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Elements of Immune Fitness

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Hradec Králové, 2013

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DECLARATION

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Hradec Králové, 2013

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TABLE OF CONTENTS

DECLARATION	2
TABLE OF CONTENTS	3
DEDICATION	5
ACKNOWLEDGEMENTS.....	6
SUMMARY.....	8
SOUHRN	9
ABBREVIATIONS.....	11
THESIS	12
BACKGROUND.....	12
OBJECTIVES OF THE THESIS.....	16
INTRODUCTION.....	18
Specific Aim 1, 2 and 3	18
Specific Aim 4 and 5.....	20
MATERIALS AND METHODS.....	22
Specific Aim 1, 2 and 3	22
Strains of mice	22
Determination of TCR V β diversity	22
CDR3 size spectratyping of TCR V β	23
Fluorescence activated cell sorting analysis.....	26
T cell priming to Pan DR reactive epitope (PADRE) peptide.....	27
Delayed-type hypersensitivity assay.....	27
T cell proliferation assay	27
Skin grafts.....	28
Pneumocystis murina infection and assessment of organismal burden.....	28
Statistical analysis	29
Specific Aim 4 and 5.....	30
Strains of mice	30
Thymectomy and gene analysis.....	30
FACS analysis and antibodies.....	31

Delayed type hypersensitivity (DTH) assay.....	32
Skin Grafts.....	32
TRECS (T cell receptor excision circles).....	32
ELISA (Enzyme-linked immunosorbent assay).....	33
ELISPOT (Enzyme-linked immunosorbent spot).....	33
TCR beta chain diversity analysis.....	33
Statistical analysis.....	34
RESULTS.....	35
Specific Aim 1, 2 and 3.....	35
Diverse B cells and Ig maintain T cell numbers in the spleen and TCR diversity.....	35
T cell function in mice with contracted TCR diversity.....	36
Lymphoid organogenesis in mice with contracted TCR repertoires.....	36
Impact of TCR diversity on cell-mediated immunity.....	37
Impact of TCR repertoire contraction in host defense.....	38
Specific Aim 4 and 5.....	41
Removal of the thymus of mature mice causes a persistent decrease in the number of CD4+ or CD8+ T cells without contracting T cell receptor diversity.....	41
Thymectomy does not impair T cell memory.....	42
Removal of the thymus in adult mice does not impair primary or secondary antibody responses but increases long-lived antibody secreting cells in the bone marrow.....	43
Removal or manipulation of the thymus impairs the generation of Ig heavy chains associated with high affinity to NP.....	44
DISCUSSION.....	47
Specific Aim 1, 2 and 3.....	47
Specific aim 4 and 5.....	50
FIGURES.....	52
TABLES.....	72
REFERENCES.....	85
CITATIONS.....	96
SUMMARY AND CONCLUSION.....	98

DEDICATION

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SUMMARY

The goal of this thesis was to define the contribution of lymphocyte receptor diversity to the functions that define adaptive immunity. Most in the field of immunology believe that immune fitness requires lymphocyte receptor diversity. In models of contracted lymphocyte diversity, we tested this idea. We tested the fitness of cell-mediated immunity in mice with contracted T cell diversity and we tested the impact of contracted T cell receptor diversity on the generation of B cell responses to model antigens.

We tested the concept of that the fitness of cell-mediated immunity depend on TCR diversity using $JH^{-/-}$ mice that lack B cells and have TCR V diversity $< 1\%$ that of wild-type mice and QuasiMonoclonal (QM) mice with oligoclonal B cells and TCR V diversity 7% that of wild-type mice. Despite having a TCR repertoire contracted $> 99\%$ and defective lymphoid organogenesis, $JH^{-/-}$ mice rejected H-Y-incompatible skin grafts as rapidly as wild-type mice. $JH^{-/-}$ mice exhibited T cell priming by peptide and delayed-type hypersensitivity, although these responses were less than normal owing either to TCR repertoire contraction or defective lymphoid organogenesis. QM mice with TCR diversity contracted $> 90\%$, and normal lymphoid organs rejected H-Y incompatible skin grafts as rapidly as wild type mice and exhibited normal T cell priming and normal delayed-type hypersensitivity reactions. QM mice also resisted *Pneumocystis murina* like wild-type mice. Thus, cell-mediated immunity can function normally despite contractions of TCR diversity $> 90\%$ and possibly $> 99\%$. Our results showed that many of the responses attributed to T cells appear to be independent of TCR diversity.

In search for an adaptive advantage of receptor diversity, we asked whether contractions of T cell receptor diversity impaired B cell responses and the generation of high affinity antibodies.

Contrary to the prediction of that in mature individuals the generation of B-cell memory would proceed independently of the thymus, we show here that removal of the thymus after the establishment of the T-cell compartment or sham surgery without removal of the thymus impairs the affinity maturation of antibodies. Because removal or manipulation of the thymus did not decrease the frequency of mutation of the Ig variable heavy chain exons encoding antigen-specific antibodies, we conclude that the thymus controls affinity maturation of antibodies in the mature individual by facilitating selection of B cells with high affinity antibodies.

Key words: repertoire, TCR, BCR, thymus, thymectomy, B cells, affinity maturation

SOUHRN

Cílem disertační práce bylo definovat podíl diverzity receptorů pro antigen na funkcích, které určují adaptivní imunitu. Obvykle se předpokládá, že imunologická způsobilost vyžaduje diverzitu lymfocytárních receptorů. My jsme tuto hypotézu ověřili na modelu omezené lymfocytární diverzity. Byla testována způsobilost buňkami zprostředkované imunity myši s omezenou diverzitou receptorů TCR. Byl ověřen vliv omezené diverzity receptorů TCR na vznik B lymfocytární odpovědi indukované modelovými antigeny.

Koncept způsobilosti buňkami zprostředkované imunity ve vazbě na diverzitu receptorů PCR byl ověřen na myších experimentálních modelech, který zahrnovaly myši $JH^{-/-}$, u kterých nejsou vyvinuty B lymfocyty a mají diverzitu receptorů TCR $< 1 \%$, kmen konvenčních myši a quasimonoklonální (QM) myši s oligoklonální B lymfocytární populací a diverzitou oblastí V TCR receptorů 7% v porovnání s konvenčním kmenem. V experimentech jsme zjistili, že myši $JH^{-/-}$ odhojují H-Y inkompatibilní kožní štěpy stejně rychle jako konvenční kmen myši, přestože mají omezený repertoár TCR receptorů $> 99 \%$ a projevují známky defektní organogeneze lymfoidních orgánů. Myši $JH^{-/-}$ reagují aktivací T lymfocytů po stimulaci peptidem a vykazují opožděný typ hypersenzitivity, přestože intenzita těchto reakcí je nižší než u konvenčních myši, buď jako důsledek omezeného repertoáru TCR nebo defektní organogeneze lymfoidních orgánů. Myši QM s repertoárem TCR omezeným na $> 90 \%$ a normálním vývojem lymfoidních orgánů, odhojují H-Y inkompatibilní kožní štěpy srovnatelně s konvenčním kmenem myši a mají zachovanou schopnost aktivace T lymfocytů i opožděné hypersenzitivní reakce. Myši QM jsou také odolné vůči infekci *Pneumocystis murina* v míře srovnatelné s konvenčním kmenem myši. Lze tedy uzavřít, že buňkami zprostředkovaná imunita u myši $JH^{-/-}$ a QM myši vykazuje normální funkce, přestože je diverzita receptorů TCR je omezena na $> 99 \%$ a $> 90 \%$. Naše výsledky ukázaly, že mnohé z aktivit připisovaných T lymfocytům se jeví být nezávislé na diverzitě receptorů TCR.

