by means of a complex series of gene rearrangement events in developing B cells [13].

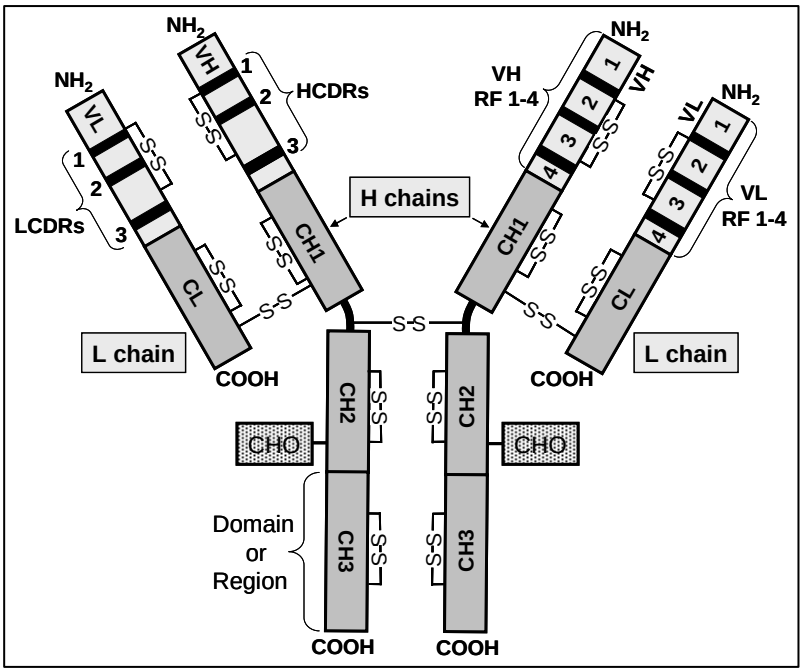


Figure 1

Schematic drawing of a typical antibody molecule

Each antibody consists of four polypeptides: two heavy and two light chains held together by disulfide brigs (S-S). The variable (VL and VH) domain gives the antibody its specificity for binding, while the constant domains (CL, CH1, CH2, and CH3) determine the antibody isotype. The variable region is further subdivided into hypervariable (hv) or complementarity-determining regions (CDR) and framework (FR) regions. Within light and heavy chains, there are three CDR regions: CDR 1, 2 and 3 and four FR regions: FR1, 2, 3, and 4 which separate the hv regions. The 6 CDRs in the N-terminal (NH2) V domain of both H and L chains form the antigen binding site, whereas at the C-terminal (COOH) of CH domain is the Fc region which mediates different physiological effector functions of antibodies. All antibodies are glycosylated at conserved positions in their constant regions. The carbohydrates are indicated by (CHO).

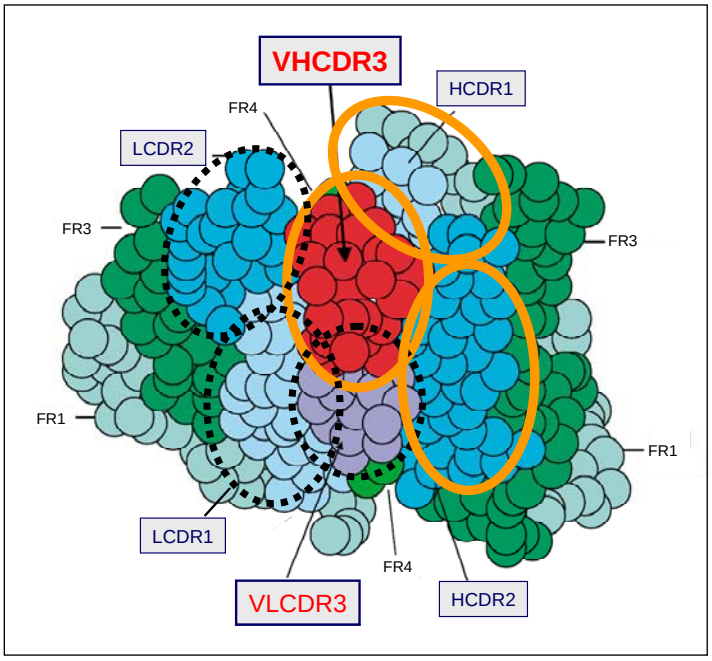


Figure 2

Schematic drawing of antigen binding site

The framework regions (FRs) form beta-sheet structures which hold the complementary-determining regions (CDRs) of L (black dotted ellipsis) and H (orange ellipsis) chains in the correct position to form the antigen binding site or paratope. The CDR3s of L and H chains make up the center of antigen binding site and they are surrounded by LCDR1 and 2 from one site, and from other site by HCDR1 and 2. The paratope makes various contacts with epitope (adpted from [29])

1.3 Immunoglobulin-coding genes

The adaptive immune system is remarkable for its ability to respond to a seemingly unlimited variety and number of antigens, including synthetic antigens which do not occur in nature. This characteristic depends on the capacity of lymphocytes to create highly diversified antigen receptor repertoires [13]. The variable regions of the 2 polypeptide chains of B as well as T cell antigen receptors are encoded in different ways. One chain of the antigen receptor, *i.e.* the BCR light chain and the TCR α chain, is encoded by variable genes (coding for about 100 amino acids) and smaller J gene segments (coding for about 10 amino acids) which, after recombination, enable joining with the constant genes. The other chain of antigen receptors, *i.e.* BCR heavy chain and the TCR β chain, is likewise encoded by variable genes and J gene segments, but between both genetic elements there are small diversity-enhancing D gene segments interspersed.

1.3.1 Organization of the immunoglobulin λ and κ gene loci

Each genetic locus coding for one of the three constant regions, *i.e.* λ and κ light chains and the heavy chain, has its own group of variable genes. This is due to the fact that the different genetic loci for Igλ, Igκ and IgH chains are located on different chromosomes.

The mouse **λ** locus on chromosome 16 contains only three functional V_λ genes ($V_\lambda 1$, $V_\lambda 2$ and $V_\lambda 3$) and two C_λ genes, each with its associated J_λ gene segments [14]. The organization of the λ locus also allows for few rounds of V_λ–J_λ rearrangement, in this case between an up-stream V_λ and a downstream J_λ–C_λ cluster. V_λ elements are flanked by 'two-turn' spacers and J_λ by `one turn´ spacers (Fig. 3), limiting the likelihood of V_κ–J_λ or V_λ–J_κ rearrangements. Because of the few variable genes in this locus, λ-encoded antibodies contribute very little to the germ line repertoire. This correlates to the minimal occurrence of only 5 % of antibodies with λ L-chains while the rest of 95 % contains κ L-chains.

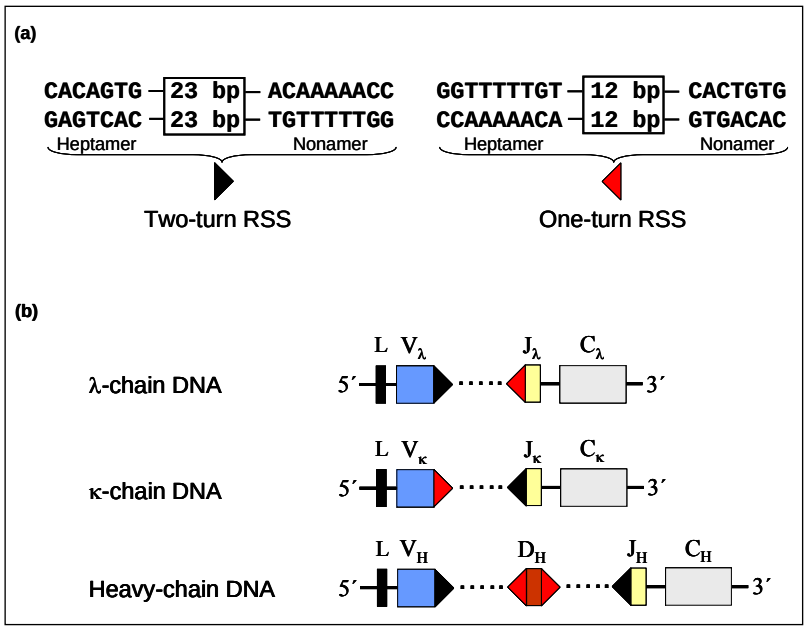


Figure 3

Recombination signal sequences

(a) DNA nucleotide sequences of recombination signal-sequences. Each recombination signal sequence contains a conserved heptamer (7 nucleotides) and a conserved nonamer (9 nucleotides) motif separated by a 'spacer' of either 12 (one-turn) or 23 (two-turn) base pairs.
(b) Location of RSSs in germ-line immunoglobulin DNA. Each V and J chains is jointed with one RSS (one-turn or two-turn depends on the isotype) at 3' and 5', respectively, whereas the DH segment is flanked by one-turn RSS at 3' as well as one-turn at 5' for joining to the J gene segment and the V gene.

The mouse V_{κ} locus is located on chromosome 6 and contains up to 211 expressed V_{κ} gene segments [14]. The J_{κ} locus contains four functional genes ($J_{\kappa}1$, $J_{\kappa}2$, $J_{\kappa}4$, $J_{\kappa}5$). $J_{\kappa}3$ is a non-functional pseudo-gene. The mouse κ locus contains only one C_{κ} element. Joining of one of the V_{κ} genes with one of the J_{κ} segments is mediated by recombination signal sequences (RSS) at the 3' end of V_{κ} genes and 5' end of J_{κ} (Fig. 3) [15]. As in the λ locus, the RSS contain a highly conserved heptamer sequence separated from a less conserved nonamer sequence by 12 or 22/23 base pair intervals termed spacers. Each spacer region serves to separate the heptamer and nonamer sequences by either one (12 base pairs) or two (22/23 base pairs) turns of the DNA helix. Rearrangement of a 'one-turn' spacer with a 'two turn' spacer is strongly favored. In contrast to the λ locus, the V_{κ} genes are flanked by a 'one-turn' spacer and Jκ gene segments are flanked by a 'two-turn' spacer (Fig. 3). Each of the V_{κ} gene segments has the potential to rearrange with any one of the J_{κ} gene segments. V_{κ} genes, as well as VH and V_{λ}, can be grouped into families of related sequences that by definition share greater than 80 % identity with each other. Expressed V_{κ} genes in mouse can be grouped into eighteen families that contain as few as one and as many as 22 different elements. The members of these families are partially clustered in the locus, and a large number of them is inverted in relation to their orientation to the J_{κ} gene segments. The organization of the κ locus provides abundant opportunity for multiple rearrangements. Up to three rounds of sequential upstream V_{κ} to downstream J_{κ} recombination can occur by deletion and inversion.

1.3.2 Genomic organization of the heavy chain locus

The murine IgH locus spans approximately 3 Mb near the telomeric end of chromosome 12. The VH genes lie about 100 kb 5′ of the JH segments, and the distance between two adjacent VH genes is 12-15 kb [16]. It has to be stressed that, among the various strains of mice, there is considerable variation in the number as well as the sequence of the IgH gene segments. In BALB/c mice (one of the best characterized mouse strains), more than 700 genes can be grouped into 15 different families arrayed over 2.7 Mb. VH genes that lie closer to the DH gene segments have been termed proximal whereas VH genes lying further upstream have been termed distal (Fig. 4) [14]. VH and JH gene segments are flanked by `two-turn′ spacers (Fig. 3), whereas DH gene segments are flanked by `one-turn′ spacers. The DH gene segments have the potential to be read in any of six different reading frames, depending on whether rearrangement proceeds by deletion or by inversion. Fusions of 2 D gene segments (D–D fusions) have also been observed, but they are rare. Importantly, the 3′ terminus of a

VH gene contains an embedded cryptic heptamer sequence that is mostly preserved after initial V/D/J rearrangement. This sequence can be used to replace a particular VH gene sequence. Such a replacement is named receptor editing when happening during the antigen-independent differentiation in the bone marrow, but receptor revision when is takes place in the periphery after stimulation by antigen [17, 18].

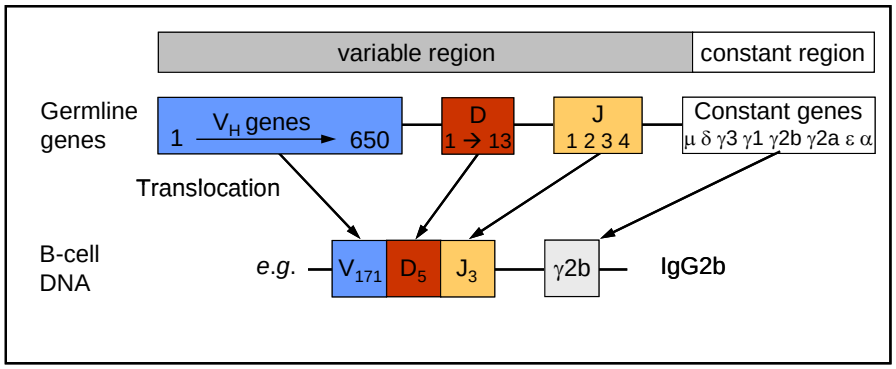


Figure 4

Construction of heavy chains

The heavy chain of an antibody is composed of V and C domains. The BALB/c genome includes 650 VH genes, 13 DH segments, 4 JH gene segments, and 8 constant genes (μ, δ, γ3, γ1, γ2b, γ2a, ε, and α). A functional heavy chain of a mature B cell is encoded by one particular VH (blue), one DH (red), one JH (orange) and a constant gene. In this example, VH 171, DH 5 and JH3 are joined to γ2b constant domain to generate an IgG2b antibody.

The members of the murine VH and DH families tend to be located in clusters of related sequences. This contrasts to the situation in humans, where in both the VH and the DH loci, family members are interspersed within other families. Murine members of the VH5 (7183) and VH2 (Q52) families are JH proximal, whereas genes of the VH1 family are most distal and account for more than 50 % of the active VH repertoire. Studies of the Ig repertoire suggested that the VH1 family of C57BL/6 mouse consists of ~ 200 closely related members [19]. However, the exact number of VH1 genes of BALB/c mouse remained unclear [20-22].

The BALB/c haploid genome contains 13 active DH and a cluster of four active JH segments, which are located about 7 kb 5′ of the CH genes [22], while the C57BL/6 haploid genome contains only an estimated 101 functional VH, 9 DH, and 4 JH gene segments. The DH gene segments can be grouped into four families: (a) DFL which in BALB/c mice consists of the 16.1 and 16.2 gene segments and in C57BL/6 only of DFL16.1, (b) DSP consists of nine gene segments in BALB/c, but only six in C57BL/6, (c) the singleton DST4 gene segment and (d)

DQ52. DFL16.1 is most distal from JH (80 kb), whereas DQ52 is adjacent to JH1 (ca. 1 kb) [23, 24].

The mouse C_H gene locus consists of the eight constant genes in the following order: 5′-C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\gamma 2b}$-$C_{\gamma 2a}$-C_ε–C_α- 3′ that cluster in a 200-kb region and code for μ, δ, γ3, γl, γ2b, γ2a, ε and α chains. In the mouse C_H gene locus there are no stop codon-containing conserved pseudogenes [22, 25].

1.4 Contribution immunoglobulin variable genes to the structure of variable regions

The VH region is encoded by the three separate DNA segments VH, DH, and JH genes or gene segments [26]. Consequently, the complete active VH domain is generated by two somatic recombinations, VH-DH and DH-JH joining. In contrast, VL regions of both types of L-chains are encoded by only two segments, VL and JL genes. Each newly formed VH and VL domain contains 4 intervals with relatively constant sequences which are named framework regions (FRs) (Fig. 1). They are separated from each other by three hypervariable intervals (complementarity determining regions or CDRs). The FRs encode the anti-parallel strands that make up not only the core β-barrel structure of the V but also the C domains. The first 2 hypervariable regions CDRs 1 and 2 of both, VH and VL regions are encoded by the V genes and form the outside borders of the antigen binding site (Fig. 2) [27]. In contrast, the third hypervariable regions HCDR3 and LCDR3 are created by VDJ and VL recombination, respectively. Both build the center of the antigen binding site (Fig. 2) [28].

1.5 Structure of the third hypervariable region of heavy chain (HCDR3)

After differentiation in the bone marrow, the initial diversity in antibodies is primarily concentrated at the joining sites of V, D and J segments thereby creating HCDR3. The extraordinary diversity of CDR3 of VH is created during the recombination process by the action of several enzyme systems (Fig. 5). The products of recombination-activating genes 1 and 2 (RAG-1 and RAG-2 proteins) accomplish the association of RSS of the involved gene segments and cutting off the loops. However, the hair-pin loops of both double-stranded DNA ends are mostly not ligated properly. Before the single DNA strands are connected by ligases, other enzymes modify both ends. First, exonucleases cut off nucleotides from the coding sequences at the 3′ end of VH, the 5′ and/or 3′ end of DH and or the 5′ end of JH (Fig. 5c).

Secondly, the hair-pins may be opened asymmetrically in such a way that sticky ends are created which together form a palindromic sequence. Therefore, the subsequently filled in nucleotides of the second strand are termed P nucleotides (Fig. 5a). Third, during the recombination process, so-called non-germ line encoded nucleotide sequences (N nucleotides) are inserted by the enzyme terminal deoxynucleotidyl transferase (TdT) at the DH-JH and the VH-DH border (Fig. 5b). Furthermore, the BALB/c DH locus encloses 13 diversity DH segments, which can be used, at least theoretically, in 6 different reading frames (RFs). As an overall result, only the middle part of the HCDR3 sequence is encoded by variable parts of a given DH gene while parts at the border are generated through random nucleotide sequences inserted during the recombination process [12]. The inserted nucleotides provide the opportunity to create totally random HCDR3 sequences, indicating that HCDR3 is not restricted to the genomic nucleotide repertoires of the DH gene segments. The recombination process at HCDR3 is so multifaceted that it is not possible to deduce the original DH germ line gene segment in a rather high percentage of antibodies [29].

In several murine immune responses, the vast majority of antibodies use DH reading frame 1 (RF1) which favors tyrosine and glycine residues [30]. It has been argued, that the preference for RF1 usage can be explained by the fact that RF1 of mice DH gene segments encodes neutral amino acids, while RF2 and RF3 are counter selected on the basis of stop codons and/or hydrophobic encoded nucleotides [31, 32]. Hence, such antibodies are eliminated by secondary regulatory mechanisms. This is indicated by the finding, that many auto-reactive antibodies are distinguished from non-pathogenic antibodies only by HCDR3 through insertion of P/N nucleotides or usage of alternative DH reading frames [33]. Hence, the restriction of HCDR3 plays a critical role in controlling autoimmune diseases [34]. Thus, under normal conditions, the central amino acids of HCDR3 are usually derived from the nucleotides encoded by RF1 of DH gene segments. Moreover, a preferentially usage of some DH gene segments restricts the HCDR3 repertoire [35]. In fact, HCDR3 of fetal antibodies are shorter than in antibodies produced in later life [36]. This is due to low or no expression of TdT (lack of N nucleotides) and usage of short DH gene segments.

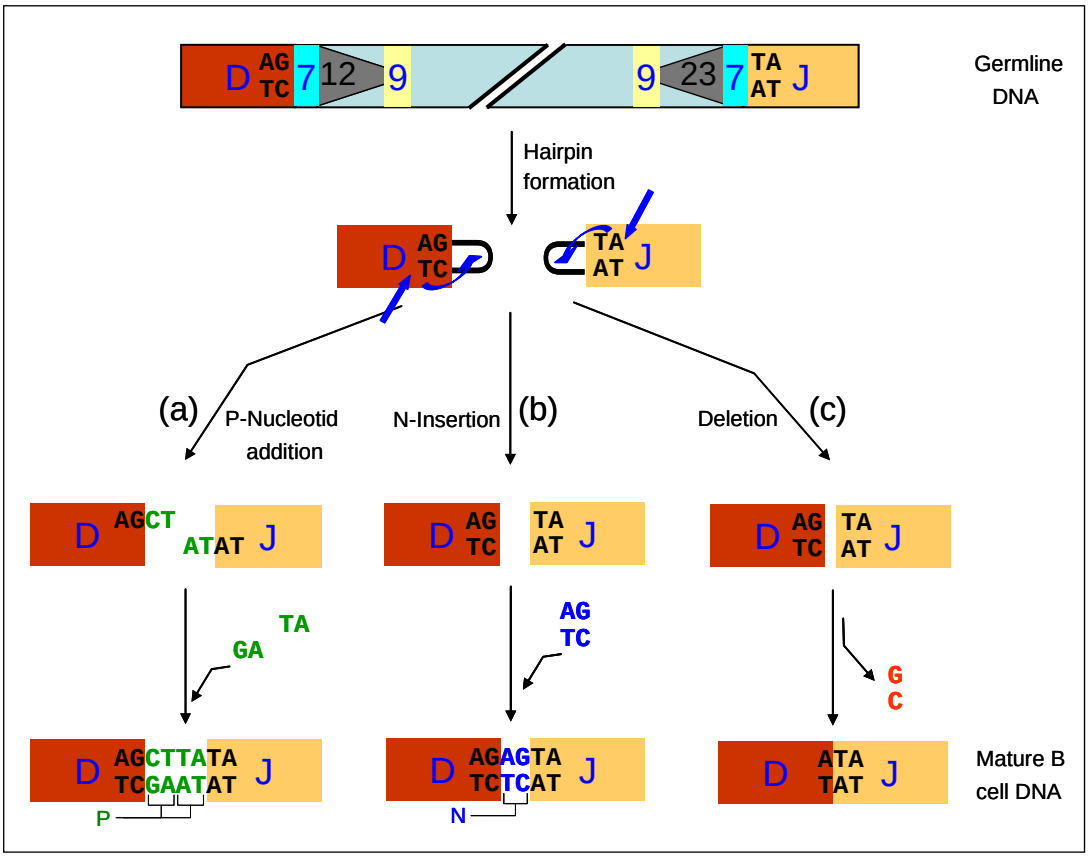


Figure 5

Imprecisions during DH-JH recombination

V(D)J recombination is mediated by the 2 RAG enzymes which cleave out the recombination signal sequences (RSS). However, during the recombination of DH-JH and its subsequent joining with a particular VH gene, the genomic sequences of the involved genetic elements are modified by three different mechanisms.
a) The resulting DNA hairpins may be opened asymmetrically in such a way that sticky ends are generated which may contain DNA palindromic sequences. Such P-nucleotides (green) are encoded by the complementary strand.
b) Nucleotides are added by terminal deoxytransferase (TdT) at random before complementary pairing occurs. Since these nucleotides are **not** encoded in the germ line they are termed N-nucleotides (blue) or N region insertions.
c) During this process, exonucleases cut off nucleotides (red) at the ends of the involved gene segments.
All three variability-enhancing mechanisms also occur during the recombination processes of joining between VH-DH and VL-JL.

Several reports have demonstrated that the HCDR3 is especially important and decisive for the determination of antigen-specificity of antibodies. Transgenic mice carrying a single functional VH gene but a full DH diversity repertoire are able to generate efficient immune responses to a variety of antigens. Antibodies generated in these mice, share the same VH and VL gene and differ only at HCDR3 [37]. Furthermore, mice lacking TdT (TdT°) [38] show

drastically reduced BCR and TCR repertoires similar to that found in fetuses, where the TdT is not or minimally expressed [29, 39]. Interestingly, the responses to TD and TI antigens are very similar in TdT° and control WT mice. Moreover, several studies have demonstrated that CDR3s of the TCR are crucial for antigen-recognition by T cells [37, 40]. Thus, it was concluded that HCDR3 determines antigen-specificity, while CDR1 and 2 play a role in the cross-reactivity [37].

1.6 B cell development

Mammalian B lymphocytes are derived from the primary lymphoid organs fetal liver and bone marrow, while B cells in birds are generated in the bursa of Fabricius. B cell development is a linear progression of defined stages characterized by assembly of the BCR from different genetic elements. This differentiation in the bone marrow is an intrinsic ability of the system and occurs independently of external antigens [41]. The assembly of the BCR begins in the H chain locus in pro-B cells. At first, one of the 13 DH segments (in BALB/c mice) is recombined with one of the available 4 JH gene segments with the aid of RAG1 and RAG2 proteins. In the early pre-B cells, a VH gene is joined to previously established DHJH segment, completing the rearrangement of the IgH locus by production of heavy chain of the μ isotype. The further successful association of the μ chain at pre-B cell stage with a surrogate light chain (SLC) correlates with the downregulation of transcription of *RAG*1 and *RAG*2 genes. SLC is a heterodimer composed of two peptides λ5 and VpreB. λ5 and VpreB show homology to lambda constant and variable regions, respectively. SLC combine with μ chain to form pre-B cell receptor complex (pre-BCR). In the late pre-B cell, reactivation of *RAG*1 and *RAG*2 allows L chain rearrangement. Typically, Vκ–Jκ recombination precedes Vλ–Jλ joining. Successful association of an in-frame L chain with the endogenous μ chain forms membrane bound molecules (mIgM), establishing immature B cells [42]. If the expressed IgM is not properly assembled or is auto-reactive, the interaction with self-antigens may induce either receptor editing by a secondary gene rearrangement to produce non-auto-reactive BCR, or the cells die by apoptosis in order to avoid autoimmunity [43]. If the cells pass this check point, an alternative splicing is induced which leads to co-expression of IgD at the surface. These mature B cells, which have survived negative selection in the bone marrow, leave the bone marrow via the blood stream and colonize the secondary lymphoid organs. In the periphery, they can receive positive selection signals necessary for further differentiation and maturation [44]. Depending on the nature of the antigen, mature naïve B follow different

destinations. Cells failing a renewed check of self-reactivity may undergo receptor revision changing the specificity of their BCR, convert to anergic (non-responsive), or may be deleted by apoptosis. B cells reacted with thymus-independent (TI) Ag will differentiate into plasma cells producing mostly IgM isotype. Anti-thymus-dependent (TD) Ag B cells receive additional stimulations from T_H cells necessary for hypermutation and class switch. Anti-TD antigen-specific B cells can differentiate into Ab secreting plasma and memory B cells.

1.7 Antibody response

The specific interaction between antigens and BCR on particular B cells is the basis for the clonal selection theory. The reactive B cells are activated to proliferation and differentiation into plasma cells that synthesize and secrete antibodies with identical specificity as the original BCR. Some antigens are capable of inducing a rapid antibody response that does not require T-cell help. There are of two types of the thymus-independent (TI) antigens. TI-1 antigens (*e.g*. bacterial lipopolysaccharide) can specifically be recognized by the B cell receptor (BCR) and, in a more non-specific way, by toll-like receptors (TLRs). Recognition by this second receptor induces proliferation and differentiation of the B cells. Another group of TI antigens is typically composed of repetitive carbohydrate (*e.g*. pneumococcal polysaccharide) or protein epitopes present at high density on the surface of a microorganism. These TI-2 antigens can extensively cross-link the antigen receptors and co-receptors on the B-cell surface. The rapid TI humoral responses are largely brought about by B-1 and marginal zone (MZ) B cells which produce predominantly IgM antibodies [45]. While TI-1 antigens do not induce generation of immunological memory, it is generally assumed that responses to TI-2 antigens include a long-term memory effect [46].

In contrast, thymus-dependent (TD) antigens require T helper (T_H) cells to stimulate antibody production. TD antigens must first be bound and internalized by antigen-presenting cells (APC) where they are enzymatically fragmented (processed). Peptide fragments are then presented by MHC class II molecules to antigen-specific T cells in order to activate them. Thus, interaction of T_H and B cells depends on recognition of the MHC class II-bound peptide by the TCR and simultaneous interaction of the MHC class-II on the B cells with CD4 on the T cell, *i.e*. T_H-B cell interaction is MHC class II-restricted. The successful interaction of both cell types leads to antibody production and secretion by plasma cells. The first antibodies made after TD antigen priming are of the IgM class. With continued T_H B-cell stimulation activated B cells undergo isotype switching, somatic hypermutation, and affinity maturation

within the germinal centers of secondary lymphoid organs, resulting in secretion of IgG antibodies with increased affinity/avidity for the inducing antigens. In addition, stimulation with TD antigens generates long lived plasma cells migrating to the bone marrow and induces B/T memory cells. While long lived plasma cells are capable to secrete specific antibodies for several months without antigen [47], memory B/T cells are fundamental for secondary and further immune responses providing efficient protection for many years [45, 48].

1.8 Internal processing of antigen-induced immune responses

The immune system does not only respond to the stimulation by external antigens, but develops a "*balance between the processing of foreign antigens and well-balanced self-reactivity"* [49]. This is achieved by idiotypic interactions: When antigen-reactive antibodies (idiotypes or Ab1) from one animal (rabbit, mouse) were used to immunize another animal, this produced Abs reacting with individual specificities of the injected idiotypes (anti-idiotypes or Ab2). Ab2 could stimulate anti-anti-idiotypes (Ab3) in a third animal, and Ab3 could stimulate anti-anti-anti-idiotypes (Ab4) in a fourth animal. This idiotypic cascade also proceeds within a particular immune system and the adaptive immune system functions as an idiotypically connected network [50]. Antibodies at each step of the idiotypic cascade represent a polyclonal collection and each clone expresses multiple idiotypic epitopes (idiotopes). Hence, every clone of a particular collection, *e.g*. Ab1, has a counterpart among Ab2s. Nevertheless is the collection of Ab3s not identical with that of Ab1s and, likewise, Ab4s are not identical with Ab2s. In a minority of investigations only, low amounts of antigen-reactive Ab1' antibodies were found among Ab3s after immunization with Ab2. Hence, the induction of anti-idiotypes does not only depend on mutual recognition, but is also dictated by regulatory properties within the idiotypic network. This regulation depends largely on helper and regulatory T cells (T_{reg}) recognizing BCR as well as TCR idiotopes. The induction of autologous anti-idiotypic (aId) immune responses demonstrates that the immune system develops *no* tolerance to the idiotopes of its own antigen-receptors. Therefore, the pre-immune repertoire as well as that of antigen-activated B and T cells is regulated by idiotypic interactions [50-52]. For instance, in the thymus-independent type 2 response to α (1>3) dextran, T_{reg} restrict the response to two major idiotypes and inhibit a class switch-recombination to IgG [53]. The involved T_{reg} cells are *not* antigen-, but Id-specific and recognize the HCDR3 region of the major J558 Id in an I-E^d-restricted manner [53]. Since these Id-specific T_{reg} do not arise in germ-free mice even when transferred to a normal environment at an age of 8 weeks it can be concluded that they were neonatally induced by contact with microbial antigens and persisted for

a long period of time. Hence, idiotypic regulation is evidently of particular importance during early ontogeny and it has been hypothesized that maternal antibodies play a major role by inducing an immunological imprinting which directs the future development of the immune system until adulthood.

1.9 Characteristic features of the anti-phOx immune response in BALB/c mice

The molecular events during the onset and the maturation of immune responses have mostly been studied with model antigens composed of hapten carrier complexes. Thymus-dependent immune responses to several hapten-coupled proteins are characterized by dominant idiotypes. It is only this circumstance that allowed tracking of the fate of particular primarily activated clones during secondary and tertiary responses. Hence, these systems were fundamental for detection of somatic hypermutation during TD responses as the underlying mechanism of immune maturation. One of the best studied systems is the TD response of BALB/c mice to the hapten 2-phenyl-oxazolone (phOx) after coupling to chicken serum albumin (CSA) as a carrier [54, 55].

The analysis of VH/VL sequences of a large number of hybridoma-derived antibodies allowed the construction of genealogical trees of antibodies activated during sequential steps from primary to memory responses [54, 56]. Analogous confirming data have been obtained in several other experimental systems including responses to other haptens like p-nitrophenyl-acetyl (NP) [57] and phenylarsonate (Ars) [58, 59], and protein antigens [60, 61].

The primary TD anti-phOx response in BALB/c mice is characterized by a dominant clone, the Ox1 idiotype or Id_{Ox1}. On day 7 after primary immunization, about 75 % of all antibodies are of this idiotype which, however, is lost within 3 weeks by somatic mutations. Ox1-idiotypic antibodies are of highest affinity while the binding strength of non-Id_{Ox1} is 10-100-times lower. Immune maturation is primarily based on accumulation of somatic mutations occurring in Id_{Ox1}, but the propensity of non-Id_{Ox1} antibodies for somatic mutations is also known. Hence, the phOx-specific antibody production is probably the best known immune response at the molecular level.

Id_{Ox1} antibodies are encoded by the VH/VL gene combination VH171/V_{κ}072 (nomenclature of the VBASE2 data base; old nomenclature VHOx1/VκOx1) [56]. However, syngeneic

monoclonal Ox1 (Id_{Ox1}) idiotype-specific antibodies have demonstrated that the idiotypic specificity entirely depends on the HCDR3 sequence Asp-Arg-Gly (DRG) [62]. This interaction is obviously extremely sensitive to conformational alterations of the idiotype and of high specificity, as documented by the observation that about half of 19 anti-Id_{Ox1} antibodies lost their reactivity already with class switching from IgM to IgG and none of the anti-Id_{Ox1} reacted with anti-phOx antibodies which carried a glycine or histidine instead of arginine as the middle amino acid of the D region [62]. During immune maturation in the late primary and the secondary response, antibodies exhibiting a DRG D-segment are first lost before they may reappear during the very late stages of tertiary and quaternary memory responses in the context of highly mutated VH and VL genes [62]. This observation was interpreted to indicate that the activation of antigen-reactive antibodies thereupon induces idiotypic responses which suppress major antigen-reactive clones, but stimulate minor clones. Concurrently, mutated clones would survive and could participate in later stages of immune maturation and memory responses. These mutations probably alter the overall structure of the paratope and in particular HCDR3 so that Id_{Ox1}-specific suppression is not active. Hence, idiotypic network reactions were viewed as the driving force of immune maturation. An involvement idiotypic regulation in clonal progression during immune maturation has also been deduced from the observation that Id_{Ox1} clones are suppressed during secondary anti-phOx responses, while non-Id_{Ox1} antibodies encoded by alternative VH/VL genes appear at an increased rate. This allowed the assumption that *`idiotypic interactions and, in particular, idiotypic suppression could be a way of dampening a dominating monoclonal response to favor the shift´* [55]. Other reports, however, did not find evidence for this possibility [63-65]. If clonal progression during immune responses is actually regulated by idiotypic regulation, this can only take place with the help of idiotypically connected T cells [52] which restrict the activation of anti-Id_{Ox1} antibodies to HCDR3-specific ones. Since the Id_{Ox1}-specific DRG-sequence is only expressed in those anti-phOx antibodies which are encoded by the VH171/Vκ072 gene combination [46], it is conceivable that the Id_{Ox1} dominance is T cell-mediated. It is generally assumed that Id_{Ox1} antibodies dominate the early response because of their superior affinity and that further clonal progression is also brought about by preferential activation of memory B cells which exhibit BCR with stepwise increasing affinities. However, this view is not quite valid since the pre-immune repertoire of BALB/c mice contains phOx-reactive non-Id_{Ox1} clones of higher affinity (Lange et al., in preparation).

1.10 Mice carrying single DH gene segments

The above data indicated a strict correlation between dominance of the Id_{Ox1} with the canonical gene combination VH171/VL072, the expression of a DRG amino acid sequence in HCDR3 and the ability of the syngeneic host to produce DRG-specific anti-idiotypic antibodies. Hence, these relationships depend on the available D gene repertoire in the BALB/c strain. Hoping to shed light on these relationships, we analyzed the anti-phOx primary and memory immune responses in two BALB/c mutant strains each of which harbor only one of two different DH gene-segments in their genome. These strains were generated in the following way.

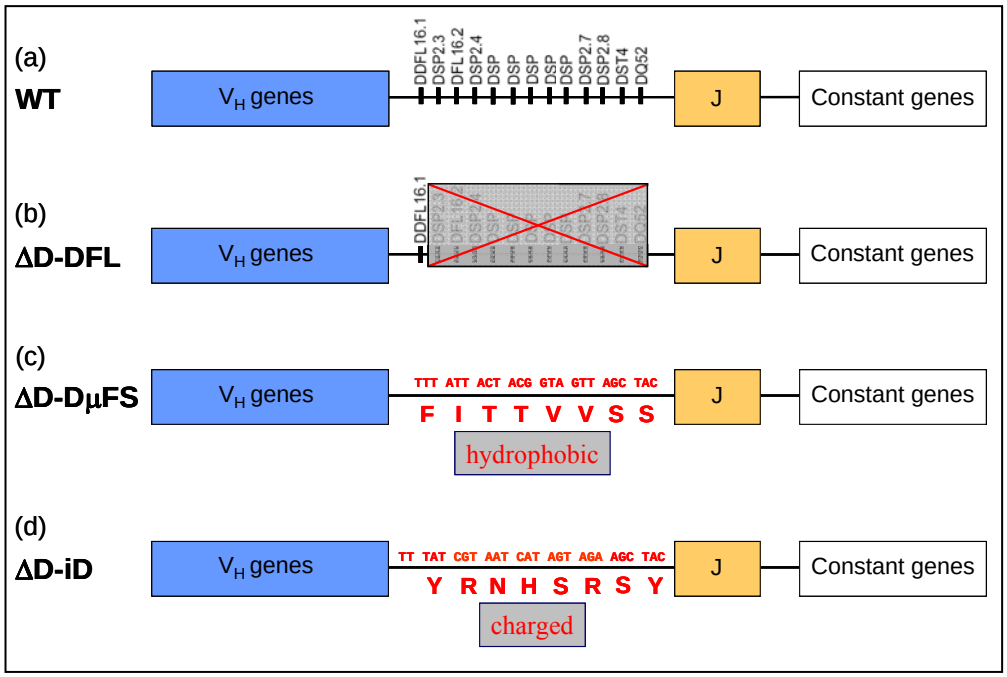


Figure 6

Generation of mice carrying a single DH gene segment

The murine IgH locus contains variable genes (blue), diversity-enhancing (D gene segments - black), joining (J gene segments - orange) and constant genes (white). (a) The D locus of BALB/c wildtype mice is made up of 13 DH genes. (b) In ΔD-DFL mice, only the DFL16.1 D gene segment remained while the other 12 of the DH gene segments are deleted by gene targeting with the aid of the Cre-*loxP* system. In ΔD-DμFS mice (c), the central amino acids of DFL16.1 in ΔD-DFL mice are replaced by hydrophobic and in ΔD-iD mice (d) by charged amino acids. For ΔD-DμFS mice (c) and ΔD-iD mice (d), the nucleotides of the respective single DH gene segments and the deduced amino acid sequences, translated in reading frame 1 (RF1) are also shown.

Cre-*loxP* gene targeting was used in a BALB/c embryonic stem cell line to create mice in which the DH locus was simplified to only one gene, *DFL16.1*, the most JH-distal DH gene segment. The other 12 DH gene segments were deleted. Thus, the ΔD-DFL mice have only

the most VH-proximal naturally DH gene *DFL16.1* in the DH locus, instead of 13 DH gene segments in WT animal [66] (Fig. 6).

The ΔD-DFL mice were used to create two other mutant strains, ΔD-iD and ΔD-DμFS mice, both of which also have only 1 DH gene segment each coding for HCDR3 amino acid sequences with very different properties.

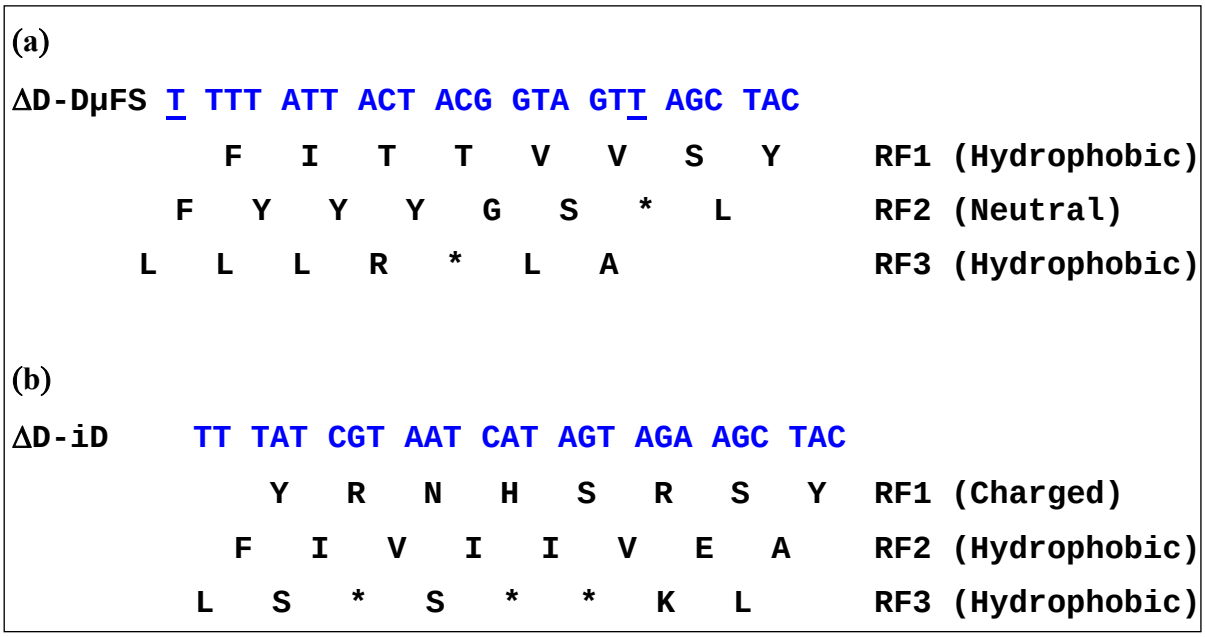


Figure 7

Reading frames of mutant DH genes in ΔD-iD and ΔD-DμFS mice

First line denotes the genomic nucleotide sequences (in blue), the followed three lines stand for the amino acid sequences in three reading frames (RF) (in black). Asterisks symbolize a stop codon. (a) and (b) represent the mutant DH gene of ΔD-DμFS and ΔD-iD mice, respectively.

In **ΔD-iD** mice, the central portion of *DFL16.1* was replaced with the complete inverted coding sequence of *DSP2.2* [67], [68]. In this way, central RF1 codons for tyrosine and glycine were replaced with those for arginine, histidine and asparagines. Thus, RF1 is coding for charged amino acids, while i-RF1 generates the original tyrosine enriched sequence of *DSP2.2*. The coding sequences for tyrosine and serine at the 5′ and 3′ termini of the *DFL16.1* DH gene segment were retained to preserve microhomology between the VDJ boundaries. RF2 produces hydrophobic stretches of HCDR3 and leads probably to new-Dμ protein expression. RF3 and i-RF3 (inverted RF3) contain termination codons (Fig. 7 b).