S cílem ověřit adaptivní výhody diverzity receptoru pro antigen, jsme ověřili hypotézu, zda omezení repertoáru TCR receptorů negativně ovlivní B lymfocytární odpověď a tvorbu vysokoafinních protilátek. V protikladu vůči předpokladu, že u dospělých jedinců probíhá vznik paměťových B lymfocytů nezávisle na thymu, prokázali jsme experimentálně, že odstranění thymu po ustavení T lymfocytárního kompartmentu nebo po „sham“ operaci bez odstranění

thymu negativně ovlivní afinitní vyžívání protilátek. Protože odstranění nebo manipulace s thymem nesnížuje frekvenci mutací v exonech kódujících variabilní část molekuly protilátek specifických pro antigen, uzavíráme, že thymus kontroluje afinitní vyžívání protilátek u dospělých jedinců mechanismem usnadnění selekce klonů B lymfocytů se schopností produkovat vysokoafinní protilátky.

Klíčová slova: repertoár, TCR, BCR, thymus, thymektomie, B lymfocyty, afinitní vyžívání

ABBREVIATIONS

ASC	Antibody secreting cells
CD	Cluster designation
CDR3	Complementarity determining region 3
CI	Confidence interval
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
IGMT	ImMunoGeneTics information system
IL	Interleukin
PADRE	Pan DR reactive epitope
QM	Quasi monoclonal
SD	Standard deviation
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TREC	T cell receptor excision circle(s)

THESIS

BACKGROUND

The immune system is the body defense mechanism. It acts by responding to any offending agent. This consists of a series of molecular and cellular events that lead to contain the aggravating agent. This agent usually is a pathologic organism, toxic substance, cellular debris or tumor cell.

Traditionally, the immune system divided in to two categories: innate and adaptive. The innate immune system responds to offending agent without specialized recognitions or previous encounter. The adaptive immune system is more sophisticated in with it requires recognition from previous encounter to mount an immune response.

Immune responses:

1. Innate immunity characterized by having a preset very standardized method of responding to offending agent that is recognized in antigen-independent matter. This reaction leads to the production and release of cytokines and mediators to attach the offending agent. Cells involved in this reaction include neutrophils and macrophages (Delves and Roitt 2006).
2. Adaptive immunity in the other hand needs a specific antigen to be activated to generate a response. This mechanism is carried on by the antigen specific effector cells (B-cell, T-cells). The first step is to recognize the foreign material and ensure that it is not-self. Then it gets broken to unique antigenes that are recognized by the immune effector cells and the processes of elimination or isolation starts (Delves and Roitt 2006).

One of the important features of the adaptive immune system is that it is adaptive. After eliminating self-reacting cells during the early development (Male 1996), it starts generating cells with receptors reactive against new unrecognized molecular structure. In this process antigen specific T cells and antigen specific antibodies are produced.

This system is contently evolving as it encounters new antigens. B cells do that by recombination, rearrangement and mutation of the naive gene (Lim et al. 2008; Schatz et al. 1989). This allows this system to have very large repertoire of receptors that potentially could recognize a huge number of antigens (Oettinger et al. 1990).

In this review, we will focus on the adaptive system component and function. The mechanism of function of the system, is thought to be triggered by antigens. This mostly proteins but could be lipids or carbohydrates(Bollum 1978). As we described earlier this response is mediated by specific T-cell and B- cells. These cells have antigen receptors (paratopes) that is presented in the T lymphocytes or on antibodies produced by B cells. These receptors are specific for each antigen segment (epitope). This series of reaction (recognition, processing and responding) explain the delay in the adaptive immune response. One of the hallmarks of adaptive immune system that it utilizes memory to previous encounter. This leads to stronger, faster secondary response to the same offender.

The mechanism of action of the adaptive immune system can be divided to three stages. In the first stage the offending molecule encounter antigen presenting cells and interaction occur. This then lead to processing of the offending molecule antigen and then presents it to (T cells, B cells, antibodies). The antigen presenting cells encounter the antigen at different sites then it migrates through lymphatics to lymph nodes. Then the effector cells and antibody's migrate to primary site of encounter to form immune complex or delayed hypersensitivity reaction.

B lymphocytes main function is the production of antibodies after getting activated by helper T lymphocytes. Antibodies are molecules that have a specific receptor for one antigen epitope and they are made of glycoproteins. Majority of B cells start as naïve lymphocytes. A certain population of B cells functions as an antigen presenting cells. They express IgM and IgD on their surface that functions as antigen receptor that recognizes antigen epitope. At this point helper T cell interacts with B cells leading to farther activation and differentiation. This include but not limited to cell division, synthesize and release antibody's, expressing of accessory molecules and change the antibody class from IgM to IgG, IgA or IgE (Gilfillan et al. 1993; Honjo et al. 2002; Wu et al. 2003). T cell cytokines play a major role in regulating these changes at the genetic level.

Classical teaching of T cells function stress that helper T cells are the main functional cell type for immune processing. CD4 is expressed on most helper T lymphocyte cell membrane. T cells recognize antigens only when it is presented on APC with HLA molecule after it have been processed. When this complex bind to antigen receptor on T lymphocyte, CD4 molecule function both, as a bond stabilizer and a signal enhancer. This leads to T cell activation and release cytokines in addition to cell division and expression cell adhesion molecule and costimulatory molecules. The release of IL-2 by this mechanism is an important factor in the progression of T cell activation and functional differentiation.

In Summary, the adaptive immune system of higher vertebrates has two distinguishing properties. These properties are specificity, and memory. Adaptive immunity has the ability of mounting responses to disparate molecules in part due to the great diversity of lymphoid receptors which in humans exceed 10 million. Responses that result from engaging one receptor with one ligand are generally specific to that pair of ligand and receptor. Once a productive response has been generated, subsequent responses directed at the same antigen will develop faster and with increased efficiency enhancing protection of the host. These properties are often referred to as memory. The goal of my thesis was to define the contribution of lymphocyte receptor diversity to the functions that define adaptive immunity.

Most in the field of immunology believe that immune fitness requires lymphocyte receptor diversity (Thompson and Neiman 1987; Yoshikawa et al. 2002). In models of contracted lymphocyte diversity we tested this idea. In Chapter 3 I tested the fitness of cell mediated immunity in mice with contracted T cell diversity and in Chapter 4 I tested the impact of contracted T cell receptor diversity on the generation of B cell responses to model antigens (Tuailleon and Capra 2000).

Fitness of cell-mediated immunity is thought to depend on TCR diversity (Cabaniols et al. 2001); however, this concept has not been tested formally. We tested the concept using $JH^{-/-}$ mice that lack B cells and have TCR V diversity $< 1\%$ that of wild-type mice and QuasiMonoclonal (QM) mice with oligoclonal B cells and TCR V diversity 7% that of wild-type mice. Despite having a TCR repertoire contracted $> 99\%$ and defective lymphoid organogenesis, $JH^{-/-}$ mice rejected H-Y-incompatible skin grafts as rapidly as wild-type mice. $JH^{-/-}$ mice exhibited T cell priming by peptide and delayed-type hypersensitivity, although these responses were less than normal owing either to TCR repertoire contraction or defective

lymphoid organogenesis. QM mice with TCR diversity contracted > 90 %, and normal lymphoid organs rejected H-Y incompatible skin grafts as rapidly as wild type mice and exhibited normal T cell priming and normal delayed-type hypersensitivity reactions. QM mice also resisted *Pneumocystis murina* like wild-type mice. Thus, cell-mediated immunity can function normally despite contractions of TCR diversity > 90 % and possibly > 99 %. Our results showed that many of the responses attributed to T cells appear to be independent of TCR diversity. In search for an adaptive advantage of receptor diversity we asked whether contractions of T cell receptor diversity impaired B cell responses and the generation of high affinity antibodies.

The generation of B-cell responses to proteins requires a functional thymus to produce CD4⁺ T cells which help in the activation and differentiation of B cells. Because the mature T-cell repertoire has abundant cells with the helper phenotype, one might predict that in mature individuals the generation of B-cell memory would proceed independently of the thymus. Contrary to that prediction, we show here that removal of the thymus after the establishment of the T-cell compartment or sham surgery without removal of the thymus impairs the affinity maturation of antibodies. Because removal or manipulation of the thymus did not decrease the frequency of mutation of the Ig variable heavy chain exons encoding antigen-specific antibodies, we conclude that the thymus controls affinity maturation of antibodies in the mature individual by facilitating selection of B cells with high affinity antibodies.

OBJECTIVES OF THE THESIS

Specific Aim 1: To determine what mechanisms maintain the number and diversity of T cells in the peripheral T cell compartment.

Rationale: Our lab recently found that TCR diversification in the thymus depends on B cell receptor or immunoglobulin (Ig) diversity. We also found that B cells help maintain the number and diversity of T cells in the peripheral T cell compartment. We will determine the extent to which thymus output, peripheral survival or proliferation maintain the size and diversity of the T cell compartment.

Specific Aim 2: To determine how the T cell compartment adapts to contraction of T cell diversity.

Rationale: We found that when T cell diversity is contracted, T cells commonly exhibit a "memory-like" phenotype; the functional significance of this phenotype in this setting is unknown. We will determine whether the T cells in mice with contracted T cell diversity exhibit memory-like function, and/or whether the cells cross-react more widely than normal T cells and/or immune-regulation is modified in this setting.

Specific Aim 3: To determine which functions of cell-mediated immunity are impaired by contraction of T cell diversity.

Rationale: We have found that human subjects and mice with profoundly decreased T cell diversity can reject allografts, even across minor antigen barriers, mount normal primary immune responses and avoid the opportunistic infections characteristic of DiGeorge syndrome and AIDS. Still these individuals have higher levels of gamma herpes viruses (in the case of humans) and *Pneumocystis murinae* (in the case of mice) than do normal individuals. We will test whether mice with severe contraction of T cell diversity can clear pathogenic microorganisms and whether these organisms cause disease, as opposed to modifying endogenous levels without causing disease. We will also test whether these mice suffer increased susceptibility to auto-immunity.

Specific Aim 4: To determine how thymectomy, T cell depletion and immunosuppression in mice, separately or in combination, compromise B cell memory responses.

Rationale: Postnatal thymectomy of mice, at 5 weeks decreases persistently the number of CD4 and CD8 T cells while maintaining the number of B cells in the periphery.

We performed thymectomy in C57BL/6 mice at 5 weeks of age. Thymectomy effectively abrogated thymic function because thymectomized mice lacked any measurable T cell receptor excision circles.

To determine if thymectomy perturbed the T and B cell compartments we enumerated T and B cells in the spleens of thymectomized, sham operated or unmanipulated mice 5 and 10 weeks after the operation. We will show that postnatal thymectomy causes a persistent 4 fold decrease in the number of CD4- or CD8-positive T cells, while the number of B cells is maintained. Our results suggest that the adult thymus contributes to the maintenance of T cells in the periphery of mice.

Specific Aim 5: If B cell memory is maintained following thymectomy in young mice B cell memory antibody responses critically depend on T cell help.

Rationale: To determine the extent to which T cell function was maintained in thymectomized mice we performed male to female skin grafts. To determine whether thymectomy perturbed primed T cell responses we tested delayed type hypersensitivity (DTH) to ovalbumin in the footpad of mice.

The median survival time of male skin grafts was 37 days in thymectomized female mice and only 25 days in sham operated and control mice. Thus, thymectomy impairs cellular immunity to minor antigens. The median survival time of secondary male skin grafts was 19 days in thymectomized female mice, 16 days in sham operated mice and 15 days in control mice. Re-transplant 30 days after shedding of the primary graft, hastened graft rejection in all mice even though thymectomized recipients had delayed graft rejection compared to controls. Accelerated secondary graft rejection indicates efficient generation of T cell memory. To test long-lived plasma cells we will determine the number of NP-specific antibody secreting cells in the spleen or in the bone marrow 6 months after boosting thymectomized, sham operated or control C57BL/6.

INTRODUCTION

Specific Aim 1, 2 and 3

B cell deficiency has been associated with defective T cell responses to intracellular microorganisms such as *Salmonella enterica* (Mastroeni et al. 2000), *Bordetella pertussis* (Elkins et al. 1999), *Plasmodium chabaudi* (Langhorne et al. 1998), *Chlamydia trachomatis* (Yang and Brunham 1998), *Leishmania major* (Hoerauf et al. 1996), coronavirus (Bergmann et al. 2001), and *Lymphocytic choriomeningitis* virus (Homann et al. 1998). Like B cell-deficient mice, humans with X-linked agammaglobulinemia, who have very few peripheral B cells and very reduced levels of serum Ig, are highly susceptible to such organisms as mycoplasma (Minegishi et al. 1999), enteroviruses (Minegishi, Rohrer and Conley 1999), and echoviruses (Wilfert et al. 1977) and to the development of poliomyelitis following vaccination with attenuated viruses (Wright et al. 1977). Defective responses to intracellular pathogens suggest the possibility that, in addition to hypogammaglobulinemia, individuals and mice with B cell deficiency may suffer intrinsic abnormalities in the T cell compartment. We recently found that B cell-deficient mice have a remarkable decrease in the number and diversity of thymocytes (Joao et al. 2004; Keshavarzi et al. 2003) and hypothesized that defects in cell-mediated immunity could result from contraction of the TCR repertoire. Because each TCR recognizes a limited number of different peptides associated with MHC, the recognition of diverse Ags, even allowing for cross-reactivity, is thought to reflect the diversity of the TCR repertoire. Thus, the competency of cellular immunity is thought to depend on the number and diversity of T cells available to mount a response (Nikolich-Zugich et al. 2004).

However, the concept that TCR diversity determines the competence of cell-mediated immunity does not explain every aspect of immune physiology. Although the TCR repertoire contracts profoundly with age (Goronzy and Weyand 2005) and elderly individuals can suffer disseminated viral infections and heightened susceptibility to tumors, most elderly individuals experience neither of these ailments (compared with those who have AIDS). Still more dramatic is the observation that those who undergo cardiac transplantation in infancy and have a profound contraction of the TCR repertoire owing to "total" thymectomy and mature T cell depletion suffer no excess of opportunistic infections or tumors (Ogle et al. 2006).

We asked whether and to what extent defects in TCR diversity impair cell-mediated immunity. Toward this end, we exploited mice that, owing to defects in the assembly of Ig genes (Cascalho et al. 1996; Chen et al. 1993) have profoundly contracted TCR repertoires. Our results indicate that extreme contractions of TCR repertoire do not impair cell-mediated immunity and host defense. These unexpected results have profound implications for transplantation and in the treatment of immune deficiencies.

Specific Aim 4 and 5

B cell memory confers lasting immunity to microorganisms and their products by ensuring rapid production of high affinity antibodies of switched isotype(s) (particularly immunoglobulin G (IgG)), distinct from those that predominate in the “natural” immune response. Antibodies opsonize microbes and neutralize toxins and viruses, precluding cell entry and damage. The high affinity of recall antibodies may be the most critical property for effective neutralization of toxins since those are toxic at very low concentrations. Production of high affinity class switched antibodies requires that activated B cells undergo somatic hypermutation and class switch recombination, followed by antigen selection of B cells expressing the receptors with enhanced affinity. B cell memory is manifested by recall antibody responses the result of plasma cells generated from B memory cells upon re-exposure to the antigen and by persisting antigen-specific antibodies secreted by long-lived plasma cells in the bone marrow (Manz et al. 2002).