ΔD-DμFS mice were also derived from ΔD-DFL mutant mice. Hence, they harbor a modified *DFL16.1* gene in the DH locus while the other 12 DH gene segments were deleted. The reading frame of *DFL16* was shifted by insertion of two thymidine nucleotides (Fig. 7 a). While RF1 is thus coding for hydrophobic amino acids of old RF2, RF2 and RF3 comprise a stop codon and recreate the old RF1 and RF3, respectively.

1.11 Aim of the work

In the present communication, we analyzed the humoral immune response to the hapten phOx in the 2 mutant mouse strains ΔD-iD and ΔD- DμFS in the hope to answer the following questions:

1.) Is the VH/VL gene repertoire of anti-phOx antibodies identical to that of WT mice when the genome harbors only 1 DH-region gene instead of 13?
2.) Is the VH/VL gene repertoire identical, but the DH gene-dependent HCDR3 repertoire of the anti-phOx response restricted when it is generated from a single DH gene segment?
3.) Is the VDJ-recombination with only 1 available DH gene segment flexible enough to create the canonical Id_{Ox1} VH/VL gene combination together with the typical DRG D-region sequence? Are there differences in this respect between the two mutant strains?
4.) Is the Id_{Ox1} still dominating the anti-phOx response, *i.e.* does the Id_{Ox1} dominance depend on the VH171/Vκ072 gene combination only or is it really dictated by the DRG D-region sequence?
5.) How does the immune maturation in the 2 mutant strains develop from primary to memory responses, *i.e.* does immune maturation still depend on accumulation of somatic mutations in one or more particular primarily activated clonotypes?
6.) Is the immune maturation in these mutant mouse strains strong enough to develop clones of highest affinity?

2 Materials and methods

2.1 Materials

2.1.1 Buffer and media

Phosphate-buffered saline, PBS

137 mM sodium chloride (NaCl)

2.7 mM potassium chloride (KCl)

4.3 mM disodium hydrogen phosphate (Na_2HPO_4)

1.4 mM potassium dihydrogen phosphate (KH_2PO_4)

Adjust the pH to 7.4 with hydrochloric acid (HCl)

Carbonate bicarbonate buffer (0.5 M, pH 9.6)

Sodium hydrogen carbonate (NaHCO3)	2.93g
0.5 M disodium carbonate (Na2CO3)	1.59g
ddH_2O to	1 liter

Standard medium for cell culture

RPMI 1640	a bottle of powder for 10 liters
GlutaMAX	2 mM
Streptomycin	100 U/ml
Penicillin	0.1 mM
2ß-mercaptoethanol	0.1 mM
FCS	10 % final concentration
NaHCO3	20 g
ddH_2O	add 10 l

Adjust the pH to 7.4 with HCl. Sterilize by filtration (0.2 µm filter)

HT-Medium

Standard medium (see above) fortified with:

Hypoxanthin (50 mM)

Thymidin (0.2 mM)

10 % J774A.1-supernatant

Freezing medium

Standard medium

+ 10 % (v/v) Dimethylsulfoxyd (DMSO)

+ 10 % (v/v) FCS (20 % final concentration)

Filter through 0.2 µm filter.

Aliquot in 15 ml conical tubes and store in –20 °C freezer for long term periods

6X Loading Dye Solution

10mM Tris-HCl (pH 7.6)

0.03 % bromophenol blue

0.03 % xylene cyanol FF

60 % glycerol

60 mM EDTA

DEPC-treated water

0.1 ml DEPC

100 ml dd H_2O

Autoclave for 15 min to remove any trace of DEPC

10xPCR buffer

200 mM Tris-HCl

500 mM KCl

pH 8.4

EB

10 mM Tris.Cl

pH 8

10x TBE Buffer (Tris-Borate-EDTA)

108 mg Tris base

55 mg Boric acid

9.3 mg Na_4EDTA

Add ddH_2O to 1 liter

The pH is 8.3 and requires no adjustment.

2.1.2 Chemicals and instruments (all companies in Germany)

ABTS	Roche Diagnostics, Mannheim
Agarose	Serva, Heidelberg
Amphotericin B	Merck Biosciences, Darmstadt
Buffer for ABTS	Roche Diagnostics, Mannheim
Casein Natrium	Roth, Karlsruhe
Dimethylsulfoxid (DMSO)	Sigma, Taufkirchen
DNA Ladder Plus	Fermentas, St. Leon-Rot
Ethanol	Roth, Karlsruhe
Ethidium bromide	Roth, Karlsruhe
Fetal calf serum (FCS)	Invitrogen, Karlsruhe
GlutaMAX	Invitrogen, Karlsruhe
HAT	Sigma, Taufkirchen
Histopaque	Sigma, Taufkirchen
HT	Sigma, Taufkirchen
Imject Alum	Perbio Science, Bonn
Isopropanol	Roth, Karlsruhe
Minocyclin	Sigma, Taufkirchen
Oligonucleotide; HPSF purified	MWG, Ebersberg
Penicillin	Sigma, Taufkirchen
Polyethylenglycol PEG1500	Roche Diagnostics, Mannheim
RPMI 1640	Biochrom, Berlin
Streptomycin	Sigma, Taufkirchen
Taq polymerase	Fermentas, Leon-Rot
Tiamulin	Sigma, Taufkirchen
Trypan Blue, 0.4 %	Promega, Mannheim
6-well culture plates	Sarstedt, Nümbrecht
24-well culture plates	Sarstedt, Nümbrecht
cDNA Synthesis	Fermentase, Leon-Rot
Electrophoresis chamber	Serva, Heidelberg
ELISA reader	Bio rad, München
Forceps	Roth, Karlsruhe

Fusion culture plates	Greiner, Frickenhausen
Gel casting trays	Serva, Heidelberg
Loading buffer	Fementas, Leon-Rot
MaxiSorp plates	Nunc, Wiesbaden,
Multiscreen HTS plates	Milliport, Billerica, USA
PCR clean-up gel extraction	Macherey-Nagel, Düren
Petri dishes	Sarstedt, Nümbrecht
Power supply	Serva, Heidelberg
RNA Extraction Kit	Quiagen, Hiden
Sample combs	Serva, Heidelberg
Scissors	Roth, Karlsruhe
Sterile 0.2 ml microfuge tubes	Sarstedt, Nümbrecht
Sterile centrifuge tubes	Sarstedt, Nümbrecht
Thermal Cycler	Serva, Heidelberg
Total IgG Kit	Roche, Karlsruhe
Transilluminator	Bio rad, München
UV spectrophotometer tubes	Eppendorf, Hamburg

2.1.3 Antibodies

APC anti-mouse CD19	NatuTec, Frankfurt
FITC anti-mouse CD3e	NatuTec, Frankfurt
Goat anti-mouse IgG	Dianova, Hamburg
Goat anti-mouse IgG/M/A	Dianova, Hamburg
Goat anti-mouse IgM	BIOZOL Diagnostica, Eching

2.2 Methods

2.2.1 Mice

ΔD-iD and ΔD-DμFS mice, carrying a targeted deletion of 12 DH gene segments and intervening sequences [66], [68], were obtained from PD. Dr. M. Zemlin (Marburg) and maintained in the animal facility of the CAU under clean conventional conditions. After arrival, the mice were kept at last two weeks in the animal house to adapt to the new environmental conditions before starting with the experiments.

2.2.2 Preparation of hapten carrier complex (phOx-CSA)

The hapten-carrier-complex (phOx-CSA) was prepared as described by Mäkelä et al [69]. CSA (fraction V; 0.5 ml) was stirred with 37.5 mg of phOx in 20 ml of 5 % $NaHCO_3$ for 24 h at 4 °C.

After incubation, the mixture was centrifuged for 30 min at 35.000 g and then excessively dialyzed against 0.15 M NaCl. The amount of non-dialyzable phOx was estimated from the optical density assuming that phOx coupled to the lysine residues of the protein has the same optical density (OD_{352} = 32.000) as phOx coupled to epsilon-aminocaproic acid.

The conjugate contained 8.5 molecules of phOx per molecule of CSA. The final solution was sterile filtered, aliquoted in 200 µl and stored by –20 °C until usage. For generation of a strong immune response, the antigen was mixed with Imject Alum (Pierce; Rockford, USA) as an adjuvant at a 1:1 ratio for 30 min. Imject Alum is an aqueous solution of aluminum hydroxide (40 mg/ml) and magnesium hydroxide (40 mg/ml) plus inactive stabilizers.

2.2.3 Immunization

ΔD-iD and ΔD-DμFS mice were injected intraperitoneally (i.p.) with 80 µg of alum-adsorbed phOx-CSA. For analysis of the early and late primary response, mice were sacrificed on day 7 and 14, respectively. Spleens were teased apart and the spleen cells were fused by conventional PEG-mediated hybridization with the immunoglobulin-negative myeloma cell line Ag8.653 [70].

For analysis of the secondary response, mice were boosted 8 weeks later by i.p. injection of the same amount of Ag and spleen cells were fused on day three. Mice were rested until the antibody titers of the secondary immune response had declined after about two months. Then,

the tertiary i.p. immunization was performed and at day three, mice were sacrificed and spleen cells were fused with Ag8.653 myeloma cells. The antibody titers were tested regularly after every immunization and compared with that of wild type animals.

2.2.4 Collection of blood samples

The mouse was isolated in a small cage or container and warmed under an infrared lamp for 10-15 min. This treatment increased the blood flow to the tail, but should not hurt the mouse. The tail was swabbed with antiseptic and nicked with a sterile scalpel on the underside across one of the lower veins that should be visible. Several drops of blood could be collected and the mouse was returned back to its cage.

The blood was incubated at 37 °C for 1 h and shaken several times to dislodge the clot. After the tube was transferred to 4 °C for 2 h or overnight and spun at 10.000 x g for 10 min at 4 °C, the cell pellet was discarded and the supernatant was spun a second time for 10 min. The serum was transferred to a new tube, preserved by adding 0.02 % sodium azide or 0.001 % to 0.01 % thimerosal and frozen at –20 °C.

2.2.5 Cell culture

Myeloma and hybridoma cell lines were cultured in RPMI 1640 medium supplemented with foetal calf serum (FCS) to a final concentration of 10 % and 11 x 10^{-5} M β-Mercaptoethanol. FCS is used normally, because it contains very low concentrations of IgG molecules that may interfere in some assays or purifications. In addition, the medium was supplemented with antibiotics; penicillin at 100 U/ml and streptomycin at 100 µg/ml. The cells were cultivated in a CO_2-incubator at a concentration of 7.5 % CO_2, 37 °C and water-saturated atmosphere.

2.2.6 Counting of cells

Cell viability was assessed by trypan blue exclusion using a Neubauer haemocytometer. An aliquot of 0.2 ml cell suspension was prepared and mixed with 0.3 ml medium and 0.5 ml (0.4 %) trypan blue. Ten µl of the mixture was transferred to the counting chamber of the haemocytometer. After 1-2 min (but not longer as viable cells may die and begin to take up the dye) the number of cells could be counted within "counting squares". The calculated percentage of unstained cells represented the percentage of viable cells.

2.2.7 Thawing of cells

It was important to arrange all reagents before starting thawing of cells. 10 ml of medium were dispensed into a sterile 15 ml tube and warmed at 37 °C in a water bath. The freezing vial was removed from the liquid nitrogen and placed on ice until thawing in a water bath by gentle agitating. The O-ring and cap should be kept out of the water to prevent contamination. The vial was removed from the warmed water as soon as the contents were thawed.

After thawing, the entire vial had to be sprayed with 70 % EtOH (or antiseptic) and the cell suspension was poured out from the vial to the 10 ml of pre-warmed medium. After centrifugation for 5 min at 1.100 x g, the supernatant was aspirated with a Pasteur pipette and the cell pellet was dispersed by gentle tapping and flicking of the tube to prevent cell clumping when medium was added and resuspended in 5 ml fresh medium. The cell suspension was then transferred to a Petri dish (TC-quality) and placed in the CO_2-incubator.

2.2.8 Freezing of Cells.

Generally, cells were frozen slowly and thawed quickly for maximal survival. For freezing, cells had to be in log phase of growth and centrifuged at 1.100 x g for 7 min. The old medium was aspirated as completely as possible with a sterile Pasteur pipette and the cells were resuspended at a concentration of 0,5 - 2 x 10^6 cells/ml in freezing medium and aliquots of 1 ml were put into freezing vials (1.8 ml cryovials). These vials were placed in a -20 °C freezer for 1 h and then transferred to –80 °C. After several days, the tubes could be removed from the freezer and transferred to liquid nitrogen.

2.2.9 Cell fusion

Before fusion, myeloma cells should be cultured for a certain period of time in the logarithmic phase and maintained at a density of about 1 x 10^6 cells/ml. The mouse was sacrificed and the abdominal area swabbed with alcohol. After opening the abdomen with sterile forceps and scissors, the spleen was removed and placed into a sterile Petri dish containing 10 ml of serum-free RPMI 1640 medium. Splenocytes were passed through sterile Falcon 0.40 µm cell filter using syringe needle and sterile forceps. The suspension was transferred to a 15 ml tube and centrifuged for 5 min. Then, the supernatant was discarded, the pellet of splenocytes was washed twice in serum free medium, resuspended in 10 ml medium and the viable lymphocytes were counted in a haemocytometer.

Concurrently with the splenocytes, Ag8.653 myeloma cells were washed twice with serum-free medium, centrifuged for 5 min at 1.100 x g, resuspended in 10 ml medium and counted. The spleen and myeloma cells were mixed in a 50 ml tube at a ratio of 2:1-5:1 and centrifuged for 5 min at 1.100 x g. The supernatant fluid was aspirated as completely as possible with a Pasteur pipette and the pellet was gently disrupted by tapping the bottom of the tube. One ml of warmed 50 % PEG 1500 (w/v) was added dropwise and slowly to the pellet over a period one min while tapping the side of the tube to achieve thorough mixing. Then, the PEG/cell mixture was gradually diluted under continuous and gentle swirling of the tube: 5 ml serum-free medium for 5 min and thereafter 25 ml serum free medium for 5 min.

After centrifugation for 5 min at 1.100 g, the supernatant was removed and the fused cells gently resuspended and mixed with 400 ml volume of standard RPMI 1640 medium supplemented with 10 % FCS, HAT selection medium, 10 % J774A.1 supernatant, 2.5 ml Ciprobay and 5 µg/ml Amphotericin B. The cell suspension was distributed in fusion plates (1.5 ml/well) and incubated for the next 10 days at 37 °C and 8-10 % CO_2.

The fusion plates were examined visually 24-48 h after fusion for any abnormalities (*e.g.* bacterial contaminations) and during the next days for growing of cell clusters. At day 10, 0.5 ml aliquots were taken from every well and tested for phOx-reactive antibodies by ELISA.

2.2.10 Determination of hybridomas secreting phOx-specific antibodies

96-well microtiter plates (Nunc; Wiesbaden, Germany) were coated with phOx-BSA using multichannel pipette (50 µl/well; 10µg protein/ml sodium carbonate buffer; 0.05 M, pH 9.6) and blocked with casein (1 % casein in PBS, 200µl/well). Antibody-containing cell culture supernatant fluids (50 µl/well) were added and incubated for 1 h. After careful washing, HRP-labeled goat anti-mouse antiserum was added as a secondary antibody and incubated for 1 h. All incubations were performed at 37 °C for 1h. Washing was performed with PBS/T using an automatic washer. ABTS was used as substrate (50 µl/well), and the absorbance was measured at a 405 nm wavelength. Extinction values exceeding those of the negative controls with PBS, instead of phOx-BSA, by a factor of 5 were considered positive. Clones of positive wells picked up and transferred separately to 24 well plates. One week later, the clones of 24 well plates were tested again for reactivity with phOx-BSA, but non-reactivity with BSA. Antibodies fulfilling this criterion were considered as phOx-specific.

2.2.11 Isolation of total RNA

Total RNA was extracted with the aid of Qiagen´s RNEasy mini kits according to manufacturer's instructions (Qiagen, Hilden, Germany). $1x10^7$ myeloma cells were used to cover the maximum binding capacity of this column (100 µg RNA). After centrifugation of the cells at 1.100 g for 7 min, the cell pellet was disrupted by addition of 600 µl lysis buffer (RLT) and homogenized by vortexing for 1 min. One volume (600 µl) of 70 % ethanol was added and mixed thoroughly by pipetting. 700 µl of the solution was transferred to an RNEasy mini column and centrifuged for 15 sec at 10.000 g. The RNA was bound to the column material and the flow-through was discarded. For cleaning of the column-bound RNA, 700 µl wash buffer (RW1) was spun through the column for 15 sec at 10.000 g. Then, the collection tube was changed to a clean 1.5 ml Eppendorf tube and 500 µl of a second wash buffer (RPE) was spun through the tube again for 15 sec at 10.000 g. Then, 500 µl of RPE buffer was added to the tube and spun for 2 min at 10.000 g to ensure that the column was dry. As an additionally precaution against contamination, the collection tube was changed again and the column was spun for 1-2 min at maximum speed (13.000 g). To elute the RNA from the tube, 70 µl of RNase-free H_2O was added directly to the filter in the column and spun for 1 min at 10.000 g. The RNA concentration was determined spectrophotometrically at 260 nm and extracted RNA was frozen at –20 ºC.

2.2.12 Synthesis of complementary DNA

The cDNA library was constructed using a "RevertAid First Strand cDNA Synthesis" kit according to the manufacturer's protocol (Fermentas**,** St. Leon-Rot, Germany). 8 µl of total RNA was added in a 50 µl sterile tube on ice, mixed gently with 1 µl oligo$(dt)_{18}$ primers (0.5 µg/µl) and 3 µl of DEPC-treated water and spun down for 5 sec in a microcentrifuge. The mixture was incubated at 70 °C for 5 min and the tube placed on ice. Then, the following components were added: 4 µl of 5 x reaction buffer, 1 µl of Ribolock ^{TM} (ribonuclease inhibitor, 20 U/µl) and 2 µl of 10 mM dNTP mix, mixed gently and incubated at 37 °C for 5 min. Now, 1 µl of RevertAid^{TM} M-Mulv (reverse transcriptase, 200 U/µl) was added and the mixture was incubated at 42 °C for 60 min. Finally, the reaction was stopped by heating at 70 °C for 10 min. The new synthesized cDNA could be used directly in PCR reaction or frozen at –20 °C.

2.2.13 Polymerase chain reaction (PCR)

The PCR reaction was performed in volumes of 50 µl and the following components were set up in 200 µl sterile tube:

5 µl	10x PCR reaction buffer
5 µl	DMSO
1 µl	dNTP (10 mM)
1.5 µl	$MgCl_2$ (50 mM)
1 µl	3´ primer (10 µM) (Tab. 1)
1 µl	5´ primer (10 µM) (Tab. 2)
0.4 µl	Taq polymerase (5 U/µl)
2 µl	cDNA (from first-strand reaction)
33 µl	autoclaved, distilled water

The sample was mixed by pipetting up and down and loaded in a thermocycler at 94 °C. The thermocycle included 1 cycle at 94 °C for 2 min of initial denaturation, and 35 cycles at 94 °C for 1 min, at 72 °C for 1.5 min, and at 54 °C for 1.5 min, followed by a final extension at 72 °C for 10 min. The amplified DNA was then chilled at 4 °C. The PCR products were separated by electrophoresis in a 1 % agarose gel, stained with ethidium bromide and the desired fragments were cut out and purified.

Table 1

Primers 3´→5´

Name of primers	Sequences	T_M[°C]
KLM13	gta aaa cga cgg cca gtg gaa gct tac tgg atg gtg	72.9
CHIgG M13	gta aaa cga cgg cca gtg gaa gct tay ctc cac aca	72.3
CM 15	gct ctc gca gga gac	53.3

KL – primer of constant L chain. CHIgG – primer of constant H chain of IgG.

CM 15 – primer of constant H chain of IgM.

T_M [°C] = 2 (n_A +n_T) + 4 (n_G + n_C), annealing temperature.

Y = C, T

Table 2

Primers 5′→3′

Name of primers	Sequences	T_M[°C]
VLFR1M13rev	gga aac agc tat gac cac aga crt cma gat ray cca	70.6
VLFR2M13rev	gga aac agc tat gac cac ara mat tkt gct gac yca	69.5
VLFR3M13rev	gga aac agc tat gac cac aga try tkt gat gac cca	70.0
VLFR4M13rev	gga aac agc tat gac cac asr aaw tst tct cwy mca	68.9
VLFR5M13rev	gga aac agc tat gac cac arr crt tst gat gwc aca	70.0
VLFR6M13rev	gga aac agc tat gac cac aga tat tgt gat rac kca	68.3
VLFR7M13rev	gga aac agc tat gac cac arr yat tgt gat gac cca	70.0
VH1M13rev	gga aac agc tat gac cag taa gac gtc mag ctt cag	71.2
VH2M13rev	gga aac agc tat gac caa att gac gtc car syg cag	71.7
VH3M13rev	gga aac agc tat gac caa att gac gtc cag ytg swg	71.2
VH4M13rev	gga aac agc tat gac cac agc tga ags ast cag gac	72.9
VH5M13rev	gga aac agc tat gac cam wgs kgg tgg agt ctg ggg	75.2
VH6M13rev	gga aac agc tat gac caa rss tgg tgg aat ctg gag	72.3
VH7M13rev	gga aac agc tat gac caa rgs tgr tsg agt ctg gag	72.9
VH8M13rev	gga aac agc tat gac cac agc tgc agc agt cwg tg	73.0
VH9M13rev	gga aac agc tat gac cam asy tgs wgg wgw	68.1
VH10M13rev	gga aac agc tat gac cac agm tsc agc agy ctg g	73.1
UmIgVH	tga ggt gca gct gga gga gtc	68.39
UmIgVK	gac att ctg atg acc cag tct g	62.74

VH – primers of variable H chains. VL – primers of variable L chains. UmIg – universal Ig primers. T_M [°C] = 2 (n_A +n_T) + 4 (n_G + n_C), annealing temperature.

N = A, C, G, T	B = G, T, C	M = A, C	S = G, C
V = G, A, C	H = A, T, C	R = A, G	Y = C, T
D = G, A, T	W = A, T	K = G, T	

2.2.14 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis is a method used in biochemistry and molecular biology for separation of DNA or RNA molecules according to size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate further than longer ones.

To pour a gel, agarose powder was mixed with electrophoresis buffer (TBE) to a final concentration of 1 %, and then heated in a microwave oven until completely melted (10 min, 400 W). After cooling the solution to about 60 °C, it was poured into a casting tray containing 2 combs, one at the middle of the gel and the other at one side, about 5 mm from the end of the gel and allowed to solidify at room temperature or in a refrigerator.

After the gel was solidified, the combs were removed using care not to rip the bottom of the wells. The gel in its plastic tray was inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA were mixed with loading buffer and then pipetted into the sample wells (30 µl), the lid and power leads were placed on the apparatus and a current was applied (100 V, 400 mA). DNA migrated towards the positive electrode, which is usually colored red.

After 35 min, the gel was stained by soaking in a dilute solution of ethidium bromide for 15 min. To visualize amplified DNA, the gel was placed on an ultraviolet transilluminator. Now, the desired DNA bands could be cut out of the gel and dissolved to retrieve the purified DNA fragments.

2.2.15 Isolation of DNA from agarose

QIAquick PCR purification kit (Qiagen, Hilden, Germany) was used to isolate and extract DNA fragments from the gels. The amplified DNA fragments were separated in horizontal agarose gels and visualized under UV light. The respective DNA fragments of about 450 pb were cut from the gel and placed into a microcentrifuge tube.

All buffers needed for the DNA isolation were included in the kit. In the first step, 3 volumes of binding buffer (QG) were added to 1 volume of gel slice and solubilized in a water bath or thermomixer at 50 ºC. After complete solubilization, 1 gel volume of isopropanol was added and the solution was immediately applied onto a mini spin column. During centrifugation (1 min, 10.000 g), the DNA was bound onto the column material. The flow-through was discarded and 500 µl of washing buffer (QC) was added to wash away all traces of agarose. The column was centrifuged for 1 min and the flow-through was discarded. In a third centrifugation step, 750 µl of washing buffer (PE) was added and spun through. Then the column was spun for a second time to ensure complete removal of the buffer. At that time, the spin column was placed into a clean and sterile microcentrifuge tube. To elute the purified DNA, 50 µl of sterile water or elution buffer (EB) was added and the column was centrifuged for 1 min at 10.000 x g. The concentration of the extracted DNA could be determined by UV spectrophotometer.

2.2.16 Determination nucleic acid concentration

Nucleic acid concentrations were determined in an UV spectrophotometer by measurement of the extinction (E) at 260 and 280 nm using UV tubes, where an E260 = 1 corresponds to a concentration of 40 ng/µl RNA or 50 ng/µl double-stranded DNA [71]. The cleanness was controlled by means of the quotients E260/E280, which should lie between 1.8 and 2.0 [71].

2.2.17 DNA sequencing

The DNA sequencing was performed using the dye-terminator Sanger sequencing method and Roche FLX sequencer provided by different companies (Agova; Berlin, Germany, Qiagen; Hilden, Germany). In addition, variable sequences of some antibodies were determined at the Institute of Clinical Molecular Biology of Kiel University.

2.2.18 Determination of serum anti-phOx antibody titers

96-well microtiter plates (Nunc; Wiesbaden, Germany) were coated with phOx-BSA with the aid of multichannel pipettes (50 µl/well; 10µg protein/ml sodium carbonate buffer; 0.05 M, pH 9.6) and blocked with casein (1 % casein in PBS, 200µl/well). The sera were diluted serially in PBS/T and 50 µl/well were added. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgM Abs were diluted 4000-fold in PBS/T and 50 µl/well were added. All incubations were performed at 37 °C for 1 h. After every incubation step, three washes with PBS/T were performed using an automatic washer. ABTS was used as substrate (50 µl/well), and the absorbance was measured at a 405 nm wavelength.

2.2.19 Determination of relative affinity of phOx specific Abs

Determination of relative affinity of phOx specific IgG and IgM antibodies was done in two steps. In the first step, the dilution factor corresponding the 80 % of maximal binding of every supernatant was determined. In the next step, the concentration of 80 % value was used in an inhibition assay. Antibodies that did not reach maximal binding plateau were excluded from further investigation.

96-well flat-bottomed microtiter plates (Nunc; Wiesbaden, Germany) were coated with phOx-BSA and then 25 µl of doubled concentration of the 80 % value of every supernatant and 25 µl of serially diluted phOx-caproic acid were incubated for 1 h at RT. After washing, 50 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgM Abs were added to

the wells and the plates were incubated for 1h at RT and then washed three times in PBS/T. ABTS (50 µl/well) was used as a HRP-substrate and incubated in the dark at RT for 10 min. The absorbance of the wells was read at 405 nm. PhOx-caproic acid concentration giving 50 % binding inhibition was considered as relative affinity of the investigated anti-phOx antibody. An affinity factor was generated as the quotient of the relative affinity of the prototype Ox1-idiotypic antibody NQ2/16.2 divided by that of a particular antibody. Hence, antibodies with an affinity factor of >l exhibited a higher while those with an affinity factor of <l a lower affinity than NQ2/16.2.

2.2.20 Idiotypic determination of phOx specific antibodies

Since the monoclonal anti-idiotypic antibody (Ab2) 8-21/W18 recognizes an Ox1 idiotype-(Id_{Ox1})-specific idiotope of anti-phOx specific antibodies, this antibody was used to characterize anti-phOx antibodies generated in ΔD-DµFS and ΔD-iD mice.

Microtiter plates were coated with phOx-BSA and residual surface of the wells were blocked with 1 % casein in PBS. After addition of 25 µl of anti-phOx antibody-containing supernatant fluid and 25 µl of serially diluted 8-21/W18 supernatant (1/1 – 1/4800), the plates were incubated for 1 h at RT. After washing, 50 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgM Abs were added to the wells and the plates were incubated for 1 h at RT and then washed three times with PBS/T. ABTS (50 µl/well) was added as a HRP-substrate and incubated in the dark at RT for 10 min. The absorbance of the wells was measured at 405 nm. Id_{Ox1} anti-phOx antibodies showed a descending curve in an 8-21/W18-depended manner, while Id-Ox1 negative Abs were not affected by addition of the anti-Id 8-21/W18 and showed a straight line in the plot (absorbance against Ab2 concentration).

2.2.21 Flow cytometric measurement

A single cell suspension was prepared from freshly isolated spleen and bone marrow using scalpel, forceps and cell strainer (0.22 µl). The cell suspension was centrifuged 5 min (4,000 x g) at 4 °C and the pellet was resuspended in 3 ml of complete RPMI 1640 medium. Red blood cells were removed by density gradient of Histopaque (Sigma-Aldrich Chemie; Munich, Germany) centrifugation. Three ml of Histopaque was filled into a 15 ml conical tube and 3 ml of the cell suspension was carefully layered on top. After centrifugation at 400 x g for exactly 30 min at RT, the upper layer was discarded and the cells lying on top of the opaque interface were quantitatively collected with a Pasteur pipette and transferred into a new 15 ml

centrifuge tube. The cells were washed two times with PBS, and then resuspended in 3 ml PBS, 5 % FCS (staining buffer) and a small sample (50 µl) was used to perform a cell count and viability analysis by Trypan Blue.

The cell suspension was adjusted to concentrations of 1-10 x 10^5 cells/sample (0.5 ml) in U bottom plastic centrifuge tube (Sarstedt; Nümbrecht, Germany). After centrifugation, the purified cells were suspended in 50 µl of staining buffer and pre-incubated with 1 µl (0.5 µg) of anti-CD16/CD32 (anti-FC) for 5-10 min at RT to block unspecific staining by reaction with Fc-receptors (FcR) of the cells. For the staining, 1.25 µl (0.25 µg) of Allophycocyanin (APC) anti-CD3e and 2 µl (1 µg) of fluorescein isothiocyanate (FITC) anti-CD19 were added pro sample. The negative controls were performed only with staining buffer without any antibodies. The samples were mixed by gently vortexing or tapping and incubated for 60 min in the dark at 4 °C. After incubation, 3 ml of staining buffer was added, mixed, and centrifuged at 400 x g for 5 min. After the cell pellet was resuspended in 500 µl of PBS, 2 % FCS on ice, the cell sample could be analyzed in the flow cytometer.

2.2.22 Flow cytometric staining of dead cells with 7-Amino-actinomycin D

7-Amino-actinomycin D (7-AAD) intercalates into double-stranded nucleic acids. Since it is excluded by viable cells but can penetrate cell membranes of dying or dead cells it is suitable for determination of dead cells in flow cytometric assays.

The cells were stained as outlined above in the protocol for dual color staining with APC and FITC monoclonal antibodies. After the last washing step, the cells were resuspended as usual in 500 µl of PBS containing 2 % FCS. Then, viability of the cell sample was assessed by addition of 1-2 µl of 7-AAD solution per tube. The samples were kept in this solution as usual at 4 °C and protected from light until analysis in the flow cytometer.

2.2.23 Flow cytometric analysis

Cell fluorescence was analyzed with a FACScan apparatus (BD Biosciences; San Jose, USA) using an excitation laser wavelength of 488 nm. Cell populations of interest were gated and analyzed using CellQuest 3.3 software. Gates were set to eliminate debris.

2.2.24 The enzyme-linked immunospot assay (Elispot)

The Elispot assay provides an effective method of measuring single plasma cells secreting antigen-specific antibodies. Each well of MultiScreen plates (Millipore; Billerica, USA) was pre-wetted with 15 µl of 35 % ethanol for 1 min and then rinsed with 150 µl sterile PBS three times before the ethanol evaporates. One plate was coated with 100 µl (10 µg/ml) phOx-BSA in carbonate buffer (0.05 M, pH 9.6). In parallel, a second plate was coated with BSA alone under the same condition as phOx-BSA. Then, the plates were incubated over night at 4 °C. After two washing steps with 150 µl bidest. H_2O per well, the membrane was blocked with 150 µl per well of culture medium (RPMI 1640, 10 % FCS, penicillin, streptomycin, glutamine) for at least 2 h at 37 °C. 100 µl (ca. 5 x 10^5 cells) of purified mononuclear cell suspension isolated from spleen and BM (as described for flow cytometry using a Histopaque density gradient) was added per well.

The cells were incubated for 18 h at 37 °C in 5 % CO_2 incubator and then removed by washing the plate 6 times with PBS/0.01 % Tween 20. 100 µl of secondary antibodies (HRP-conjugated anti-IgG or anti-IgM, diluted 1/40,000 in PBS) was added per well and incubated for 1 hr at 37 °C in an atmosphere containing 5 % CO_2 and 95 % humidity. After this incubation period, the secondary antibodies were decanted and the plate was washed 3 times with PBS-0.01 % Tween 20, followed by 3 washes with PBS. 100 µl/well of 3-Amino-9-Ethylcarbazol (AEC) was added and incubated until dark spots emerge. Spot development was stopped after 10 min under running water and left to dry. After complete drying, the spots were counted under a dissecting microscope. The number of phOx-specific antibody-secreting plasma cells was calculated by subtracting the number of spots of BSA- from phOx-BSA-coated wells.

2.2.25 Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison post test, using GraphPad Prism for Windows (version 4.02, GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

2.2.26 Hydropathicity of the HCDR3

The hydropathicity of the HCDR3 interval was determined with normalized Kyte-Doolitle hydropathicity index [72, 73]. The average hydropathicity was calculated by adding the hydropathicity value for each amino acid in the HCDR3 divining by the number of amino acid.

3 Results

Investigations of 3 genetically engineered mouse strains have demonstrated that B cell development and immune responses strongly depend on the number of available DH gene segments and the sequence of particular remaining single DH genes. ΔD-DFL mice containing only the natural occurring DFL16.1 gene segment in the DH locus showed, in comparison to WT mice, a rather normal B cell development and an almost unaltered response to the TD antigen NP-CGG [66]. Although the number of immature IgM^+IgD^- B cells in the bone marrow was decreased, numbers of mature IgM^+IgD^+ B cells in BM and spleen as well as B1a, B1b and B2 in the peritoneal cavity were as in WT mice. Length and amino acid compositions of DFL-16.1-encoded HCDR3 of BM B cells resembled those of WT mice, but differed in non-DFL16.1-related D regions. In ΔD-iD mice with an inverted DH gene segment encoding for the charged amino acids asparagine, histidine and arginines (instead of the normally predominating tyrosine and glycine), marginal zone (MZ) B cells were increased, but the numbers of mature B cells in BM and spleen as well as B1 cells in the peritoneum were reduced to half and immune responses to thymus-dependent (TD) as well as thymus-independent (TI) antigens were substantially reduced [68]. A similar strong impact on B cell development and TI-2 antigen-induced IgM immune responses was observed in ΔD-DμFS mice containing a frame-shifted DFL16.1 gene in the DH locus [74, 75]. In these mice, kept under specific pathogen-free conditions, immunoglobulin serum concentrations of IgM, all IgG subclasses and IgA and the development of primary and secondary immune responses to TD antigen NP-CGG were identical to those of WT mice. The latter 2 strains, ΔD-iD and ΔD-DμFS mice, are under investigation here.

3.1 Composition of B and T cell compartments in mutant mouse strains

For a first broad characterization of the immune systems of our mutant mice and a comparison to published data [66, 68, 74, 75], we examined the compartment of B cells in BM and spleen. Moreover, we determined the size of the T cell compartment and compared it with that of WTe mice for two reasons: 1.) Since we surmised that the selection of HCDR3 amino acid composition might be connected with T cell regulation (see 1.9), the reduced B cell compartment may also cause a reduction of the total number of T cells. 2.) The development of B and T cells are not independent of each other but mutually regulatory as demonstrated in several investigations: a) B cells and antibodies participate in the selection of the T cell repertoire [76] and this immunoglobulin-dependent T cell selection operates only during the first 3

weeks of life [77]. b) Spleens of B cell deficient mice show a three- to fivefold reduction in T cell numbers [78]. c) Studies of B cell deficient mice demonstrate that B cells are not only important for antibodies production, but also required for efficient T cell responses [79, 80].

Total mononuclear cells were isolated from BM and spleen of WT, homozygous ΔD-DμFS and homozygous ΔD-iD mice. After purification by Histopaque gradient centrifugation, cells were analyzed by fluorescence-activated cell sorting (FACS).

The average number of splenic mononuclear cells of ΔD-DμFS and ΔD-iD mice was about 2.5 and 2 fold lower than in WT mice. A similar reduction was also seen for lymphocyte counts. Lymphocytes in the spleen of ΔD-DμFS mice were 2.6 times lower and those of ΔD-iD mice were 2.4 times lower as in WT mice. In contrast, in the bone marrow, numbers of total mononuclear cells as well as lymphocytes of both mutant strains and WT mice were rather identical (Tab. 3).

The flow cytometric analysis of total T and B cell numbers in the spleen were determined with goat-anti-mouse CD3 and CD19, respectively, as shown in Fig. 8 and summarized in Tab. 3. In the spleen of ΔD-DμFS mice, the total number of 8.1 x 10^5 T and 20.6 x 10^5 B cells was approximately two and fourfold lower than in WT mice (29.2 x 10^5 and 74.4 x 10^5, respectively). The splenic T and B cells of ΔD-iD mice were reduced to a similar extent (Tab. 3). When relating the proportion of T and B cells to total numbers of mononuclear cells, the following picture merged. Compared to WT mice with 14.7 %, the contribution of T cells of ΔD-DμFS (10.3 %) and ΔD-iD (13.2 %) among mononuclear cells remained almost unchanged. However, the percentages of B cell were relatively decreased, i.e. 26.3 % in ΔD-DμFS and 25.4 % in ΔD-iD mice compared to 37.5 % in WT mice.

Flow cytometric analysis of bone marrow cells demonstrated a much more heterogeneous picture of cells (Fig. 9), but numbers of B and T lymphocytes were rather similar in both mutant mouse strains as well as in WT mice (Tab. 3).

Hence, compared to WT mice, both mutant mouse strains have significantly reduced amounts of total mononuclear cells as well as T and B lymphocytes in the spleen. However, the proportion of T cells among mononuclear cells was rather unchanged while B cell counts were significantly reduced. We therefore compared the concentrations of total IgM and IgG in mutant and BALB/c WT mice. Total IgM concentration of ΔD-DμFS mice (~144 μg/ml) was comparable with that of WT (~153 μg/ml), while ΔD-iD mice showed a slightly decreased IgM level of about 99 μg/ml (Fig. 10). Thus, amounts of IgM immunoglobulin were in the

same range in all 3 strains. IgG concentrations, however, were significantly reduced in both mutant strains (Fig. 10).

Table 3

FACS analysis of spleen and bone marrow cells of BALB/c wild-type and ΔD-iD and ΔD-DμFS mutant mice

	Total mononuclear cell x E+05	Lymphocytes x E+05	T cell x E+05	B Cell x E+05
Spleen				
WT	197.6 ± 24.5	118.2 ± 15	29.2 ± 8.3 (14.7 %)	74.2 ± 17.6 (37.5 %)
ΔD-DμFS	78.2 ± 17.6	45.2 ± 7.8	8.1 ± 3.2 (10.3 %)	20.6 ± 6.3 (26.3 %)
ΔD-iD	99 ± 12.8	48.5 ± 8.1	13.1 ± 6.4 (13.2 %)	25.2 ± 10.2 (25.4 %)
Bone marrow				
WT	8.02 ± 1.46	0.42 ± 0.105	0.037 ± 0.012 (0.46 %)	0.33 ± 0.12 (4.1 %)
ΔD-DμFS	8.22 ± 1.12	0.53 ± 0.11	0.046 ± 0.011 (0.55 %)	0.44 ± 0.08 (5.3 %)
ΔD-iD	7.16 ± 1.43	0.48 ± 0.12	0.033 ± 0.013 (0.46 %)	0.40 ± 0.09 (5.5 %)

Legend

Spleen and bone marrow mononuclear cells were isolated by Histopaque gradient centrifugation. Cell numbers ± standard error of the mean of five separately analyzed cell preparations are given for 1 spleen or refer to the bone marrow of two femurs of five 10-wk-old ΔD-iD, ΔD-DμFS or wild-type BALB/c mice. The numbers and percentages of B ($CD19^+$) and T ($CD3^+$) cells are related to the amount of total mononuclear cells. Lymphocytes are calculated from gate P1 in Fig. 6 and 7.

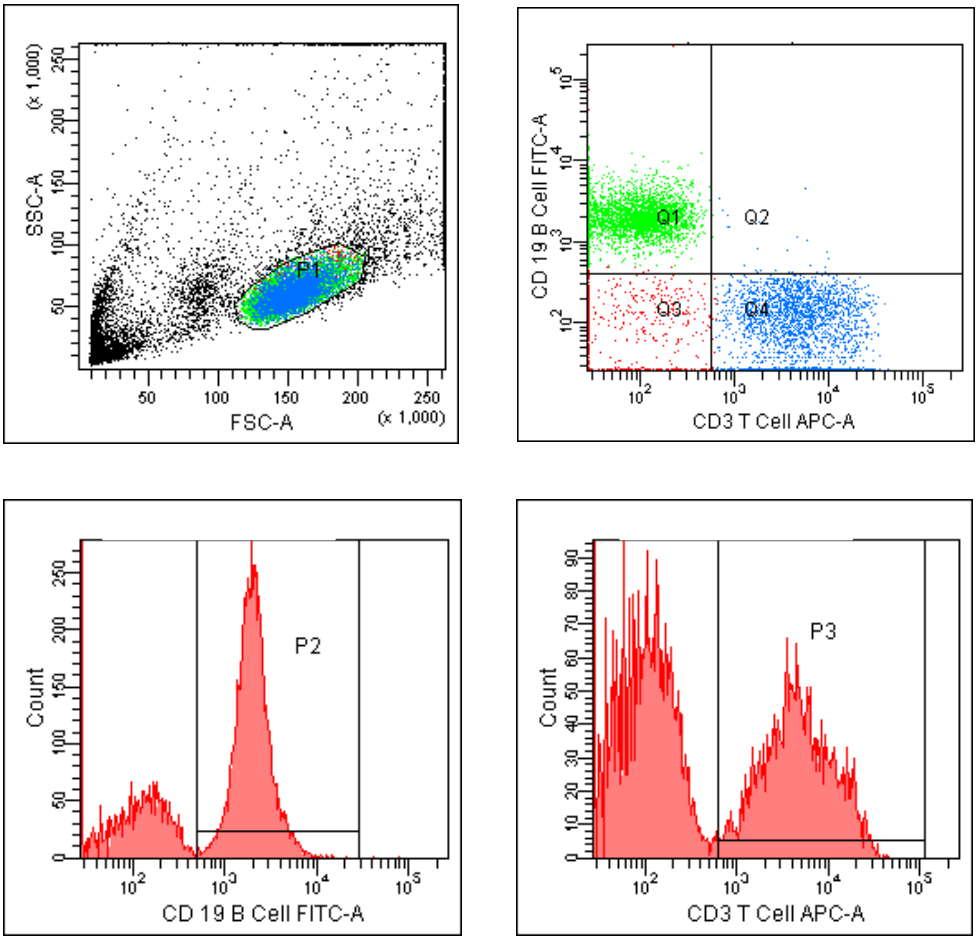


Figure 8

Flow cytometric analysis of splenic lymphocytes of homozygous ΔD-iD mice

Cells within the lymphocyte gate of spleen cells (upper left panel) were differentiated on the basis of CD19 and CD3 expression. The top right histogram shows the dot plot analysis of splenic B lymphocytes stained with FITC-labeled anti-CD19 versus T cells stained with APC-labeled anti-CD3. The lower two panels of gated splenic lymphocytes show the intensity of staining with both specific antibodies. P1, P2 and P3 represent the total number of lymphocytes, B and T cells, respectively. The data show representative staining of five separately analyzed spleen cell preparations. Cell viability (> 98 % data not shown) was estimated by staining with fluorescent marker 7-Aminoactinomycin D. The analysis of splenocytes of wildtype as well as homozygous ΔD-DμFS mice gave similar results (data not shown). The average total numbers of different cells type in the spleen are depicted in Tab. 1.

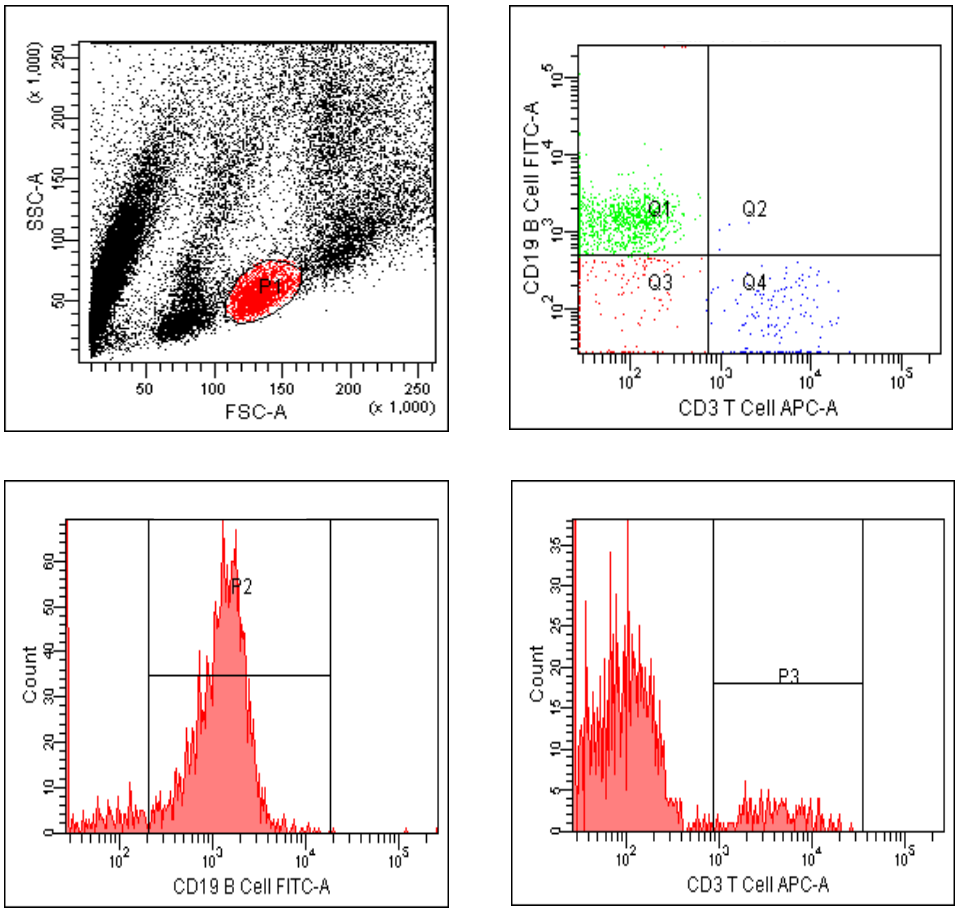


Figure 9

Flow cytometric analysis of bone marrow lymphocytes of homozygous ΔD-iD mice

Cells within the lymphocyte gate in the bone marrow (upper left panel) were differentiated on the basis of CD19 and CD3 expression. The top right histogram shows the dot plot analysis of BM B cells stained with FITC-labeled anti-CD19 versus T cells stained with APC-labeled anti-CD3. The lower two panels of gated BM lymphocytes show the intensity of staining with both specific antibodies. P1, P2 and P3 represent the total number of lymphocytes, B and T cells, respectively. The data show representative staining of five separately analyzed BM cell preparations. Staining with fluorescent marker 7-Aminoactinomycin D showed a cell viability of > 98 % (data not shown). The analysis of bone marrow cells of wildtype as well as homozygous ΔD-DμFS mutant mice gave similar results (data not shown). The average total numbers of different cells type in the spleen are depicted in Tab. 3.

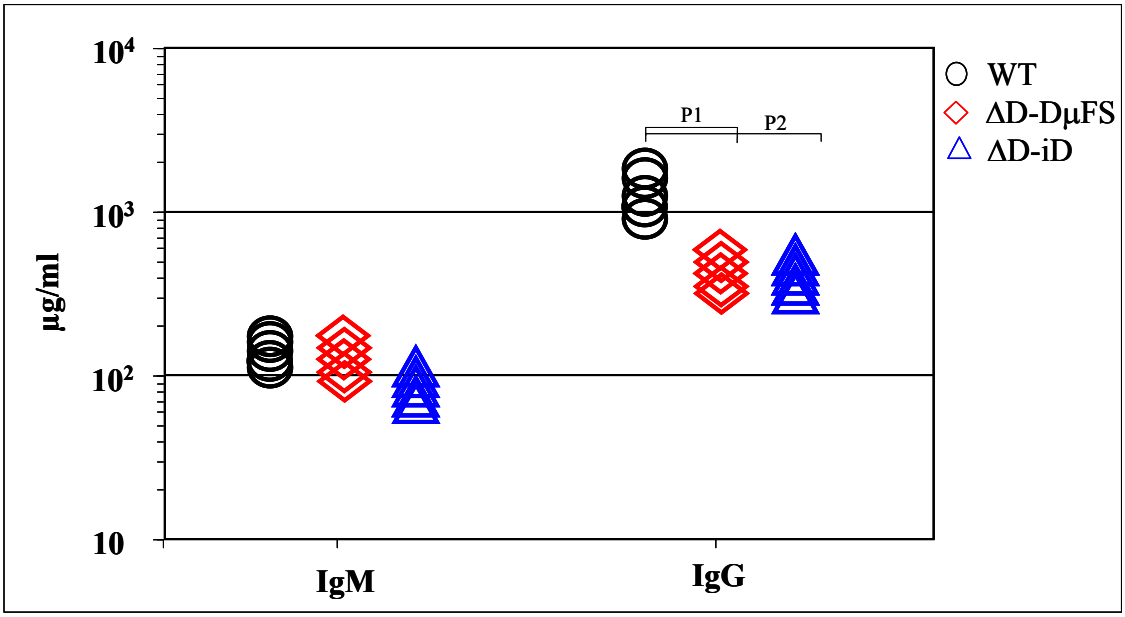


Figure 10

Total IgM and IgG concentrations in normal sera

Immunoglobulin concentrations of 10-weeks old ΔD-DµFS (red lozenge), ΔD-iD (blue triangle) and WT (black circle) mice were determined with isotype-specific secondary antisera. P1 < 0.0003, P2 < 0.0004.

3.2 Comparison of phOx-specific background plasma cells and serum antibody concentrations

Hence, the question arose as to whether the observed global changes in the T and B cell compartments may influence the starting position for a specific immune response. Since WT BALB/c mice contain considerable concentrations of phOx-reactive antibodies in the serum [81], we first compared the background anti-phOx-secreting plasma cells and serum antibodies in mutant mice with that of WT mice. Plasma cells secreting phOx-specific IgM or IgG were determined with the Eli-spot assay. In this test, cells are incubated in culture wells on antigen-coated membranes so that secreted antibody can bind to the antigen-coated surface. After washing off the cells, bound antibody is detected with enzyme-labeled secondary antibody. Plasma cells are indicated by coloured spots as shown in Fig. 11. Surprisingly, compared to WT control mice, numbers of phOx-reactive IgM-secreting plasma cells were significantly enhanced in spleens as well as in the bone marrow of both mutant strains (Fig. 12). However, plasma cells secreting phOx-reactive IgG antibodies were missing in both transgenic strains as well as in WT mice (Fig. 12).

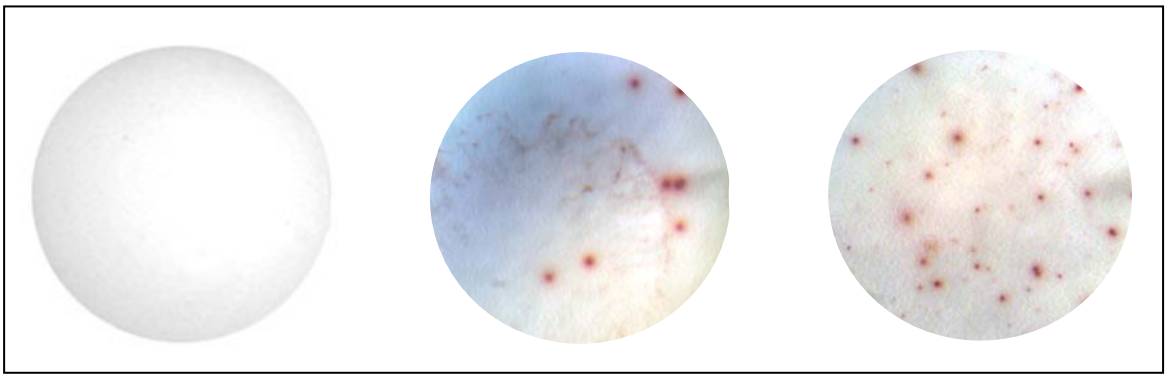


Figure 11

Determination of anti-phOx secreting splenic plasma cells with the ELI-spot assay

At an age of 10 weeks, BALB/c mice received a primary immunization with the TD antigen phOx-CSA. On day 7, mononuclear spleen cells were isolated by Histopaque gradient centrifugation. For determination of IgM and IgG anti-phOx secreting plasma cells, graded concentrations of mononuclear cells were incubated for 18 hr in phOx-BSA- or BSA-coated wells. Bound antibodies were detected with secondary antiserum to IgG (B) or IgM (C). In the negative control (A), wells were reacted with all reagents, but mononuclear cells were omitted. Splenocytes and bone marrow cells of ΔD-iD and ΔD-DμFS mice provided a similar pattern (data not shown). The average numbers of phOx-specific IgG- and IgM-secreting plasma cells were calculated as the difference between ELI-spots on phOx-BSA- and BSA-coated wells. ELI-spot numbers of BSA-reactive IgG as well as IgM were usually 10-15 % of phOx-BSA-reactive spots.

3.3 Comparison of the humoral anti-phOx immune response in D-mutant and wildtype BALB/c mice

We then compared the primary immune responses of all 3 mouse strains.

First, mice were stimulated with the type 2 thymus-independent form of the antigen, *i.e.* immunization was performed with the hapten phOx coupled to Ficoll in a ration of about 60:1. Despite the enhanced numbers of IgM-anti-phOx-secreting background plasma cells (Fig. 12), primary IgM (Fig. 13A) as well as IgG (Fig. 13B) response to this TI-2 antigen of all three mouse strains were rather similar and there was no significant difference.

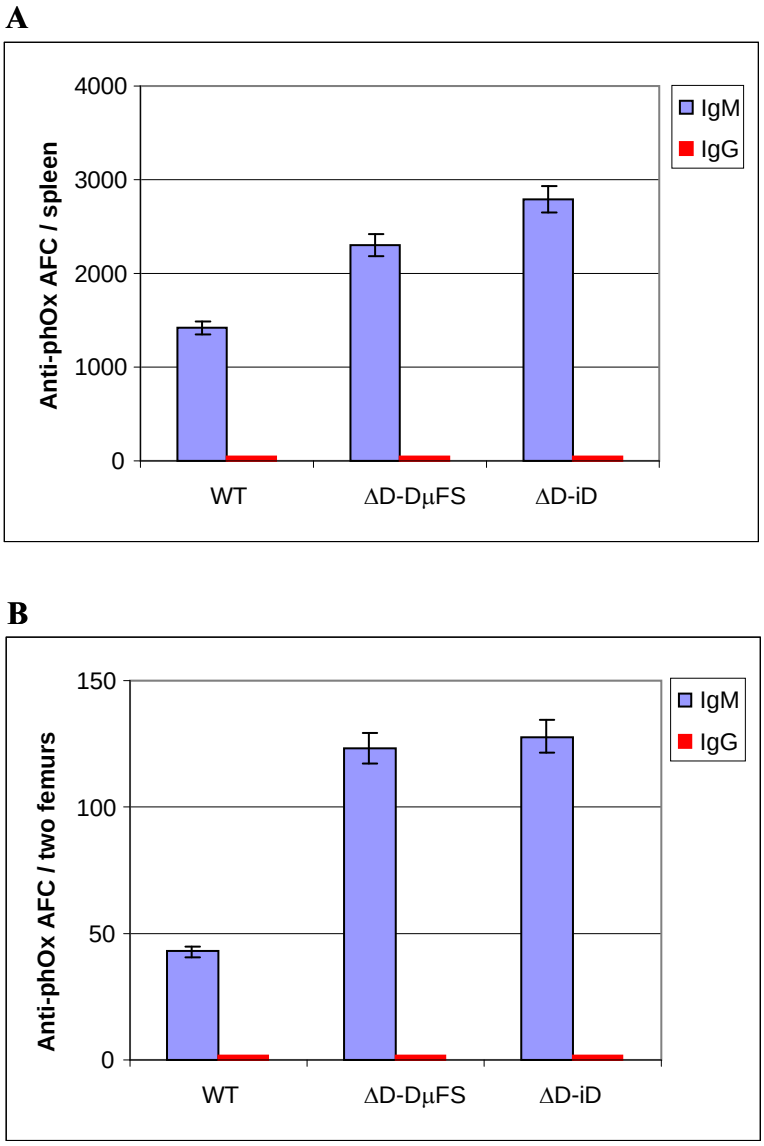


Figure 12

Anti-phOx antibody-secreting plasma cells in spleen (A) and bone marrow (B) of non-immunized mutant and wildtype BALB/c mice

For determination of IgM and IgG anti-phOx antibody-forming plasma cells (AFC), graded concentrations of Histopaque-isolated mononuclear cells were incubated for 18 hr in phOx-BSA- or BSA-coated wells and bound antibodies were detected with secondary antiserum to IgG or IgM. The number of phOx-specific antibody-secreting plasma cells was calculated as the difference of spots in phOx-BSA- and BSA-coated wells. The error bars indicate the standard error of the mean of five separate determinations.

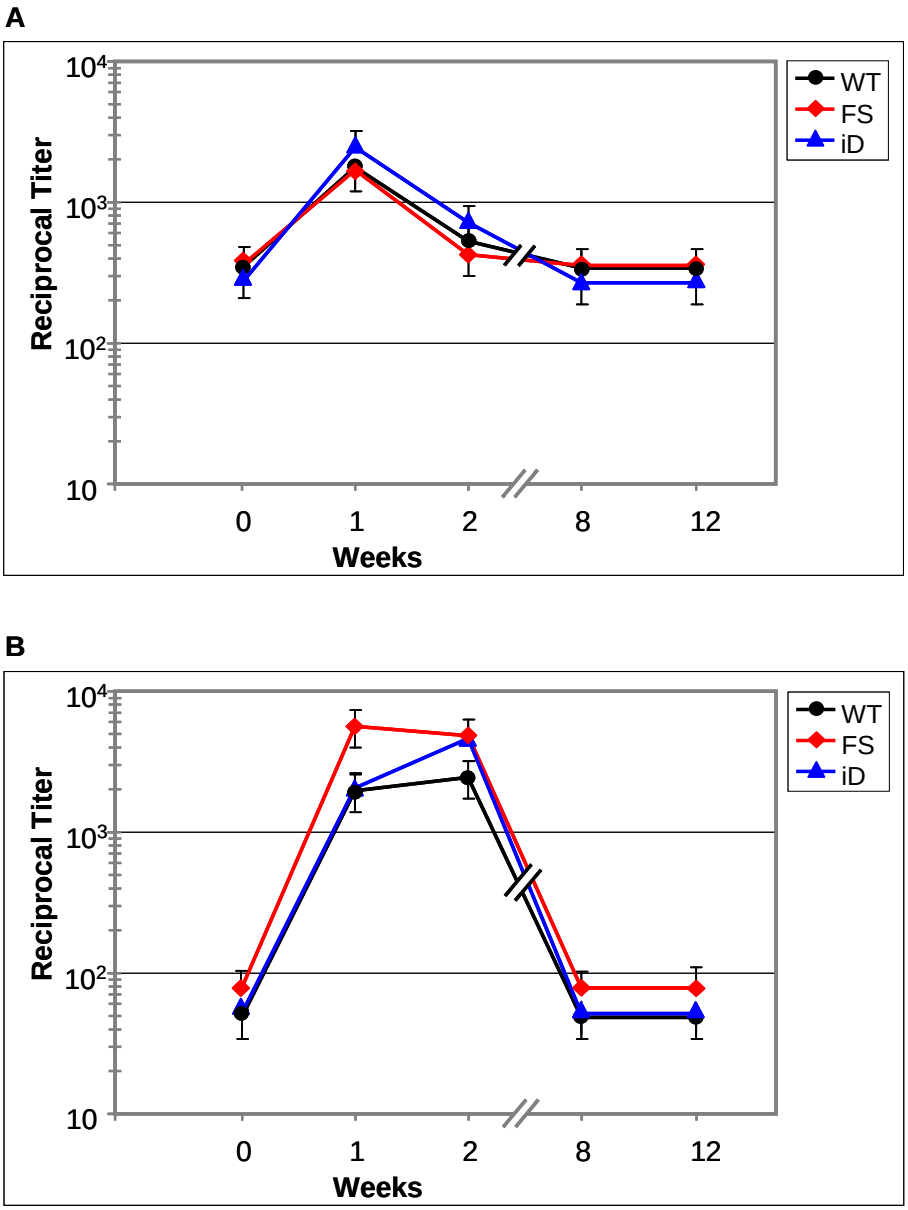


Figure 13

Primary anti-phOx humoral response after immunization with the TI-2 antigen phOx-Ficoll

IgM (A) and IgG (B) anti-phOx antibodies in the sera of 10-weeks old ΔD-DμFS (red curve), ΔD-iD (blue curve) and WT mice (black curve) were determined at the given times points (weeks) after primary immunization with phOx-Ficoll at day 0. Background anti-phOx titers were evaluated in normal pre-immune sera.

Second, the TD response was induced with phOx-coupled CSA (phOx-CSA) and measured at the single cell level [antibody-forming cell response (AFC); Fig. 14] and by determination of serum antibody concentrations (Fig. 15). In WT BALB/c mice (Fig. 14), the primary IgM AFC-response on day 7 was hardly distinguishable from the level of background plasma cells shown in Fig. 12. In contrast, mice of both mutant strains ΔD-DμFS and ΔD-iD showed a substantial increase of IgM AFC (Fig. 14A) which was at least twofold higher than that of WT mice. These IgM-secreting plasma cells seem to be recruited from the bone marrow since anti-phOx-secreting plasma cells in this organ are reduced 7 days after immunization (Fig. 14B). Interestingly, the average number of phOx-specific IgG-secreting cells of WT mice (276 pro spleen) was approximately fourfold higher than that of ΔD-DμFS (64, $p < 0.0001$) and ΔD-iD mice (74, $p < 0.0001$) (Fig. 14A). This result seems to indicate an impaired class switch recombination in both mutant mouse strains.
In bone marrow, phOx-specific IgG-secreting cells were neither detectable in WT nor in transgenic mice (Fig. 14B).

The phOx-CSA-induced TD response was also measured by determining the humoral antibody concentrations after immunization. Despite the enhanced numbers of IgM anti-phOx-secreting plasma cells (Fig. 14), ΔD-DμFS as well as ΔD-iD mutant mice developed reduced primary IgM TD responses and secondary IgM responses were barely visible in all three strains (Fig. 15A). The primary IgG response of both mutant strains developed with slower kinetics and maximal titers reached 2 weeks after immunization were about 5 times lower than in WT mice. In all three strains, the primary maximal titers remained for several weeks. When a secondary immunization with phOx-CSA was performed 14 weeks after primary stimulation, the difference between mutant and WT mice remained. Two weeks after secondary immunization, the mean anti-phOx IgG titer of WT (302000 [250000-350000]) was approximately sevenfold higher than ΔD-DμFS (45800 [36000-60000]) and ΔD-iD mice (40200 [28000-45000]) (Fig. 15).
These data may allow the conclusion that in transgenic mice lower numbers of memory B cells are generated in the primary response and that this drawback can not be compensated for during the secondary response.

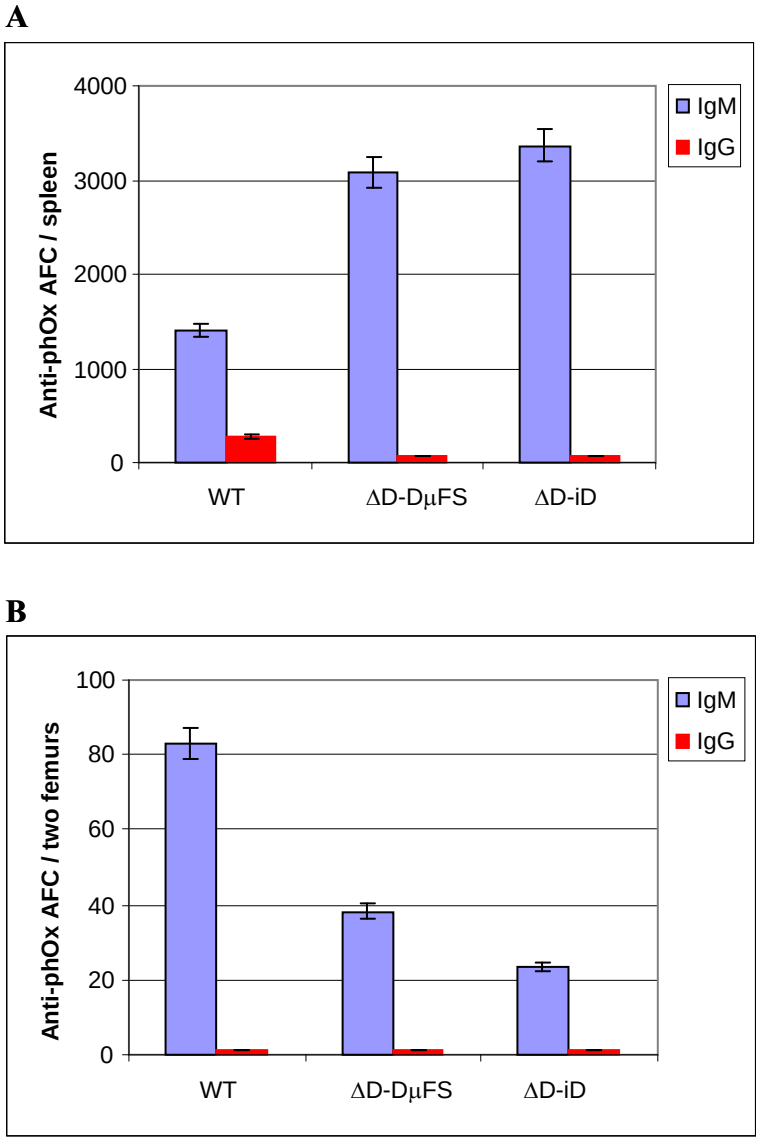


Figure 14

Primary Anti-phOx-secreting plasma cells in spleen (A) and bone marrow (B) of mutant and wild-type BALB/c mice

Seven day after primary i.p. immunization with the TD antigen phOx-CSA, spleen and bone marrow cells were isolated by Histopaque gradient centrifugation and phOx-reactive antibody-forming plasma cells (AFC) were determined with the ELI-spot assay. For further description see legend to Fig. 12. The error bars indicate the standard error of the mean of five separate determinations.

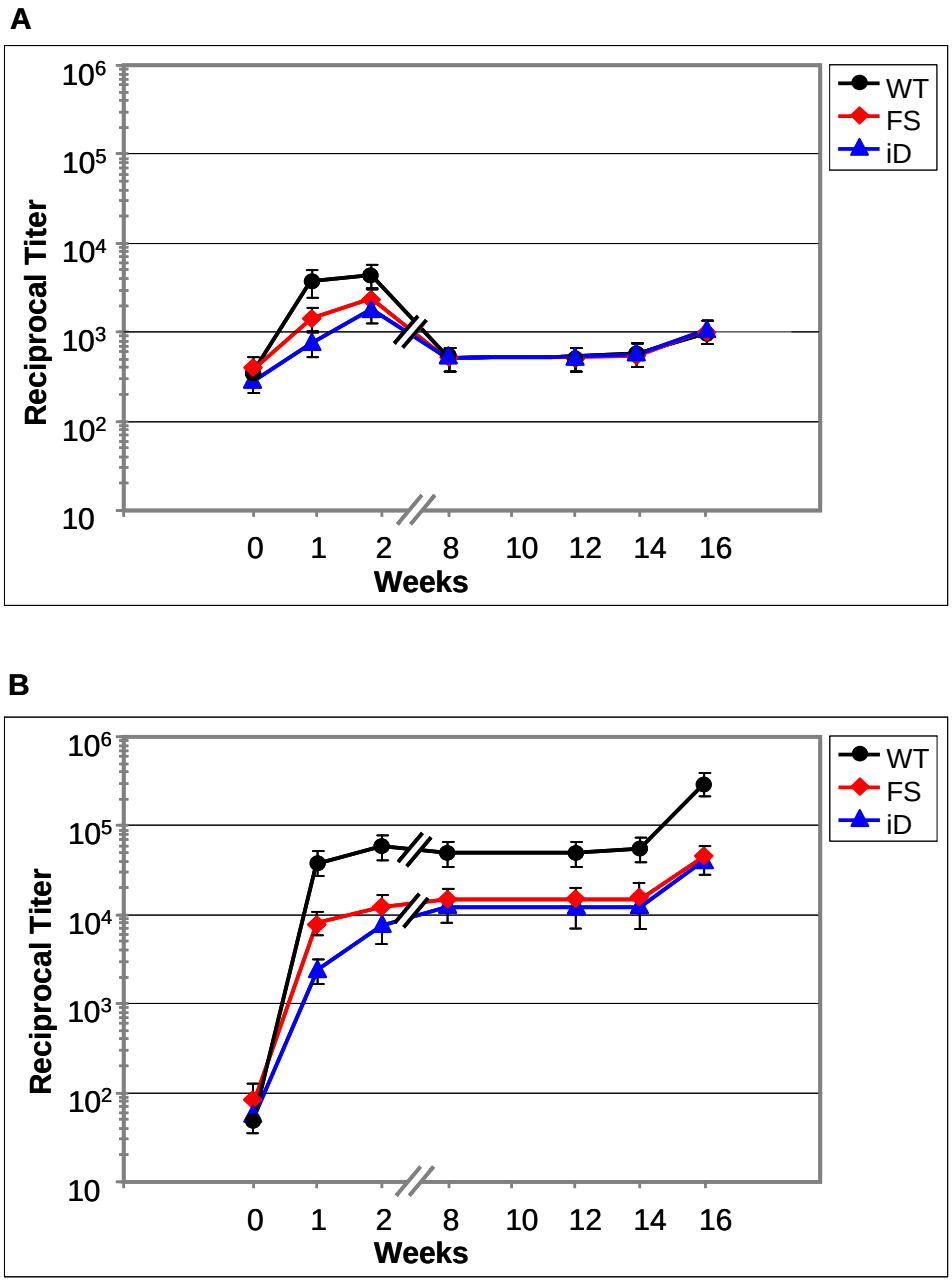


Figure 15

Primary and secondary anti-phOx humoral immune response of wildtype and mutant mice to the TD antigen phOx-CSA

IgM (A) and IgG (B) anti-phOx antibodies in the sera of 10-weeks old ΔD-DμFS (red curve), ΔD-iD (blue curve) and WT mice (black curve) were determined after primary immunization at day 0 and secondary immunization 14 weeks later with phOx-CSA. Back ground anti-phOx titers were evaluated in pre-immune sera.

3.4 Analysis of variable genes of anti-phOx antibodies generated in ΔD-DμFS and ΔD-iD mice

In the order to study the influence of DH gene segments on VH/VL gene usage, somatic mutation and affinity maturation, the TD immune response to the hapten-carrier complex phOx-CSA in ΔD-DμFS and ΔD-iD mice, each of which carry a single modified DH gene segment, was studied at single cell level. To this end, mice of both strains were first immunized by i.p. injection of 80 µg of phOx-CSA adsorbed to $AL(OH)_3$ and hybridomas were generated by fusion of immune spleen cells with the Ig non-secretor myeloma cell line Ag8.653. The primary immune response was analyzed at an early stage on day 7 and a late stage on day 14.

Furthermore, since the alteration in the DH locus obviously impaired the antibody production in the memory response (see 3.3), a set of hybridomas were also prepared after secondary and tertiary immunizations. Secondary immunization was performed 8 weeks after primary immunization also by i.p. injection of the same amount of antigen and the fusion was done three days later. The tertiary immunization followed two months later and cell hybridization was again performed on day 3.

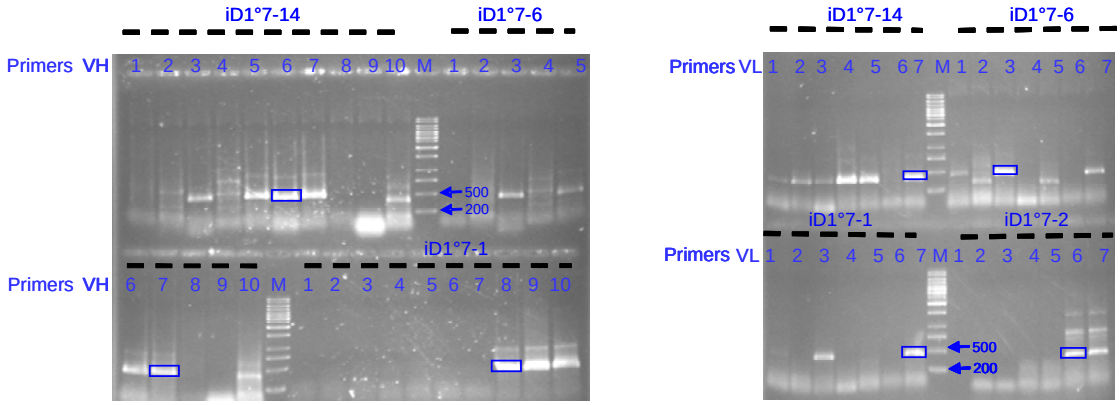


Figure 16

Amplification of variable region-coding sequences of anti-phOx antibodies

Here, the amplification of the variable region-coding sequences of heavy and light chains of antibodies iD°7-1, iD°7-2, iD°7-6 and iD°7-14 is shown. Amplification was performed with one of 10 VH and 7 VL variable primers in combination with respective constant primers.
(A) Amplification of heavy chain. (B) Amplification of light chain. The molecular size of the amplified DNA fragment can be estimated at approximately 450 base pair (bp). DNA fragments of interest are sliced out of the gel purified and used for sequencing (blue rectangle).
M - DNA marker.

Anti-phOx antibody-secreting hybridomas could be established from all stages of the primary to the tertiary immune response. RT-PCR was performed to amplify heavy and light chains of every positive clone (Fig. 16). Ten different primers were included for amplification of VH and seven different primers for amplification of VL. The size of amplified segments lies between 430 and 460 bp (Fig.16). Despite amplification of all the clones, some of them could not be sequenced successfully, *i.e*. incomplete or bad quality of the nucleotide sequence. In those cases, repetition of the sequencing reaction was also ineffective.

3.5 Genetic analysis of phOx-reactive antibodies generated from ΔD-DμFS mice

For an assessment of the genetic repertoire of both DH-mutant BALB/c variants it is essential to remember that it is to be compared with that of the anti-phOx repertoire of BALB/c WT animals. Therefore, the different characteristic molecular features of this response are repeated:

1.) The primary response is characterized by expression of a dominant clone with the canonical gene combination VH171/VL072. This Ox1-named idiotype (Id_{Ox1}) makes up 75 % of the IgG antibodies on day 7.
2.) The specific idiotope of Id_{Ox1} is related with the characteristic sequence aspartic acid-arginine-glycine (DRG) in the third hypervariable region of the heavy chain (HCDR3).
3.) The reactivity of Id_{Ox1} antibodies with syngeneic Id_{Ox1}-specific monoclonal anti-idiotypic antibodies is lost within 3 weeks by somatic mutations.
3.) Id_{Ox1} antibodies are of highest affinity while non-Id_{Ox1} have a 10-100-times lower one.
4.) Immune maturation is primarily based on the accumulation of somatic mutations occurring in Id_{Ox1} antibodies.
5.) Non-Id_{Ox1} antibodies encoded by alternative VH/VL genes appear during secondary and tertiary immune responses.
6.) The propensity of non-Id_{Ox1} antibodies for somatic mutations is known.

Hence, the primary genetic repertoire and the clonal development during memory responses in both mutant strains will be compared with these findings.

3.5.1 Genetic analysis of the phOx-reactive repertoire of ΔD-DμFS mice

First, V region-coding sequences of anti-phOx antibodies will be described which were generated from TD responses of ΔD-DμFS mice. These mice can only make use of a single DH

gene coding for hydrophobic amino acids in HCDR3. The complete amino acid sequences of V regions are showed in Fig. 1 in the appendix.

3.5.1.1 Analysis of primary anti-phOx antibodies

Primary repertoire

From the early primary day 7 TD response, 36 antibodies could be produced from two independent fusions (Tab. 4). The nomenclature of the antibodies is as follows: FS stands for ΔD-DμFS mice and 1°7 indicates that the antibodies were generated after primary immunization and fusion on day 7. In both fusions, about half of the antibodies were of the IgM and IgG class, respectively.

The repertoire of the 18 IgM antibodies was extremely heterogeneous and every gene combination was unique. Hence, there was no dominant clone = idiotype. The Id_{Ox1} was not realized among IgM antibodies. Two IgM antibodies (FS1°7-09, FS1°7-10) expressed the VHOx1 gene VH171, but in combination with alternative light chains, namely Vκ078 and Vκ121, respectively. Antibody FS1°7-03 is encoded by the VκOx1 gene Vκ072 in association with alternative heavy chain gene VH627.

The repertoire of 17 IgG antibodies was as heterogeneous as that of IgM antibodies and again every gene combination was unique. However, antibody FS1°7-24 used the canonical Id_{Ox1} combination (VH171/Vκ072). The 2 antibodies FS1°7-27 and FS1°7-34 used the VκOx1 gene Vκ072 but in combination with a VH163 gene and an unknown VH gene, respectively. In addition, one IgA antibody was obtained.

- In conclusion, the TD antigen-activated repertoire of ΔD-DμFS mice does not contain the Id_{Ox1} gene combination as a major component, neither among IgM nor among IgG antibodies.

Affinity of primary antibodies

Since the primary immune response reflects the first encounter of naive B cells with antigen, the dominance of Id_{Ox1} antibodies in the primary anti-phOx response of BALB/c WT mice was ascribed to their superior affinity for the hapten. Indeed, non-Id_{Ox1} antibodies of the IgM class were generally of much lower affinity. To elucidate the influence of a restriction to a single DH gene, coding for hydrophobic amino acids, on the affinity of the primarily stimulated antibodies, we measured the relative affinities of our antibodies in comparison to the IgM Id_{Ox1} H11.5 (μ,κ) and IgG Id_{Ox1} NQ2.16.2 (γ,κ) (Tab. 4). It is interesting to see that only

3 IgM were of very low affinity (FS1°7-01, -05 and -13), 3 exhibited slightly reduced affinities and 3 had similar affinities as the Id_{Ox1} control antibody H11.5 (μ,κ). Surprisingly, antibodies FS1°7-09 and -14 had significantly enhanced affinities.

Table 4

VH/VL gene combinations of phOx-CSA-induced primary day 7 anti-phOx antibodies of ΔD-DμFS mice

MAb[a]		VH chain genes					VL chain genes					rel.
FS1°7	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
01[h]	μ	1	2	673	8/2	1	9B	1	121	0/0	2	0,01
02[h]	μ	1	1	671	4/4	1	10	1	139	0/1	1	0,4
03[h]	μ	1	2	627	12/3	1	4/5	1	**072**[k]	1/0	4	0,4
04[h]	μ	1	2	627	3/0	2	10	1	139	0/0	1	0,6
05[h]	μ	1	1	532	4/1	2	4/5	1	078	0/0	2	0,03
06[h]	μ	1	2	506	13/3	2	9B	1	121	1/0	1	0,8
07[h]	μ	1	3	396	0/0	2	19/28	1	201	2/0	4	-
08[h]	μ	1	3	386	5/1	3	9A	1	112	2/0	2	0,1
09[h]	μ	2	1	**171**[k]	1/0	3	4/5	1	078	-/-	4	4,5
10[h]	μ	2	1	**171**[k]	0/0	1	9B	1	121	0/0	2	0,6
11[h]	μ	3	1	138	0/0	1	4/5	1	163	0/0	5	0,3
12[i]	μ	3	1	138	1/1	2	19/28	1	157	0/0	2	-
13[h]	μ	3	1	128	1/2	1	-	-	-	-/-	-	0,04
14[h]	μ	3	1	120	0/1	3	21	1	066	2/2	1	5,2
15[h]	μ	6	1	114	2/0	3	9B	1	106	1/0	2	-
16[h]	μ	9	1	155	1/0	4	1	1	122	0/0	1	2,2
17[i]	μ	-	-	-	-/-	-	4/5	1	154	0/0	2	-
18[h]	μ	-	-	-	-/-	-	9B	1	121	0/0	2	-
19[h]	γ	1	2	690	5/1	2	21	1	210	0/0	1	2,0
20[h]	γ	1	2	690	5/5	1	24/25	1	123	0/0	2	1,8
21[h]	γ	1	2	643	8/5	2	21	1	210	0/0	1	0,5
22[h]	γ	1	1	532	3/0	2	4/5	1	077	1/1	2	0,5
23[h]	γ	1	2	307	-/-	1	4/5	1	078	0/0	1	0,1
24[h]	γ	2	1	**171**[k]	1/0	3	4/5	1	**072**[k]	0/0	5	0,5
25[h]	γ	3	1	128	0/2	1	-	-	-	-/-	-	0,1
26[h]	γ	3	1	128	3/1	1	4/5	1	143	10/1	5	2,7
27[i]	γ	5	1	163	1/1	3	4/5	1	**072**[k]	2/0	2	13,6
28[h]	γ	5	1	163	1/2	4	23	1	168	1/1	4	14,1
29[h]	γ	5	1	139	2/0	2	2	1	099	0/0	2	5,7
30[i]	γ	6	1	114	1/0	3	1	1	115	0/0	2	1,8
31[h]	γ	7	1	158	1/0	4	21	1	210	2/1	2	0,08
32[h]	γ	14	1	125	4/0	3	10	1	108	0/0	1	-
33[h]	γ	14	1	125	1/0	3	12/13	1	172	4/4	4	2,0
34[h]	γ	-	-	-	-/-	-	4/5	1	**072**[k]	1/0	4	-
35[h]	γ	-	-	-	-/-	-	1	1	179	-/-	1	115
36[h]	α	14	1	125	3/0	3	12/13	1	172	2/0	2	-

Legend

[a] – The annotation of antibodies indicates their generation from ΔD- DμFS mice (FS), after

primary immunization (1°) on day 7 (7) and is followed by a sequential number.
[b] – Isotype of the monoclonal antibody.
[c] – Indication of VH or VL gene family in the VBASE2 integrative database.
[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.
[e] – VH and VL gene numbers according to the integrative database VBASE2.
[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.
[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.
[h, i] – Indication of different fusions.
[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold.
- – Sequence not determined.
Grey background shows the Id_{Ox1} gene combination VH171/Vκ072.

A similar picture emerged in IgG antibodies. Some of them had lower and some had rather similar affinities as the IgG Id_{Ox1} control antibody NQ2/16.2. Strikingly, 4 non-Id_{Ox1} antibodies exhibited clearly enhanced affinities and one of them (FS1°7-35) had an even 100-times higher affinity.

Conclusion:

- Thus, the pre-immune repertoire contains clonotypes exhibiting clearly higher affinities than the normally dominant Id_{Ox1}.

Mutations in early primary antibodies

Somatic mutation is targeted to the rearranged immunoglobulin V-gene itself and their intronic flanking sequences. In cells harbouring hypermutated V-genes, the constant (C) and unrearranged V genes are usually not modified. After intraperitoneal injection of a TD antigen, activated B cells migrate into the primary follicles of peripheral lymphoid organs where they undergo intense proliferation and form germinal canters, which appear towards the end of the first week after challenge and provide the location for both hypermutation and antigen selection.

We compared the V region sequences with those of the next related known V genes and counted the numbers of silent and productive mutations (Tab. 4). This comparison did not reveal any alteration from mutation rates seen in WT mice. The majority of light chain sequences of IgM anti-phOx antibodies was not mutated or contained less than 2 mutations per chain. In contrast, few VL genes of IgG antibodies showed clearly mutated Vκ genes, *i.e.* FS1°7-26 and -33 with 11 and 8 mutations, respectively.

When comparing mutations in VH, it is striking to see that all IgM encoded by VH1 family genes contain rather high mutation rates except antibody FS1°7-07 with no mutations. Aston-

ishingly, VH1 family-encoded IgM showed no or very few mutations in VL. This disparity is not seen in the other IgM antibodies, but again in VH1 family-encoded IgG antibodies FS1°7-19 to -22. Since the entire number of VH1 family genes is not known it can be assumed that the observed differences do not represent real mutations, but indicate hitherto unknown genes of this family.

It is widely accepted that VH genes show higher mutation rates than VL genes [64, 82-84]. One example of this kind seems to be IgG antibody FS1°7-32 with 4 productive mutations in VH, but no mutations in VL. However, the opposite situation is found in antibody FS1°7-33 with only 1 productive mutation in VH, but 4 productive plus 4 silent mutations in VL. This situation has already been observed in a previous investigation [46].