The generation of B cell memory requires T cells. Thus, removal of the thymus in newborn mice (during the first 16 hours of life) causes severe cellular immunity defects (Miller 1961) and abolishes antibody responses to protein antigens (Miller et al. 1965). However, removal of the thymus of mature mice (between 5 and 8 weeks of age) has no immediate effect on the primary antibody responses to protein antigens or cellular immune responses (Metcalf 1965; Miller 1965; Taylor 1965). Whether or not removal of the thymus in mature individuals perturbs B cell memory is not known.

T cells promote B cell responses to protein antigens by directly interacting with B cells. Thus, deficiencies in the CD40 or CD154 or blocking their interaction by antibodies impairs antibody responses to protein antigens, immunoglobulin isotype class switch, somatic hypermutation and B cell memory (Davies and Thrasher 2010; Kawabe et al. 1994; Korthauer et al. 1993). Because primary responses to protein antigens proceed to establish memory, the specific requirements for the generation and/or maintenance of B cell memory cannot be exploited in their absence. We have recently found that individuals with severe contraction of the T cell repertoire owing to removal of the thymus and depletion of mature T cells before cardiac transplantation in infancy do not develop hyper immunoglobulin M (IgM) syndrome and/or hypo-gamma-globulinemia, indicating some level of T cell help. Preliminary studies in subjects of cardiac transplantation in

infancy suggested defective B cell memory to vaccination with protein antigens in spite of normal primary antibody responses. These observations suggested that the T cell help required to generate primary antibody responses might differ in some respects from T cell help necessary to establish and/or evoke B cell memory responses (Ogle, West, Driscoll, Strome, Razonable, Paya, Cascalho and Platt 2006). Here we report that selection of affinity mature antibodies generated in response to protein antigens requires the integrity of the thymus.

MATERIALS AND METHODS

Specific Aim 1, 2 and 3

Strains of mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). B cell-deficient strains of mice used included $JH^{-/-}$ mice, obtained by gene-targeted deletion of the JH segments (Chen, Trounstein, Alt, Young, Kurahara, Loring and Huszar 1993), and QuasiMonoclonal (QM) mice, generated by gene-targeted replacement of the endogenous JH elements with a VDJ rearranged region from a 4-hydroxy-3-nitrophenylacetate-specific hybridoma (Cascalho, Ma, Lee, Masat and Wabl 1996). The $JH^{-/-}$ mice lack mature B cells and Ig (Chen, Trounstein, Alt, Young, Kurahara, Loring and Huszar 1993). QM mice have 80 % of B cells that are 4-hydroxy-3-nitrophenylacetate specific (Cascalho, Ma, Lee, Masat and Wabl 1996). Monoclonal B cell-T cell mice have monoclonal B and T cell compartments; the T cells express a $\alpha\beta$ DO 11.10 transgenic cell receptor restricted to MHC class II^B (Keshavarzi, Rietz, Simoes, Shih, Platt, Wong, Wabl and Cascalho 2003).

$JH^{-/-}$ and QM mice were bred and all mice were housed in a specific pathogen-free facility at the Mayo Clinic. All mice were between 6 and 18 wk of age, and all experiments were conducted in accordance with protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Determination of TCR V β diversity

Isolation of RNA. Spleens harvested from mice were placed in RPMI 1640 and pushed through a 70- μ m cell strainer. Leukocytes were isolated by Ficoll-Paque (Amersham Biosciences) gradient. Total RNA was obtained with an RNeasy kit (Qiagen) per the manufacturer's instructions.

Generation of diversity standards. Diversity standards were prepared by generating oligonucleotide mixtures of known diversity, as previously described (Ogle et al. 2003). For example, to generate an oligonucleotide sequence with diversity of 10^6 , 18-mer oligonucleotides were synthesized with 10 sites of random assignment generating 4^{10} or 1,040,526 different

oligomers. Similarly, we created oligomer mixtures with 1, 10^3 and 10^9 variants. Oligonucleotides were biotin-labeled and hybridized to the gene chips as explained below.

Generation of lymphocyte receptor-specific cRNA. First strand cDNA was obtained by reverse transcription with a mouse TCR C β reverse primer, T7 plus C β (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGCTTGGGTGGAGTCACATTTCTC-3'). Second strand synthesis and preparation of biotin-labeled cRNA was conducted according to Affymetrix standard protocols.

Application of cRNA to the gene chip. Equal amounts of cRNA from different samples and diversity standards were hybridized to U133B gene chips (Affymetrix). Gene chips were processed at the Microarray Core Facility, Mayo Clinic, Rochester, MN.

Data analysis. Raw data corresponding to oligo location and hybridization intensity were obtained. The number of oligo locations with intensity above background (i.e., number of hits) was summed. A standard curve was generated by hybridizing samples with known numbers of different oligomers. Diversity of the test samples was estimated by comparison with the standard curve.

CDR3 size spectratyping of TCR V β

PCR primers. Primers were synthesized by Mayo Molecular Biology Core Facility (Pannetier et al. 1995). Two C β primers were designed to be homologous to the 3' end of the constant region of the β -TCR for the initial RT-PCR and a second nested constant region primer near the 5' end of the constant region was end labeled with Well RED D4 fluorescent dye for detection on a CEQ 8000 DNA fragment analyzer (Beckman Coulter). Twenty-four V β specific primers were synthesized to distinguish individual V β genes (Rodriguez et al. 1993).

cDNA production. cDNA was produced using a C β constant region primer in a RT-PCR amplification. The reverse transcriptase reaction was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) by incubating heat-denatured RNA template and the constant region primer (Nest I) at 37°C for 40 min and 42°C for 20 min followed by a heat-deactivating incubation at 100°C for 5 min. The final reaction concentrations

contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM MgCl₂, and 0.25 mM dNTP in a final volume of 5 µl per reaction.

cDNA amplification. Following cDNA synthesis, provision for a subsequent "hot-start" PCR was made by adding an AmpliWax PCR Gem 100 tablet (PerkinElmer) to each tube before incubation in the cycler at 100° for 5 min. This incubation inactivates the Moloney murine leukemia virus reverse transcriptase and melts the wax tablet. After removing the tubes from the cycler, the wax layer was allowed to set for 1 min and an upper PCR mix was added. This layer consisted of 5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 pmol of a specific V β variable region primer, and 0.5 U of *Taq* polymerase (Promega) in a final volume of 20 µl. PCR cycling parameters were one cycle of denaturation at 94° for 5 min, annealing at 56° for 30 s, and extension at 72° for 1 min followed by 29 cycles with denaturation at 94° for 40 s and the same annealing and extension parameters. A final extension was conducted at 7° for 5 min.

Second nested PCR for labeling. A second nested PCR was performed to label the products from the first amplification reaction. One microliter of the first PCR was used as template for the second reaction. The same specific V β variable region primers were used and a nested constant region primer (GAGGGTAGCCTTTTGTGGT) with a fluorescent tag, WellRED D4 (Proligo), for detection on a Beckman CEQ 8000 DNA fragment analyzer for all reaction products. This reaction consisted of 5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 pmol of a specific V β variable region primer, and 0.6 U of *Taq* polymerase in a final volume of 25 µl. PCR cycling parameters were one cycle of denaturation at 94° for 2 min, annealing at 56° for 30 s, and extension at 72° for 1 min followed by 29 cycles with denaturation at 94° for 40 s and the same annealing and extension parameters. A final extension was conducted at 72° for 5 min. Data acquisition and peak detection were handled by the manufacturer's supplied software for the CEQ 8000 (Beckman Coulter).

TCR V β gene sequencing. Total RNA was isolated from splenocytes of C57BL/6, QM, and JH^{-/-} mice with an RNeasy mini kit (Qiagen). cDNA was obtained by reverse transcription with a ThermoScript RT-PCR system (Invitrogen Life Technologies). Amplification of V β sequences were done using 10 pmol of the V β 8.1 specific (forward) primer (CATTACTCATATGTCGCTGAC), 10 pmol of the C β (reverse) primer

(GAGACCTTGGGTGGAGTCAC), and 1.25 U of *Pfu*Turbo DNA polymerase (Stratagene) in 50 μ l. PCR conditions were 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 7 min. The presence of a PCR product was detected by visualizing the appropriate size bands on 1.5 % agarose following gel electrophoresis. The PCR products were cloned using TOPO TA cloning (Invitrogen Life Technologies). Sequencing was performed at the Mayo Clinic Molecular Biology Core Facility using an M13 (forward and reverse) primer. Analysis of the sequences was done with Sequencher software (Gene Codes Inc.), and the sequences were aligned using the software provided by the international information system of ImMunoGeneTics, IMGT (M.-P. Lefranc, Montpellier, France; <http://imgt.cines.fr>) (Bosc and Lefranc 2000; Giudicelli et al. 2005). The following is a list of the specific V β variable primers used and their sequences (5' to 3') with the IMGT nomenclature according to Bosc and Lefranc (Bosc and Lefranc 2000) in parentheses:

V β 1 (IMGT V β 5), CTGAATGCCAGACAGCTCCAAGC;

V β 2 (IMGT V β 1), CAAAGAGGTCAAATCTCTTCCCGGTG;

V β 3 (IMGT V β 26), GTTCTTCAGCAAATAGACATCACTG;

V β 4 (IMGT V β 2), CTTATGGACAATCAGACTGCCTCA;

V β 5.1 (IMGT V β 12-2), CATTATGATAAAATGGAGAGAGAT;

V β 5.2 (IMGT V β 12-1), AAGGTGGAGAGAGACAAAGGATTC;

V β 5.3 (IMGT V β 12-3), AGAAAGGAAACCTGCCTGGTT;

V β 6 (IMGT V β 19), TCAATAACTGAAAACGATCTT;

V β 7 (IMGT V β 29), TACGATGTTGATAGTACCAGCG;

V β 8.1 (IMGT V β 13-3), CATTACTCATATGTCGCTGAC;

V β 8.2 (IMGT V β 13-2), CATTATTCATATGGTGCTGGC;

V β 8.3 (IMGT V β 13-1), TGCTGGCAACCTTCGAATAGGA;
V β 9 (IMGT V β 17), ATGATAAGATTTTGAACAGGGA;
V β 10 (IMGT V β 4), GCAATCCATTGTAAACGAAACAG;
V β 11 (IMGT V β 16), CAAGCTCCTATAGATGATTCAGGG;
V β 12 (IMGT V β 15), AAGTCTCTTATGGAAGATGGTGG;
V β 13 (IMGT V β 14), TCCTCTATAACAGTTGCCCTCG;
V β 14 (IMGT V β 31), TGTTGGCCAGGTAGAGTCGGTGCAA;
V β 15 (IMGT V β 20), GCACTTTCTACTGTGAACTCAGC;
V β 16 (IMGT V β 3), GGTAAGATCATGGAGAAGTCTAAAC;
V β 17 (IMGT V β 1), AGAGATTCTCAGCTAAGTGTTCCCTCG;
V β 18 (IMGT V β 30), CAGCCGGCCAAACCTAACATTCTC;
V β 19 (IMGT V β 121), CTGCTAAGAAACCATGTACCA;
V β 20 (IMGT V β 23), TCTGCAGCCTGGGAATCAGAA.

Fluorescence activated cell sorting analysis

Splenocytes were obtained by pushing minced spleen tissue through a 0.70- μ m mesh followed by hemolysis in an NH₄Cl lysis buffer. The total number of splenocytes was determined using a Neubauer chamber. Cells were stained with one, two or three of the following mAbs (all the Abs were from BD Pharmingen) as described (Cascalho, Ma, Lee, Masat and Wabl 1996): FITC-conjugated rat anti-mouse CD4 (clone GK1.5), rat anti-mouse CD8 α (clone Ly-2), and rat anti-mouse CD19 (clone 1D3); PE-conjugated rat anti-mouse CD8 α (clone 53-6.7) and rat anti-mouse CD44 (Pgp-1, Ly-24); and biotin-conjugated rat anti-mouse CD62L (LECAM-1, Ly22) and rat anti-mouse CD3 ϵ (clone 145-2C11). PE-conjugated anti-mouse Forkhead box P3 (clone FJK-

16S) was bought from eBioscience. Lymphocytes were gated on the light scatter plot by back gating onto CD4⁺CD3⁺ and CD8⁺CD3⁺ cells; numbers of the splenocytes in the subpopulations were determined by multiplying their total number by the percentage as defined by gating on the FACS plot. Data were collected on a FACScalibur (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

T cell priming to Pan DR reactive epitope (PADRE) peptide

Age-matched B6 mice were injected subcutaneously with the 140- μ g PADRE peptide aK(X)VAAWTLKAAa, where "a" is alanine and X is cyclohexyl alanine (Wei et al. 2001) in PBS. Three weeks later, CD4⁺ Th cells were purified from draining lymph node and spleens and cultured with dendritic cells isolated according to Kodaira et al. (Kodaira et al. 2000) and matured by incubation with LPS (from *Escherichia coli* 0111:B4; Sigma-Aldrich) at 5 μ g/ml overnight in the presence of PADRE (35 μ g/ml) for 5 days. Data represent the mean counts per minute of three wells \pm SE in one representative experiment.

Delayed-type hypersensitivity assay

Mice were primed by the injection of 100 μ g of OVA subcutaneously and challenged by intradermal injection of 20 μ g of OVA in the footpad 6 days after priming. Non primed mice controls were included. Effective swelling was indicated by the difference in thickness measured with a caliper between Ag-injected footpad and a PBS-injected footpad. Responses were recorded at 24, 36, and 60 h.

T cell proliferation assay

Isolated CD4⁺ T cells from age-matched (B6, QM, and JH^{-/-}) were cultured in a 96-well plate coated with anti-CD3 (clone H57-597) (in three different concentrations: 0.2, 1, and 10 μ g/ml) in the presence of anti-CD28 at 10 μ g/ml for 48 h. Alternatively, T cells were cultured with Con A (BD Biosciences) (in three different concentrations (ConA to medium): \sim 1/20, 1/10, and 1/5) in the presence of anti-CD28 at 10 μ g/ml for 48 h. Proliferation was measured by (³H)thymidine incorporation. Data represent the mean counts per minute of three wells \pm SE in one representative experiment.

Skin grafts

Skin grafts were performed according to a modified technique of Billingham et al. (Billingham et al. 1953). Briefly, full thickness tail skin (0.5 x 0.5 cm) was grafted onto the lateral flank. Grafts were observed daily after removal of the bandage at day 8. Grafts were considered rejected when 90 % or more of the graft lacked any viable signs (hair, pigment, and scale pattern). All mice were grafted between 6 and 18 wk after birth. Re-transplants were performed 16–20 wk after the primary graft was shed.