Conclusions:

- The mutation rates of early primary phOx-CSA-induced antibodies in ΔD-DμFS mice differed not significantly from those of BALB/c WT mice. Thus, the alteration in the DH locus did not influence the mutation rate at this stage of immune response.

HCDR3 nucleotide sequences of early primary antibodies

The comparison of D-region nucleotide sequences of the early primary anti-phOx antibodies of ΔD-DμFS mice with the genomic DH gene sequence is depicted in Tab. 5. As proposed by Kaartinen and Mäkelä [30], we assumed that an identity of at least 6 successive nucleotides with the genomic sequence, interrupted by no more than 1 mismatched nucleotide, proves that the genomic DH gene is verified in a particular antibody. If so, HCDR3 sequences of 7/13 IgM and 11/18 IgG could be traced back to the genomic D gene. However, one IgM and 3 IgG had identical sequences of 4 nucleotides (marked in blue in Tab. 5). It is difficult to decide if this sequence is derived from the genome or generated during the recombination process.

As stated above, the vast majority of antibodies, including those with phOx-specificity, use DH reading frame 1 (RF1) which favors tyrosine and glycine residues [30] while RF2 and RF3 are counter selected on the basis of stop codons and/or hydrophobic encoded nucleotides. However, the analysis of CDR3 sequences of the early primary anti-phOx antibodies from ΔD-DμFS mice showed quite a different picture. Only 3/13 IgM and 4/18 IgG antibodies used RF1 in HCDR3, 4/13 IgM and 7/18 IgG used RF2 and in 6/13 IgM and 7/18 IgG the reading frame could not be assigned.

Table 5

Nucleotide sequences of HCDR3 of phOx-CSA-induced primary day 7 anti-phOx antibodies generated from ΔD-DμFS

FS1°7		----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene			T TTT ATT ACT ACG GTA GTT AGC TAC		1
05	μ	TGT GCA AGA	TGG GGC TCT ATT ACT AAG GTA GTT AGT	GTC TAC TGG	1
06	μ	TGT GCA AGT	G TGT ATT ACT ACG GTA GA	GAC TGC TGG	2
10	μ	TGT GCA GGA	G TCC ATA ATT ACT ACG GTA GTT ACG GGT GGT ACT TC	GAT GTC TG	2
12	μ	TGT GCA CTC	TTT ACT ACG GTA GTT AGC TAC GTA CTT TTT	GAC TAC TGG	1
14	μ	TGT GCA AGA	G TTT ACT ACG AAG ACG GGT TT	GCT TAC TGG	2
15	μ	TGT GCG GGC	GTA GTT AGG TTT	GCT TAC TGG	1
16	μ	TGT GCA AGA	G ATT ATT ACT ACG AGG GTA TG	GAC TAC TGG	2
03	μ	TGT GCA AGG	G GGT ACT TC	GAT GTC TGG	nf
04	μ	TGT GCA AGA	GCG AAC TTT	GAC TAC TGG	nf
07	μ	TGT GCA ATA	CGA	GAC TAC TGG	nf
08	μ	TGT GCA AGG	GCT ACG GCC ATG GGG GCC TGG TTT	GCT TAC TGG	nf
09	μ	TGT GCC AGA	CTG ACC CAG ACG TTT	GCT TAC TGG	nf
13	μ	TGT GCA AGG	CGG TAC TTC	GAT GTC TGG	nf
11	γ	TGT GCA AGA	TTT ACT ACG GTA GTT TGC TAC TGG TAC TTC	GAT GTC TGG	1
19	γ	TGT GCA AGT	G TAG CTA ACT ACC GTA GTA ATA AAC GAG GGG AA	AAC TAC TGG	2
20	γ	TGT GCA GTT	ATT ACT ACG GTA GCC CCC	GAT GTC TGG	1
21	γ	TGT GCA AGA	AGA GTT AGC TAC GGA AAC TAC TTT	GAC TAC TGG	1
22	γ	TGT GCA AAT	T ACT ACG GTA CCT CCT TT	GAC TAC TGG	2
23	γ	TGT GCA AGG	G CCT ATT ACT ACG GC	GAT GTC TGG	2
27	γ	TGT GCA AGC	C ATT ACT ACG GTA CCC CTT TT	GCT TAC TGG	2
30	γ	TGT GCC AGG	C CGG GTT ACG GTA GTC CCT GGT TT	GCT TAC TGG	2
31	γ	TGT GCA AGA	CCT ACG GTA GTT AGC TTC TAT GCT ATG	GAC TAC TGG	1
33	γ	TGT GCT CTT	T ATT ACT ACG GTA GTG GGA CCT GGT TT	TCT TAC TGG	2
36	γ	TGT ACT AGC	T ACC GGC AGG CCT ACG GTA GTT CCT GGT TT	GCT TAC TGG	2

01	γ	TGT GCA AGA	GGG TAC TTC		GAT GTC TGG	nf
24	γ	TGT GCC AGA	GAT CCC GGG		GCT TAC TGG	nf
25	γ	TGT GCA AGG	CGG TAC TTC		GAT GTC TGG	nf
26	γ	TGT GCA AGA	GGA CTC TTA GTT TAC TGG TAC TTC		GAT GTC TGG	nf
28	γ	TGT GCA AGA	CAT AAC AAC TAT GCT ATG		GAC TAC TGG	nf
29	γ	TGT GCA AGA	TCG CCC GGA		GAC TAC TGG	nf
32	γ	TGT GTC CCG	GTG GCC TGG TTT		GCT TAC TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.
Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-DμFS mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-DμFS mice.
DH germ line coding sequence of ΔD-DμFS is outlined in first line in green colour.

Conclusions:

- It seems that RF1 which codes for hydrophobic amino acids is counter selected during the generation of the primary repertoire ΔD-DμFS mice, harbouring only 1 non-natural = artificial gene in the DH locus.

HCDR3 amino acids in early primary antibodies in ΔD-DμFS mice

The lengths of the HCDR3-loops of the early primary anti-phOx antibodies from ΔD-DμFS mice are shown in Tab. 6. The HCDR3 lengths vary in a wide range from only 1 to 12 amino acids. It seems that the average length correlates with the used reading frame, *i.e*. antibodies in which the D region is encoded by RF1 or RF2, *i.e*. with `green amino acids´, have longer HCDR3 loops than those antibodies in which the reading frame could not assigned, *i.e*. they only contain `red amino acids´. HCDR3 loops of `green´ IgM and IgG antibodies have an average length of 7.85 and 8.09 amino acids, respectively. In IgM and IgG antibodies containing only `red´ amino acids, the D region loops have on average 3.83 and 4.1 amino acids, respectively.

Antibody FS1°7-24 which is encoded by the canonical Id_{Ox1} gene combination VH171/Vκ072 has a D loop with amino acids DPG which is rather similar to the DRG of Id_{Ox1} antibodies. Therefore, we tested these primary antibodies with our prototypic Id_{Ox1}-specific monoclonal antibody 8-21/W18. It turned out that none of these primary antibodies were Id_{Ox1} positive. Hence, the substitution of the middle R with P destroys the idiotope recognized by syngeneic Id_{Ox1}-specific antibodies, although the following base of the loop contains alanine and tyrosine (AY) which are also expressed in Id_{Ox1} antibodies. This confirms a previous observation that an exchange of R with P destroys the Id_{Ox1} quality (Lange et al., 2008).

In conclusion:

- The extreme variability of HCDR3 length suggests that the D region is not of prime importance for the phOx-specificity of ΔD-DμFS mice primary antibodies.
- The more D gene nucleotides are eliminated by exonucleases during VDJ recombination in the course of generation of the pre-immune repertoire, the shorter the HCDR3 length will be.

Table 6

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced primary day 7 anti-phOx antibodies generated from ΔD-DμFS mice

FS1°7		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
05	μ	CAR	WGSITKVVS	VYW	1	0.38
06	μ	CAS	VYYYGR	DCW	2	-0.08
10	μ	CAG	VHNYYGSYGWYF	DVW	2	-0.04
12	μ	CAL	FTTVVSYVLF	DYW	1	0.78
14	μ	CAR	VYYEDGF	AYW	2	-0.28
15	μ	CAG	VVRF	AYW	1	0.75
16	μ	CAR	DYYYEGM	DYW	2	-0.28
03	μ	CAR	GYF	DVW	nf	0.28
04	μ	CAR	ANF	DYW	nf	0.28
07	μ	CAI	R	DYW	nf	-1.3
08	μ	CAR	ATAMGAWF	AYW	nf	0.5
09	μ	CAR	LTQTF	AYW	nf	0.27
13	μ	CAR	RYF	DVW	nf	-0.15
11	γ	CAR	FTTVVCYWYF	DVW	1	0.55
19	γ	CAS	VANYRSNKRGE	NYW	2	-0.42
20	γ	CAV	ITTVAP	DVW	1	0.59
21	γ	CAR	RVSYGNYF	DYW	1	-0.026
22	γ	CAN	YYGTSF	DYW	2	0.07
23	γ	CAR	AYYYG	DVW	2	-0.002
27	γ	CAS	HYYGTPF	AYW	2	-0.1
31	γ	CAR	PTVVSFYAM	DYW	1	0.56
30	γ	CAR	PGYGSPWF	AYW	2	0.03
33	γ	CAL	YYYGSGTWF	SYW	2	0.004
36	γ	CTS	YRQAYGSSWF	AYW	2	-0.12
01	γ	CAR	GYF	DVW	nf	0.28
24	γ	CAR	DPG	AYW	nf	-0.44
25	γ	CAR	RYF	DVW	nf	-0.15
26	γ	CAR	GLLVYWYF	DVW	nf	0.6
28	γ	CAR	HNNYAM	DYW	nf	-0.26
29	γ	CAR	SPG	DYW	nf	-0.14
32	γ	CVP	VAWF	AYW	nf	0.83

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].

Amino acids marked in green correspond to DH germline sequence in ΔD-DμFS mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

3.5.1.2 Analysis of late primary anti-phOx antibodies

Clonal development in the late primary response

Next, we prepared anti-phOx antibody-secreting hybridomas 14 days after primary immunization with the TD antigen phOx-CSA. From the established 16 clones, 9 (56 %) secreted IgM and 7 (44 %) secreted IgG antibodies (Tab. 7). This is in clear contrast to the control response in BALB/c WT mice where the vast majority of the anti-phOx forming cells at this stage of immune response produce IgG antibodies. Furthermore, while in WT mice there is a clear preference for Id_{Ox1} antibodies as a dominant clonotype, ΔD-DμFS mice did not develop any dominant clone. The VHOx1 gene VH171 is only expressed in antibody FS1°14-11 and the VκOx1 gene Vκ072 was not used at all. Thus, no clone was encoded by the Id_{Ox1} canonical gene combination VH171/Vκ072.

Conclusions:

- The development of the dominant Id_{Ox1} clonotype obviously depends on the available DH gene repertoire and can not or only very rarely (see previous section) be generated from the non-natural DH gene of ΔD-DμFS mice.

Mutations in late primary antibodies

The mutation rates of these late primary anti-phOx antibodies were higher than those of the early primary antibodies (Tab. 4). This is especially the case if one only looks at those antibodies which are encoded by VH and VL class 1 genes and disregards uncertain mutations in antibodies encoded by class 2 genes. (Classification of variable genes as *class 1* rests on genomic as well as rearranged evidence while for class 2 genes, there is only genomic evidence and so far such genes have not been observed in known antibodies.) However, the numbers of mutations in VH differ greatly and range from 0 in antibody FS1°14-06 and -07 to 11 mutations in antibody FS1°14-08. Collectively, IgG antibodies seem to contain more mutations than IgM antibodies. Moreover, the observed mutation rates in these late primary anti-phOx antibodies of ΔD-DμFS mice are comparable with those of BALB/c WT mice. The VH/VL ratio of mutations is in accord with the above mentioned rule that VH contain more mutations than VL. A ratio of productive to silent mutations greater than 1 (Tab. 7) indicates an antigenic selection of the produced clones.

Conclusions:

- Late primary anti-phOx antibodies in ΔD-DμFS mice contain an astonishingly high number of non-switched IgM antibodies.

- An immune maturation is indicated by enhanced mutations rates, but the mutations do not accumulate in a particular clone.

Table 7

VH/VL gene combinations of phOx-CSA-induced primary day 14 anti-phOx antibodies in ΔD-DμFS mice

MAb[a]		VH chain genes					VL chain genes					rel.
FS1°14	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
01	μ	1	2	569	2/0	2	4/5	1	077	0/0	2	-
02	μ	1	1	532	1/0	3	2	1	097	1/0	4	-
03	μ	1	2	495	5/0	3	4/5	1	166	0/0	1	-
04	μ	1	2	286	1/0	1	4/5	1	166	0/0	2	-
05	μ	1	2	073	4/1	3	10	1	129	3/0	2	-
06	μ	5	1	147	0/0	2	23	1	179	0/0	4	-
07	μ	9	1	155	0/0	3	4/5	1	082	0/0	4	-
08	μ	9	1	118	6/5	4	2	1	097	0/0	1	-
09	μ	-	–	-	-/-	-	4/5	1	166	0/0	2	-
10	γ	1	1	480	3/0	3	1	1	115	2/1	5	-
11	γ	2	1	**171**[k]	5/4	3	4/5	1	142	5/1	4	-
12	γ	5	1	192	1/2	2	-	1	-	-/-	-	-
13	γ	5	1	139	1/1	3	9A	1	103	1/0	4	-
14	γ	7	1	168	6/1	3	1	1	115	4/0	2	-
15	γ	7	1	158	5/2	3	-	1	-	-/-	-	-
16	γ	14	1	125	3/0	3	12/13	1	172	2/0	2	-

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-DμFS mice (FS), after primary immunization (1°) on day 14 (14) and is followed by a sequential number. All clones are generated from the same fusion.

[b] – Isotype of the monoclonal antibody.

[c] – Indication of VH or VL gene family in the VBASE2 integrative database.

[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.

[e] – VH and VL gene numbers according to the integrative database VBASE2.

[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.

[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold.

- – Sequence not determined.

Table 8

Nucleotide sequences of HCDR3 of phOx-CSA-induced primary on day fourteen anti-phOx antibodies generated from ΔD-DμFS

FS1°14	----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene		T TTT ATT ACT ACG GTA GTT AGC TAC		1
01 μ TGT	GCA AGA	T CCT ATT ACT ACG GTA GGG CCT TT	GAC TAC TGG	2
02 μ TGT	GCA AGA	G GTT ACT ACG GTA GCT CCT GGT TT	GCT TAC TGG	2
04 μ TGT	GCA AGA	T CAG ACT ACG GTA GTT ACG ACT GGT ACT TC	GAT GTC TGG	2
06 μ TGT	GCA AGA C ATC CGG TAG TTA GCA TGT ACT ACT TT		GAC TAC TGG	2
03 μ TGT	GCA AGA TGG GAG		GCT TAC TGG	nf
05 μ TGT	GCA AGA GAC GGG GGT		GCT TAC TGG	nf
07 μ TGT	GCA AGA AAG GGG GCT ACC GTC ACT TTT AGG GTG TTT		GCT TAC TGG	nf
08 μ TGT	GCC AGA TTT ATG GTA GTT TGG GGT TCT ATG		GAC TAC TGG	nf
10 γ TGT	GCA AGA TCT GAT TTT ATT ACT CAC GCC TGG TTT		GCT TAC TGG	1
12 γ TGT	GCA AGA CTG AGT ACT ACG GCG		GAC TAC TGG	1
14 γ TGT	TCA AGA G GCC ATT ACT ACG GCT CCT GGT TT		GCT TAC TGG	2
15 γ TGT	GCA AGA G GGG ATT ACT ACG GTT GTT GGT TT		GCT TAC TGG	2
16 γ TGT	GCT AGC TTT TTT ATT ACT ACG GTA GTT AGG		GCT TAC TGG	1
11 γ TGT	GCC AGA GAT TTC GGT		AAG GAC TGG	nf
13 γ TGT	GCA AAA GAC TAC GGT		GTC GGG TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH-nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.
Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-DμFS mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-DμFS mice.
DH germ line coding sequence of ΔD-DμFS is outlined in first line in green colour.

Table 9

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced primary on day fourteen anti-phOx antibodies generated from ΔD-DμFS mice

FS1°14		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CAR	SYYYGRAF	DYW	2	-0.04
02	μ	CAR	GYYGSSWF	AYW	2	0.04
04	μ	CAR	SDYGSYDWYF	DVW	2	-0.2
06	μ	CAR	HPVVSMYYF	DYW	2	0.35
03	μ	CAR	WE	AYW	nf	-0.57
05	μ	CAR	DGG	AYW	nf	-0.31
07	μ	CAR	KGATVTFRVF	AYW	nf	0.37
08	μ	CAR	FMVVWGSM	DYW	nf	0.71
10	γ	CAR	SDFITHAWF	AYW	1	0.27
12	γ	CAR	LSTTA	DYW	1	0.39
14	γ	CSR	GHYYGSWF	AYW	2	-0.07
15	γ	CAR	GDYYGCWF	AYW	2	0.06
16	γ	CAS	FFITTVVR	AYW	1	0.71
11	γ	CAR	DFG	KDW	nf	0.04
13	γ	CAK	DYG	VGW	nf	-0.41

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].

Amino acids marked in green correspond to DH germline sequence in ΔD-DμFS mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

HCDR3 nucleotide sequences of late primary antibodies

The nucleotide sequences of the late primary antibodies are depicted in Tab. 8. Ten of these 16 antibodies (62 %) contained 6 or more successive nucleotides derived from the germ line DH gene segment with more or less modifications at both boundaries of VDJ joining sites. The other 6 antibodies (37 %) showed no homology with the germ line DH segment.

As already observed in early primary antibodies of ΔD-DμFS mice, RF1 usage was reduced in late primary antibodies in favor of RF2 (37 %) or a situation that the reading frame could not be determined. With antibodies FS1°14-07 and -8 as exceptions, HCDR3 loops were longer in antibodies with germ line-related than in those with non-related DH nucleotide sequences.

Conclusions:

- Late primary anti-phOx antibodies of ΔD-DμFS mice preferably use germ line-related HCDR3 nucleotides sequences, but `try to avoid´ reading frame 1.

HCDR3 amino acids in late primary antibodies in ΔD-DμFS mice

The deduced amino acid sequences from the HCDR3 nucleotide sequences prove the above conclusions (Tab. 9). With the exceptions of antibodies FS1°14-07 and -08, antibodies with germ line-related HCDR3 sequences have clearly longer D region loops than antibodies with non-related sequences. Among the antibodies with germ line-related HCDR3 sequences, only 3 (FS1°14-10, 12 and 16) used the DH RF1 coding for hydrophobic amino acids. This is indicated by a positive hydrophobicity index (Tab. 9). Only RF2 in HCDR3 encoded the normally preferred amino acids glycine and tyrosine. Since 6 clones (37 %) used a germ line non-related reading frame in HCDR3 intervals, antibodies with DH RF1 represent only 19 % instead of 90 % in WT.

Analysis of hydropathic score of the HCDR3 demonstrated that the germ line DH non-related clones used hydrophobic as well as hydrophilic amino acids and some of this clones (FS1°14-5, FS1°14-11, FS1°14-13) presented a similar HCDR3-loop (DXG) found in WT mice. However, clones using RF1 in DH showed a hydrophobic HCDR3, while that using RF2 presented mostly a hydrophilic HCDR3.

In conclusion:

- As concluded from the early primary anti-phOx antibodies of ΔD-DμFS mice, the variability of HCDR3 length suggests that the D region is not of prime importance for the phOx-specificity.
- In general, the more DH gene nucleotides are eliminated by exonucleases during VDJ recombination in the course of generation of the pre-immune repertoire, the shorter the HCDR3 length will be.

3.5.1.3 Analysis of secondary anti-phOx antibodies

Clonal composition of the secondary response

Eight weeks after primary immunization, a group of ΔD-DμFS mice were immunized a second time with the TD antigen phOx-CSA and phOx-reactive antibody-secreting hybridomas were prepared 3 days later by cell fusion. From 2 such fusions, 12 anti-phOx specific hybridomas were established (Tab. 10). From the first fusion of one mouse, only 4 IgM anti-phOx

antibodies were obtained and from the other mouse only 8 IgG-secreting clones. Compared to BALB/c WT mice, isolation of IgM antibodies in the first fusion is a rather strange event, particularly since they were obtained already on day 3 after immunization. The collection of antibodies obtained in the second fusion also present curious features. In wildtype mice, the secondary response is characterized by a clonal shift away from the primary dominant Id_{Ox1} to other clonotypes. In these secondary antibodies from ΔD-DμFS mice, 3 antibodies (FS2°-05 to -07) were encoded by the Id_{Ox1} gene combination VH171/Vκ072. Hence, they dominated the secondary response of this mouse with 37 %. In addition, antibody FS2°-08 was also encoded by VH171, but in combination with VL gene Vκ137.

Conclusions:

- The very different outcome in these 2 fusions indicates that the repertoire of B cells in ΔD-DμFS mice which can be activated by phOx-CSA develops at random. Therefore, the normally dominant Id_{Ox1} may even be generated in very few mice.
- Because of the probably non-sufficient amount of secondary antibodies, a clear repertoire switch as in BALB/c WT mice can not be concluded.

Mutations in secondary antibodies

The IgM antibodies were almost non-mutated and the 7 productive and 5 silent mutations in antibody FS2°-01 are problematic since the coding gene VH623 is `only´ a class 2 gene. Since the complete repertoire of the VH1 family is not known, the VH gene used FS2°-01 may belong to the group of hitherto unknown VH1 family genes. Thus, we can regard the associated cells as naive and not as memory B cells.

As mutation rates of secondary IgG antibodies are higher than in late primary antibodies, it can be concluded that these antibodies have undergone immune maturation through somatic hypermutation. Indeed, VL-coding gene Vκ072 of antibodies FS2°-06 and -07 contain the well known classical affinity-enhancing early mutations H>N in position 33 and Y> F in position 35 [55]. In addition, the VH171 gene of antibody FS2°-07 contains the known mutation S>T in position 31 [55]. Hence, the 4 antibodies encoded by VH171 are immune maturated and this correlates well with their considerably enhanced affinities (Tab. 10).

Conclusion:

- Immune maturation can occur in individual, but not all ΔD-DμFS mice.

HCDR3 nucleotide sequences of secondary antibodies

Two of the IgM antibodies could be sequenced in VH. Both of them used germ line-related

nucleotide sequences in HCDR3, 1 in RF1 and the other in RF2 (Tab. 11). HCDR3 sequences of 3 IgG antibodies are also germ line-related. Two of them are translated in RF2 and the in RF3. Interestingly, the other 4 IgG antibodies which are encoded by one or both Id_{Ox1} variable genes have rather short HCDR3 loops (Tab. 11).

Conclusion:

- In ΔD-DμFS antibodies encoded by one or both genes of the canonical Id_{Ox1} combination, the HCDR3 loop could obviously not be generated from germ line-related nucleotide sequences.

Table 10

VH/VL gene combinations of phOx-CSA-induced secondary anti-phOx antibodies in ΔD-DμFS mice

MAb[a]		VH chain genes					VL chain genes					rel.
FS2°	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
01[h]	μ	1	2	623	7/5	3	1	1	115	0/0	2	-
02[h]	μ	1	2	591	0/0	3	-	-	-	-/-	-	-
03[h]	μ	-	-	-	-/-	-	1	1	097	0/0	1	-
04[h]	μ	-	-	-	-/-	-	9B	1	121	0/0	2	-
05[i]	γ	2	1	**171**[k]	3/0	4	4/5	1	**072**[k]	1/0	5	16,4
06[i]	γ	2	1	**171**[k]	6/0	3	4/5	1	**072**[k]	5/0	5	127,7
07[i]	γ	2	1	**171**[k]	5/2	3	4/5	1	**072**[k]	4/2	5	65,7
08[i]	γ	2	1	**171**[k]	6/0	4	10	1	137	3/3	2	19,1
09[i]	γ	2	1	162	10/6	3	1	1	115	3/3	1	-
10[i]	γ	7	1	158	9/5	3	1	1	115	2/1	2	2,0
11[i]	γ	15	1	125	3/1	2	10	1	137	4/0	1	10,4
12[i]	γ	-	-	-	-/-	-	19/28	1	202	1/0	2	-

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-DμFS mice (FS), after secondary immunization (2°) and is followed by a sequential number.
[b] – Isotype of the monoclonal antibody.
[c] – Indication of VH or VL gene family in the VBASE2 integrative database.
[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.
[e] – VH and VL gene numbers according to the integrative database VBASE2.
[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.
[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.
[h, i] – Indication of different fusions.
[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold.
- – Sequence not determined.

Grey background shows the Id_{Ox1} gene combination VH171/Vκ072.

Table 11

Nucleotide sequences of HCDR3 of phOx-CSA-induced secondary anti-phOx antibodies generated from ΔD-FμFS

FS2°		----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene			T TTT ATT ACT ACG GTA GTT AGC TAC		1
01	μ	TGT GCA AGA	GGG TGG AGT ATT ACT ACG GTA GTT AGC TAC	GCT TAC TGG	1
02	μ	TGT GCA AGA	G AGA GGT CTT TTT ACT ACG GTA GGG AGT GGC TGG TT	TGC TTA TGG	2
09	γ	TGT GCC AGA	CA TGA ATT TTT ATT ACT ACG GAG TTA CCT ACG C	GCT TAC TGG	3
10	γ	TGT GCA CGA	G GGC ATT ACT ACG GTA CTT GGT TT	GCT TAC TGG	2
11	γ	TGT GTT AGA	T ATT ACT ACG GTA GGC CT	GAC TAC TGG	2
05	γ	TGT GCC AGA	GAC TCC GGG	GAC TAC TGG	nf
06	γ	TGT GCC AGA	GAT GGG GGG	GCT TAC TGG	nf
07	γ	TGT GCC AGA	G ATT ACG GA	ATT TAC TGG	nf
08	γ	TGT GCC AGA	GAT GGG GGG	GAC TAC TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.

Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-DμFS mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-DμFS mice.
DH germ line coding sequence of ΔD-DμFS is outlined in first line in green colour.

Table 12

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced secondary anti-phOx antibodies generated from ΔD-DμFS mice

FS2°		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CAR	GWSITTVVSY	AYW	1	0.42
02	μ	CAR	ERSFYYGREWLV	CLW	2	-0.1
09	γ	CAR	HEFLLLRSYLR	AYW	3	0,17
10	γ	CAR	GHYYGTWF	AYW	2	-0,06
11	γ	CVR	YYYGRP	DYW	2	-0,41
05	γ	CAR	DSG	DYW	nf	-0,36
06	γ	CAR	DGG	AYW	nf	-0,31
07	γ	CAR	DYG	IYW	nf	-0,41
08	γ	CAR	DGG	DYW	nf	-0,36

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].

Amino acids marked in green correspond to DH germline sequence in ΔD-DμFS mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

HCDR3 amino acids in secondary antibodies in ΔD-DμFS mice

A conspicuous character of the secondary response is the reduced usage of DH RF1-encoded hydrophobic amino acids in the HCDR3 loop. Antibodies FS2°-2, -10 and -11 used tyrosine-rich germ line-related DH sequences and only antibody FS2°-01 used hydrophobic amino acids encoded by the DH RF1 (Tab. 12). Thus, the single clone using DH RF1 represents only 11 % of our secondary Abs.

Thus, tyrosine- and glycine-rich HCDR3s were seen in all clones except antibodies FS2°-01 and FS2°-09. The prevalence of these 2 amino acids shifted the average hydrophobicity of the HCDR3s strongly into the hydrophilic range (average hydrophobicity less than -0.3) (Tab. 10). In contrast, hydrophobicity of HCDR3 in antibodies FS2°-01 and FS2°-09 had average hydropathicity scores in the normalized Kyte-Doolittle scale of 0.17 and 0.42, respectively.

Moreover, the usage of VHOx1 in antibodies FS2°-05 to -08 was accompanied by the expression of germ line-non-related Ox1 motive (DXG), pointing out the minimal role of DH genes in the constraining of amino acid composition of the HCDR3. However, the DRG sequence

determining the Id_{Ox1} idiotope was not found. Hence, these antibodies did not react with our Id_{Ox1}-specific prototypic monoclonal anti-idiotype 8-21/W18.

Conclusions:

- In principle, ΔD-DμFS mice can generate the genetic VH/VL combination of Id_{Ox1} antibodies.
- However, the idiotypic motive DRG in which the middle arginine is essential for recognition by syngeneic anti-idiotypes can either not or only extremely rarely be generated.

3.5.1.4 Analysis of tertiary anti-phOx antibodies

Clonal composition of the tertiary response

Two months after secondary immunization mice received a tertiary immunization and on day 3 thereafter, hybridomas secreting phOx-specific antibodies were prepared by cell fusion with the non-secretor myeloma cell line Ag8.653. From one fusion, 35 hybridoma lines could be established (Tab. 13). Strikingly and in accord with secondary fusions (see above), even after tertiary immunization, 12/35 clones (34 %) secreted antibodies of the IgM class. This is in sharp contrast to BALB/c WT mice in which only IgG antibodies can be established at this stage of a memory response [46].

Among IgM antibodies, FS3°-04 was encoded by the VHOx1 gene VH171 and antibodies FS3°-07 used the VκOx1 gene Vκ072. However, the classical full VHOx1/VκOx1 combination was not found (Tab. 13). IgG antibodies FS3°-18 and -19 were encoded by the VHOx1 gene VH171, but only FS3°-18 utilized the VκOx1 gene Vκ072. This gene was also used in 2 other IgG antibodies, in FS3°-15 in combination with the VH2 family gene VH175 and in FS3°-23 in combination with the VH5 family gene VH139. Interestingly, 7 IgG clones (FS°3-24 to -30, representing 30 % of IgG antibodies) were encoded by the VH/VL gene combination VH158/Vκ115. Thus, this combination can be regarded as a dominating clone in the tertiary response of ΔD-DμFS mice.

Conclusions:

- In contrast to BALB/c WT mice with a full DH gene repertoire, the Id_{Ox1} does not prevail the tertiary TD response of ΔD-DμFS mice which can only make use of one artificial DH gene coding for hydrophobic amino acids.
- Instead, the VH158/Vκ115 genetic combination may develop into a dominating clone. Interestingly, this gene combination was also a major clone when wildtype mice were

immunized first with the TI-2 antigen phOx-Ficoll, 3 months later with the TD antigen phOx-CSA and a second TD immunization 2 months later [46].

Table 13

VH/VL gene combinations of phOx-CSA-induced tertiary anti-phOx antibodies in ΔD-DμFS mice

MAb[a]		VH chain genes					VL chain genes				
FS3°	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J
01	μ	1	2	627	2/0	2	24/25	1	096	0/0	5
02	μ	1	2	495	2/0	2	-	-	-	-/-	-
03	μ	1	2	286	1/0	3	9A	1	112	1/0	2
04	μ	2	1	**171**[k]	0/1	2	10	1	137	3/0	2
05	μ	5	1	178	2/0	1	21	1	210	0/0	1
06	μ	5	1	176	0/0	3	8	1	195	0/0	2
07	μ	5	1	139	1/0	2	4/5	1	**072**[k]	4/0	5
08	μ	5	1	139	2/0	2	1	1	231	5/3	4
09	μ	6	1	494	8/3	3	2	1	099	0/0	5
10	μ	7	1	158	10/1	3	1	1	115	1/3	2
11	μ	-	–	-	-/-	-	10	1	137	3/0	2
12	μ	-	-	-	-/-	-	1	1	115	0/1	1
13	γ	1	2	627	1/0	2	9A	1	103	6/0	2
14	γ	1	2	627	1/0	2	12/13	1	174	0/0	5
16	γ	1	2	073	3/2	2	4/5	1	078	2/1	5
17	γ	1	2	073	6/0	2	-	-	-	-/-	-
15	γ	2	1	175	3/1	3	4/5	1	**072**[k]	6/0	5
18	γ	2	1	**171**[k]	7/4	3	4/5	1	**072**[k]	1/0	5
19	γ	2	1	**171**[k]	8/3	3	10	1	137	3/3	2
20	γ	3	1	138	7/0	1	24/25	1	123	2/0	1
21	γ	5	1	139	3/1	3	4/5	1	**072**[k]	5/2	5
22	γ	6	1	114	3/2	2	9A	1	108	3/2	2
23	γ	6	1	114	2/1	2	9A	1	108	2/1	2
36	γ	7	1	663	4/1	3	1	1	115	3/1	4
24	γ	7	1	158	8/1	3	1	1	115	0/0	2
25	γ	7	1	158	9/1	3	1	1	115	1/1	2
26	γ	7	1	158	5/5	3	1	1	115	4/1	4
27	γ	7	1	158	10/2	3	1	1	115	0/0	2
28	γ	7	1	158	10/2	3	1	1	115	0/0	2
29	γ	7	1	158	11/2	3	1	1	115	0/0	2
30	γ	7	1	158	4/1	3	1	1	115	1/1	4
31	γ	14	1	125	8/1	2	10	1	129	2/2	1
32	γ	14	1	125	8/1	2	10	1	129	2/1	1
33	γ	14	1	125	3/0	2	12/13	1	172	0/1	4
34	γ	-	-	-	-/-	-	4/5	1	078	2/0	1
35	γ	-	-	-	-/-	-	10	1	137	3/3	2

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-DμFS mice (FS), after tertiary immunization (3°) and is followed by a sequential number. All clones are generated from the same fusion.
[b] – Isotype of the monoclonal antibody.
[c] – Indication of VH or VL gene family in the VBASE2 integrative database.
[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.
[e] – VH and VL gene numbers according to the integrative database VBASE2.
[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.
[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.
[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold.
- – Sequence not determined.
Grey background shows the Id_{Ox1} gene combination VH171/Vκ072.

Mutations in tertiary antibodies

Mutations further accumulated in tertiary IgG antibodies as compared to secondary antibodies while IgM antibodies remained rather non-mutated. Only IgM FS3°-09 and -10 contained considerable numbers of mutations. This might indicate that both antibodies had undergone immune maturation. However, if this were the case it has to be asked why they did not traverse class switch recombination.

Among IgG antibodies, the 2 VH171 genes in antibodies FS3°-18 and -19 exhibited rather high numbers of mutations, but the typical S>T mutation in position 31 is not seen. However, antibody FS3°-19 showed a silent mutation in this position. Highest mutation rates in IgG antibodies occur in antibodies encoded by the VH158/Vκ115 gene combination (Tab. 13). In 4 positions (52, 56, 61 and 76), the VH158 gene in all of these antibodies had a variable number of silent and productive mutations in common with previously established anti-phOx antibodies [46]. Those antibodies were obtained in mice which had been immunized first with the TI-2 antigen phOx-Ficoll followed by a primary and then a secondary immunization with phOx-CSA. This further proves that tertiary ΔD-DμFS antibodies with the VH158/Vκ115 gene combination had undergone immune maturation.

Conclusions:

- The tertiary anti-phOx antibodies from ΔD-DμFS mice underscore that the single artificial DH gene in these mice does not allow the Id_{Ox1} to become a dominant clone.
- However, ΔD-DμFS mice can develop antibodies with the VH158/Vκ115 gene combination as a dominant clone in the tertiary response.

Table 14

Nucleotide sequences of HCDR3 of phOx-CSA-induced tertiary anti-phOx antibodies generated from ΔD-DμFS

FS3°		----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene			T TTT ATT ACT ACG GTA GTT AGC TAC		1
01	μ	TGT GCA AGC	GGG GGT ACT ACG GTA GCC TTT	GAC TAC TGG	1
02	μ	TGT GCA AGA	G ACT ACG GCA AAA GAG GT	GGC TAC TGG	2
05	μ	TGT ATT ACT	ACG GTA GTT AGC TAC TGG TAC TTC	GAT GTC TGG	1
06	μ	TGT GCA AGA	GGC AGG GCT ACG GTA GTT AGC TAC GCC TGG TTT	GCT TAC TGG	1
07	μ	TGT GCA AGA	G ACT ACG GT	GAC TAC TGG	2
08	μ	TGT GCA AGA	G ACT ACG GT	GAC TAC TGG	2
03	μ	TGT GCA AGA	AGA GAT GGG	GCT TAT TGG	nf
04	μ	TGT GCC AGA	GAT TCG GGG	GAC TAC TGG	nf
09	μ	TGT ACG GGG	GGT CCC TGG TTT	GCT TAC TGG	nf
10	μ	TGT GCA AGA	GGG GCC GGT AGT GGA GCC TGG TTT	GCT TAC TGG	nf
13	γ	TGT GCA AGG	T TTT ACT ACG GTA GGG GGG GGC TCG TCTACTTT	GAC TAC TGG	2
14	γ	TGT GCA AGG	T TTT ACT ACG GTA GGG GGG GGC TCG TCTACTTT	GAC TAC TGG	2
16	γ	TGT GCA AGA	GGA GGC TTT ATT AGG ACG ACG GTA GCC GGG	GAC TAC TGG	1
17	γ	TGT ACA ACA	G GGG ATT ACT ACG GT	AGT TAC TGG	2
20	γ	TGT GCA AGA	TTT ACT ACG GTA GTT AGC TAC TGG TACTTC	GAT GTC TGG	1
21	γ	TGT GCA AGA	G ACT ACG GG	GCT TAC TGG	2
25	γ	TGT GCA AGA	G GTT ATT ACT ACG GTA GTT GGT TT	GCT TAC TGG	2
26	γ	TGT GCA AGA	G GGC ATT ACT ACG GTG CCT GGT TT	GCT TAC TGG	2
27	γ	TGT GCA AGA	G GTC ATT ACT ACG GTA CTT GGT TT	GCT TAC TGG	2
28	γ	TGT GCA AGA	G GTC ATT ACT ACG GTA CTT GGT TT	GCT TAC TGG	2
29	γ	TGT GCA AGA	G GTC ATT ACT ACG GTA CTT GGT TT	GCT TAC TGG	2
30	γ	TGT GCA AGA	G GGG ATT ACT ACG GTG CCT GGT TT	GCT TAC TGG	2
31	γ	TGT GCT AGT	G ACT ACG GC	CTC TAC TGG	2

32	γ	TGT GCT AGT	G ACT ACG GC		CTC TAC TGG	2
33	γ	TGT GCT AGA	TAT GAG ATT ACT ACG GTA GTT ACC		TCT ATC TGG	1
36	γ	TGT GCA AGA	G GGG ATT ACT ACG GTG CCT GGT TT		GCT TAC TGG	2
15	γ	TGT GCC AGA	GAT TGG GGG		GAT TAC TGG	nf
18	γ	TGT GCC AGA	GAT GGG GGG		ACT TAT TGG	nf
19	γ	TGT GCC AGA	GAT GAG GGT		GTG AAC TGG	nf
22	γ	TGT ACA ACT	AGA GGG		GAC TAT TGG	nf
23	γ	TGT ACA ACT	AGA GGG		GAC TAT TGG	nf
24	γ	TGT GCA AGA	G GGG ACG GTC GTG GAG CCT GGT TT		GCT TAC TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.
Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-DμFS mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-DμFS mice.
DH germ line coding sequence of ΔD-DμFS is outlined in first line in green colour.

HCDR3 nucleotide sequences of tertiary antibodies

60 % of the IgM antibodies contain germ line-related HCDR3 sequences of variable length (Tab. 14). Among IgG antibodies this value is even higher with 72 %. As in all previous antibodies of this investigation, antibodies with RF1 (~ 19 %) usage in HCDR3 are clearly counter selected. RF2 dominates with 50 % and in 31 % of the antibodies the reading frame could not be ascribed. The HCDR3 loop of antibody FS3°-16 seems to interesting since to separated germ line-related sequences occur which are separated from each other by non-ascribable nucleotides. It may be speculated that this D region may be generated by D-D duplication.

Conclusion:

- Reading frame used in these tertiary anti-phOx antibodies from ΔD-DμFS mice thus confirm findings from the earlier stages of this response.

HCDR3 amino acids in tertiary antibodies in ΔD-DμFS mice

The deduced amino acid HCDR3 sequences in the tertiary anti-phOx antibodies are shown in Tab. 15. Only 3 IgM and 3 IgG contained hydrophobic amino acids encoded by DH RF1, while 3 IgM and 13 IgG antibodies had RF2-encoded hydrophilic amino acids. In 4 IgM and 6 IgG, the reading frame could not be assigned and the newly generated HCDR3 nucleotide sequences encoded mostly hydrophilic amino acids. Interestingly, only the germ line-related HCDR3 sequences coded for tyrosines, which was missing in germ line-non-related HCDR3. The negative selection of hydrophobic amino acid encoded by DH RF1 is also reflected in the hydropathicity scores of the HCDR3 loops, *i.e.* negative values prevail while positive scores are very rare. Thus, the majority of the HCDR3-loops resembled those seen in BALB/c WT mice with hydrophilic amino acids like tyrosine and glycine (Tab. 15).

The lengths of HCDR3-loops of these tertiary ΔD-DμFS anti-phOx antibodies showed the same characteristics as before, namely that germ line-related coding caused longer loops than non-related coding. In IgG antibodies, the length of HCDR3-loops ranged from 2 to 11, while those of IgM antibodies varied between 3 and 11 amino acids. Interestingly, the middle Id_{Ox1} related motive DXG was present in four clones of IgM class (FS3°-3, -7, -8 and -41) and in 5 IgG antibodies (FS3°-15, -18, -19, -21, -31 and -32). The HCDR3-loops of these clones were encoded by N nucleotides generated during the recombination processes and did not show any homology with the germ line DH segment.

Conclusions:

- Also in the tertiary response, ΔD-DμFS mice `try to avoid´ coding of HCDR3 in RF1. Instead, RF2 usage and generation of newly form nucleotide sequences during recombination favor HCDR3 loops which are rich in glycine and tyrosine.