Pneumocystis murina infection and assessment of organismal burden

P. murina was isolated from the lungs of heavily infected, SCID mice as previously described (Keely et al. 2004). The infected lungs were aseptically minced and disaggregated in a Stomacher laboratory blender. *Pneumocystis* organisms were isolated by differential centrifugation, washing, and filtration through micropore filters containing 10- μ m pores as previously reported (O’Riordan et al. 1995). The organisms were resuspended in freezing medium (RPMI 1640 with glutamine containing 10 % FCS and 7.5 % DMSO), aliquoted, and frozen in liquid nitrogen. All mice were infected with the same frozen stock of *Pneumocystis*.

As described by Shellito et al. (Shellito et al. 1990), inoculation was performed by inserting a 22-gauge feeding tube into the trachea of anesthetized animal, visualizing its position through the 5-mm long incision of the skin, and injecting 75 μ l of *Pneumocystis* suspension (containing 10^7 organisms) followed by 300 μ l of air. Control animals were injected with 75 μ l of saline followed by 300 μ l of air. Two weeks after the infection the mice were sacrificed and the lungs were removed and frozen until analysis for *Pneumocystis* burden by quantitative RT-PCR was conducted.

Quantitative RT-PCR to enumerate *Pneumocystis* was performed using the Bio-Rad iCycler System, SYBR Green detection software, and primers targeting the *Pneumocystis* large mitochondrial subunit (Wakefield et al. 1990). Lung DNA was isolated by phenol-chloroform extraction and ethanol precipitation and finally resuspended in Tris-EDTA buffer. Amplifications of unknown samples were compared with plasmid standards containing mouse *Pneumocystis*-specific mitochondrial DNA. All samples were run in triplicate.

Statistical analysis

Statistical analysis for comparison of the TCR V β diversity of groups was performed using natural log transformation of the data and subsequent one-way ANOVA. The comparison of the groups was performed by an unpaired, two-sided Student's *t* test on the natural log transformed data. Group comparisons of the numbers of T cells in the splenocyte subpopulations were performed using the Student's *t* test after testing the global difference with a one-way ANOVA. Comparison of skin graft survivals was performed by a log-rank test. A value of $p < 0.05$ was considered significant.

Specific Aim 4 and 5

Strains of mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a specific pathogen-free facility at the University of Michigan. All mice were between 5 and 25 weeks of age and all experiments were carried out in accordance with protocols approved by UCUCA.

Thymectomy and gene analysis

Thymectomy: Thymuses were removed surgically from mice or sham-surgery was performed at 5 weeks of age. Mice were anesthetized with ketamine (120-200 mg/kg) + xylazine (10 mg/kg) i.p. An incision was made on the ventral neck midline extending from 0.5 cm cranial of the sternal notch. The clavicle was cut along the sternum to the second rib and retracted to expose the trachea, sternohyoid and sternothyroid muscles which were gently separated to expose the superior end of the thymic lobes. The thymus was then gently dissected with blunt instruments and excised by vacuum. The thorax was closed using 6-0 absorbable suture placed through the dorsal thorax to draw the clavicle and ribs together. The fat pad with the submaxillary gland was returned to its original position and held in place by liquid skin adhesive. Skin was closed using 6-0 absorbable suture. Mice were monitored every 12h for the first 48h, and daily thereafter. Sham-operated mice underwent the same surgical procedure, except for the fact that the thymus was not excised, just manipulated with the tip of blunt scissors.

Blood collection: Done following the recommendations of the University of Michigan Committee on Use and Care of Animals (UCUCA).

Immunizations: T-independent immunizations were performed as explained by Mantchev et al. (Mantchev et al. 2007) by injecting mice i.p. with 30 µg of NP-Ficoll (NP41- AECM-Ficoll; Biosearch Technologies, Novato, CA, USA) diluted in 100 µl PBS once. Primary T-dependent immunizations were performed by i.p. injection of 100 µl of an emulsion of incomplete Freund's adjuvant containing 100 µg NP (25)-ovalbumin (Biosearch Technologies, Novato, CA, USA)

and boost immunizations performed by i.p. injection of 100 µl of a PBS solution containing 10 µg NP (25)-ovalbumin. To obtain RNA from memory B cells, mice were boosted a second time by i.v. injection of 50 µg NP (25)-ovalbumin dissolved in 100 µl of PBS.

Ig gene analysis: RNA was obtained from spleen cells and extracted with QIAGEN RNeasy (Qiagen, Inc., Valencia, CA, USA). cDNA was obtained from 0.2 µg of RNA using oligo(dT) primed reverse transcription. VH186.2 gene sequences joined to the IgG1 constant region were amplified with VH186.2 and C1 specific primers in a nested reaction and with Pfu polymerase, followed by cloning with pCR4-TOPO (Invitrogen, USA). Sequencing of cloned PCR fragments was done by the Mayo Clinic Sequencing Core. Forward primer: CATGCTCTTCTTGGCAGCAACAGC (specific for VH186.2), reverse primer: GTGCACACCGCTGGACAGGGATCC (specific for C 1). PCR was performed for 30 cycles of 1 minute at 94⁰ Celsius, 2 minutes at 55⁰ Celsius, and 3 minutes at 72⁰ Celsius. Nested PCR amplification forward primer: CAGGTCCAACCTGCAGCAG, and reverse primer, AGTTTGGGCAGCAGA. Sequences were aligned and analyzed using Sequencher software (Gene Codes, MI, USA) and with a software program developed by Dr. Calvacoli at the University of Michigan Sequencing Core. VH, D and JH gene CDR3 sequence assignments were done according to the international ImMunoGeneTics (IMGT) system software developed by Dr Lefrank at the CNRS, France (Giudicelli, Chaume and Lefranc 2005). Complementary determining regions were determined according to Kabat et al. (Kabat et al. 1991).

FACS analysis and antibodies

Splenocytes were obtained and prepared for flow cytometry analysis as in (Cascalho, Ma, Lee, Masat and Wabl 1996). Fluorescently conjugated or biotinylated antibodies were purchased from BD Biosciences unless noted: rat anti-mouse CD4 (GK 1.5), rat anti-mouse CD21a/CD35 (7G6), rat anti-mouse CD3 (145-2C11), rat anti-mouse IgD^b (AMS9.1), rat anti-mouse CD4 (GK1.5), rat anti-mouse CD44 (Pgp-1, Ly-24), rat anti-mouse CD23 (Fc ε RII), rat anti-mouse IgMb (DS-1), rat anti-mouse CD8α (Ly-2), rat anti-mouse CD19 (1D3), rat anti-mouse CD62L (LECAM-1, Ly22), rat anti-mouse CD25 (IL-2R α chain, p55) and rat anti mouse Foxp3 (FJK-16S, eBiosciences). Biotinylated antibodies (Abs) were revealed by streptavidin-PE-Cy5 purchased from BD Biosciences, USA. Data were collected on a FACScalibur (BD Biosciences)

and analyzed with CellQuest software (BD Biosciences). Isotype controls were used to define gates.

Delayed type hypersensitivity (DTH) assay

Mice were primed by subcutaneous injection of 100 µg of ovalbumin dissolved in PBS, and challenged by intra-dermal injection of 20 µg of ovalbumin in PBS in the footpad, 6 days after priming. Non-primed mice challenged with PBS were included as controls. Footpad swelling was measured with a caliper. Effective swelling indicates the difference in the thickness of footpads in the same mice (one injected with the antigen, another injected with PBS). Responses were recorded at 24 and 48 hours post-challenge.

Skin Grafts

Skin grafts were performed according to a modified technique of Billingham et al. (Billingham, Brent and Medawar 1953). Secondary transplants were 30 days after the primary graft was shed.

TRECS (T cell receptor excision circles)

DNA was obtained from splenocytes with a DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA). PCR amplification of sjTREC DNA was done from 100 ng of DNA in a Mastercycler ep realplex real-time PCR system (Eppendorf) using specific primers targeting murine δ Rec- ψ J α excision circles. Real-time PCR cyclers conditions were set for 95⁰C for 10' followed by 40 cycles of 95⁰C for 15'' and 60⁰C for 1' with 5µM forward and reverse primers and 0.05µl of 100µM FAM-QSY probe. Forward primer (upstream of ψ J α segment): 5'CAT TGC CTT TGA ACC AAG CTG3'; Reverse primer (downstream of the δ Rec1 segment): 5'TTA TGC ACA GGG TGC AGG TG3' according to (Sempowski and Rhein 2004). A fluorescent probe for RT-PCR: FAM - CAG GGC AGG TTT TTG TAA AGG TGC TCA CTT - QSY (Applied Biosystems). The mouse transferring receptor gene *Tfrc* gene (TaqMan Copy Number Reference Assay, Applied Biosystems) was amplified to quantify cell number in mouse DNA samples. Each sample was run in triplicate. Standard curves were created with either serial dilutions sjTREC plasmid DNA or of C57BL/6J DNA followed by *Tfrc* gene amplification.

ELISA (Enzyme-linked immunosorbent assay)

MaxiSorp-treated or PolySorp-treated polystyrene 96-well plates (Thermo Scientific, Rochester, NY, USA) were coated with 4 µg/mL of goat anti-mouse Ig (SouthernBiotech, Birmingham, AL, USA) in PBS to measure total Ig, or with 5 µg/mL of NP-BSA in borate saline buffer to detect NP-specific antibodies, for 1 hour at room temperature. ELISA was performed according to previously described protocols (Cascalho, Ma, Lee, Masat and Wabl 1996; Cascalho et al. 1997). Plates were developed with ABST (SouthernBiotech, Birmingham, AL, USA) read at 405 nm in microplate reader Synergy 2 (BioTec Laboratories Ltd., Suffolk, UK) and analyzed using Gen 5 software version 1.04.5 (BioTek, VT, USA). The 17.2.25 IgG1 was used as a standard for quantification.

ELISPOT (Enzyme-linked immunosorbent spot)

Done according to standard procedures in the laboratory (Mantchev, Cortesao, Rebrovich, Cascalho and Bram 2007). MultiScreen HTS-HA 96-well plates (Millipore, Billerica, MA, USA) were coated with 5 µg/mL NP-BSA or 5 µg/mL BSA in sodium carbonate buffer overnight at 4°C and blocked with 5 % milk in TBS-Tween for 2 hours at 37°C. B cells isolated from the spleen by negative selection were serially diluted, seeded in the wells and cultured in complete RPMI-1640, overnight at 37°C in 5 % CO₂ atmosphere. ELISPOT analyses of antibody secreting cells obtained from adoptively transferred recipients were done with splenocytes. To detect NP-specific antibody secreting cells, each well was washed and incubated with AP-conjugated goat anti-mouse IgM or IgG antibody (SouthernBiotech, Birmingham, AL, USA) for 2 hours at 37°C. Each well was developed with BCIP/NBT (Sigma-Aldrich, St. Louis, MO, USA). The number of spots of NP-specific IgM or IgG secreting cells was counted by ImmunoSpot Professional Analyzer version 5.0.9 (Cellular Technology Ltd., Shaker Heights, OH, USA) and confirmed by direct observation.

TCR beta chain diversity analysis

TCR beta chain diversity analysis was done as reported (Wettstein et al. 2008). Briefly, RNA was obtained from spleens using a RNeasy Protect Minikit (Qiagen, CA). Residual DNA was

removed from RNA samples using a RNase-Free DNase Set (Qiagen). cDNA was produced from 15 ng of RNA with a 20 pmol of a 5'biotynilated TCR C β β b primer and pools of 21 different TCR V β primers homologous to the CDR 1 region providing 66 pmol of each (three pools of 5 and one pool of 6 primers), at 50⁰C for 32' followed by incubation at 94⁰C to inactivate the reverse transcriptase. cDNA synthesis was followed by PCR amplification at 1' at 94⁰C, 30'' at 60⁰C, and 1' at 72⁰C for 25 cycles. RT-PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and biotynilated products separated with MyOneTM Streptavidin C1 Dynabeads (DynaL Biotech ASA, Oslo, Norway) according to the manufacturers' instructions. TCR V β diversity was determined by real time PCR in a total of 240 individual reactions using combinations of 20 TCR V β and 20 TCR J β primers, as described (Wettstein, Strausbauch, Therneau and Borson 2008). Reactions were performed in a 10 ml volume containing 10 pmol of a nested TCR V β primer homologous to TCR V β CDR2, 10 pmol of a TCR J β primer, 1 μ l of purified PCR products and 5 μ l of Power SYBR Green PCR master mix (2x) (Applied Biosystems). Cycling was preceded by incubation at 50⁰C for 2' and at 95⁰C for 10', followed by 40 cycles of 15'' at 95⁰C and 1' at 60⁰C. Data were analyzed with the 7900HT Sequence Detection System Version 2.3 software (Applied Biosystems) to estimate the cycle threshold (Ct) for all reactions. Ct values are fractional cycle numbers at which fluorescence passes the threshold set to be within the exponential region of the amplification curve corresponding to a linear relationship between the log of change in fluorescence and cycle number. Primers were as published (Wettstein, Strausbauch, Therneau and Borson 2008) and synthesized by Invitrogen (Carlsbad, CA, USA).

Statistical analysis

Performed using Prism software (Prism Software Corporation, Irvine, CA). Group comparisons were performed using the unpaired, two-sided Student's *t* test after testing the global difference with a one-way analysis of variance (ANOVA). Comparison of skin graft survival was performed by a log rank test. A value of $p < 0.05$ was considered significant.

RESULTS

Specific Aim 1, 2 and 3

Diverse B cells and Ig maintain T cell numbers in the spleen and TCR diversity

Competence of the T cell compartment, particularly cell-mediated immunity, is thought to depend upon the number and diversity of T cells available to respond to antigenic challenge (Nikolich-Zugich, Slifka and Messaoudi 2004). T cell diversity would seem to assure that one or more clones of T cells will bear a TCR capable of recognizing a peptide(s) from a microorganism, a toxin, or a minor histocompatibility Ag associated with self-MHCs, thus allowing the activation of rare Ag-specific T cells (Kanagawa et al. 1982; Lindahl and Wilson 1977; Suchin et al. 2001). To test this concept, we studied the structure of the T cell compartment and cell-mediated immunity in mice with defects in Ig assembly.

We asked whether mice with B cell defects have normal numbers of T cells. The numbers of CD3⁺, CD4⁺, or CD8⁺ T cells in the spleen of QM mice were not significantly changed compared with those of wild-type mice ($p > 0.05$), indicating that QM mice with oligoclonal B cells maintain normal numbers of T cells in the adult spleen (Figure. 1). In contrast, numbers of CD3⁺ or CD4⁺ but not CD8⁺ T cells were significantly decreased in JH^{-/-} mice ($p < 0.05$). This finding is in agreement with the observations of Ngo et al. (Ngo et al. 2001), who showed reduced T cell numbers in the spleen of B cell deficient μ MT mice.