Table 15

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced tertiary anti-phOx Abs generated from ΔD-DμFS mice

FS3°		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CAS	GGTTVAF	DYW	1	0.48
02	μ	CAR	DYGKRG	GYW	2	-0.6
05	μ	CIT	TVVSYWYF	DVW	1	0.43
06	μ	CAR	GRATVVSYAWF	AYW	1	0.36
07	μ	CAR	DYG	DYW	2	-0.64
08	μ	CAR	DYG	DYW	2	-0.64
03	μ	CAR	RDG	AYW	nf	-0.76
04	μ	CAR	DSG	DYW	nf	-0.36
09	μ	CTG	GPWF	AYW	nf	0.16
10	μ	CAR	GAGSGAWF	AYW	nf	0.31
13	γ	CAR	FYYGRGGLVYF	DYW	2	0.29
14	γ	CAR	FYYGRGGLVYF	DYW	2	0.29
16	γ	CAR	GGFIRTTVAG	DYW	1	0.38
17	γ	CTT	GDYYG	SYW	2	-0.3
20	γ	CAR	FTTVVSYWYF	DVW	1	0.45
21	γ	CAR	DYG	AYW	2	-0.41
25	γ	CAR	GYYYGSWF	AYW	2	0.01
26	γ	CAR	GHYYGAWF	AYW	2	0.04
27	γ	CAR	GHYYGTWF	AYW	2	-0.06
28	γ	CAR	GHYYGTWF	AYW	2	-0.06
29	γ	CAR	GHYYGTWF	AYW	2	-0.06
30	γ	CAR	GDYYGAWF	AYW	2	0.03
31	γ	CAS	DYG	LYW	2	-0.41
32	γ	CAS	DYG	LYW	2	-0.41
33	γ	CAR	YEITTVVT	SIW	1	0.43
36	γ	CAR	GDYYGAWF	AYW	2	0.03
15	γ	CAR	DWG	DYW	nf	-0.37
18	γ	CAR	DGG	TYW	nf	-0.31
19	γ	CAR	DEG	VNW	nf	-0.66
22	γ	CTT	RG	DYW	nf	-0.64
23	γ	CTT	RG	DYW	nf	-0.64
24	γ	CAR	GDGRGAWF	AYW	nf	-0.06

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].
Amino acids marked in green correspond to DH germline sequence in ΔD-DμFS mice.
Amino acids marked in red are generated by N and P region insertions. nf – Not found

As general conclusions from the analysis of the thymus-dependent anti-phOx response in ΔD-DμFS mice it can be stated:

1.) The restriction of the number of DH gene segments to one artificial DH gene demonstrates the extraordinary variability at the third hypervariable region which is enzymatically created during VDJ-recombination by exonucleases and the insertion of non-templated (N) and palindromic (P) nucleotides.
2.) The restriction of the number of DH gene segments to one artificial DH gene, coding for hydrophobic amino acids, causes a substantial reduction of the antibody repertoire of the mice.
3.) The canonical VH/VL gene combination VH171/Vκ072 together with Id_{Ox1}-typical amino acid sequence DRG in HCDR3 is extremely rarely verified and, if so, only in particular animals.

3.5.2 Genetic analysis of the phOx-reactive repertoire of ΔD-iD mice

Next, ΔD-iD mice were immunized with the TD antigen phOx-CSA. These mice can only make use of a single DH gene coding for charged amino acids in HCDR3. Anti-phOx antibody-secreting hybridomas were generated by cell fusion from the primary, secondary and tertiary of immune response. Here, the V region-coding sequences of these antibodies will be described.

3.5.2.1 Analysis of early primary anti-phOx antibodies

Primary repertoire

From the early primary response of ΔD-iD mice, *i.e.* 7 days after immunization, 18 hybridomas could be established. Eleven of them (61 %) were of the IgM and 39 % belonged to the IgG class (Tab. 16). The nomenclature of these antibodies is analogous to that of ΔD-DμFS mice.

This mutant strain is abbreviated by iD and early primary antibodies are marked with 1° followed by the day 7 of the fusion. IgM anti-phOx antibodies iD1°7-05, -06 and -07 and IgG antibodies iD1°7-13 and -14 expressed a VHOx1 heavy chain (VH171) in association with alternative light chains. Only IgG antibody iD1°7-12 used the VκOx1 light chain (Vκ072) in combination with VH183. Hence, the Id_{Ox1} VH/VL gene combination VH171/Vκ072, dominating the primary TD response of BALB/c WT mice, is not expressed among early primary antibodies of ΔD-iD mice. Moreover, the normally dominant Id_{Ox1} is not replaced by another dominant VH/VL gene combination. Most of the variable genes in this primary response in the ΔD-iD mice have been observed in the WT mice, but in different combinations.

Conclusions:

- Hence, the restriction to a single DH gene segment coding for charged amino acids changes the pattern of VH/VL combinations in ΔD-iD mice, but not the V gene usage of heavy and light chains.
- There is no dominant idiotype in the early primary anti-phOx antibodies.

Affinity of primary antibodies

The relative affinity of the majority of IgM antibodies was 10-20 lower than that of the IgM Id_{Ox1} antibody H11.5 (μ, κ). In contrast, the affinities of the 2 IgG antibodies iD1°7-16 and -17 were in the range of the Id_{Ox1} control antibody NQ2/16.2, and antibodies iD1°7-13, -14 and -15 exhibited clearly higher affinities (Tab. 16).

Conclusion:

- Even a restricted pre-immune repertoire generated from the single D gene segment of ΔD-iD mice may contain antibodies with VH/VL gene combinations which confer higher affinities than that of Id_{Ox1} antibodies.

Mutations in early primary antibodies

Most of the early primary anti-phOx antibodies from ΔD-iD mice showed no or low mutation numbers, with some exceptions. The 2 IgM antibodies iD1°7-03 and -04 had enhanced mutations in the VH1 family gene VH286. However, since this is a class 2 gene, these mutations are doubtful. Three other IgM antibodies, iD1°7-01, -05 and -10, contain some productive as well as silent mutations in their light chain which can not be explained at this early stage of the primary response.

Conclusion:

- We conclude that the mutation rates of early primary phOx-CSA-induced antibodies in ΔD-iD mice did not differ significantly from those of BALB/c WT mice. Thus, the alteration in the DH locus did not influence the mutation rate at this stage of immune response.

Table 16

VH/VL gene combinations of phOx-CSA-induced primary day 7 anti-phOx antibodies in ΔD-iD mice

MAb[a]		VH chain genes					VL chain genes					rel.
iD1°7	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
01	μ	1	1	532	0/0	1	2	1	097	7/1	1	-
02	μ	1	1	532	1/0	3	23	1	171	0/0	2	0,05
03	μ	1	2	286	5/1	2	9A	1	108	1/0	2	-
04	μ	1	2	286	3/0	2	1	1	122	1/0	2	0,07
05	μ	2	1	**171**[k]	1/0	4	4/5	1	078	2/3	2	0,2
06	μ	2	1	**171**[k]	3/0	2	10	1	137	1/1	1	0,1
07	μ	2	1	**171**[k]	2/0	3	21	1	210	0/1	2	0,1
08	μ	5	1	131	0/1	1	1	1	122	1/0	2	0,05
09	μ	6	1	114	1/0	3	9A	1	108	0/0	2	0,06
10	μ	-	-	-	-/-	-	8	1	195	6/3	5	0,07
11	μ	-	-	-	-/-	-	21	1	064	2/0	4	-
12	γ	2	1	183	1/0	4	4/5	1	**072**[k]	1/0	5	-
13	γ	2	1	**171**[k]	0/1	4	1	1	115	0/0	2	4,1
14	γ	2	1	**171**[k]	1/1	3	4/5	1	154	0/0	5	5,7
15	γ	5	1	176	1/0	3	8	1	195	0/1	1	9,2
16	γ	5	1	139	0/0	4	4/5	1	050	0/0	2	0,8
17	γ	6	1	114	0/0	3	8	1	188	0/0	4	0,9
18	γ	-	-	-	-/-	-	19/281		186	0/0	2	-

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-iD mice (iD), after primary immunization (1°) on day 7 (7) and is followed by a sequential number. All clones are generated from the same fusion.
[b] – Isotype of the monoclonal antibody.
[c] – Indication of VH or VL gene family in the VBASE2 integrative database.
[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.
[e] – VH and VL gene numbers according to the integrative database VBASE2.
[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.
[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.
[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold.
- – Sequence not determined.

Table 17

Nucleotide sequences of HCDR3 of phOx-CSA-induced primary day 7 anti-phOx antibodies generated from ΔD-iD mice

iD1°7		----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene			TT TAT CGT AAT CAT AGT AGA AGC TAC		1
01	μ	TGT GCA AGA	AGG GGT CGT AAT CAT AGT AGA AGC TAC GTA GGC GGG TAC TTC	GAT GTC TGG	1
02	μ	TGT GCA AGG	ATT TAT CTG ATC	CCT TAC TGG	1
08	μ	TGT GCA AGA	CAG GGG CGT AAT CAT AGT AGA AGC TAC CCC TCA CTG GTA CTT	CGA TGT CTG	1
03	μ	TGT GCA AGT	ATT AGT ACG GTA	GTC ATC TGG	nf
04	μ	TGT GCA AGA	TGG GGC AAC	GAC TAC TGG	nf
05	μ	TGT GCC AGA	GAT CGG GGG	GAC TAC TGG	nf
06	μ	TGT TCC AGA	GAT CGG GGG	GAC TAC TGG	nf
07	μ	TGT GCC AGA	GAT CGG GGG	GCT TAC TGG	nf
09	μ	TGT TCC AGG	CGA GGG	ACT CAC TGG	nf
14	γ	TGT GCC AGG	TCT TAT CGT AAT CAT AGT AGA ACC	GCT TAC TGG	1
15	γ	TGT GCA AGT	AGC TTC TAC TAT GAT TAC GAT AAA TCG TTT	GCT TAC TGG	inv
16	γ	TGT GCA AGA	CC CGT AAT CAG ATA TTA CTA TGC TAT G	GAC TAC TGG	2
17	γ	TGT ACC AGG	CC TCT TAT CGT AAG GGT TCA CTT T	GCT TAC TGG	2
12	γ	TGT GCC AGA	AAT TGG GGG	GAC TAC TGG	nf
13	γ	TGT GCC AGA	GAT CGA GGG	GAC TAC TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.

Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-iD mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-iD mice.
DH germ line coding sequence of ΔD-iD is outlined in first line in green colour.

HCDR3 nucleotide sequences of early primary antibodies

From 15 antibodies we obtained full sequences of VH regions (Tab. 17). In 3/9 of IgM and in 1/6 IgG of the 15 VH sequences, HCDR3 were translated in RF1. In another IgG antibody (iD1°7-15), HCDR3 was in an inverted RF1. Only the 2 IgG antibodies iD1°7-16 and -17 used RF2 in HCDR3. In the majority of the antibodies, *i.e.* 6 IgM and 2 IgG, the D regions the reading frames could not be assigned.

Conclusions:

- The bias against RF1 usage is not as pronounced as in ΔD-DμFS mice. RF2 is not really dominant and in most of the antibodies the reading frame could not be assigned.
- Invariably, non-assignable reading frames code for shorter D-regions lengths.
- B cells seem to escape the usage of charged HCDR3-loop in two mechanisms:
  - First, by shifting the RF, so that 2 antibodies (iD1°7-16 and 17) contain RF2-encoded DH segments and 1 antibody (iD1°7-15) contained DH sequence encoded by an inverted RF1 (i-RF1).
  - Second, by an enhanced loss of nucleotides at the termini of DH gene, so that 53 % of the HCDR3 intervals contained no germ line-encoded DH nucleotides.

HCDR3 amino acids in early primary antibodies in ΔD-iD mice

Three IgM antibodies (iD1°7-5, -06 and -07) and 1 IgG antibody (iD1°7-13), exhibiting germ line non-related readings frames in HCDR3, contained the DRG motive of wildtype Id_{Ox1} antibodies, but only one of them (iD1°7-13) reacted with our Id_{Ox1}–specific prototypic monoclonal anti-idiotype 8-21/W18. This is astonishing, since only antibody iD1°7-07 showed the complete Id_{Ox1}-typical sequence CAR-DRG-AYW (Tab. 18). However, the 3 IgM antibodies iD1°7-05, -06 and -07 contained the DRG motive and used the VHOx1 in combination with alternative light chain genes as the other VH171-encoded IgG antibodies.

Conclusions:

- This shows that the Id_{Ox1} specificity relies not only on the DRG motive in the HCDR3 loop, but is also influenced in its 3-dimensional structure by other parts of the complete molecule, *i.e.* including the L chain.
- With the only exception of antibody iD1°7-02, short HCDR3-loops are only seen in antibodies in which the reading frame could not assigned.

Table 18

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced primary day 7 anti-phOx antibodies generated from ΔD-iD mice

iD1°7		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CAR	RGRNHSRSYVGGYF	DVW	1	-0,27
02	μ	CAR	IYLI	PYW	1	1,13
08	μ	CAR	QGRNHSRSYPSLVLRC	LGR	1	-0,4
03	μ	CAS	ISSV	VIW	nf	0,78
04	μ	CAR	WGN	DYW	nf	-0,37
05	μ	CAR	DRG	DYW	nf	-0,76
06	μ	CSR	DRG	DYW	nf	-0,76
07	μ	CAR	DRG	AYW	nf	-0,76
09	μ	CSR	RG	THW	nf	-0,64
14	γ	CAR	SYRNHSRT	AYW	1	-0,63
15	γ	CAS	SFYYDYDKSF	AYW	1inv	-0,19
16	γ	CAR	PVIRYYYAM	DYW	2	0,27
17	γ	CTR	PLIVRVHF	AYW	2	0,6
12	γ	CAR	NWG	DYW	nf	-0,37
13	γ	CAR	DRG	DYW	nf	-0,76

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].
Amino acids marked in green correspond to DH germline sequence in ΔD-iD mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

3.5.2.2 Analysis of late primary anti-phOx antibodies

Clonal development in the late primary response

After primary immunization of ΔD-iD mice with phOx-CSA, 20 anti-phOx antibody-secreting hybridomas could be established from the late primary response in 2 separate fusions on day 14 (Tab. 19). As the most remarkable finding, the absolute majority of 19 antibodies were of the IgM and only 2 antibodies of the IgG class. This is in sharp contrast to BALB/c WT mice in which only IgG-secreting hybridomas can be generated at this late time point of the primary immune response. It is also striking that the Id_{Ox1} variable genes were almost absent. The VHOx1 gene VH171 is found not at all in antibodies of both fusions, and VκOx1 is only found once in the IgM antibody iD1°14-11. Hence, as among the early primary antibodies, the

Id_{Ox1} is not expressed (Tab. 17). In addition, there is no other dominant VH/VL combination. The repertoire of these 20 late primary antibodies is with 15 VH and 14 VL genes extremely heterogeneous and every gene combination is unique.

Conclusions:

- As already observed in ΔD-DμFS mice, antibodies of the late primary response of ΔD-iD mice revealed an impaired class switch recombination.
- The genetic repertoire is quite heterogeneous.
- There is no dominant VH/VL gene combination

Mutations in late primary antibodies

It is also surprising that the majority of H and L chains were not mutated. In fact, except the 3 antibodies iD1°14-06, -09and -20, none of the VL genes showed any mutation. In addition, 5 VH genes did not present any deviation from the germ line configuration. These data indicate that the late primary IgM-secreting activated B cells neither undergo class switch recombination nor do they show somatic mutation.

Conclusion:

- Hence, the restriction in ΔD-iD mice to a single DH gene coding for charged amino acids in HCDR3 drastically inhibits the early immune maturation through somatic mutation.

HCDR3 nucleotide sequences of late primary antibodies

The nucleotide sequences of the late primary anti-phOx antibodies from ΔD-iD mice are depicted in Tab. 20. In 11/18 IgM antibodies (61 %), the HCDR3 nucleotide sequences derived from the germ line DH gene and in another antibody (iD1°14-05), the germ line relation of the HCDR3 sequence was doubtful since only 5 successive nucleotides were identical with the germ line DH gene. A germ line related HCDR3 sequence is also observed in 1 IgG antibody, but not the other.

As already observed in the early primary antibodies of ΔD-iD mice, RF1 was not decreased to such an extent as in ΔD-DμFS mice. In fact, 8/18 antibodies (~44 %) used RF1 in HCDR3, 2 antibodies used RF2 and RF3, respectively, and in 6 antibodies (~33 %) the reading frame could not be assigned (Tab. 20).

Conclusions:

- The bias against RF1 usage is not as pronounced as in ΔD-DμFS mice. RF2 and RF3

are least frequently used and antibodies with germ line non-related HCDR3 sequences are rather common.

- Non-assignable reading frames code for shorter D-regions lengths.

Table 19

VH/VL gene combinations of phOx-CSA-induced primary day 14 anti-phOx antibodies in ΔD-iD mice

MAb[a]		VH chain genes					VL chain genes					rel.
iD1°14	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
05[h]	μ	1	1	706	0/0	2	4/5	1	142	0/0	2	-
02[h]	μ	1	2	654	1/2	3	2	1	097	0/0	2	-
03[h]	μ	1	2	499	0/0	3	19/28	1	198	0/0	1	-
04[h]	μ	1	2	286	1/1	1	19/28	1	201	0/0	1	-
01[h]	μ	1	3	014	0/0	1	10	1	137	0/0	1	-
06[i]	μ	3	1	128	4/1	2	4/5	1	050	3/1	4	-
07[h]	μ	3	1	128	1/0	1	10	1	137	0/0	4	-
08[i]	μ	5	1	192	1/1	2	23	1	168	0/0	1	-
13[h]	μ	5	1	185	1/1	2	4/5	1	082	0/0	5	-
12[h]	μ	5	1	178	2/0	2	8	1	069	0/0	5	-
09[i]	μ	5	1	147	0/0	2	38C	1	140	1/0	5	-
10[i]	μ	5	1	135	0/0	1	23	1	168	0/0	4	-
11[h]	μ	5	1	135	1/0	1	4/5	1	**072**[k]	0/0	5	-
14[h]	μ	5	1	135	1/1	2	23	1	179	0/0	5	-
15[i]	μ	6	1	114	2/2	3	1	1	115	0/0	1	-
16[h]	μ	10	1	028	1/0	2	12/13	1	170	0/0	5	-
17[i]	μ	-	-	-	-/-	-	12/13	1	170	0/0	1	-
18[h]	μ	-	-	-	-/-	-	2	1	097	0/0	1	-
19[h]	μ	-	-	-	-/-	-	4/5	1	078	0/0	5	-
21[h]	γ	1	1	528	8/0	3	-	-	-	-/-	-	-
20[i]	γ	1	2	455	3/1	3	1	1	115	1/1	1	-

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-iD mice (iD), after primary immunization (1°) on day 14 (14) and is followed by a sequential number.
[b] – Isotype of the monoclonal antibody.
[c] – Indication of VH or VL gene family in the VBASE2 integrative database.
[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.
[e] – VH and VL gene numbers according to the integrative database VBASE2.
[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.
[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.
[h, i] – Indication of different fusions.
[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold. - – Sequence not determined.

Table 20

Nucleotide sequences of HCDR3 of phOx-CSA-induced primary day 14 anti-phOx antibodies generated from ΔD-iD mice

iD1°14	----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene		TT TAT CGT AAT CAT AGT AGA AGC TAC		1
01 μ	TGT GCA AGA	CGA GAT CGT AAT CAT AGT AGA AGC TTC CTC TAC TGG TAC TTC	GAT GTC TGG	1
03 μ	TGT GCA AGA	T TAT CGT ACT CAT ATT TTA AGC TCC TGG TT	TGC TTA TGG	3
04 μ	TGT GCA AGA	CAT CAT AGT AGA AGC TAC TGG TAC TTC	GAT GTC TGG	1
06 μ	TGT GCA AGG	GG CGT AAT CAT ACT ACT AGC TAG ATA CTT T	GAC TAC TGG	2
07 μ	TGT GCA AGA	AAG GAT CGT AAT CAT AGT AGA AGG GAC TGG TAC TTC	GAT GTC TGG	1
08 μ	TGT GCA AGA	CCT TTT TAT CGT AAT CAT AGG GGA TAC TTT	GAC TAC TGG	1
10 μ	TGT GCA AGA	CAT CGG CGT AAT CAT AGT AGA AGA GAG ACT GAC TGG TAC TTC	GAT GTC TGG	1
11 μ	TGT GCA AAT	CGT AAT CAT AGT AGA AGC TAC TGG TAC TTC	GAT GTC TGG	1
12 μ	TGT ACA AGA	GTA GCT TCT ACT ATG ATT ACG GTA AAG AGG GTC CTT	GAC TAC TGG	3inv
13 μ	TGT GCA AGG	TC AAT CGT AAT CAT AGT AGA AGC T	AAA CTA TGG	2
14 μ	TGT GCA AGA	CCA GGA CGT AAT CAT AGT AGA AGC TAC CCC TAC TTT	GAC TAC TGG	1
02 μ	TGT GCA AGA	GGG GGG ATT CAT CGT AAA AAC CTG GTT	TGC TTA TGG	nf
05 μ	TGT GCA AGA	AGA GAT	GCC TAC TGG	nf
09 μ	TGT GCA AGA	CAC CCG CTA CGA CGC TAC TTT	GAC TAC TGG	nf
15 μ	TGT ACC AGG	CCC GTC ATC CCA GAG GCC TGG TTT	GCT TAC TGG	nf
16 μ	TGT GTG AGA	CAT GGG ACG TAC TCA TGG AGA	GGA CTA TGG	nf
20 γ	TGT ACA AGA	TCA TGG GGG GAT CAT AGT AAA ACC TAC GGT TCC TGG TTT	GCT TAC TGG	1
21 γ	TGT GCA AGA	CGC TTT	GCT TAC TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.

[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.
Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-iD mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-iD mice.
DH germ line coding sequence of ΔD-iD is outlined in first line in green colour.

Table 21

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced primary day 14 anti-phOx antibodies generated from ΔD-iD mice

iD1°14		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CAR	RDRNHSRSFLYWYF	DVW	1	-0.29
03	μ	CAR	LSYSYFKLLV	CLW	3	0.51
04	μ	CAR	HHSRSYWYF	DVW	1	-0.32
06	μ	CAR	GVIILLARYF	DYW	2	0.81
07	μ	CAR	KDRNHSRRDWYF	DVW	1	-0.69
08	μ	CAR	PFYRNHRGYF	DYW	1	-0.32
10	μ	CAR	HRRNHSRRETDWYF	DVW	1	-0.68
11	μ	CAN	RNHSRSYWYF	DVW	1	-0.43
12	μ	CTR	VASTMITVKRVL	DYW	3inv	0.57
13	μ	CAR	SIVIIVEA	KLW	2	1
14	μ	CAR	PGRNHSRSYPYF	DYW	1	-0.41
02	μ	CAR	GGIHRKNLV	CLW	nf	0.05
05	μ	CAR	RD	AYW	nf	-1.15
09	μ	CAR	HPLRRYF	DYW	nf	-0.24
15	μ	CTR	PVIPEAWF	AYW	nf	0.41
16	μ	CVR	HGTYSWR	GLW	nf	-0.39
20	γ	CTR	SWGDHSKTYGSWF	AYW	nf	-0.21
21	γ	CAR	RF	AYW	nf	-0.1

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].
Amino acids marked in green correspond to DH germline sequence in ΔD-iD mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

HCDR3 amino acids in late primary antibodies in ΔD-iD mice

As expected, the majority of these antibodies expressed mainly hydrophilic amino acids in HCDR3 so that the average hydrophatic score of the D loops became negative (hydropathy index lower than - 0.1; Tab. 21). In fact, all RF1 translated HCDR3 had the lowest hydropathic values and antibodies with positive scores were translated in RF2 and RF3 or the reading frame could not be assigned. In contrast to the early primary antibodies, HCDR3 sequences of all these antibodies showed no similarities to the typical D-loops of Id_{Ox1} antibodies and also the middle DRG motive was not verified. This finding supports again the conclusion that the selection of phOx-repertoires at the different stages of the immune response is individually specific for every mouse.

Conclusions:

- In the late primary anti-phOx antibodies of ΔD-iD mice, hydrophilic amino acids are selectively expressed in HCDR3.
- The divergence of the HCDR3 sequences from those of Id_{Ox1} antibodies confirm findings from the early response in this mouse strain.

3.5.2.3 Analysis of secondary anti-phOx antibodies

Although 4 fusions were performed after secondary immunization which all exhibited normal cell growth, we obtained only 2 anti-phOx antibody-secreting hybridomas from one fusion, 1 IgM and the other an IgG antibody (Tab. 22). The IgM antibody iD2°-01 exhibited 1 silent and 3 productive mutations in VH as well as in VL. The IgG antibody iD2°-02 showed a high number of mutations in VH and half as many mutations in VL. Hence, it can be concluded that especially this IgG antibody had undergone immune maturation. This is also indicated by its substantially enhanced relative affinity in comparison to the Id_{Ox1} IgG antibody NQ2/16.2 (Tab. 22).

While the HCDR3 nucleotide sequence of the IgG antibody was germ line-related, the reading frame of the IgM antibody could not be assigned (Tab. 23) and the deduced HCDR3 amino acid sequences were not related to those of Id_{Ox1} antibodies (Tab. 24).

Conclusions:

- Although it is not possible to draw firm conclusion from only 2 secondary antibodies, it is again astonishing that an IgM antibody could be isolated 3 days after secondary immunization with the TD antigen phOx-CSA.

- Enhanced mutations and an increased affinity of the IgG antibody indicated that secondary antibodies in ΔD-iD mice are derived from an immune maturated repertoire.

Table 22

VH/VL gene combinations of phOx-CSA-induced secondary anti-phOx antibodies in ΔD-iD mice

MAb[a]		VH chain genes					VL chain genes					rel.
iD2°	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
01	μ	6	1	114	3/1	2	9A	1	108	3/1	5	0,5
02	γ	1	3	386	13/4	2	9A	1	108	6/1	2	306,6

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-iD mice (iD), after secondary immunization (2°) and is followed by a sequential number. All clones are generated from the same fusion.
[b] – Isotype of the monoclonal antibody.
[c] – Indication of VH or VL gene family in the VBASE2 integrative database.
[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.
[e] – VH and VL gene numbers according to the integrative database VBASE2.
[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.
[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.

Table 23

Nucleotide sequences of HCDR3 of phOx-CSA-induced secondary anti-phOx antibodies

iD2° ----VH[a]---	-----------DH[b]-----------	----JH[c]---	RF[d]
DH gene	TT TAT CGT AAT CAT AGT AGA AGC TAC		
01 μ TGT ACC AGG	CGG GGC	GAC TAC TGG	nf
02 γ TGT GCA AGA	CTA CGT AAT CAT GGA	GAC TCT TGG	1

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.
Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-iD mice.
Nucleotides marked in red are generated by N and P region insertions.
DH germ line coding sequence of ΔD-iD is outlined in first line in green colour.

Table 24

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced secondary anti-phOx antibodies generated from ΔD-iD mice

iD2°		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CTR	RG	DYW	nf	-0,64
02	γ	CAR	LRNHG	DSW	1	-0,36

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].

Amino acids marked in green correspond to DH germline sequence in ΔD-iD mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

3.5.2.4 Analysis of tertiary anti-phOx antibodies

Clonal development in the tertiary response

Two months after the secondary immunization, mice were immunized once more with phOx-CSA and hybridomas secreting anti-phOx antibodies were prepared by cell fusion 3 days later. From 2 fusions, we obtained 16 monoclonal antibodies (Tab. 25). In one fusion, we established 9 phOx-specific hybridomas; seven of them were of the IgG class and two were IgM antibodies. From the other animal seven hybridomas were derived all of which produced IgM antibodies. The 3 IgM antibodies iD3°-02, -4 and -04 and IgG antibody iD3°-12 were encoded by the VHOx1 gene VH171. While IgM antibody iD3°-3 and IgG antibody iD3°-12 showed the canonical VHOx1/VκOx1 gene combination, characteristic for phOx-specific antibody, the 2 IgM antibodies iD3°-02 and -04 were encoded by alternative VL genes. Antibody iD3°-09 was encoded by the VκOx1 gene Vκ072 in combination with an unknown VH gene.

Conclusions:

- In contrast to anti-phOx antibodies from earlier stages of TD immune responses, tertiary antibodies contained again VH171/Vκ072-encoded Id_{Ox1} antibodies.
- However, the Id_{Ox1} clonotype was not dominant and there was no other dominant clone.

Table 25

VH/VL gene combinations of phOx-CSA-induced tertiary anti-phOx Abs in ΔD-iD mice

MAb[a]		VH chain genes					VL chain genes					rel.
iD3°	Is[b]	Fam[c]	Cl[d]	IGHV[e]	p/s[f]	J	Fam[c]	Cl[d]	IGKV[e]	p/s[f]	J	Aff.[g]
01[h]	μ	2	1	201	0/0	2	4/5	1	050	0/0	5	-
02[h]	μ	2	1	**171**[k]	1/0	2	9/10	1	137	0/0	2	0,3
03[h]	μ	2	1	**171**[k]	0/0	3	4/5	1	**072**[k]	0/0	5	1,0
04[h]	μ	2	1	**171**[k]	0/0	1	4/5	1	079	0/0	2	-
05[i]	μ	5	1	192	1/0	4	4/5	1	157	0/0	5	0,03
06[i]	μ	5	1	148	-/-	4	38C	1	140	1/0	1	0,01
07[h]	μ	5	1	147	0/1	4	10	1	137	0/0	1	-
08[h]	μ	5	1	135	2/1	1	21	1	210	0/0	1	0,01
09[h]	μ	-	-	-	-/-	-	4/5	1	**072**[k]	0/0	2	-
10[i]	γ	1	3	386	7/1	2	9A	1	108	0/1	2	3,8
11[i]	γ	1	3	386	12/3	2	9A	1	108	-/-	2	2,8
12[i]	γ	2	1	**171**[k]	5/3	3	4/5	1	**072**[k]	4/1	5	143,7
13[i]	γ	3	1	120	-/-	2	23	1	168	8/2	1	13,9
14[i]	γ	7	1	158	14/8	3	1	1	115	2/1	2	4,6
15[i]	γ	-	-	-	-/-	-	23	1	168	0/1	2	2,5
16[i]	γ	-	-	-	-/-	-	VRF	1	128	4/3	1	4,6

Legend

[a] – The annotation of antibodies indicates their generation from ΔD-iD mice (iD), after tertiary immunization (3°) and is followed by a sequential number.

[b] – Isotype of the monoclonal antibody.

[c] – Indication of VH or VL gene family in the VBASE2 integrative database.

[d] – In the integrative database VBASE2, the genes are classified: for class 1 genes there is genomic and rearranged evidence, for class 2 genes only genomic evidence and for class 3 genes only rearranged evidence.

[e] – VH and VL gene numbers according to the integrative database VBASE2.

[f] – Productive (p) and silent (s) mutations of corresponding VH and VL chains.

[g] – The relative affinities were measured in a hapten inhibition test in comparison to Id_{Ox1} IgM and IgG anti-phOx antibodies, respectively.

[h, i] – Indication of different fusions.

[k] – Id_{Ox1} genes VH171 and Vκ072 are marked in bold.

\- – Sequence not determined.

Grey background shows the Id_{Ox1} gene combination VH171/Vκ072.

Table 26

Nucleotide sequences of HCDR3 of phOx-CSA-induced tertiary anti-phOx Abs generated from ΔD-iD mice

iD3°		----VH[a]----	----------DH[b]----------	----JH[c]----	RF[d]
DH gene			TT TAT CGT AAT CAT AGT AGA AGC TAC		1
01	μ	TGT GCC AAA	ATG GGT CGT AAT CAT AGT AGA AGC TAC TTT	GAC TAC TGG	1
04	μ	TGT GCC AGA	GCG GGT AGA AGC TAC GGG TGG TAC TTC	GAT GTC TGG	1
07	μ	TGT GCA AGA	ATC AAT CGT AAT CAT AGT AGA AGC TAC CCG GGG TAC TTT	GAC TAC TGG	1
08	μ	TGT GCA AGT	ATT TAT CGT AAT CAT AGT AGA AGT CAC TGG TAC TTC	GAT GTC TGG	1
02	μ	TGT GCC AGA	GAT GGG GGG	GAC TAC TGG	nf
03	μ	TGT GCC AGA	GAT GGA GGG	ATT TCC TGG	nf
05	μ	TGT GCA AGA	TCC CGT TAC GAT TAC TAT GCT ATG	GAC TAC TGG	nf
06	μ	TGT GCA AGA	CAT GGA GCT CCT TAT TAC TAC GGT AGT AGC TAC TAT GCT ATG	GAC TAC TGG	nf
10	γ	TGT GTA AGA	AAG TTC CGA GGG GGT	GAC TAC TGG	nf
11	γ	TGT GTA AGA	AAG TTC CGA GGG GGT	GAC TAC TGG	nf
12	γ	TGT GCC AGA	GAT GGA GGT	GCT TTC TGG	nf
13	γ	TGT GTA AGA	GGA GGG ACG GTA GTA GCT TTT	GAC TAC TGG	nf
14	γ	TGT GCA AGA	GGG TTG TAC GAG GGA GCC TGG TTT	TCT TAT TGG	nf

Legend

[a] – VH-encoded base of HCDR3.
[b] – DH nucleotides of the HCDR3-loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
inv – inverted.
nf – not found.

Nucleotides marked in green (≥ 6 successive nucleotides) correspond to DH germline sequence in ΔD-iD mice.
Nucleotides marked in red are generated by N and P region insertions.
Nucleotides marked in blue (4 or 5 successive nucleotides) correspond probably to DH germline sequence in ΔD-iD mice.
DH germ line coding sequence of ΔD-iD is outlined in first line in green colour.

Mutations in tertiary antibodies

The tertiary IgM antibodies were either non- or almost non-mutated. In fact, VH genes of antibodies iD3°-01, -03 and -04 and all VL genes, except antibody iD°3-6, did not present any deviation from the respective germ line genes. In contrast, the IgG antibodies showed high numbers of productive and few silent mutations (Tab. 25). The relative affinity of the Id_{Ox1} antibody iD3°-03 was identical to the control Id_{Ox1} antibody H11.5 (μ, κ), while affinities of the non-Id_{Ox1} antibodies was 10-100-times lower. In contrast, although IgG antibodies displayed many mutations the affinity of most of them remained comparably low. The relative affinities of antibodies iD3°-10, -11, -14, -15 and -16 were only slightly higher than that of the primary IgG Id_{Ox1} antibody NQ2/16.2 (γ, κ). The affinity of antibody iD3°-13 was ~14-times higher than that of NQ2/16.2 and only the Id_{Ox1} antibody iD3°-12 exhibited an affinity comparable to those of BALB/c WT mice, *i.e*. about 150-times higher than primary Id_{Ox1} antibody NQ2/16.2 (Tab. 25). The affinity maturation of antibody iD3°-12 can also be deduced from the fact that the VL-coding gene Vκ072 shows the well known classical affinity-enhancing early mutations H>N in position 33 and Y>F in position 35 (Fig. 2 in the appendix). However, the known mutation S>T in position 31 in VH171 gene of antibody iD3°-12 was not seen.

Conclusions:

- In the tertiary response of ΔD-iD mice, a high percentage of naïve, non-mutated and non-maturated IgM antibodies are obtained by cell fusion.
- These IgM antibodies resemble antibodies from the primary response.
- In individual ΔD-iD mice, the tertiary response may contain somatically mutated and affinity-maturated Id_{Ox1} antibodies.

HCDR3 nucleotide sequences of tertiary antibodies

The HCDR3-coding nucleotide sequences of the tertiary anti-phOx antibodies are depicted in Tab. 26. As observed before, RF1-encoded sequences are not totally avoided, but verified in 4 IgM antibodies. In the other 4 IgM and 5 IgG antibodies, the HCDR3 reading frames could not be assigned. RF2 and RF3 were not observed. Except in antibodies iD3°-02, -03 and -12, the lengths of the germ line non-related HCDR3 sequences were surprisingly long, especially in antibodies iD3°-13, -14 and -06.

Conclusions:

- Since the germ line DH gene segment of ΔD-iD mice codes for charged, *i.e*. hydrophilic amino acids, RF1 usage is suppressed, but not avoided.

- In a rather high proportion of antibodies, the HCDR3 reading frames could not be assigned.

Table 27

Predicted amino acid sequences of HCDR3 of phOx-CSA-induced tertiary anti-phOx antibodies generated from ΔD-iD mice

iD3°		Base[a]	CDRH3-loop[b]	Base[c]	RF[d]	H[e]
01	μ	CAK	MGRNHSRSYF	DYW	1	-0,31
04	μ	CAR	AGRSYGWYF	DVW	1	-0,02
07	μ	CAR	INRNHSRSYPGYF	DYW	1	-0,29
08	μ	CAS	IYRNHSRSHWYF	DVW	1	-0,29
02	μ	CAR	DGG	DYW	nf	-0,31
03	μ	CAR	DGG	ISW	nf	-0,31
05	μ	CAR	SRYDYYAM	DYW	nf	-0,21
06	μ	CAR	HGAPYYYGSSYYAM	DYW	nf	-0,03
10	γ	CVR	KFRGG	DYW	nf	-0,25
11	γ	CVR	KFRGG	DYW	nf	-0,25
12	γ	CAR	DGG	AFW	nf	-0,31
13	γ	CVR	GGTVVAF	DYW	nf	0,72
14	γ	CAR	GLYEGAWF	SYW	nf	0,24

Legend

[a] – VH-encoded base of HCDR3.
[b] – Predicted amino acid sequence of the HCDR3 loop.
[c] – JH-encoded base of HCDR3.
[d] – Reading frame of DH gene.
[e] – The average, normalized Kyte-Doolittle hydrophobicity of the HCDR3 loop [72, 73].

Amino acids marked in green correspond to DH germline sequence in ΔD-iD mice.
Amino acids marked in red are generated by N and P region insertions.
nf – not found

HCDR3 amino acids in tertiary antibodies in ΔD-iD mice

The deduced amino acids for HCDR3 of the tertiary antibodies are hydrophilic to such an extent that the average hydrophatic score for the D-loops are mostly negative (Tab. 27). Only in antibodies iD3°-13 and – 14 they are slightly positive. Hence, this result presents further evidence for a positive selection of hydrophilic HCDR3-loops.

Three of the 4 antibodies encoded by the VHOx1 gene VH171 showed the Id_{Ox1}-related DH motive DGG, while in the fourth antibody (iD3°-04) the HCDR3 sequence show no such relation. Interestingly, in antibody iD3°-12 which show highest affinity, the entire CDR3-loop, *i.e.* the VH-encoded base plus the HCDR3-loop plus the second base = CAR-DGG-

AFW, is most similar to the respective prototypic sequence of true Id_{Ox1} antibodies (CAR-DRG-AYW).

Conclusions:

- The expression of the original Id_{Ox1} motive DRG or its modified forms DXG is rather independent of the available germ line DH segments.
- Moreover, these findings confirm that this motive is completely associated with the VHOx1gene VH171.

As general conclusions from the analysis of the thymus-dependent anti-phOx response in ΔD-iD mice it can be stated:

1.) As in ΔD-DμFS mice, the restriction of the number of DH gene segments to a non-natural D gene segment which encodes for charged amino acids in HCDR3, demonstrates the extraordinary variability at the third hypervariable region which is enzymatically created during VDJ-recombination by exonucleases and the insertion of non-templated (N) and palindromic (P) nucleotides.

2.) The restriction of the number of DH gene segments to one artificial D gene, coding for charged amino acids, causes a substantial reduction of the antibody repertoire of these mice.

3.) The canonical VH/VL gene combination VH171/Vκ072 together with Id_{Ox1}-typical amino acid sequence DRG in HCDR3 is extremely rarely verified and, if so, only in particular animals.

4 Discussion

Adaptive immune responses are generated by B and T lymphocytes. Their ability to recognize a huge multitude of different molecular structures on antigens relies on a similarly huge repertoire of clonally distributed antigen receptors, BCR on B and TCR on T cells. The variable portions of antigen receptors of both lymphocyte populations are created by V(D)J recombination, pairing of immunoglobulin H and L chains in B cells and α and β or γ plus δ chains in T cells as well as imprecisions during the recombination process. These imprecisions are based on a) the action of exonucleases which delete variable numbers of nucleotides from the coding ends of the involved genes, b) addition of palindromic (P) nucleotides and c) random addition of non-template (N) nucleotides by the enzyme terminal deoxynucleotidyl transferase (TdT). Due to the fact that the diversity-enhancing DH genes are rather short mini-genes, the recombinatorial imprecisions create a huge non-genetic repertoire at the third hypervariable regions of the BCR (HCDR3) as well as the TCR (VβCDR3). In addition, the size of this repertoire depends on the possibility that the modified D genes can be translated in all 6 reading frames. Together, these mechanisms enable the immune repertoire to recognize a seemingly unlimited universe of antigens. Imprecisions during V(D)J recombination can modify a particular DH gene to such an extent that it can not be deduced from the final nucleotide sequence. Hence, since the HCDR3 is the most diverse component of antibodies and lies at the centre of antigen-binding site, it plays a significant role in the development of cellular and humoral immune responses.

The relative contributions to the entire antibody pre-immune repertoire of the genomic repertoire of V(D)J genes on the one hand and the non-genetic generation of DH gene-associated variability on the other have only incompletely been studied. In a previous study [28], it has been demonstrated that mice with a single VH gene but complete DH and JH gene clusters are able to respond to a variety of quite different protein antigens like keyhole limpet hemocyanin (KLH), ovalbumin (OVA), hen egg-white lysozyme (HEL), cholera toxin B subunit (CTB) and haptens like 2,4-dinitrophenyl (DNP, coupled to KLH), phOx-conjugated CSA and phosphorylcholine (PC, coupled to KLH). It was observed that antibodies specific for these antigens differed only in HCDR3. Therefore, the authors concluded that the diversity at HCDR3 is decisive for determining the fine specificity of the antigen binding site while the genome-encoded CDR1 and 2 are much more cross-reactive. Thus, this model would argue that the high number of variable genes only supplies this underlying background reactivity

whereas the precise specificity for the epitope is provided by HCDR3. In the study by Xu and Davis [28], it was also observed that memory antibodies developed surprisingly high affinities through somatic mutations.

The contribution of recombinatorial non-genetic imprecisions to the pre-immune repertoire of specific immune responses has so far only been investigated in TdT knock-out mice which, because of this genetic defect, lack N-region diversity [29, 85]. Therefore, HCDR3 loops of TdT^- mice are shorter and less diverse than in WT mice. These mice harbor an immature repertoire [86] resembling that of fetal mice [85]. Nevertheless, with regard to efficiency as well as specificity, TdT^- mice develop quasi normal immune responses [38]. Hence, it seems necessary to reassess the general assumption that N-region diversity is required for an effective T and B cell repertoire. However, other results give better insights into the significance of TdT-mediated junctional diversification. Since HCDR3 is of central importance for expression of idiotypic determinants [52] it is not surprising that particular idiotypes are lost in TdT^- mice [87]. Moreover, HCDR3 sequences are important for binding of self antigens by autoantibodies and autoreactive T cells. Accordingly, compared to wildtype control mice, TdT^- mice showed lower serum concentrations of DNA-reactive autoantibodies and rheumatoid factor activity and had shorter HCDR3 with fewer arginines [88]. These lower autoantibody concentrations seem to be due to a lower incidence of polyreactivity and lower affinity of anti-DNA autoantibodies [89]. A physiological significance of this finding is indicated by the fact that, in an animal model, TdT deficiency reduces autoimmune nephritis and prolongs survival although the concentrations of anti-DNA and anti-histone autoantibodies were similar as in WT control mice [90]. Hence, either the autoantibodies under consideration are not the indicative parameter or the protective effect of TdT deficiency seems to be mediated indirectly. In relation to the above mentioned findings by Mi and co-workers [87], idiotypic regulation and regulatory T cells may be considered as possible mechanistic pathways.

The influence of DH gene restriction on the immune system has been investigated in different mutant strains (Tab. 28). The restriction to the single natural DH gene DFL16.1 in ΔD-DFL mice had no severe impact on several parameters of B cell development. Although the number of IgM^+/IgD^- pre-B cell in the bone marrow was decreased, numbers of mature IgM^+/IgD^+ B cells in bone marrow and spleen as well as of B1a, B1b and B2 in the peritoneum were as in WT mice [66]. In addition, the HCDR3 repertoire deduced from DFL16.1 was similar as in WT mice, but differed in HCDR3 sequences not related to DFL16.1. The total serum

immunoglobulin concentrations were slightly enhanced for IgM, IgG1 and 2a, but slightly decreased for IgG2b and similar for IgG3 and IgA (Tab. 28). Humoral IgG immune response to the two TD antigens NP-CGG and Tetanus toxoid (TT) were almost identical to wildtype control mice [66].

In contrast, DH mutant mice harbouring one of two artificial DH genes exhibited more severe alterations of B cell development. In one of these strains, ΔD-iD mice, the replacement of the central portion of *DFL16.1* by the complete inverted coding sequence of *DSP2.2*. resulted in coding for charged amino acids in HCDR3 [67, 68]. In ΔD-DμFS mice, the reading frame of *DFL16* was shifted by insertion of two thymidine nucleotides. In this way, the preferentially used RF1 leads to a predominant usage of hydrophobic amino acids in HCDR3 [74]. ΔD-iD and ΔD-DμFS mice showed an altered B cell development and reduced B cell numbers in bone marrow as well as in the spleen (Tab. 28). However, numbers of natural B cells in the peritoneum of ΔD-DμFS mice were identical to those of wildtype mice. Under specific pathogen-free (SPF) conditions, concentrations of total IgM and IgA in ΔD-iD and ΔD-DμFS were as in wildtype mice; however, all IgG subclasses were decreased in ΔD-iD, but alike in ΔD-DμFS mice (Tab. 28) [68, 74]. In part, these findings contrast our own results. While the IgM concentration in sera of ΔD-DμFS mice was identical to wildtype mice, they were slightly lower in ΔD-iD mice (Fig. 13). Total IgG concentrations were reduced in both strains and not only in ΔD-iD as observed by Ippolito, Schelonka and co-workers [68, 74]. In their experiments, the immune response to the TI-2 antigen dextran was impaired in both DH mutant strains, while the IgM response to the TD antigen tetanus toxin (TT) in ΔD-iD mice equalled that of WT mice and was even enhanced in ΔD-DμFS mice [74]. Compared to WT mice, IgG responses to NP-chicken serum gamma-globulin (CGG) was decreased in ΔD-iD, but unchanged in ΔD-DμFS mice, while the anti-TT IgG response was impaired in both strains. The general overview, given in Tab. 28, reveals that ΔD-DFL mice with a single natural DH gene showed the least while ΔD-iD mice with an artificial DH gene the most severe deviations from BALB/c WT mice.

Table 28

Comparison of the immune responses of 3 mutant mouse strains carrying different single DH gene segments relative to BALB/c wildtype mice

	ΔD-DFL [66]	**ΔD-iD** [68]	**ΔD-DμFS** [74]
Total nucleated cells			
Bone marrow	⇔	⇔	↓
Spleen	⇔	↓	↓
Peritoneal cavity	⇔	↓	⇔
B cells total			
Bone marrow	⇔	↓	↓
Spleen	⇔	↓	↓
Peritoneal cavity	⇔	↓	⇔
B cells mature			
Bone marrow	⇔	↓	↓
Spleen	⇔	↓	n.d.
Total Ig			
IgM	↑	⇔	⇔
IgG1	↑	↓	⇔
IgG2a	↑	↓	⇔
IgG2b	↓	↓	⇔
IgG3	⇔	↓	⇔
IgA	⇔	⇔	⇔
Anti-NP-CGG			
IgG	⇔	↓	⇔
Anti-TT			
IgM	↑	⇔	↑
IgG	⇔	↓	↓
IgA	⇔	n.d.	⇔
Anti-Dex			
IgM	↓	↓	↓
IgG	↓	↓	n.d.

Legend

The different indicated parameters were compared with those of BALB/c wildtype mice.
Double arrows (⇔) indicate no difference while green and red (↑/↓) arrows symbolize an increase and decrease, respectively.
Total ($CD19^+$) as well as mature ($CD19^+CD21^{lo}CD23^{hi}$) B cells were determined by FACS analysis.
The humoral immune responses were measured by ELISA.

Dex - dextran
NP-CGG - 4-hydroxy-3-nitrophenylacety-chicken gamma globulin
TT - Tetanus Toxin
n.d - not determined

However, the quality of a particular immune response in the latter 2 mutant mouse strains has so far not been investigated at the level of single B cell clones. Therefore, the data presented herein describe for the first time the connection between clonal development during the TD immune response and a particular DH gene.

4.1 B cell development in DH mutant mice

How do these data relate to our observations? In both of our DH mutant strains, the total lymphocyte and B cell counts in the bone marrow were rather enhanced than decreased (Tab. 3). In spleen, however, B cell numbers were clearly decreased as observed in the investigations by Schroeder and colleagues [66, 68, 74]. Hence, our data would indicate that simplifying the genomic DH segments to a single gene, coding for hydrophobic HCDR3 amino acids in ΔD-DμFS or charged amino acids in ΔD-iD mice, does not influence the development of B cells in the antigen independent phase in the bone marrow and does not prevent an efficient V(D)J rearrangement, an assembling of H and L chain and the formation of pre-BCR in the bone marrow. Thus, the reduction of splenic B cells is most likely first induced in the periphery and does not reflect a lower influx of newly generated naïve B cells from the bone marrow. The progression from immature to mature naïve B cells is a critical phase in B cell development. Mainly non-autoreactive immature B cells are allowed to move from the bone marrow towards the periphery, where they undergo a second round of selection against peripheral auto-antigens. Is it possible that this negative selection in the periphery which eliminates autoreactive and B cells with non-functional BCR is responsible for the reduced numbers of B cells in the periphery?

As pointed out in the introduction, B and T cells mutually regulate the development of their repertoires [76, 77]. This finding is confirmed by the observation that spleens of B-cell deficient mice show a three- to fivefold reduction in T cell numbers [78]. Other studies in B cell deficient mice proved that the function of B cells is not limited to antibody production, but are also required for efficient T cell responses [79, 80]. In line with these observations, T cell numbers in the spleen of ΔD-DμFS as well as ΔD-iD mice were drastically reduced (Tab. 3) although the genetic alteration in both strains of mice is restricted to Ig genes and does not affect the germ line repertoire of TCR.

After selection in the thymus, thymic emigrants mature to naïve immunocompetent T cells in secondary lymphoid organs. In the spleen, T cells are located in the periarteriolar lymphoid

sheath (PALS), which also contains low numbers of plasma cells, macrophages, and interdigitating dendritic cells (DC) [91]. The PALS is surrounded by a region containing primary follicles, which are filled with B cells and macrophages. Naïve T cells become activated when they are exposed to antigen-MHC complexes presented by resident DCs or other antigen presenting cells [92].

On the other hand, studies in B cell-deficient mice generated by μ-chain gene disruption showed that B cells provide signals which promote T cell accumulation in the spleen and support the development of splenic T-zone stromal cells as well as the accumulation of DCs [80]. Ngo et al. [78] reported that the numbers of T cells in the spleen of B cell-deficient mice were reduced threefold in comparison with WT controls. Our study provides further evidence for the necessity of B cells in normal development of splenic T cells. Thus, it can be assumed that the decrease of T cell numbers in ΔD-DμFS and ΔD-iD mice results from an insufficient stimulus by a reduced number of B cells, which emerged from a simplified DH gene repertoire. Furthermore, it has been documented that B cells can act as APC in the initiation of T cell maturation [93, 94]. Indeed, B cells are highly efficient for the presentation of their BCR-specific antigen in association with MHC molecules to mature T_H cells, and can stimulate proliferation and differentiation of naïve T cells. Therefore the decrease in antigen presentation affects the T cell maturation. Together, reduced splenic T cells in ΔD-DμFS and ΔD-iD mice reflect insufficient chemokine-producing B cells and / or the impairment of antigen presentation by B cells or both.

In contrast, the numbers of T and B cells in the bone marrow of mutant mice were similar to that of WT controls. In the bone marrow, the B cells include all stages of B cell development as well as memory and long lived plasma cells [95, 96]. Concerning the T cells, various studies showed that memory T cells ($CD4^+$ and $CD8^+$) migrate to the bone marrow of immunized mice and are sustained there over long periods of time [97-99]. Therefore our data suggest that the memory T cells are not affected by the alteration in the DH locus.

4.2 Humoral immune responses in DH mutant mice

The murine peripheral B cell pool consists of mature B cells and a small subset of three different cell types: long lived plasma cells, memory and B1 cells. Mature IgM^+/IgD^+ B cells are the main source of cells participating in the primary immune response. They differentiate into specific antibody-producing plasma cells and in case of TD antigens also into memory B cells

[100]. Our data show that the total population of splenic B cells, including MZ, B-1 and follicular B cells are reduced in ΔD-DμFS and ΔD-iD mice, but does this cause impaired humoral immune responses? For approaching an answer to this question, we first analyzed the initial situation and determined a) the total IgM and IgG serum concentrations, b) phOx-specific background antibodies and c) anti-phOx antibody-secreting cells before any immunization, namely in non-immunized animals.

As shown in Fig. 13, the concentrations of total IgM were rather similar in WT and ΔD-DμFS mice, but reduced in ΔD-iD mice. In contrast, IgG concentrations were significantly reduced in both DH mutant strains. Surprisingly, spleens of the DH mutant strains contained almost twice as many IgM-anti-phOx secreting cells than wildtype mice (Fig. 11). Also in the bone marrow, IgM-anti-phOx secreting cells were almost threefold higher in mutant as compared to wildtype control mice (Fig. 11). These natural IgM antibodies are known to be produced by B-1 cells without antigenic stimulation. Natural antibodies recognize antigens of many common pathogens and they are capable of self-renewal [101]. In addition to the protective function against pathogens, antibodies produced by B-1 cells play an important role in the clearance of apoptotic cells. Moreover, B-1 and MZ B cells are proposed to provide protection during responses against bacterial TI-2 antigens [102, 103]. The B-1 cells predominantly reside in peritoneal and pleural cavities and only a small number is found in the spleen. Natural antibodies are mostly of the IgM class, they are auto- as well as poly-reactive, encoded by immunoglobulin V genes in germ line configuration, and they react with low affinity with a variety of endogenous and exogenous antigens [104]. Thus, B-1 cells and their natural antibodies represent part of the pre-immune repertoire.

Own data have demonstrated that natural phOx-reactive antibodies do not participate in the TD phOx-CSA-induced response (Lange et al., in preparation). This view is supported by the observation that the enhanced amounts of background anti-phOx-secreting cells in spleen and bone marrow of DH mutant mice does neither correlate with the phOx-Ficoll-induced TI-2 nor with the phOx-CSA-induced TD antibody response. The TD-induced IgM-anti-phOx serum concentrations measured 1 week after stimulation with phOx-CSA were slightly lower in both DH mutant mouse strains (Fig. 15). This is astonishing, since the number of IgM-anti-phOx-secreting plasma cells in the spleen of DH mutant mice were much higher than in WT mice (Fig. 12A). This can only be explained by the assumption that the amount of Ig produced by a single antibody-forming cell (AFC), as indicated by the size of the ELI-spot, varies considerably especially for IgM-AFC (Fig. 10). The TD-induced IgG response was

strongly suppressed in correlation with reduced number of IgG-AFC (Fig. 15). Antibody production in both DH mutant mouse strains after stimulation with phOx-Ficoll, however, was almost identical for IgM and developed slightly faster IgG response in ΔD-DμFS mice, but was not significantly different in ΔD-iD and wildtype mice (Fig. 14).

IgG antibodies are produced by short and long lived plasma cells. The short lived plasma cells, continuously generated from activated memory B cells after re-stimulation with the antigen, are responsible for an immediate increase of the IgG serum titer and have a lifespan of a few days. The long lived plasma cells are responsible for long-term production of serum antibodies. They can survive in the BM (95 %) or in the spleen (5 %) for extended periods of time, apparently from several months to years [105]. These long-lived plasma cells or their precursors are generated in germinal centres of secondary lymphoid organs like the spleen, and then migrate to the bone marrow, where they take up residence in a complex molecular microenvironment, a survival niche. Due to their antigen independent survival, they can maintain the humoral memory to previously encountered antigens without antigenic stimulation [106, 107]. However the switch from IgM to IgG occurs usually in the germinal centre and is driven by T_H cell cytokines [108]. Thus, the low IgG concentration in our DH mutant mice probably reflects the reduction in the IgM>IgG switched plasma cells. A reduced number of phOx-specific IgG memory B cells can also be concluded from the observation that the difference in antibody production between WT on the one hand and DH mutant mice on the other remained after secondary renewed stimulation with phOx-CSA (Fig. 15). The data, however, do not give any information about the cellular components of the primary and further immune responses. It is important to remember that a substantial number of IgM-secreting hybridomas could be established at times of the TD immune response when in WT mice such IgM-producers have never been seen, *i.e.* in the late primary, secondary and tertiary response (see below). Hence, the restriction of HCDR3 by using a single DH segment encoding for hydrophobic or charged amino acids severely impairs all stages of TD immune responses. In contrast, pre-immune sera and TI-2 responses seem to be not affected to a great extent.

4.3 Comparison of anti-phOx repertoires in wildtype and DH mutant mice

It was of interest to compare the repertoire of all anti-phOx antibodies generated in this study from DH mutant mice with that of BALB/c wildtype mice. Fortunately, the VH/VL gene combinations of almost 200 anti-phOx antibodies from WT mice were available (Lange et al.,

in preparation). The repertoire of these antibodies represents a) natural antibodies, *i.e.* the hybridomas were obtained from non-immunized mice, b) antibodies obtained after immunization with the TI-2 antigen phOx-Ficoll, c) anti-phOx antibodies received after primary to quaternary immunizations with the TD antigen phOx-CSA and d) various combinations of initial TI-2 and following TD immunizations or *vice versa*. Altogether, this WT anti-phOx repertoire was encoded by 44 VH and 40 VL genes (Tab. 29). It is striking to see that IgM antibodies are distributed all over the table, whereas IgG antibodies are concentrated in certain areas of VH/VL combinations. Most of them are encoded by one of the Id_{Ox1} genes VH171 or Vκ072. The highest proportion of IgG antibodies is encoded by the Id_{Ox1} combination VH171/Vκ072. This demonstrates that most IgM-anti-phOx antibodies, activated during the early phases of primary TI-2 and TD responses, did **not** undergo class switch recombination. This is especially striking for IgM antibodies which are encoded by VH1 family genes. With the exception of four VH532/Vκ115-encoded antibodies, none of them switched to the IgG class.

Hence, the question was as to whether the new anti-phOx antibodies obtained from DH mutant mice were encoded within this panel of VH and VL genes. Therefore, we extended Tab. 29 and included all anti-phOx antibodies from the 2 DH mutant strains (Tab. 30). In this table, the light colours represent antibodies from the early stages of the response whereas dark colours symbolized antibodies from the late stages. The darkest colours show antibodies from tertiary responses. To our surprise, these antibodies were encoded by 24 more VH and 12 more VL genes. However, the new VH genes belonged to the same families already seen in WT mice. Most of the additional 24 VH genes (15 = 62 %) belonged to the VH1 family and the rest pertained to VH families 2, 3, 5, 6, 7, 9 and 14. The new VL genes belonged to families 1, 4/5, 8, 9/10, 19/28, 21 and 23, already seen in wildtype mice, and to the new VL families 24/25 and 38C. In contrast to WT antibodies (Tab. 29), IgG antibodies of DH mutant mice were more evenly distributed over the whole table and a comparably high proportion of IgG antibodies were encoded by VH1 family genes (Tab. 30). From Tab. 30 it is already obvious that Id_{Ox1} antibodies did not dominate any phase of the response, neither in ΔD-iD (Tab. 30, yellow to reddish colours) nor in ΔD-DμFS mice (Tab. 30, blue colours).

Hence, in principle, anti-phOx antibodies from ΔD-iD and ΔD-DμFS DH mutant mice can make use of VH and VL genes already known from BALB/c WT mice. But in addition, a large fraction of them is encoded by V genes hitherto not observed in wildtype mice. Is it possible to explain this fact by a correlation between HCDR3 sequence and usage of VH/VL gene combinations? It is conceivable that antibodies encoded by `new´ VH or VL genes carry new sequences in HCDR3 or that these sequences exhibit new properties which require an as-

sociation with new V genes to create a reactivity for the hapten phOx. However, the variability at HCDR3 is so high that we so far could not find such an association

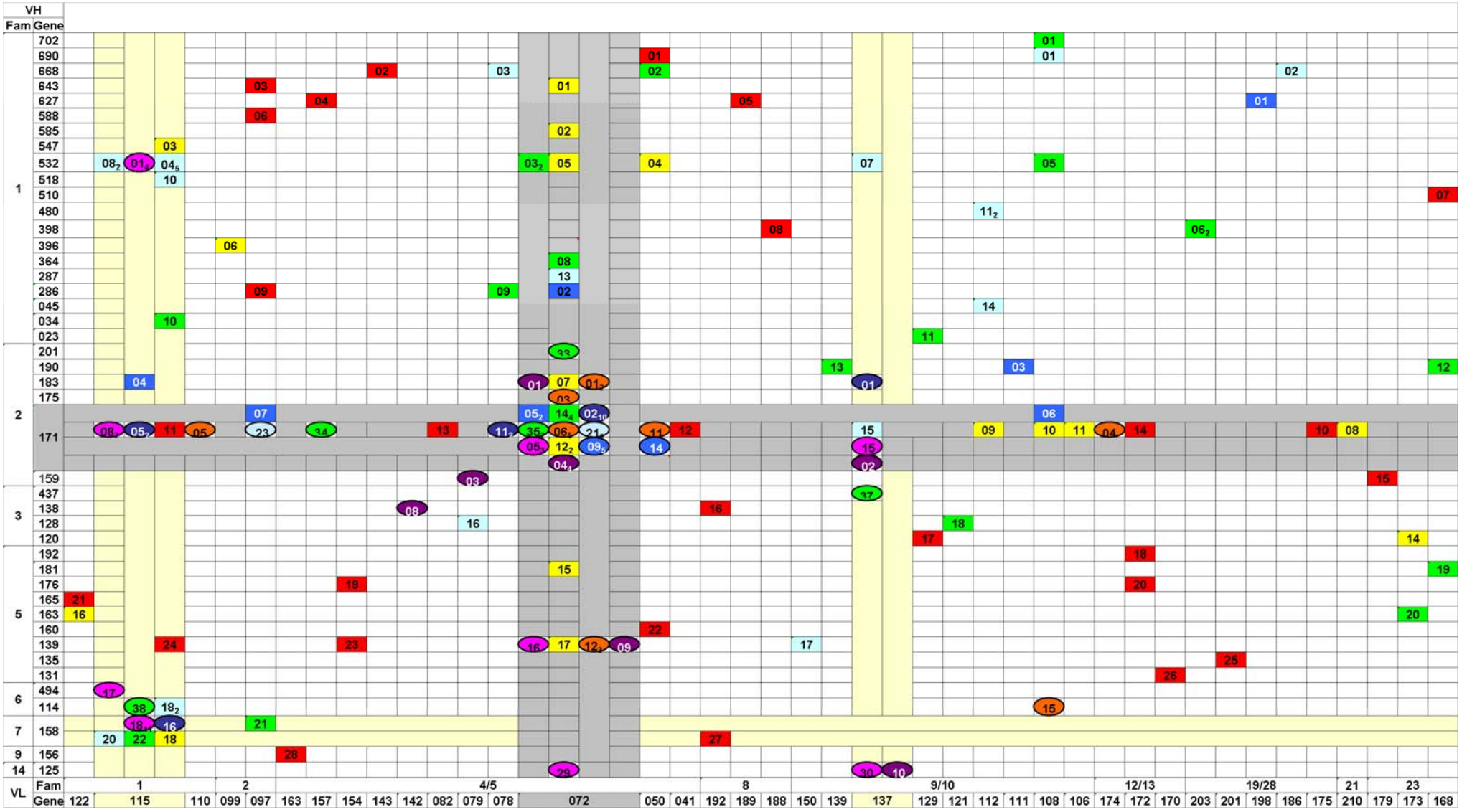
4.4 D gene segments and homogeneity of immune responses

Despite the potential to create an enormous antibody repertoire, certain mouse strains respond to the immunization with some haptens and simple antigens with a remarkably homogenous population of antibodies. For instance, such dominant clonotypes or idiotypes are observed in the response of BALB/c mice to phosphorylcholine (PC) [109], p-arzophenylarsonate [110], (4-hydroxy-3-nitrophenyl)acetyl (NP) [111] and phOx [69]. As already mentioned above, the early stage (day 7) of the primary TD anti-phOx response of BALB/c mice is characterized by the dominant Ox1 idiotype with the VHOx1/VκOx1 = VH171/Vκ072 gene combination. In addition, the Id_{Ox1} specificity is correlated with the DRG sequence in the middle of HCDR3. The idiotypic characteristic is destroyed by somatic mutations during immune maturation and a repertoire shift is observed during the secondary response [112]. In clear contrast to these findings in WT mice, primary to tertiary anti-phOx antibodies from ΔD-DμFS and ΔD-iD mice did not contain the Id_{Ox1} or any other dominant clonotype. Moreover, there was no increased usage of the Id_{Ox1} genes in combination with alternative VH or VL genes. The Id_{Ox1}-typical DRG sequence in HCDR3 was only observed 3-times among early primary antibodies in ΔD-iD mice in the VH171 gene which, however, was associated with 3 different alternative VL genes (Fig. 18). Only in the tertiary response of a particular animal of ΔD-DμFS mice we obtained a major clonotype with the VH158/Vκ115 gene combination (Tab. 13).

This combination has already been observed as major clonotype in the secondary response of BALB/c WT mice [46]. However, a comparison of HCDR3 sequences in Tab. 15 with those in [46] revealed that some antibodies showed rather similar sequences while others differed considerably and we could not detect a common motive. Thus, the genomic elimination of all but one DH gene, coding for hydrophobic or charged amino acids in HCDR3, does not allow the development of dominant clonotypes.

Table 29

VH/VL gene combinations of natural and antigen-induced anti-phOx antibodies of BALB/c wildtype mice



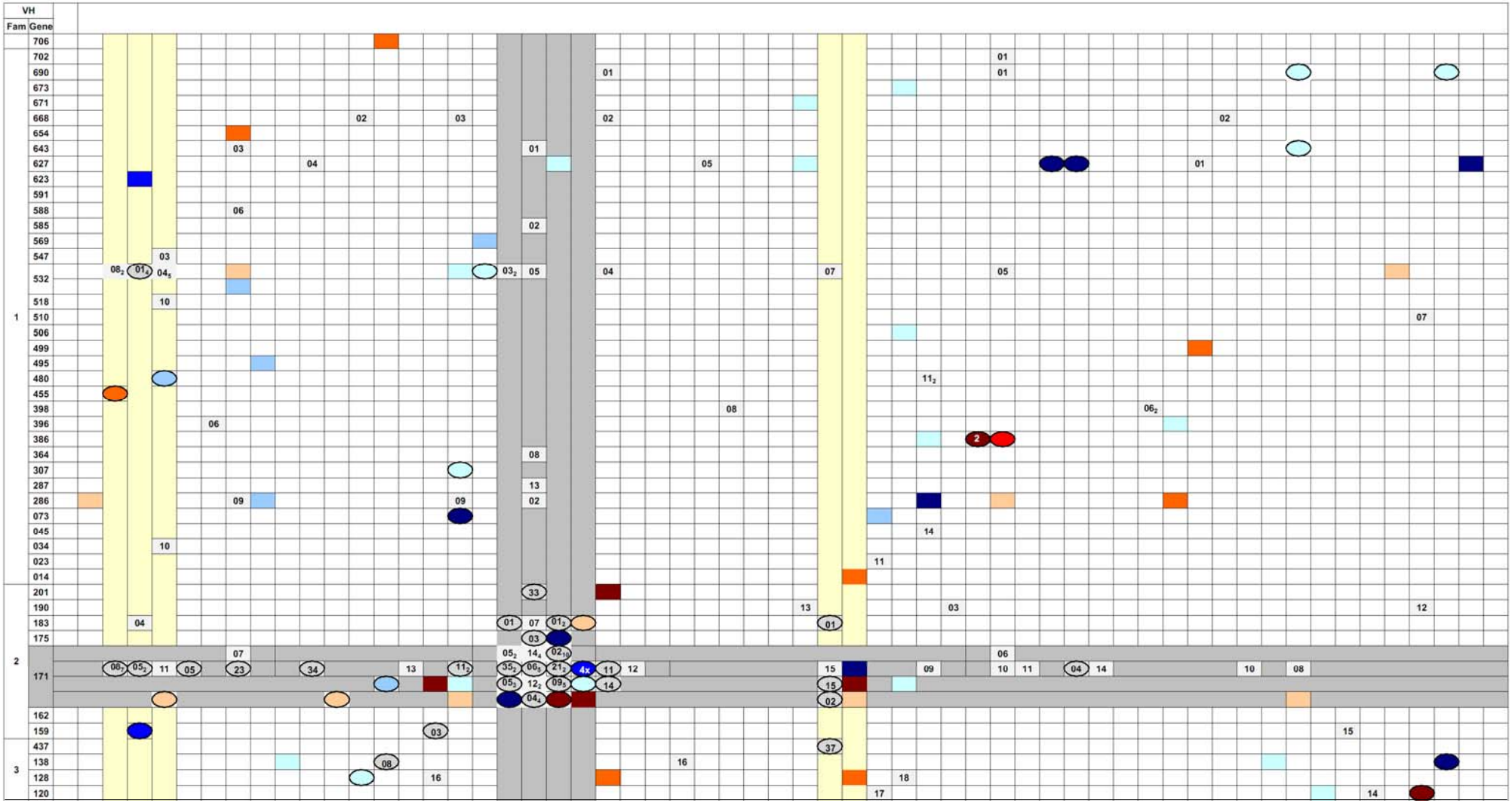
Legend

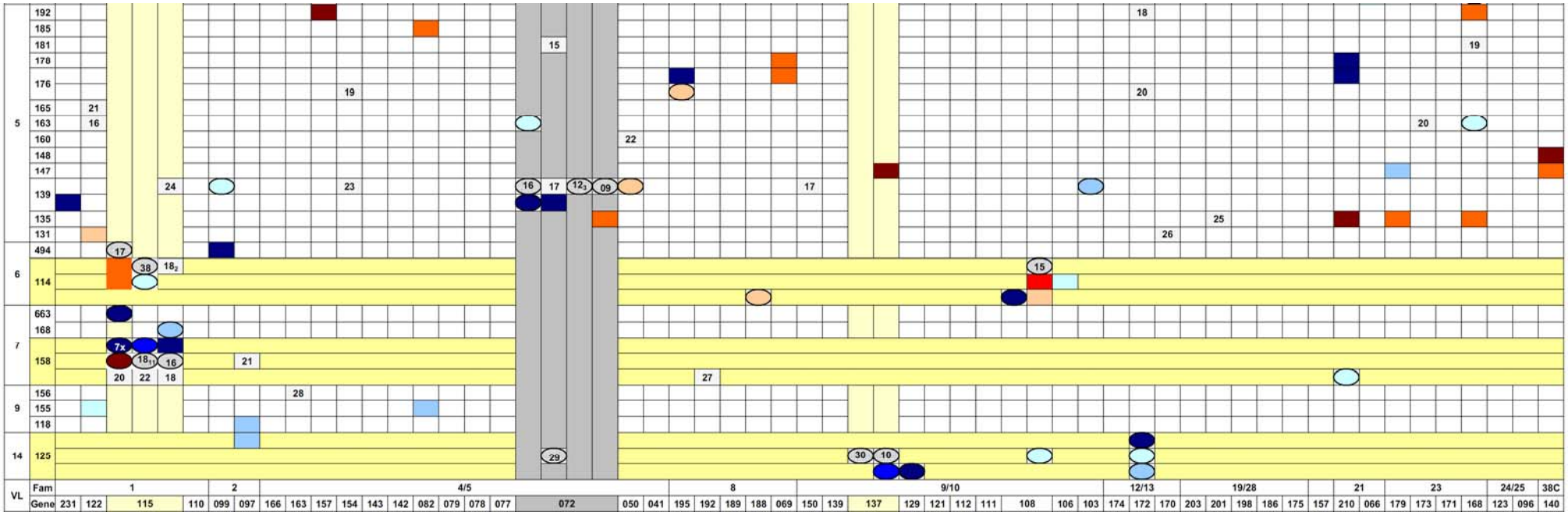
Sequences of variable region genes of heavy (VH) and light chains (VL) of anti-phOx hybridoma antibodies from BALB/c wildtype mice were analyzed with the integrative database VBASE2. IgM antibodies are indicated by filled rectangles and IgG antibodies by ellipses. Large numbers indicate the sequential numbers of a particular group of antibodies and subscript numbers specify the amount of antibodies with a particular VH/VL combination. Antibodies were obtained in the following way

- natural antibodies from spleens of non-immunized mice
- primary antibodies after immunization with the TI-2 antigen phOx-Ficoll
- primary antibodies after immunization with the TD antigen phOx-CSA
- antibodies obtained after tertiary TD and a final TI-2 immunization
- antibodies obtained after quaternary TD immunization
- antibodies obtained after initial TI-2 immunization and a primary TD immunization 1 month later and fusion on day 7
- antibodies were obtained after an initial TI-2 immunization and a primary TD immunization 3 months later and fusion on day 7
- initial TI-2 immunization, 3 months pause, followed by a primary TD immunization and fusion on day 14
- initial TI-2 immunization, 3 months pause, primary TD followed by a secondary TD immunization 8 weeks later and fusion on day 3.

Tab. 30

VH/VL gene combinations of thymus-dependent antigen-induced anti-phOx antibodies of DH mutant ΔD-DμFS and ΔD-iD mice





Legend

Sequences of variable region genes of heavy (VH) and light chains (VL) of anti-phOx hybridoma antibodies from ΔD-DμFS and ΔD-iD mice were analyzed with the integrative database VBASE2 and compared with those of BALB/c wildtype mice. All wildtype antibodies of Tab. 29 are marked by a grey-dotted background.
IgM antibodies are indicated by filled rectangles and IgG antibodies by ellipses in white rectangles. The number of antibodies encoded by a particular VH/VL gene combination is also indicated. All antibodies were obtained after immunization with the thymus-dependent antigen phOx-CSA in the following way:

ΔD-DμFS mice

- primary immunization and fusion on day 7; - primary immunization and fusion on day 14;
- secondary immunization, fusion on day 3; - tertiary immunization, fusion day3.

ΔD-iD mice

- primary immunization, fusion day 7; - primary immunization, fusion day 14;
- secondary immunization, fusion day 3; - tertiary immunization, fusion day 3.

Hitherto, the dominance of particular clonotypic VH/VL gene combinations has been explained by their superior affinity. However, this argument does not seem to be really valid since we observed early primary clones of higher affinity than Id_{Ox1} antibodies in BALB/c WT as well as in both DH mutant mice. In ΔD-DμFS mice, the affinity of 2 IgM non-Id_{Ox1} clones was enhanced by factors of 5, in 2 IgG antibodies by a factor of 14 and 1 IgG even by a factor of 115 (Tab. 4). In ΔD-iD mice, there were no early primary IgM non-Id_{Ox1} with enhanced affinities, but those of 3 IgG were enhanced by factors of 4, 6 and 9, respectively (Tab. 16). Hence, if affinity is a decisive or the only factor for activation of reactive clones, it is difficult to understand why such clones are not generally activated and become dominant clonotypes. Moreover, it has to be considered that the dominance of particular clones first becomes visible after class switch recombination to IgG (Lange et al., in preparation). Since this process is initiated and regulated by T cells and T cell-derived cytokines, it is tempting to speculate that the preferred selection of certain B cell clones over others is T cell-mediated and not only driven by affinity of the BCR. This view is in line with repertoire analyses after immunization with TI-2 antigens. The primary repertoire of C57BL/6 mice induced with NP-coupled Ficoll, a TI-2 carrier, is very heterogeneous while that of the TD response, induced with NP coupled to a protein as a TD carrier, is characterized by a dominant idiotype which is composed of a V_H186.2 heavy chain in combination with a lambda-1 light chain [113]. Complementing investigations have demonstrated that C57BL/6 mice harbor B cells with much higher affinity than the dominant clonotype encoded by the VH186.2-lambda 1 gene combination [114]. Hence, why are those higher affinity clones not activated in the TD response and how can T cells perform such a clonotypic selection not depending on the BCR affinity?

Physiological recognition of protein antigens (including idiotypes) by T cell antigen receptors requires a processing by antigen-presenting cells followed by the presentation of small peptide fragments through MHC molecules on the surface of these cells. B cells are particularly efficient in presenting processed peptides since they can bind antigen specifically through their BCR. Therefore, antigen-specific B cells can stimulate T cells at antigen concentrations several orders of magnitude lower than those required for activation by nonspecific APC. B cells, however, do not only process BCR-bound antigen and present its fragments, but at the same time they present peptides of their intrinsic immunoglobulin H and L chains which can then be recognized by T cells (reviewed in [52]). Does this idiotypic MHC-restricted T-B interaction take place during TD antigen-induced immune responses? This is indeed the case as older investigations have shown. Antibody secretion by idiotype$^+$ B memory cells requires

two signals: one is provided by "carrier"-specific and the other by idiotype-specific T helper cells. Both signals are required for optimal induction of idiotype$^+$ B memory clones to secrete antibody [115]. From other studies it has also been concluded that a successful T-B cooperation requires two types of T helper cells, one recognizing the antigen and the other recognizing the idiotype of the B cell [116-118]. Hence, T cell regulation is probably responsible for the dominance of Id_{Ox1} antibodies and can not only be ascribed to their superior affinity. This allows concluding that a restricted DH gene repertoire causes a restricted T helper cell repertoire and in this way interferes with clonal progression during TD immune responses. The involvement of idiotype-specific T cells in the clonal development during TD immune responses has been proposed since a long time. It has been argued that a) idiotypic interaction / suppression could be a way of dampening a dominating monoclonal response to favor the observed mutation drift and repertoire shift in the maturation of the immune response [55], b) a very dominant negative selection may act against the idiotype of primary antibodies [119] and c) HCDR3-associated idiotypic suppression may function as a driving force for diversification and maturation of the antigen-induced immune response [120].

Our sequence analyses demonstrated that the Id_{Ox1}-specific VH/VL gene combination plus the HCDR3-typical middle DRG sequence is only very rarely produced in a few ΔD-DμFS and ΔD-iD mice. Since the T and the B cell system undergo a mutually selective expansion during early life, it is conceivable that our DH mutant mice with a drastically reduced HCDR3 repertoire fail to develop corresponding idiotype-specific T cells and that this failure reduces both repertoires equally. This reduced T cell repertoire causes also an impaired class switch recombination, *i.e*. more than halve of the hybridomas of both mouse strains generated at different stages of immune responses (primary, secondary and tertiary) produce IgM antibodies. Isotype switching is promoted by cytokines in the immediate microenvironment of the activated B cells inside or outside the germinal centre [121]. These cytokines are usually derived from T_H cells. Thus, the type of T_H cells which secret a set of cytokines in the immediate microenvironment of the activated B cells, determine which antibody isotype will be produced. Without intervention of the T_H cells, the B cells `consider´ a TD as being TI antigen, and consequently, the majority of antibodies against this antigen will belong to the IgM isotype [122]. A similar scenario seems to happen in ΔD-DμFS and ΔD-iD mice. The low numbers of T_H cells may produce too low amounts of cytokines to force isotype switching of all antigen-specific B cells. Consequently, only part of the IgM-secreting activated B cells switch to IgG after primary injection of TD antigen, whereas the majority of the IgM activated B cells die

by apoptosis after elimination of the antigen. As a result, the primary antigen-specific IgG as well as the total IgG concentration is reduced in both mutant mice strains. Furthermore, since the memory B cells develop only from the switched IgG cells in germinal centres, a second injection with the same antigen activates only reduced numbers of the memory IgG and virgin IgM B cells.

4.5 Selection of reading frames

Despite its overwhelming variability, there are some limitations in HCDR3 which seem to be necessary for a proper function of the adaptive immune system [123]. In murine as well as in human sera, immunoglobulins with neutral or slight hydrophilic HCDR3 are dominant, while HCDR3 with hydrophobic as well as charged residues are infrequent in WT mice. In part, this bias is due to a preferred usage of DH reading frame 1 (RF1) which codes for neutral and hydrophilic amino acids [124]. As RF1 of all 13 BALB/c DH segments codes for neutral and hydrophilic amino acids, it is not astonishing that 90 % of murine rearranged antibodies use the DH segments in RF1 [125, 126]. Consequently certain amino acids like glycine and tyrosine are overutilized, whereas hydrophobic and charged residues like arginine and lysine are underrepresented. Hence, despite the high plasticity at HCDR3 by insertion or deletion of some nucleotides during VDJ recombination, usage of hydrophobic or charged amino acids is relatively rare. However, it has been observed that auto-antibodies reacting with double-stranded DNA contain enhanced numbers of arginines in HCDR3 [127]. Such unusual rearrangements are not only observed in anti-DNA and but also in anti-nucleosome auto-antibodies and include alternative DH reading frames, inverted D segments, and D-D fusions, all coding for hydrophobic and charged residues [128]. Hence, it could generally be concluded that antibodies with hydrophobic or charged HCDR3-loops show auto-reactivity [129]. In addition, the porcine respiratory and reproductive syndrome virus (PRRSV) causes auto-immunity in neonatal piglets by inducing expansion of B cell clones bearing hydrophobic HCDR3s encoded by reading frame 3 of one particular DH segment [130]. On the other hand, the expression of auto-reactive BCRs can disrupt the development from immature to mature B cells and to promote receptor editing [131, 132].

In ΔD-DμFS mice, ½ to ²/₃ of the antibodies use germline-encoded HCDR3 sequences, but only a minority of them is translated in RF1 and, as expected, this correlates with positive hydrophobicity scores (Tab. 6, 9, 12 and 15). Also in ΔD-iD mice, a significant number of B

cells express the germ line DH gene which, however, codes for charged HCDR3 amino acid sequences. Therefore, germline-encoded as well as germline non-related sequences, being translated in RF1 or non-RF1, are correlated with sometimes strongly negative hydrophobicity scores in HCDR3 (Tab. 18, 21, 24 and 27). However, some of the anti-phOx antibodies in ΔD-iD mice exhibit even strong hydrophobic scores, like the early primary antibodies ID1°7-02 (IgM) with a score of 1.13 and ID1°7-17 (IgG) with a score of 0.6 (Tab. 18), the late primary IgM antibody ID1°14-03 with a score of 0.51 (Tab. 21) and the tertiary IgG antibody ID3°-13 with a score of 0.72 (Tab. 27). Whether these B cells escaped negative selection in the periphery and exhibit, besides their anti-phOx reactivity, auto-reactivity remains a speculation. The first step of negative selection of auto-reactive B cells is thought to occur at the stage of pre-B cells when they are confronted with auto-antigens present in the bone marrow. A second round of negative selection is initiated when mature B cells encounter peripheral auto-antigens not expressed in bone marrow. If a BCR binds to this auto-antigen with high affinity, the maturation will be abrogated and the B cell dies by apoptosis (clonal elimination). Hence, negative selection of hydrophobic and charged HCDR3 clones in the periphery explains, at least in part, the reduction of B splenocytes of ΔD-DμFS and ΔD-iD mice.

It can be assumed that the germ line non-related HCDR3 nucleotide sequences of ΔD-DμFS and ΔD-iD antibodies result from the complete deletion of the whole germ line DH segment by exonucleases and a subsequent random addition of non-germ line (N) nucleotides inserted during VDJ joining processes. These N nucleotides are inserted by the terminal deoxynucleotidyl transferase (TdT), a unique enzyme capable of adding from one to twenty nucleotides to rearranging V, D, and J termini of immunoglobulins as well as of TCR [133]. As TdT is not expressed in early ontogeny, fetal BCR and TCR repertoires lack N regions and, as a consequence, perinatal HCDR3 are significantly less variable than those of adults, and the average length of HCDR3 increases with the development until adulthood [29, 39]. Hence, mice lacking TdT (TdT^-) show a drastically reduced BCR and TCR repertoire. However, responses to TD and TI antigens are very similar in TdT^- and control mice [38], suggesting that N regions might not be required for an effective T and B cell immune response. Our data support the assumption that N nucleotides may have a function other than a source of diversity. N region insertions promote, in some cases, the construction of germ line independent HCDR3, *e*.g. when the DH segment codes for unfavourable residues (hydrophobic or charged amino acids). However, because DH segments are evolutionary pre-selected for nucleotides coding for neutral amino acids, the construction of DH independent HCDR3 is rarely used and only 5

% of the antibodies in sera of wildtype mice contain HCDR3 motives unrelated to any DH segment.

Table 31

Genomic and non-genomic nucleotide sequences in HCDR3 of anti-phOx antibodies of DH mutant mice ΔD-DμFS and ΔD-iD

	ΔD-DμFS				ΔD-iD			
Ab	Class	genomic yes	genomic no	portion of non-genomic	Class	genomic yes	genomic no	portion of non-genomic
1°7 [a)]	IgM	7	6	46 %	IgM	3	6	66 %
	IgG	11	7	39 %	IgG	4	2	33 %
1°14 [b)]	IgM	4	4	50 %	IgM	11	5	31 %
	IgG	5	2	29 %	IgG	1	1	50 %
2° [c)]	IgM	2	0	0 %	IgM	0	1	100 %
	IgG	3	4	57 %	IgG	1	0	0 %
3° [d)]	IgM	6	4	40 %	IgM	4	4	50 %
	IgG	16	6	27 %	IgG	0	5	100 %

Legend

[a)] Primary antibodies obtained in fusion 7 days after immunization.
[b)] Primary antibodies obtained 14 days after immunization.
[c)] Secondary antibodies.
[d)] Tertiary antibodies.

In both DH mutant strains, this picture is completely different. At all stages of the primary to the tertiary immune response in ΔD-DμFS and ΔD-iD mice, the proportion of antibodies exhibiting genome non-related HCDR3 sequences ranges from about 30 to 66 % (Tab. 31). This applies for IgM as well as IgG antibodies. In tertiary anti-phOx antibodies of ΔD-iD mice, even 100 % of IgG antibodies (n = 5) had HCDR3 nucleotide sequences showing no relationship to the genomic DH gene. As mentioned above, these sequences may be generated by an initial removal of the DH gene and a subsequent addition of N and P nucleotides. A second possibility may result from a direct V to J joining, likewise associated with insertion of N and P nucleotides. Such a direct joining of VH to JH genes has been observed in

BALB/c mice *in vivo*, but at low frequency [134]. In addition, the rare V-J joining event seems to be unlikely because of the genetic restriction during the recombination process (one and two-turn roll). Hence, usage of any germ line DH gene will automatically generate a neutral or hydrophilic HCDR3. In the case of ΔD-DμFS and ΔD-iD mice, the central nucleotides of the single DH gene code for hydrophobic and charged amino acids, respectively. Thus, a direct V-J joining is also one way to escape unfavourable HCDR3 interval.

4.6 Concluding remarks

The data presented demonstrate that the reduction of normally 13 DH genes in BALB/c wild-type mice to single artificial genes in ΔD- DμFS and ΔD-iD mutant mice has a severe impact on the humoral immune response to the hapten phOx. This is astonishing since both mutant strains harbor the full repertoire of hundreds of variable and all joining genes which are needed for the generation of B cell antigen receptors. In addition, they have full access to create the vast repertoire of T cell antigen receptors. Is was striking to see that the reduction of DH genes impaired not only immunoglobulin-related parameters of the immune response, but also suppressed associated T cell functions. The overall T cell numbers were reduced, the TD response was severely suppressed and T cell-mediated class switch recombination was impaired in all phases of immune maturation. As the switch- and T cell-dependent development of Id_{Ox1} dominance was abrogated in both DH mutant strains and no other dominant clonotype developed, it can be concluded that idiotype dominance does not depend on canonical VH/VL gene combinations, but on certain HCDR3-related idiotopes. Moreover, this finding supports our earlier assumption that clonal selection during immune maturation is not only mediated by BCR-affinity but is also regulated by HCDR3-specific, *i.e*. idiotype-specific T cells.

The analysis of HCDR3 sequences documented the extreme non-genetic variability at this region. In principle, HCDR3 sequences identical or similar to the Id_{Ox1}-specific sequence CAR-DRG-AYW could be created in both DH mutant strains. However, this event was obviously so rare that it only occurred in a few individual mice. The extraordinary enzymatic activity of exonucleases during the V(D)J recombination process can be deduced from the finding that 30-66 % of antibodies (in one case even 100 %) from all phases of immune maturation used HCDR3 sequences showing no relation to the respective germline DH genes.

Future work is necessary to elucidate to precise mechanism of T cell-mediated regulation of the thymus-dependent immune response.

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6 Abbreviations

AA	Amino acid
Ab	Antibody
ABTS	2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)
AEC	3-Amino-9-Ethylcarbazol
Ag	Antigen
APC	Antigen presenting cell
BCR	B-cell receptor
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDR	Complementarily-determining region
CSA	Chicken serum albumin
DH	Diversity segment
DNA	Deoxyribonucleic acid
FITC	Fluoroisothiocyanate
H	Heavy chain
HRP	Horseradish peroxidise enzyme
i.p	Intraperitoneal
JH	Joining segment
kDa	Kilo Dalton
L	Light chain
MHC	Major histocompatibility
ORF	Open reading frame
PBS	Phosphate- buffered saline
PCR	Polymerase chain reaction
phOx	2-phenyloxazolone
RF	Reading frame
TCR	T-cell receptor
TD	Thymus-dependent
TdT	Terminal deoxynucleotidyltransferase
T_H	T helper cells
TI	Thymus-independent

UV	Ultraviolet
V	Variable domain
WT	Wildtype

7 Appendix

Figure 1

Amino acid sequences of VH/VL gene-encoded variable regions of TD antigen-induced anti-phOx antibodies in ΔD-DμFS mice

(Legend see below)

Amino acid sequences of TD antigen-induced primary day 7 antibodies of ΔD-DμFS mice

```
IGHV673  QVQLQQSGPE LVKPGASVKI SCKASGSSFT SYYIHWVKQS PGQGLEWIGW IYPGSGNTKY NEKFKGKATL TADTSSSTAY MQLSSLTSED SAVYFCAR
01H 673  ------P--- ---------- ------R--N----R -----+---- -F-----S-- ---------- +--------- -H-------E --------
01L 121  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV121  DIKMTQSPSS MYASLGERVT ITCKASQDIN SYLSWFQQKP GKSPKTLIYR ANRLVDGVPS RFSGSGSGQD YSLTISSLEY EDMGIYYCLQ YDEF

IGHV671  EVQLQQSGTV LARPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGA IYPGNSDTSY NQKFKGKAKL TAVTSTSTAY MELSSLTNED SAVYYCTR
02H 671  ---------- ------+--- -----S---- ------- ----+---+- --------- ---------- -----A---- ---------- -----+VI
02L 139  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----+---- --------
IGKV139  DIQMTQTTSS LSASLGDRVT ISCSASQGIS NYLNWYQQKP DGTVKLLIYY TSSLHSGVPS RFSGSGSGTD YSLTISNLEP EDIATYYCQQ YSKL

IGHV627  QVQLQQSGPE LVKPGASVRI SCKASGYTFT SYYIHWVKQR PGQGLEWIGW IYPGNGNTKY NEKFKGKATL TADKSSSTAY MQLSSLTSED SAVYFCAR
03H 627  ---------- +--T---LKM ----------+ G---------- ---------- ----D--S-- -------T-- -E----N--- -L-------- --I----+
03L 072  ---------- ---------- ---------- ---H------ ---------- ---------- ---------- ---------- ---------- --------
IGKV072  QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN

IGHV627  QVQLQQSGPE LVKPGASVRI SCKASGYTFT SYYIHWVKQR PGQGLEWIGW IYPGNGNTKY NEKFKGKATL TADKSSSTAY MQLSSLTSED SAVYFCAR
04H 627  ---------- ---------- ---------- ---------- ---------- -----V---- ---------- ---------- -------F-- F-------
04L 139  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV139  DIQMTQTTSS LSASLGDRVT ISCSASQGIS NYLNWYQQKP DGTVKLLIYY TSSLHSGVPS RFSGSGSGTD YSLTISNLEP EDIATYYCQQ YSKL
```


IGHV532 QVQLQQSGAE LAKPGASVKM SCKAS GYTFT SYW MHWVKQR PGQGLEWIGY INPSTGYT EY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
05H 532 RI NN +
05L 078
IGKV078 ENVLTQSPAI MSASPGEKVT MTCRAS SSVS SSY LHWYQQK SGASPKLWIY STS NLASGVP ARFSGSGSGT SYSLTISSVE AEDAATYYCQ QYSGY
IGHV506 EIQLQQSGPE LVKPGASVKV SCKAS GYAFT SYN MYWVKQS HGKSLEWIGY IDPYNGGT SY NQKFKGKATL TIDKSSSTAY MHLNSLTSED SAVYYCAR
06H 506 K + M I S T Y H F T + V S A S
06L 121 C
IGKV121 DIKMTQSPSS MYASLGERVT ITCKAS QDIN SY LSWFQQKP GKSPKTLIY R AN RLVDGVPS RFSGSGSGQD YSLTISSLEY EDMGIYYCLQ YDEF
IGHV396 EVQLQQSGPE LEKPGASVKI SCKAS GYSFT GYN MNWVKQS NGKSLEWIGN IDPYYGGT SY NQKFKGKATL TVDKSSSTAY MQLKSLTSED SAVYYC
07H 396
07L 201 I R
IGKV201 DIVMTQSQKF MSTSVGDRVS VTCKAS QNVG TN VAWYQQKP GQSPKALIY S AS YRYSGVPD RFTGSGSGTD FTLTISNVQS EDLAEYFCQQ YNSY
IGHV386 QVQLQQSGAE LMKPGASVKI SCKAT GYTFS SYW IEWVKQR PGHGLEWIGE ILPGSGST NY NEKFKGKATF TADTSSNTAY MQLSSLTSED SAVYYC
08H 386 +T N N I K
08L 112 W N
IGKV112 DIQMTQSPSS LSASLGERVS LTCRAS QDIG SS LNWLQQEP DGTIKRLIY A TS SLDSGVPK RFSGSRSGSD YSLTISSLES EDFVDYYCLQ YASS
IGHV117 SQMQLQESGP GLVKPSQSLF LACSIT GFPI TSGYY WIWIR QSPGKPLEWM GY ITHSGET F YNPSLQSPIS ITRETSKNQF FLQLNSVTTE DTAMYYCAG
10H 117
10L 121
IGKV121 DIKMTQSPSS MYASLGERVT ITCKAS QDIN SY LSWFQQKP GKSPKTLIY R AN RLVDGVPS RFSGSGSGQD YSLTISSLEY EDMGIYYCLQ YDEF
IGHV138 SDVQLQESGP DLVKPSQSLS LTCTVT GYSI TSGYS WHWIR QFPGNKLEWM GY IHYSGST N YNPSLKSRIS ITRDTSKNQF FLQLNSVTTE DTATYYCAR
11H 138
11L 163
IGKV163 ENVLTQSPAI MAASLGQKVT MTCSAS SSVS SSY LHWYQQK SGASPKPLIH RTS NLASGVP ARFSGSGSGT SYSLTISSVE AEDDATYYCQ QWSGY


IGHV138 SDVQLQESGP DLVKPSQSLS LTCTVTGYSI TSGYSWHWIR QFPGNKLEWM GYIHYSGSTN YNPSLKSRIS ITRDTSKNQF FLQLNSVTTE DTATYYCAR
12H 138 -------+-- ---------- ------ ---------- ---------- ---------- ---------- ---------- ---------- --------L
12L 175 ---------- ---------- ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV175 DIVMTQSHKF MSTSVGDRVS ITCKASQDVG TAVAWYQQKP GQSPKLLIYW ASTRHTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCQQ YSSY
IGHV120 SDVQLQESGP GLVKPSQSLS LTCSVTGYSI TSGYYWNWIR QFPGNKLEWM GYISYDGSNN YNPSLKNRIS ITRDTSKNQF FLKLNSVTTE DTATYYCAR
14H 120 -------+-- ---------- ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
14L 066 --M++---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV066 DIVLTQSPAS LAVSLGQRAT ISCRASQSVS TSSYSYMHWY QQKPGQPPKL LIKYASNLES GVPARFSGSG SGTDFTLNIH PVEEEDTATY YCQHSWEI
IGHV114 EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
15H 114 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------AG
15L 106 ---------- ---------- ---------- ---------- ---------F ---------- ---------- ---------- ---------- ----------
IGKV106 DIKMTQSPSS MYASLGERVT ITCKASQDIK SYLSWYQQKP WKSPKTLIYY ATSLADGVPS RFSGSGSGQD YSLTISSLES DDTATYYCLQ HGES
IGHV155 QIQLVQSGPE LKKPGETVKI SCKASGYTFT NYGMNWVKQA PGKGLKWMGW INTNTGEPTY AEEFKGRFAF SLETSASTAY LQINNLKNED TATYFCAR
16H 155 ----E----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
16L 122 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV122 DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHV
IGHV690 EIQLQQTGPE LVKPGASVKI SCKASGYSFT DYIMLWVKQS HGKSLEWIGN INPYYGSTSY NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
19H 690 ---------- ---------- ---------- ---------- ---------- ---------- -L-------- -V--+----- ---N----D- -------S
19L 210 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV210 DIVLTQSPAS LAVSLGQRAT ISCRASESVD NYGISFMNWF QQKPGQPPKL LIYAASNQGS GVPARFSGSG SGTDFSLNIH PMEEDDTAMY FCQQSKEV
IGHV690 EIQLQQTGPE LVKPGASVKI SCKASGYSFT DYIMLWVKQS HGKSLEWIGN INPYYGSTSY NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
20H 690 ------S--- ---------- ---------- ---------- ---------- ---------- -L-------- -V--+----- ---N------ -------V-
20L 123 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV123 DIVMTQAAFS NPVTLGTSAS ISCRSSKSLL HSNGITYLYW YLQKPGQSPQ LLIYQMSNLA SGVPDRFSSS GSGTDFTLRI SRVEAEDVGV YYCAQNLEL


IGHV643 EIQLQQSGPE LVKPGASVKI SCKASGYSFT GYIMHWMKQS HGKCLEWIGY ISCYIGATSY NQKFKGKAIF TVDKSSSTAY MQFNSLTFED SAVYYCAR
21H 643 ------+--- +--T------ -----------+-Y---V--- ---S------ -----N----- ----+---T- ---T------ -------S+- --------
21L 210 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV210 DIVLTQSPAS LAVSLGQRAT ISCRASESVD NYGISFMNWF QQKPGQPPKL LIYAASNQGS GVPARFSGSG SGTDFSLNIH PMEEDDTAMY FCQQSKEV
IGHV532 QVQLQQSGAE LAKPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGY INPSTGYTEY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
22H 532 ---------- ---------- ----------+---------- ---------- ---------- ---------- ---------- -------F-- F------N
22L 077 ---------- ---------- ----------+---------- -----R---- ---------- ---------- ---------- -----+---- --------
IGKV077 QIVLTQSPAI MSASPGEKVT ISCSASSSVS YMYWYQQKPG SSPKPWIYRT SNLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQY HSY
IGHV171 QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
24H 171 -----Q---- ---------- ----------+---------- ---------- ----------+---------- ---------- ---------- -------
24L 072 ---------- ---------- ----------+---------- ---------- ----------+---------- ---------- ---------- -------
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
IGHV128 EVQLQESGPS LVKPSQTLSL TCSVTGDSIT SGYWNWIRKF PGNKLEYMGY ISYSGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTTEDT ATYYCAR
26H 128 ------+--- ---------- ----------+---------- ---------F -R---I--- ---------- ---------- ---------- -------
26L 143 ---------- -G-------- -S----K------R -----R---- ----VF--H-+ -----C---L ---------- ---------- -------
IGKV143 EILLTQSPAI IAASPGEKVT ITCSASSSVS YMNWYQQKPG SSPKIWIYGI SNLASGVPAR FSGSGSGTSF SFTINSMEAE DVATYYCQQR SSY
IGHV163 DVKLVESGGG LVKLGGSLKL SCAASGFTFS SYYMSWVRQT PEKRLELVAA INSNGGSTYY PDTVKGRFTI SRDNAKNTLY LQMSSLKSED TALYYCAR
27H 163 ---------- ---+------ ----------+---------- ---------- ----------+---------- ---------- ---------- -------S
27L 072 ---------- ---------- ----------T ---Q------ ---------- ----------+---------- ---------- ---------- --------
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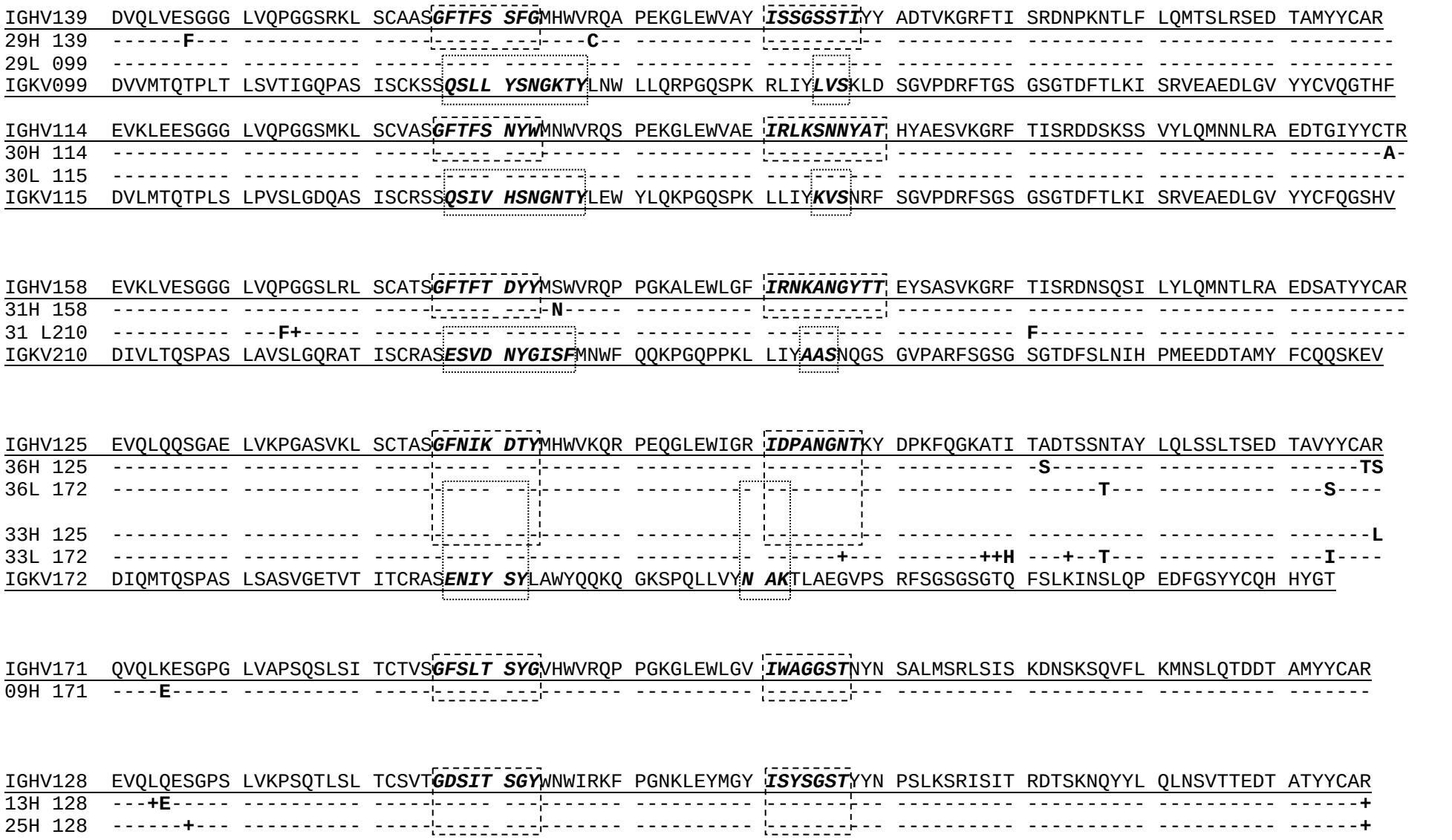
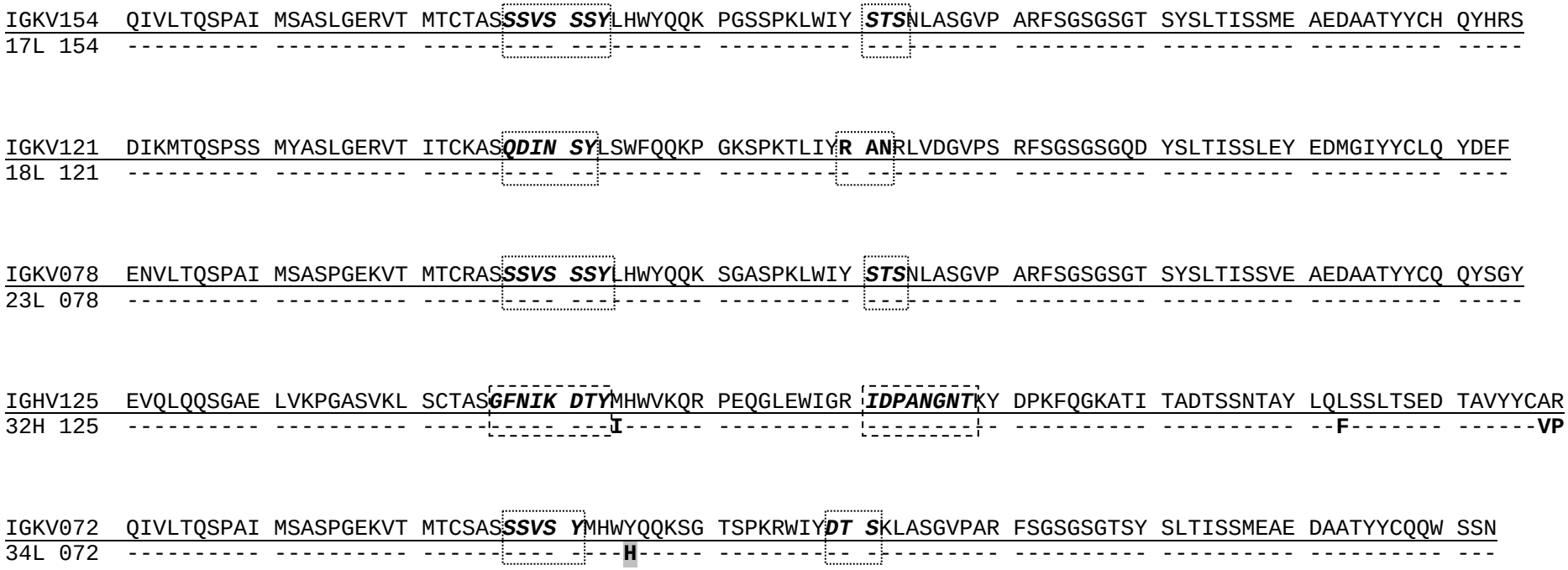
IGHV163 DVKLVESGGG LVKLGGSLKL SCAASGFTFS SYYMSWVRQT PEKRLELVAA INSNGGSTYY PDTVKGRFTI SRDNAKNTLY LQMSSLKSED TALYYCAR
28H 163 -------+-- ----------+---------- ---------- -----+---- ----------+---------- ---------- ---------G --------
28L 168 ---------- ----------+---------- ---------- ---------- ----------+---------- ---+------ ---------- --T-----
IGKV168 DILLTQSPAI LSVSPGERVS FSCRASQSIG TSIHWYQQRT NGSPRLLIKY ASESISGIPS RFSGSGSGTD FTLSINSVES EDIADYYCQQ SNSW


IGHV139 DVQLVESGGG LVQPGGSRKL SCAASGFTFS SFGMHWVRQA PEKGLEWVAY ISSGSSTIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCAR
29H 139 ------F--- ---------- ----------- ---------C-- ---------- ---------- ---------- ---------- ---------
29L 099 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV099 DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL YSNGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCVQGTHF
IGHV114 EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
30H 114 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- --------A-
30L 115 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
IGHV158 EVKLVESGGG LVQPGGSLRL SCATSGFTFT DYYMSWVRQP PGKALEWLGF IRNKANGYTT EYSASVKGRF TISRDNSQSI LYLQMNTLRA EDSATYYCAR
31H 158 ---------- ---------- ----------- ---------N ---------- ---------- ---------- ---------- ----------
31 L210 ---------- ---F+----- ----------- ---------- ---------- ---------- ---------- F--------- ----------
IGKV210 DIVLTQSPAS LAVSLGQRAT ISCRASESVD NYGISFMNWF QQKPGQPPKL LIYAASNQGS GVPARFSGSG SGTDFSLNIH PMEEDDTAMY FCQQSKEV
IGHV125 EVQLQQSGAE LVKPGASVKL SCTASGFNIK DTYMHWVKQR PEQGLEWIGR IDPANGNTKY DPKFQGKATI TADTSSNTAY LQLSSLTSED TAVYYCAR
36H 125 ---------- ---------- ----------- ---------- ---------- ---------- ---------- -S-------- ---------- ------TS
36L 172 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ------T--- ---------- ---S----
33H 125 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------- -------L
33L 172 ---------- ---------- ----------- ---------- ---------- ----------+--- -------++H ---+--T--- ---------- ---I----
IGKV172 DIQMTQSPAS LSASVGETVT ITCRASENIY SYLAWYQQKQ GKSPQLLVYN AKTLAEGVPS RFSGSGSGTQ FSLKINSLQP EDFGSYYCQH HYGT
IGHV171 QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
09H 171 ----E----- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------- -------
IGHV128 EVQLQESGPS LVKPSQTLSL TCSVTGDSIT SGYWNWIRKF PGNKLEYMGY ISYSGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTTEDT ATYYCAR
13H 128 ---+E----- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------- ------+
25H 128 ------+--- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------- ------+


IGKV154 QIVLTQSPAI MSASLGERVT MTCTASSSVS SSYLHWYQQK PGSSPKLWIY STSNLASGVP ARFSGSGSGT SYSLTISSME AEDAATYYCH QYHRS
17L 154 ---------- ---------- ------ ---- --- ------- ---------- --- ------- ---------- ---------- ---------- -----
IGKV121 DIKMTQSPSS MYASLGERVT ITCKASQDIN SYLSWFQQKP GKSPKTLIYR ANRLVDGVPS RFSGSGSGQD YSLTISSLEY EDMGIYYCLQ YDEF
18L 121 ---------- ---------- ------ ---- -- ------- ---------- - -- ------- ---------- ---------- ---------- ----
IGKV078 ENVLTQSPAI MSASPGEKVT MTCRASSSVS SSYLHWYQQK SGASPKLWIY STSNLASGVP ARFSGSGSGT SYSLTISSVE AEDAATYYCQ QYSGY
23L 078 ---------- ---------- ------ ---- --- ------- ---------- --- ------- ---------- ---------- ---------- -----
IGHV125 EVQLQQSGAE LVKPGASVKL SCTASGFNIK DTYMHWVKQR PEQGLEWIGR IDPANGNTKY DPKFQGKATI TADTSSNTAY LQLSSLTSED TAVYYCAR
32H 125 ---------- ---------- ------ ---- ---I------ ---------- ---------- - ---------- ---------- --F------- ------VP
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
34L 072 ---------- ---------- ------ ---- ---H----- ---------- - --------- ---------- ---------- ---------- ---

Amino acid sequences of TD antigen-induced primary day 14 antibodies of ΔD-DμFS mice

```
IGHV569  QVQLQQSGAE LMKPGSSVKL SCKATGYTFT GYWIEWVKQR PGHGLEWIGE ILPGSGSTNY NEKFKGKATF TADTSSNTAY MQLSSLTSED SAVYYCAR
01H 569  ---------- ---------- -----S-S-- -------- ---------- ---------- ---------- ---------- ---------- --------
01L 077  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV077  QIVLTQSPAI MSASPGEKVT ISCSASSSVS YMYWYQQKPG SSPKPWIYRT SNLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQY HSY

IGHV532  QVQLQQSGAE LAKPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGY INPSTGYTEY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
02H 532  ---------- ---------- -N-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
02L 097  ---------- ---------T ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV097  DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCWQGTHF

IGHV495  QVQLQQSGAE LARPGASVKM SCKASGYTFT SYWMQWVKQR PGQGLEWIGA IYPGDGDTRY TQKFKGRATL TADKSSSTAY MQLSSLTSED SAVYYCAT
03H 495  ---------- ---------- ---------- ---------- ---------- ---------- ----T-K--- --N------- ------A--- -------R
03L 166  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV166  ENVLTQSPAI MSASLGEKVT MSCRASSSVN YMYWYQQKSD ASPKLWIYYT SNLAPGVPAR FSGSGSGNSY SLTISSMEGE DAATYYCQQF TSS

IGHV286  EFQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY IYPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCAR
04H 286  ---------- ---------- ---------- ---------- ---------- -N-------- ---------- ---------- ---------- --------
04L 166  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV166  ENVLTQSPAI MSASLGEKVT MSCRASSSVN YMYWYQQKSD ASPKLWIYYT SNLAPGVPAR FSGSGSGNSY SLTISSMEGE DAATYYCQQF TSS

IGHV073  QVQLQQSGAE LVKTGASVKM SCKASGYTFT SYTMHWVKQR PGQGLEWIGY INPSSGYTNY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
05H 073  ---------- ---------- ---------- ---------- ---------- ---------- ----M----M -------S-- ----I-+--- --------
05L 129  ---------- ---------- ----------N------ -----Q---- ---------- ---------- ---S------ ---------- --------
IGKV129  DIQMNQSPSS LSASLGDTIT ITCHASQNIN VWLSWYQQKP GNIPKLLIYK ASNLHTGVPS RFSGSGSGTG FTLTISSLQP EDIATYYCQQ GQSY
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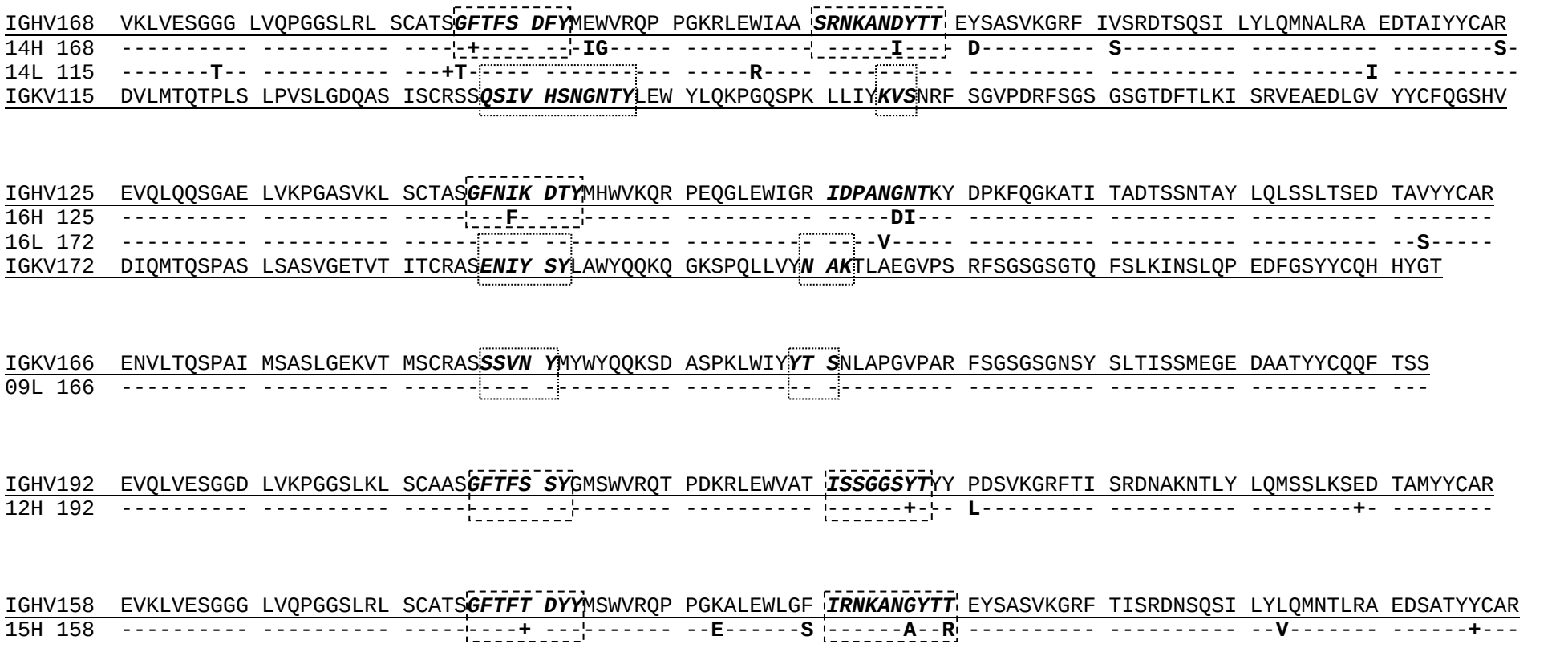
IGHV147 EVMLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
06H 147 ---------- ---------- ----------
06L 179 ---------- ---------- ----------
IGKV179 DIVMTQSPAT LSVTPGDRVS LSCRASQSIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQN GHSF
IGHV155 QIQLVQSGPE LKKPGETVKI SCKASGYTFT NYGMNWVKQA PGKGLKWMGW INTNTGEPTY AEEFKGRFAF SLETSASTAY LQINNLKNED TATYFCAR
07H 155 ---------- ---------- ----------
07L 082 ---------- ---------- ----------
IGKV082 QIVLTQSPAI MSASPGEKVT ITCSASSSVS YMHWFQQKPG TSPKLWIYST SNLASGVPAR FSGSGSGTSY SLTISRMEAE DAATYYCQQR SSY
IGHV118 QIQLVQSGPE LKKPGETVRI SCKASGYTFT TAGMQWVQKM PGKGLKWIGW INTHSGVPKY AEDFKGRFAF SLETSASTAY LQISNLKNED TATYFCAR
08H 118 ---------- ---------- ----------
08L 097 ---------- ---------- ----------
IGKV097 DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCWQGTHF
IGHV480 EVQLQQSGPE LVKPGASVKI SCKASGYSFT GYYMHWVKQS HVKSLEWIGR INPYNGATSY NQNFKDKASL TVDKSSSTAY MELHSLTSED SAVYYCAR
10H 480 ---------- ---------- ----------
10L 115 ---------- ---------- ----------
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
IGHV171 QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
11H 171 ---------- ---------- ----------
11L 142 ---------- ---------- ----------
IGKV142 EIVLTQSPTT MAASPGEKIT ITCSASSSIS SNYLHWYQQK PGFSPKLLIY RTSNLASGVP ARFSGSGSGT SYSLTIGTME AEDVATYYCQ QGSSI
IGHV139 DVQLVESGGG LVQPGGSRKL SCAASGFTFS SFGMHWVRQA PEKGLEWVAY ISSGSSTIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCAR
13H 139 XXXXXXXXXX XXXXXXXXX- ----------
13L 103 ---------- ---------- ----------
IGKV103 DIQMTQSPSS LSASLGERVS LTCRASQDIH GYLNLFQQKP GETIKHLIYE TSNLDSGVPK RFSGSRSGSD YSLIIGSLES EDFADYYCLQ YASS


IGHV168 VKLVESGGG LVQPGGSLRL SCATSGFTFS DFYMEWVRQP PGKRLEWIAA SRNKANDYTT EYSASVKGRF IVSRDTSQSI LYLQMNALRA EDTAIYYCAR
14H 168 ---------- ---------- ----+----- --IG----- ---------- -----I---- D--------- S--------- ---------- --------S-
14L 115 -------T-- ---------- ---+T----- -------- ----R----- ---------- ---------- ---------- ---------I ----------
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
IGHV125 EVQLQQSGAE LVKPGASVKL SCTASGFNIK DTYMHWVKQR PEQGLEWIGR IDPANGNTKY DPKFQGKATI TADTSSNTAY LQLSSLTSED TAVYYCAR
16H 125 ---------- ---------- -----F---- -------- ---------- -----DI--- ---------- ---------- ---------- --------
16L 172 ---------- ---------- ---------- -------- ---------- ----V----- ---------- ---------- ---------- --S-----
IGKV172 DIQMTQSPAS LSASVGETVT ITCRASENIY SYLAWYQQKQ GKSPQLLVYN AKTLAEGVPS RFSGSGSGTQ FSLKINSLQP EDFGSYYCQH HYGT
IGKV166 ENVLTQSPAI MSASLGEKVT MSCRASSSVN YMYWYQQKSD ASPKLWIYYT SNLAPGVPAR FSGSGSGNSY SLTISSMEGE DAATYYCQQF TSS
09L 166 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---
IGHV192 EVQLVESGGD LVKPGGSLKL SCAASGFTFS SYGMSWVRQT PDKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLKSED TAMYYCAR
12H 192 ---------- ---------- ---------- -------- ---------- ------+--- L--------- ---------- --------+- --------
IGHV158 EVKLVESGGG LVQPGGSLRL SCATSGFTFT DYYMSWVRQP PGKALEWLGF IRNKANGYTT EYSASVKGRF TISRDNSQSI LYLQMNTLRA EDSATYYCAR
15H 158 ---------- ---------- ----------+ --------- --E------S ------A--R ---------- ---------- --V------- ------+---

Amino acid sequences of TD antigen-induced secondary antibodies of ΔD-DμFS mice

```
IGHV623  EIQLQQSGPE LVKPGASVKM SCKASGYTFT DYYMHWVKQS HGKSLEWIRR VNPNNGGTSY NQKFKDKATL TVEKSSITAY MELRSLTSED SAVYYCAR
01H 623  ---------- ---------- -----+-S-- G-------- --------G- ---------- -----G--I+ --D-+-S--- ---+------ -+-------
01L 115  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV115  DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
05H 171  ----E----- ---------- ---------- ---R-------- ---------- -----T---- ---------- ---------- ---------- -------
05L 072  -------T-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------C--- -------

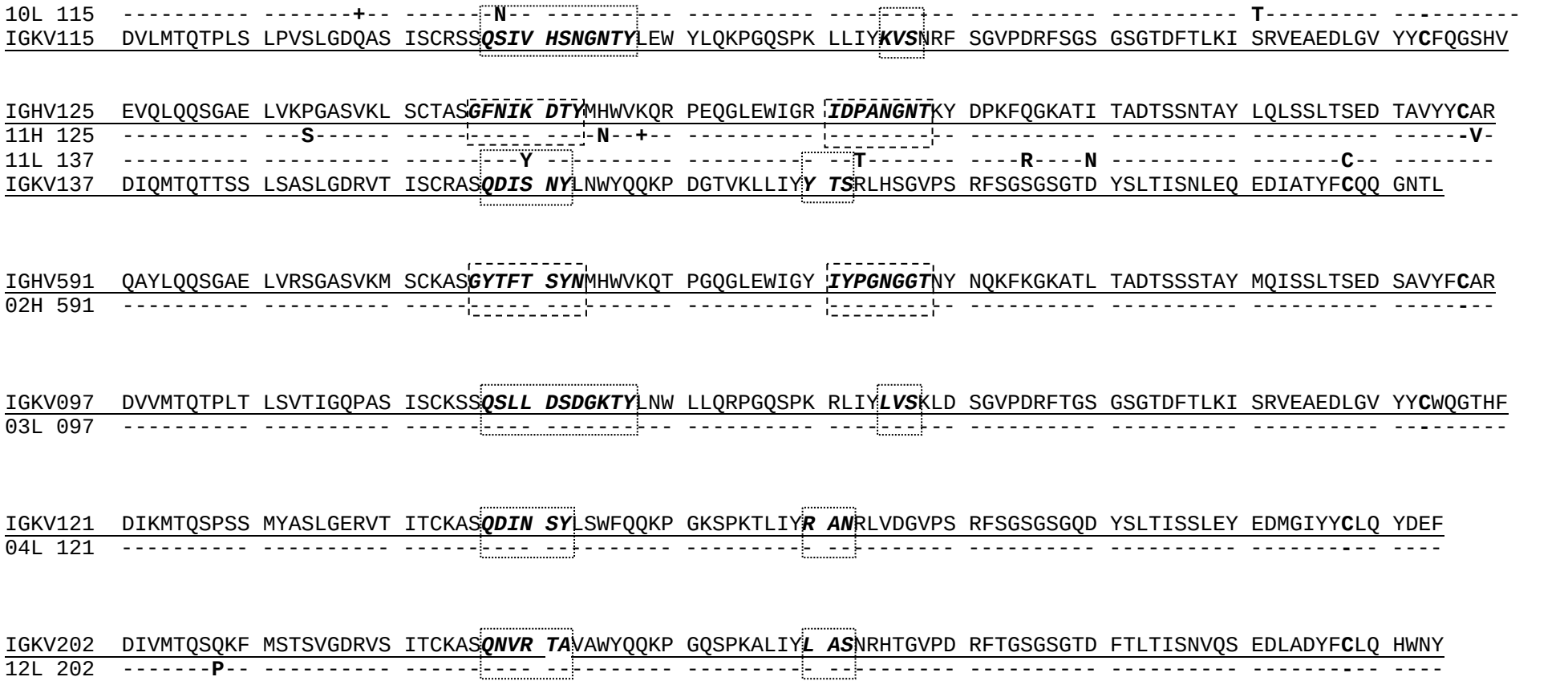
06H 171  ----E----- ---------- ---------- N--------- ---------- --T--I---- ---------R ---------- ------A--- -------
06L 072  ---------- ---------- ---------- -IN-F----- ---------- -------P-- ---------- ---------- ------C--- -T-----

07H 171  ---------- ---------- ---------- T-AI------ ---------- --T------- ---------- --+--+---- -----R---- -------
07L 072  ---------- ---------- ---------+N --N-F----- ---------- ---------- --------F- -----+---- ------C--- -------
IGKV072  QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
08H 171  ---------- ---------- ----------+---------- ---------- +---------- -+---+---- --+------- --+------- -------
08L 137  ---------- ---------- ---------- ---F------ -------+-- ----++---- ---------N ------D--- ---------- -------
IGKV137  DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL

IGHV162  QVQLKESGPD LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLVV IWSDGSTTYN SALKSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
09H 162  ---------- ---------- -----L+-- T---N-G-+- --------+- -----I---S -L+---+--R R-----+--- ------S--- ---------
09L 115  ---------- ---------- ---------- ---------- ++-----F-+ ---------- ---------- ---------- T--A------ --------
IGKV115  DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV

IGHV158  EVKLVESGGG LVQPGGSLRL SCATSGFTFT DYYMSWVRQP PGKALEWLGF IRNKANGYTT EYSASVKGRF TISRDNSQSI LYLQMNTLRA EDSATYYCAR
10H 158  ----E----- ---+------ -----+---- -T----- -----+--A- -K-R-YA--- D--T------ ---------- ---+----+- --------+
```


10L 115 ---------- -------+-- ------N-- ---------- ---------- ---------- ---------- ---------- T--------- ---------
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
IGHV125 EVQLQQSGAE LVKPGASVKL SCTASGFNIK DTYMHWVKQR PEQGLEWIGR IDPANGNTKY DPKFQGKATI TADTSSNTAY LQLSSLTSED TAVYYCAR
11H 125 ---------- ---S------ ---------- ---------- ---N--+-- ---------- ---------- ---------- ---------- ---------- -----V-
11L 137 ---------- ---------- ---------- ---Y------ ---------- ---------- --T------- ----R----N ---------- -------C-- --------
IGKV137 DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL
IGHV591 QAYLQQSGAE LVRSGASVKM SCKASGYTFT SYNMHWVKQT PGQGLEWIGY IYPGNGGTNY NQKFKGKATL TADTSSSTAY MQISSLTSED SAVYFCAR
02H 591 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV097 DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCWQGTHF
03L 097 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV121 DIKMTQSPSS MYASLGERVT ITCKASQDIN SYLSWFQQKP GKSPKTLIYR ANRLVDGVPS RFSGSGSGQD YSLTISSLEY EDMGIYYCLQ YDEF
04L 121 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----
IGKV202 DIVMTQSQKF MSTSVGDRVS ITCKASQNVR TAVAWYQQKP GQSPKALIYL ASNRHTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCLQ HWNY
12L 202 -------P-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----

Amino acid sequences of TD antigen-induced tertiary antibodies of ΔD-DμFS mice

```
IGHV627    QVQLQQSGPE LVKPGASVRI SCKASGYTFT SYYIHWVKQR PGQGLEWIGW IYPGNGNTKY NEKFKGKATL TADKSSSTAY MQLSSLTSED SAVYFCAR
01H 627    ---------- ---------- ---------- ---------- ---------- -----V---- ---------- ---------- ---------- -------S-
01L 096    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV096    DIVMTQAAPS VPVTPGESVS ISCRSSKSLL HSNGNTYLYW FLQRPGQSPQ LLIYRMSNLA SGVPDRFSGS GSGTAFTLRI SRVEAEDVGV YYCMQHLEY

IGHV286    EFQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY IYPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCAR
03H 286    ---------- ---------- ---------- ---------- ---------- ---------- -N-------- ---------- ---------- --------
03L 112    ---------- ---------- ---------- --H------- ---------- ---------- ---------- ---------- ---------- --------
IGKV112    DIQMTQSPSS LSASLGERVS LTCRASQDIG SSLNWLQQEP DGTIKRLIYA TSSLDSGVPK RFSGSRSGSD YSLTISSLES EDFVDYYCLQ YASS

IGHV171    QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
04H 171    ---------- ---------- ---------- ---------- ---------- ---------- ---------- --+------- ---------- -------
04L 137    ---------- ---------- ---------- ----F----- ---------- ----Q----- ---------- ---------- --A------- -------
IGKV137    DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL

IGHV178    DVKLVESGGG LVKPGGSLKL SCAASGFTFS SYTMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLKSED TAMYYCTR
05H 178    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------IT
05L 210    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV210    DIVLTQSPAS LAVSLGQRAT ISCRASESVD NYGISFMNWF QQKPGQPPKL LIYAASNQGS GVPARFSGSG SGTDFSLNIH PMEEDDTAMY FCQQSKEV

IGHV176    EVKLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAS ISSGGSTYYP DSVKGRFTIS RDNARNILYL QMSSLRSEDT AMYYCAR
06H 176    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
06L 195    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV195    DIVMTQSPSS LAMSVGQKVT MSCKSSQSLL NSSNQKNYLA WYQQKPGQSP KLLVYFASTR ESGVPDRFIG SGSGTDFTLT ISSVQAEDLA DYFCQQHYS
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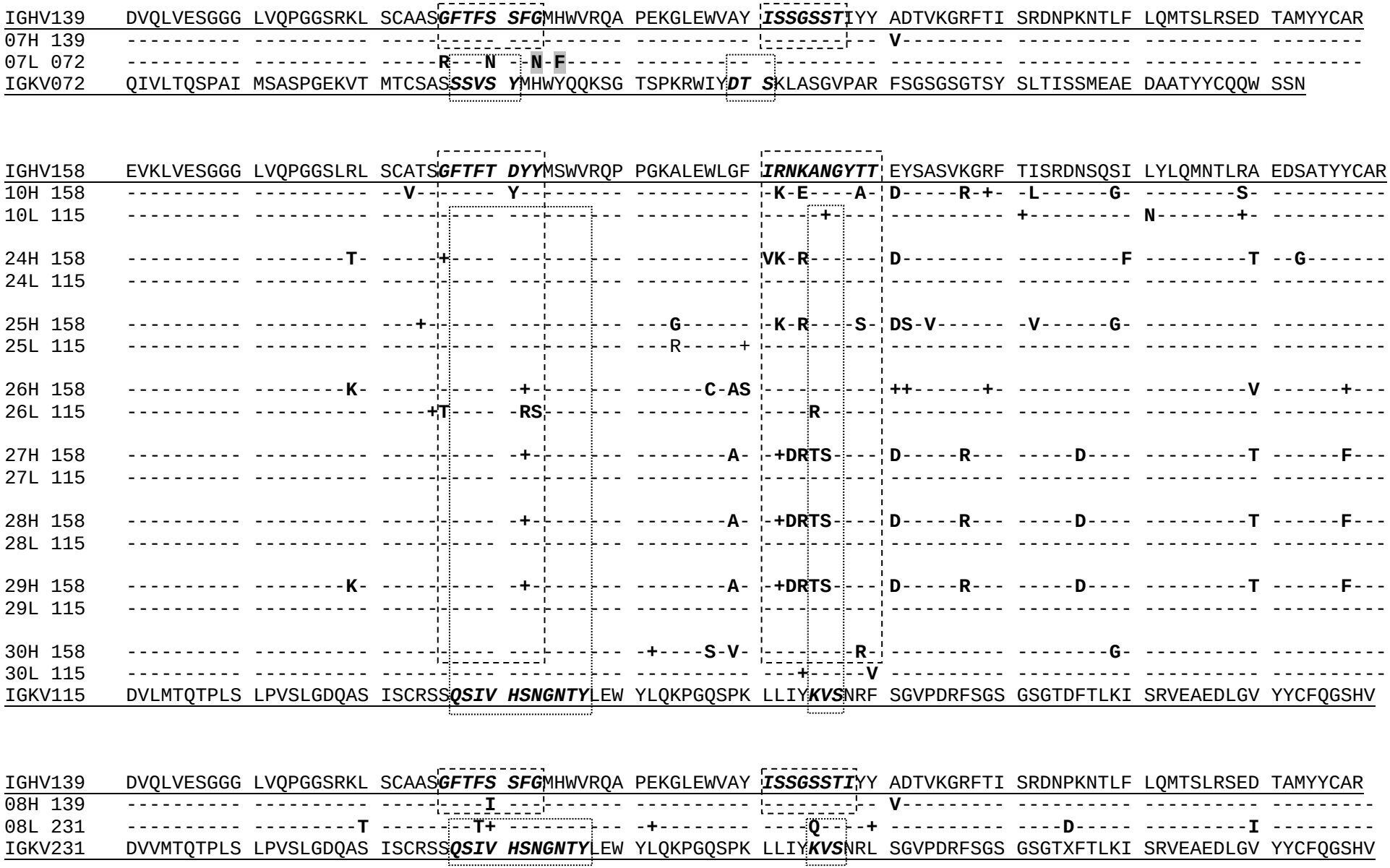
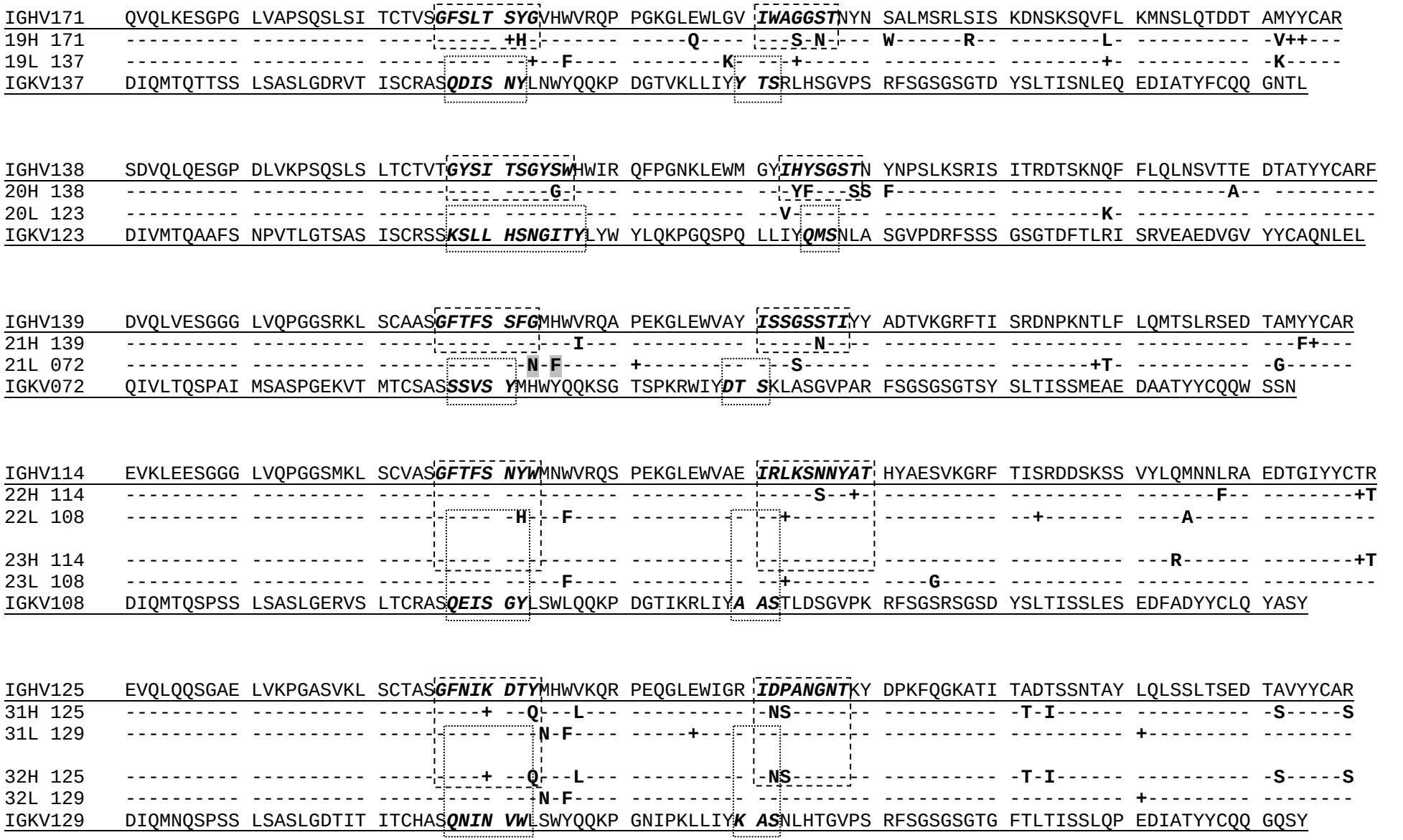

IGHV139 DVQLVESGGG LVQPGGSRKL SCAASGFTFS SFGMHWVRQA PEKGLEWVAY ISSGSSTIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCAR
07H 139 ---------- ---------- ----------
07L 072 ---------- ---------- -----R---N --N-F----- ---------- ---------- ---------- ---------- ---------- --------
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
IGHV158 EVKLVESGGG LVQPGGSLRL SCATSGFTFT DYYMSWVRQP PGKALEWLGF IRNKANGYTT EYSASVKGRF TISRDNSQSI LYLQMNTLRA EDSATYYCAR
10H 158 ---------- ---------- --V------- Y--------- ---------- -K-E----A- D-----R-+- -L------G- --------S- ----------
10L 115 ---------- ---------- ---------- ---------- ---------- ----+----- ---------- +--------- N-------+- ----------
24H 158 ---------- --------T- -----+---- ---------- ---------- VK-R------ D--------- ---------F ---------T --G-------
24L 115 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
25H 158 ---------- ---------- ---+------ ---------- ---G------ -K-R----S- DS-V------ -V------G- ---------- ----------
25L 115 ---------- ---------- ---------- ---------- ---R-----+ ---------- ---------- ---------- ---------- ----------
26H 158 ---------- --------K- ---------- -+-------- ------C-AS ---------- ++------+- ---------- ---------V ------+---
26L 115 ---------- ---------- ----+T---- -RS------- ---------- ----R----- ---------- ---------- ---------- ----------
27H 158 ---------- ---------- ---------- -+-------- --------A- -+DRTS---- D-----R--- -----D---- ---------T ------F---
27L 115 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
28H 158 ---------- ---------- ---------- -+-------- --------A- -+DRTS---- D-----R--- -----D---- ---------T ------F---
28L 115 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
29H 158 ---------- --------K- ---------- -+-------- --------A- -+DRTS---- D-----R--- -----D---- ---------T ------F---
29L 115 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
30H 158 ---------- ---------- ---------- ---------- -+----S-V- ---------R ---------- --------G- ---------- ----------
30L 115 ---------- ---------- ---------- ---------- ---------- ----+-----V ---------- ---------- ---------- ----------
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
IGHV139 DVQLVESGGG LVQPGGSRKL SCAASGFTFS SFGMHWVRQA PEKGLEWVAY ISSGSSTIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCAR
08H 139 ---------- ---------- ----------
08L 231 ---------- ---------T ----------
IGKV231 DVVMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRL SGVPDRFSGS GSGTXFTLKI SRVEAEDLGV YYCFQGSHV


IGHV114 EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
09H 114 ---------- ---------- -----+-- S--S----- ---------- -----D---- --------K- ---------R L-----S--- ---+----+G
09L 099 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV099 DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL YSNGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCVQGTHF
IGHV627 QVQLQQSGPE LVKPGASVRI SCKASGYTFT SYYIHWVKQR PGQGLEWIGW IYPGNGNTKY NEKFKGKATL TADKSSSTAY MQLSSLTSED SAVYFCAR
13H 627 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- -------+
13L 103 ---------- ---------- ----------S ----Q----- ---------- --------T- S--------- ---------- DG------
IGKV103 DIQMTQSPSS LSASLGERVS LTCRASQDIH GYLNLFQQKP GETIKHLIYE TSNLDSGVPK RFSGSRSGSD YSLIIGSLES EDFADYYCLQ YASS
IGHV627 QVQLQQSGPE LVKPGASVRI SCKASGYTFT SYYIHWVKQR PGQGLEWIGW IYPGNGNTKY NEKFKGKATL TADKSSSTAY MQLSSLTSED SAVYFCAR
14H 627 ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- -------+
14L 174 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV174 DIQMTQSPAS LSASVGETVT ITCRASGNIH NYLAWYQQKQ GKSPQLLVYN AKTLADGVPS RFSGSGSGTQ YSLKINSLQP EDFGSYYCQH FWST
IGHV175 QVQLKESGPG LVAPSQSLSI TCTVSGFSLS RYSVHWVRQP PGKGLEWLGM IWGGGSTDYN SALKSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
15H 175 ---------- ---------- -----+---- -NN------ ---------- ---------- -------I-- ---------- ---------- -------
15L 072 ---------- ---------- ---------- -IQ-F----- ---------- ---------- ----G----- ---------- N-Y----
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
IGHV073 QVQLQQSGAE LVKTGASVKM SCKASGYTFT SYTMHWVKQR PGQGLEWIGY INPSSGYTNY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
16H 073 ---------- ---------- ----------T -+------- -----+---- ---------- ----N----- I--------- ---------- --------
16L 078 ---------- ---------- ----------T ---------- ---------- -+-------- T--------- ---------- ---------- --------
IGKV078 ENVLTQSPAI MSASPGEKVT MTCRASSSVS SSYLHWYQQK SGASPKLWIY STSNLASGVP ARFSGSGSGT SYSLTISSVE AEDAATYYCQ QYSGY
IGHV171 QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
18H 171 ---------- ---------- -----VI ---+--I--- ---------+ -P--+----- -------T-T -----N---- ----+----- -------
18L 072 ---------- ---------- ---------- ---------- ---------- -------T- ---------- ---------- ---------- -------
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN


IGHV171 QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
19H 171 ---------- ---------- -----+H---- ------- -----Q---- ---S-N---- W------R-- --------L- ---------- -V++---
19L 137 ---------- ---------- -----+---- --+--F---- --------K- --+------ ---------- --------+- ---------- -K-----
IGKV137 DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL
IGHV138 SDVQLQESGP DLVKPSQSLS LTCTVTGYSI TSGYSWHWIR QFPGNKLEWM GYIHYSGSTN YNPSLKSRIS ITRDTSKNQF FLQLNSVTTE DTATYYCARF
20H 138 ---------- ---------- -----+---- -----G---- ---------- --YF---SS F--------- ---------- --------A-- ---------
20L 123 ---------- ---------- -----+---- ---------- ---------- --V+---+-- ---------- --------K- ---------- ----------
IGKV123 DIVMTQAAFS NPVTLGTSAS ISCRSSKSLL HSNGITYLYW YLQKPGQSPQ LLIYQMSNLA SGVPDRFSSS GSGTDFTLRI SRVEAEDVGV YYCAQNLEL
IGHV139 DVQLVESGGG LVQPGGSRKL SCAASGFTFS SFGMHWVRQA PEKGLEWVAY ISSGSSTIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCAR
21H 139 ---------- ---------- -----+---- ---I--- ---------- -----N---- ---------- ---------- ---------- ---F+---
21L 072 ---------- ---------- -----+---- --N-F--- -+-------- --S------ ---------- -------+T- ---------- -G------
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
IGHV114 EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
22H 114 ---------- ---------- -----+---- ---------- ---------- -----S--+- ---------- ---------- -------F-- --------+T
22L 108 ---------- ---------- -----+---- ----H---F- ---------- ---------+ ---------- --+------- ----A----- ----------
23H 114 ---------- ---------- -----+---- ---------- ---------- ---------+ ---------- ---------- ---R------ --------+T
23L 108 ---------- ---------- -----+---- -----F---- ---------- --+------- ----G----- ---------- ---------- ----------
IGKV108 DIQMTQSPSS LSASLGERVS LTCRASQEIS GYLSWLQQKP DGTIKRLIYA ASTLDSGVPK RFSGSRSGSD YSLTISSLES EDFADYYCLQ YASY
IGHV125 EVQLQQSGAE LVKPGASVKL SCTASGFNIK DTYMHWVKQR PEQGLEWIGR IDPANGNTKY DPKFQGKATI TADTSSNTAY LQLSSLTSED TAVYYCAR
31H 125 ---------- ---------- -----+---- --Q--L--- ---------- -NS------ ---------- -T-I------ ---------- -S-----S
31L 129 ---------- ---------- -----+---- --N-F---- -----+---- ---------- ---------- ---------- +--------- --------
32H 125 ---------- ---------- -----+---- --Q--L--- ---------- -NS------ ---------- -T-I------ ---------- -S-----S
32L 129 ---------- ---------- -----+---- --N-F---- ---------- ---------- ---------- ---------- +--------- --------
IGKV129 DIQMNQSPSS LSASLGDTIT ITCHASQNIN VWLSWYQQKP GNIPKLLIYK ASNLHTGVPS RFSGSGSGTG FTLTISSLQP EDIATYYCQQ GQSY

Legend for Figure 1

Antibodies are specified by numbers (01, 02, etc.) and the chains are indicated by H or L followed by the coding gene, according to the integrative data base VBASE2. The deduced amino acid sequences of antibody V genes are compared with the respective germline VH (IGVH, full sequence shown above a group of Ab) and VL sequence (IGKV, full sequence shown below a group of Ab). A dash indicates identity with the respective germline-encoded amino acid, productive mutations are indicated by the new aa in the one-letter code and silent mutations are indicated by a (+). Mutations correlate with affinity enhancement are showed in grey background.

Figure 2

Amino acid sequences of VH/VL gene-encoded variable regions of TD antigen-induced anti-phOx antibodies in ΔD-ID mice

(Legend see below)

Amino acid sequences of TD antigen-induced primary day 7 antibodies of ΔD-ID mice

```
IGHV532     QVQLQQSGAE LAKPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGY INPSTGYTEY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
01H 532  ---------- ---------- -----+----- ---+------ ---------- ----------+- ---------- ---------- ---------- ---------
01L 097  ---------- ---------- -----+----- ---+---C + ---------- ----PGT---+- ---L-WL--- ---------- ---------- ---------
IGKV097  DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCWQGTHF

IGHV532  QVQLQQSGAE LAKPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGY INPSTGYTEY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
02H 532  ---------- ---------- -----+----- ---+------ ----------+- ---------- ---------- ---------- -------+
02L 171  ---------- ---------- -----+----- -+------ ----------+- ---------- ---------- ---------- --------
IGKV171  DIVLTQSPAT LSVTPGDRVS LSCRASQSIS NYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGTD FTLSINSVET EDFGMYFCQQ SNSW

IGHV286  EFQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY IYPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCAR
03H 286  ---------- ---------- -----+----- ---+------ ----------+- -N----P-- ---------- S-------+- ---------- Y------S-
03L 195  ---------- ---------- -N---+----- ---------- ----------+- ---------- ---------- ---------- ---------
IGKV195  DIVMTQSPSS LAMSVGQKVT MSCKSSQSLL NSSNQKNYLA WYQQKPGQSP KLLVYFASTR ESGVPDRFIG SGSGTDFTLT ISSVQAEDLA DYFCQQHYS

IGHV286  EFQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY IYPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCAR
04H 286  ---------- ---------- -----+----- ---+------ ----------+- ---------S ---L------ -L-------- ---------- ---------
04L 122  ------S--- ---------- -----+----- ---------- ----------+- ---------- ---------- ---------- ---------
IGKV122  DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHV

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
05H 171  ---------- ---------- -----+----- ---+---C-- ----------+- ---------- ---------- ---------- -------
05L 078  ---------- ---------- -------+- --F--F--- ----------+- ---------- ---------- -----++--- ---------- -------
IGKV078  ENVLTQSPAI MSASPGEKVT MTCRASSVS SSYLHWYQQK SGASPKLWIY STSNLASGVP ARFSGSGSGT SYSLTISSVE AEDAATYYCQ QYSGY
```

```
IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
06H 171  ---------- ---------- -----|||||| |||||| ---------- ||||||||-- ---------- ------K--- ---------- S----S-
06L 137  ---------- ---------- ------+--- -------- ---------- ----Q----- ---------- ---------- ---------- -------
IGKV137  DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
07H 171  ---------- ---------- -----|||||| |||||| ---------+ ||||||||-- ---------- -------+-- ---------- --------
07L 210  ---------- ---------- ----------- ---------- ---------- ---------- ----+----- ---------- ---------- --------
IGKV210  DIVLTQSPAS LAVSLGQRAT ISCRASESVD NYGISFMNWF QQKPGQPPKL LIYAASNQGS GVPARFSGSG SGTDFSLNIH PMEEDDTAMY FCQQSKEV

IGHV131  EVKLVESGGG LVKPGGSLKL SCAASGFAFS SYDMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNARNTLY LQMSSLRSED TALYYCAR
08H 131  -------+-- ---------- -----|||||| |||||| ---------- ---------- ---------- ---------- ---------- ---------
08L 122  ------S--- ---------- -----------| |||||| ---------- ----------| ---------- ---------- ---------- ---------
IGKV122  DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHV

IGHV114  EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
09H 114  ---------- ---------- -----|||||| |||||| ---------- ||||||||-- ---------- P--------- ---------- ----------
09L 108  ---------- ---------- -----------| |||||| ---------- ----------| ---------- ---------- ---------- ----------
IGKV108  DIQMTQSPSS LSASLGERVS LTCRASQEIS GYLSWLQQKP DGTIKRLIYA ASTLDSGVPK RFSGSRSGSD YSLTISSLES EDFADYYCLQ YASY

IGHV183  QVQLKQSGPG LVQPSQSLSI TCTVSGFSLT SYGVHWVRQS PGKGLEWLGV IWSGGSTDYN AAFISRLSIS KDNSKSQVFF KMNSLQANDT AIYYCAR
12H 183  ---------- ---------- -----|||||| |||||| ---------L ||||||||-- ---------- ---------- ---------- -------
12L 072  ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- -------H-- -------
IGKV072  QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
13H 171  ------+--- ---------- -----|||||| |||||| ---------- ||||||||-- ---------- ---------- ---------- ---------
13L 115  ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV115  DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
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IGHV171 QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
14H 171 ---------- ---------- -----R---- ---------- ---------- ---------- ---------- ---------- ------+
14L 154 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
IGKV154 QIVLTQSPAI MSASLGERVT MTCTASSSVS SSYLHWYQQK PGSSPKLWIY STSNLASGVP ARFSGSGSGT SYSLTISSME AEDAATYYCH QYHRS
IGHV176 EVKLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAS ISSGGSTYYP DSVKGRFTIS RDNARNILYL QMSSLRSEDT AMYYCAR
15H 176 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------S--
15L 195 ---------- ---------- ---------- ---------- ---------- --+------- ---------- ---------- ---------
IGKV195 DIVMTQSPSS LAMSVGQKVT MSCKSSQSLL NSSNQKNYLA WYQQKPGQSP KLLVYFASTR ESGVPDRFIG SGSGTDFTLT ISSVQAEDLA DYFCQQHYS
IGHV139 DVQLVESGGG LVQPGGSRKL SCAASGFTFS SFGMHWVRQA PEKGLEWVAY ISSGSSTIYY ADTVKGRFTI SRDNPKNTLF LQMTSLRSED TAMYYCAR
16H 139 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
16L 050 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV050 QIVLSQSPAI LSASPGEKVT MTCRASSSVS YMHWYQQKPG SSPKPWIYAT SNLASGVPAR FSGSGSGTSY SLTISRVEAE DAATYYCQQW SSN
IGHV114 EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
17H 114 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
17L 188 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
IGKV188 DIVMTQSPSS LTVTAGEKVT MSCKSSQSLL NSGNQKNYLT WYQQKPGQPP KLLIYWASTR ESGVPDRFTG SGSGTDFTLT ISSVQAEDLA VYYCQNDYSY
IGKV195 DIVMTQSPSS LAMSVGQKVT MSCKSSQSLL NSSNQKNYLA WYQQKPGQSP KLLVYFASTR ESGVPDRFIG SGSGTDFTLT ISSVQAEDLA DYFCQQHYS
10L 195 ---------- -V-------- -N---------- ---------- --------F- --+------- -F-----+-- --F-----F- ---------- --+------
IGKV064 DIVLTQSPAS LAVSLGQRAT ISCRASESVD SYGNSFMHWY QQKPGQPPKL LIYRASNLES GIPARFSGSG SRTDFTLTIN PVEADDVATY YCQQSNED
11L 064 ---------- ---------- ---------- ---------- ---------- ---------- ---------- F--------- ---G------ --------
IGKV186 SIVMTQTPKF LLVSAGDRVT ITCKASQSVS NDVAWYQQKP GQSPKLLIYY ASNRYTGVPD RFTGSGYGTD FTFTISTVQA EDLAVYFCQQ DYSS
18L 186 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----

Amino acid sequences of TD antigen-induced primary day 14 antibodies of ΔD-ID mice

```
IGHV014   QVQLQQSGAE LVRPGTSVKI SCKASGYTFT NYWLGWVKQR PGHGLEWIGD IYPGGGYTNY NEKFKGKATL TADTSSSTAY MQLSSLTSED SAVYFC
01H 014   ---------- ---------- ----------- ---------- ---------- ---------- ---------- ---------- ---------- ------
01L 137   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------
IGKV137   DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL

IGHV654   QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGQGLEWIGA IYPGNGDTSY NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
02H 654   ---------- ---------- ---------- ---------- ---------- ---------- --P--+----- -+-------- ---------- ---------
02L 097   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
IGKV097   DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCWQGTHF

IGHV499   QVQLQQSGAE LAKPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGY INPSSGYTKY NQKFKDKATL TADKSSSTAY MQLSSLTSED SAVYYCAR
03H 499   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
03L 198   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV198   DIVMTQSHKF MSTSVGDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYRYTGVPD RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYST

IGHV286   EFQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY IYPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCAR
04H 286   ---------- ---------- ---------- ---------- ---------- ----N----- ---------- ---------- ---------- --------
04L 201   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV201   DIVMTQSQKF MSTSVGDRVS VTCKASQNVG TNVAWYQQKP GQSPKALIYS ASYRYSGVPD RFTGSGSGTD FTLTISNVQS EDLAEYFCQQ YNSY

IGHV128   EVQLQESGPS LVKPSQTLSL TCSVTGDSIT SGYWNWIRKF PGNKLEYMGY ISYSGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTTEDT ATYYCAR
06H 128   ---------- ---------- ---------- ---------- ---------- ----RN--N-H-- ---------- ---------- ---------- ------+
06L 050   ---------- ---------- -----N---- --Q------- ---------- ---------- ---------+ --------T- ---------- -------
IGKV050   QIVLSQSPAI LSASPGEKVT MTCRASSSVS YMHWYQQKPG SSPKPWIYAT SNLASGVPAR FSGSGSGTSY SLTISRVEAE DAATYYCQQW SSN
```

```
IGHV706   QVQLQQSGAE LVKPGASVKL SCKTSGYTFT SYWIQWVKQR PGQGLGWIGE IFPGTGTTYY NEKFKGKATL TIDTSSSTAY MQLSSLTSED SAVYFCAR
05H 706   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
05L 142   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
IGKV142   EIVLTQSPTT MAASPGEKIT ITCSASSSIS SNYLHWYQQK PGFSPKLLIY RTSNLASGVP ARFSGSGSGT SYSLTIGTME AEDVATYYCQ QGSSI

IGHV128   EVQLQESGPS LVKPSQTLSL TCSVTGDSIT SGYWNWIRKF PGNKLEYMGY ISYSGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTTEDT ATYYCAR
07H 128   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- -------
07L 137   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- -------
IGKV137   DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL

IGHV192   EVQLVESGGD LVKPGGSLKL SCAASGFTFS SYGMSWVRQT PDKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLKSED TAMYYCAR
08H 192   ----E-+--- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
08L 168   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
IGKV168   DILLTQSPAI LSVSPGERVS FSCRASQSIG TSIHWYQQRT NGSPRLLIKY ASESISGIPS RFSGSGSGTD FTLSINSVES EDIADYYCQQ SNSW

IGHV147   EVMLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
09H 147   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
09L 140   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- A--------- --------
IGKV140   DIQMTQSPSS LSASLGGKVT ITCKASQDIN KYIAWYQHKP GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNL

IGHV135   EVQLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
10H 135   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
10L 168   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
IGKV168   DILLTQSPAI LSVSPGERVS FSCRASQSIG TSIHWYQQRT NGSPRLLIKY ASESISGIPS RFSGSGSGTD FTLSINSVES EDIADYYCQQ SNSW

IGHV135   EVQLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
11H 135   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- -------N
11L 072   ---------- ---------- ----------- --------- ---------- --------- ---------- ---------- ---------- --------
IGKV072   QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
```


IGHV178 VKLVESGGG LVKPGGSLKL SCAASGFTFS SYTMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLKSED TAMYYCTR
12H 178 T G
12L 069
IGKV069 IVMTQSPSS LSVSAGEKVT MSCKSSQSLL NSGNQKNYLA WYQQKPGQPP KLLIYGASTR ESGVPDRFTG SGSGTDFTLT ISSVQAEDLA VYYCQNDHSY
IGHV185 EVQLVESGGG LVQPGESLKL SCESNEYEFP SHDMSWVRKT PEKRLELVAA INSDGGSTYY PDTMERRFII SRDNTKKTLY LQMSSLRSED TALYYCAR
13H 185 +
13L 082
IGKV082 QIVLTQSPAI MSASPGEKVT ITCSASSSVS YMHWFQQKPG TSPKLWIYST SNLASGVPAR FSGSGSGTSY SLTISRMEAE DAATYYCQQR SSY
IGHV135 EVQLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
14H 135 E + +
14L 179
IGKV179 DIVMTQSPAT LSVTPGDRVS LSCRASQSIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQN GHSF
IGHV114 VKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
15H 114 I Q + +
15L 115
IGKV115 VLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV
IGHV028 VQLVESGGG LVQPKGSLKL SCAASGFTFN TYAMNWVRQA PGKGLEWVAR IRSKSNNYAT YYADSVKDRF TISRDDSQSM LYLQMNNLKT EDTAMYYCVS
16H 028 R
16L 170
IGKV170 IQMTQSPAS LSVSVGETVT ITCRASENIY SNLAWYQQKQ GKSPQLLVYA ATNLADGVPS RFSGSGSGTQ YSLKINSLQS EDFGSYYCQH FWGT
IGHV455 QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYWMHWVKQR PGQGLEWIGM IDPSDSYTSY NQKFKGKATL TVDTSSSTAY MQLSSLTSED SAVYYCTR
20H 455 + T +N
20L 115 + H
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV

```
IGKV170   DIQMTQSPAS LSVSVGETVT ITCRASENIY SNLAWYQQKQ GKSPQLLVYA ATNLADGVPS RFSGSGSGTQ YSLKINSLQS EDFGSYYCQH FWGT
17L 170   ---------- ---------- ---------- -------- ---------- -------- ---------- ---------- ---------- ----

IGKV097   DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL DSDGKTYLNW LLQRPGQSPK RLIYLVSKLD SGVPDRFTGS GSGTDFTLKI SRVEAEDLGV YYCWQGTHF
18L 097   ---------- ---------- ------------ -------- ---------- ---------- ---------- ---------- ---------

IGKV078   ENVLTQSPAI MSASPGEKVT MTCRASSSVS SSYLHWYQQK SGASPKLWIY STSNLASGVP ARFSGSGSGT SYSLTISSVE AEDAATYYCQ QYSGY
19L 078   ---------- ---------- ------------ -------- ---------- -------- ---------- ---------- ---------- -----

IGHV528   EVQLQQSGPE LVKPGASVKM SCKASGYTFT DYYMKWVKQS HGKSLEWIGD INPNNGDTFY NQKFKGKATL TVDKSSSTAY MQLNSLTSED SAVYYCAR
21H 528   ---------- ---------- ------------ ---L+-+ ---------- ------V-I- ---------- ---R--N--- -H-SR----- --------
```

Amino acid sequences of TD antigen-induced secondary antibodies of ΔD-iD mice

```
IGHV114  EVKLEESGGG LVQPGGSMKL SCVASGFTFS NYWMNWVRQS PEKGLEWVAE IRLKSNNYAT HYAESVKGRF TISRDDSKSS VYLQMNNLRA EDTGIYYCTR
01H 114  ---------- --+------- ------------ -F-------- ---------- ---R-Y---- ---------- ---------- ---------- ----------
01L 108  -------T-- ---------- ------------ --------F- ---------- ---------P ---------- -----+---- ---------- ----------
IGKV108  DIQMTQSPSS LSASLGERVS LTCRASQEIS GYLSWLQQKP DGTIKRLIYA ASTLDSGVPK RFSGSRSGSD YSLTISSLES EDFADYYCLQ YASY

IGHV386   QVQLQQSGAE LMKPGASVKI SCKATGYTFS SYWIEWVKQR PGHGLEWIGE ILPGSGSTNY NEKFKGKATF TADTSSNTAY MQLSSLTSED SAVYYCAR
02H 386   ---------- V-R------- ------I- RF-D----- -----+---- -F--N+-YK+ --+------I ---------- ---W------ ----F---
02l 108   ---------- ---------- ------S--- --+--- ----N---G- ---------- --------T- ---------- G-----F--- --------
IGKV108   DIQMTQSPSS LSASLGERVS LTCRASQEIS GYLSWLQQKP DGTIKRLIYA ASTLDSGVPK RFSGSRSGSD YSLTISSLES EDFADYYCLQ YASY
```

Amino acid sequences of TD antigen-induced tertiary antibodies of ΔD-ID mice

```
IGHV201  QVQLKQSGPG LVQPSQSLSI TCTVSGFSLT SYGVHWVRQS PGKGLEWLGV IWRGGSTDYN AAFMSRLSIT KDNSKSQVFF KMNSLQADDT AIYYCAK
01H 201  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
01L 050  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
IGKV050  QIVLSQSPAI LSASPGEKVT MTCRASSSVS YMHWYQQKPG SSPKPWIYAT SNLASGVPAR FSGSGSGTSY SLTISRVEAE DAATYYCQQW SSN

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
02H 171  ----E----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
02L 137  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
IGKV137  DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
03H 171  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
03L 072  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------

12H 171  x----K---- ---------- ----------N ---I------ ---------- --V--N---- -------+-- -----++--- ---------- -------
12L 072  XXXXX--T-- ------+--- ----------- -IN-F----- ---------- ---------- ---------- ---------- ---------- -------
IGKV072  QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN

IGHV171  QVQLKESGPG LVAPSQSLSI TCTVSGFSLT SYGVHWVRQP PGKGLEWLGV IWAGGSTNYN SALMSRLSIS KDNSKSQVFL KMNSLQTDDT AMYYCAR
04H 171  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
04L 079  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
IGKV079  QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMYWYQQKPG SSPRLLIYDT SNLASGVPVR FSGSGSGTSY SLTISRMEAE DAATYYCQQW SSY

IGHV192  EVQLVESGGD LVKPGGSLKL SCAASGFTFS SYGMSWVRQT PDKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLKSED TAMYYCAR
05H 192  ----E----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
05L 157  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV157  QIVLTQSPAL MSASPGEKVT MTCSASSSVS YMYWYQQKPR SSPKPWIYLT SNLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
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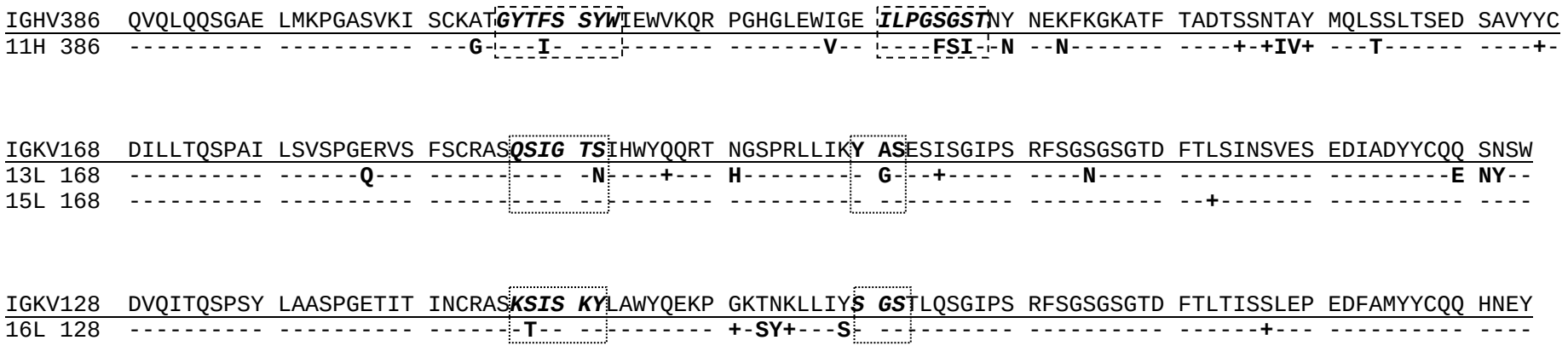
IGHV147 EVMLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
07H 147 ---------- -------+-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
07L 137 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV137 DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTL
IGHV135 EVQLVESGGG LVKPGGSLKL SCAASGFTFS SYAMSWVRQT PEKRLEWVAT ISSGGSYTYY PDSVKGRFTI SRDNAKNTLY LQMSSLRSED TAMYYCAR
08H 135 ----E----- ---------- ---------- ---------- ---------- ---------- -----+---- ---------- ---------- -------S
08L 210 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
IGKV210 DIVLTQSPAS LAVSLGQRAT ISCRASESVD NYGISFMNWF QQKPGQPPKL LIYAASNQGS GVPARFSGSG SGTDFSLNIH PMEEDDTAMY FCQQSKEV
IGHV386 QVQLQQSGAE LMKPGASVKI SCKATGYTFS SYWIEWVKQR PGHGLEWIGE ILPGSGSTNY NEKFKGKATF TADTSSNTAY MQLSSLTSED SAVYYC
10H 386 ---------- ---------- ---------- ---------- -------V-- ----FSI--- --N------- S--------- ---T------ ----+-
10L 108 ---------- ---------- ---------- ---+------ ---------- ---------- ---------- ---------- ---------- ------
IGKV108 DIQMTQSPSS LSASLGERVS LTCRASQEIS GYLSWLQQKP DGTIKRLIYA ASTLDSGVPK RFSGSRSGSD YSLTISSLES EDFADYYCLQ YASY
IGHV158 EVKLVESGGG LVQPGGSLRL SCATSGFTFT DYYMSWVRQP PGKALEWLGF IRNKANGYTT EYSASVKGRF TISRDNSQSI LYLQMNTLRA EDSATYYCAR
14H 158 --Q-E----- -----D-+-- ----------L- N---+----- -+---+-MA- MK--L---A- -+-+----+- -----D---- -----+---T -T--------
14L 115 ---------- ---------- ----------T- -R-----+- ---------- ---------- ---------- ---------- ---------- ----------
IGKV115 DVLMTQTPLS LPVSLGDQAS ISCRSSQSIV HSNGNTYLEW YLQKPGQSPK LLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YYCFQGSHV-
IGKV140 DIQMTQSPSS LSASLGGKVT ITCKASQDIN KYIAWYQHKP GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNL
06L 140 ---------- ---------- ---------- ---------- ---------- -F-------- ---------- ---------- ---------- ----
IGKV072 QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT SKLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSN
09L 072 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---



Legend for Figure 2

Antibodies are specified by numbers (01, 02, etc.) and the chains are indicated by H or L followed by the coding gene, according to the integrative data base VBASE2. The deduced amino acid sequences of antibody V genes are compared with the respective germline VH (IGVH, full sequence shown above a group of Ab) and VL sequence (IGKV, full sequence shown below a group of Ab). A dash indicates identity with the respective germline-encoded amino acid, productive mutations are indicated by the new aa in the one-letter code and silent mutations are indicated by a (+). Mutations correlate with affinity enhancement are showed in grey background.

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Curriculum vitae

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Study of biochemistry

1997-2001	Study of biochemistry at the Lebanese university in Tripolie
	Degree: Maitrise in biochemistry
	Maitrise thesis: Smoking and metabolic effects
2002-2003	German courses at the University of Kiel (PNDS)
2003-2005	Diploma study of biochemistry at university of Kiel
	Degree: Diploma in biochemistry
	Grade: 1.7 („good“)
	Diploma thesis: Affinity determination of syngenic anti-idiotypic antibody

Ph.D. Thesis

2006 - Oktober 2009:	Ph.D. thesis at the Christian Albrecht University (CAU), Kiel, Chair of Professor Dr. Hilmar Lemke
	Ph.D. thesis: Significance of the third hypervariable region of the antibody heavy chain for antigen-specificity and expression of idiotypes during the thymus-dependent immune response

Publications

R. Tanasa, **A. Trad,** H. Lange, J. Grötzinger, and H. Lemke. ‘’Allergen IgE-isotype-specific suppression by maternally derived monoclonal anti-IgG-idiotype’’*Allergy, 2009.* Epub ahead of print: DOI: 10.1111/j.1398-9995.2009.02104.x

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Declaration

Herewith I affirm that I composed the present doctoral thesis single all by my self without utilizing illegitimate resources, except for scientific advice giving by Prof. Dr Hilmar Lemke. I used no other aids besides the cited references.

Also, this doctoral thesis has not already been submitted to any other faculty.

Kiel 01.09.2009

Ahmad Trad