We next tested whether mice with B cell defects and low diversity of thymocytes (Joao, Ogle, Gay-Rabinstein, Platt and Cascalho 2004) also have decreased diversity in the periphery. TCR V β diversity was measured according to Ogle et al. (Ogle, Cascalho, Joao, Taylor, West and Platt 2003). In this assay, T cell diversity is proportional to the number of hybridization hits of TCR V β cRNA on a gene chip and quantified by comparison to a standard curve obtained with DNA oligomers of known diversity. TCR V β diversity of JH^{-/-} splenocytes was 1.1×10^3 per 10 μ g of RNA, whereas TCR V β diversity of C57BL/6 splenocytes was 1.3×10^6 per 10 μ g of RNA ($p < 0.05$). TCR V β diversity of QM splenocytes was intermediate, 8.8×10^4 per 10 μ g of RNA ($p < 0.05$, compared with C57BL/6). In addition to the specificity of the primers, we

assured that the assay detected TCR V β diversity and not B cell diversity, because sorted 1.5 x 10⁷ B cells from C57BL/6 splenocytes containing 0.09 % of CD3⁺ cells yielded a mean diversity of only 136 corresponding to 0.023 % of the diversity obtained from equal number of splenocytes containing 20 % of CD3 positive cells. Because QM mice are B cell and Ig proficient, our results indicate that T cell diversity in the spleen is not a function of B cell number and/or of serum Ig concentration (Joao, Ogle, Gay-Rabinstein, Platt and Cascalho 2004) but may rather depend on the diversity of Ig in the serum and/or on the surface of B cells.

T cell function in mice with contracted TCR diversity

We next tested whether T cells from mice with a contracted T cell repertoire exhibit normal functions at a cellular level. As Figure. 2 shows, T cells from JH^{-/-} and QM mice proliferated in response to anti-CD3 (Figure. 2A) or Con A (Figure. 2B), as did T cells from C57BL/6 mice. Next, we asked whether T cells in JH^{-/-} and QM mice could be primed *in vivo*. To this end, mice were injected with 140 μ g of the PADRE peptide and 14 days later CD4⁺ T cells purified from the spleen were cocultured with mature dendritic cells in the presence of 35 μ g/ml PADRE peptide. Figure. 3 shows that QM T cells mount robust proliferation and JH^{-/-} T cells have detectable albeit reduced proliferation (26 % of QM or 27 % of C57BL/6 values) to the PADRE peptide. The results indicate that 90 % contraction of the TCR repertoire (in QM mice) does not impair priming of T cells and that 99 % contraction, as in JH^{-/-} mice, does not preclude T cell priming.

Lymphoid organogenesis in mice with contracted TCR repertoires

Aberrant T cell response in JH^{-/-} mice could be due to the contraction of TCR diversity or to defective lymphoid organogenesis. We questioned whether T cells from JH^{-/-} mice respond normally to mitogens but fail to undergo priming because of defective lymphoid organogenesis. Golovkina et al. (Golovkina et al. 1999) showed that lack of B cells in JH^{-/-} mice causes defective peripheral lymphoid organogenesis with an absence of Peyer patches and a follicular dendritic cell network. Consistent with this possibility, Figure. 4 shows that although JH^{-/-} lymph nodes lack a follicular dendritic cell network, QM mice have a normal one, suggesting

that abnormal lymph nodes rather than contracted T cell diversity in JH^{-/-} mice could contribute to defective T cell priming.

Impact of TCR diversity on cell-mediated immunity

We next asked to which extent contraction of the T cell repertoire per se impairs cell-mediated immunity. To avoid the confounding influence of impaired lymphoid organogenesis, we addressed the question using QM mice that show nearly normal lymph nodes. First, we compared the rate of rejection of skin allografts by QM and C57BL/6 mice. The outcome of skin grafts is thought to be independent of Ab responses directed against the graft (Bogman et al. 1984; Cascalho and Platt 2001; Parker et al. 1996) and, hence, this test could be conducted in mice with oligoclonal B cells. The kinetics of rejection of allografts is modified in animals with defective lymphoid organogenesis (Lakkis et al. 2000) or T cell signaling (Harmsen and Stankiewicz 1990; Ito et al. 2005) and, therefore, the assay would help exclude these problems. Table 1 shows that MHC-disparate skin grafts were rejected with similar kinetics by QM and wild-type recipients. This result suggests that the QM mice have functional lymph nodes and that their T cells have the capacity to function like T cells from wild-type mice.

We next tested the outcome of grafts across a minor histocompatibility barrier. We reasoned that contracted TCR diversity would more likely hinder responses to minor than to major histocompatibility Ags, because the frequency of T cells specific for those Ags is less by orders of magnitude than the frequency of T cells specific for allogeneic MHC (Kanagawa, Louis and Cerottini 1982; Lindahl and Wilson 1977; Suchin, Langmuir, Palmer, Sayegh, Wells and Turka 2001). For this test, skin from the tails of male mice was transplanted onto female mice flanks. As Table 1 shows, QM or JH^{-/-} females rejected male skin grafts as reliably as wild-type mice and with the same kinetics at 26 or 23 days (median), respectively, while C57BL/6 females rejected male skin grafts at 23 days (median) (Table 1 and Figure. 5). QM and wild-type mice rejected secondary skin grafts always faster than primary grafts (Table 2). These results indicate that contraction of the repertoire of T cells has little or no measurable impact on primary or secondary alloimmune responses.

To confirm that contraction of the TCR repertoire has little impact on cell-mediated immunity, we tested the responses to OVA in QM, JH^{-/-}, and C57BL/6 mice. Responses to OVA were examined in mice primed 6 days earlier by measuring the footpad thickness at 24, 36, and 60 h following intradermic injection of 20 µg of OVA. Mice were either primed by s.c. injection with 100 µg of OVA or injected with PBS (not primed controls). Footpad swelling did not differ between QM and wild type mice (Figure. 6) at 60 h following challenge. However, footpad swelling was decreased in QM mice at 36 h after challenge, suggesting a delayed kinetics compared with that of C57BL/6 mice. JH^{-/-} mice had decreased footpad swelling 36 and 60 h after challenge, which could be due to defective lymphoid organogenesis (see above). The footpad swelling reflected cell-mediated immunity because it was fully prevented by daily injection with cyclosporin A (600 µg/day i.p. for 3 days) (data not shown).

Impact of TCR repertoire contraction in host defense

As a further test of cell-mediated immunity, we asked whether host defense was challenged by TCR repertoire contractions. To test the impact of contracted TCR repertoire on host defense, we infected QM or C57BL/6 mice with *P. murina* and measured the organism burden in the lungs 2 wk later. *Pneumocystis* is an intracellular pathogen, the elimination of which depends on T cells; T cell-deficient mice fail to clear the agent, ultimately causing pneumonia and the death of the host (Harmsen and Stankiewicz 1990). Figure. 7 shows that the level of *Pneumocystis* in the lungs of QM or C57BL/6 mice 2 wk postinfection was similar. Thus, contraction of the TCR repertoire did not compromise host defense.

TCR repertoire contraction in B cell-deficient mice is balanced

Those with impaired cell-mediated immunity caused by AIDS or DiGeorge syndrome have both TCR diversity contractions and gaps and/or oligoclonal expansions (Giacchia-Gripp et al. 2005). Whether these changes impair immunity is unknown. To address that question, we evaluated the TCR V β repertoire in mice with severely contracted diversity by spectratyping and sequencing.

TCR β -chain CDR3 lengths for each V β gene spectratype were generated for each of the V β genes using RNA derived from splenocytes obtained from three JH^{-/-} and three C57BL/6 mice according to Pannetier et al. (Pannetier, Even and Kourilsky 1995). This analysis showed that the

majority of the 24 $V\beta$ genes were associated with spectratypes exhibiting Gaussian distributions. There were a limited number of exceptions as described in Figure. 8, where single peaks were over-represented against a background of normally distributed CDR3 lengths. Thus, the contracted repertoire in $JH^{-/-}$ mice did not alter the distribution of CDR3 lengths, indicating that contraction was balanced in contrast to what is observed in immunodeficiencies such as AIDS (Connors et al. 1997; Gorochov et al. 1998).

To determine whether a normal spectratype profile reflects a balanced contraction of the repertoire, we sequenced the TCR CDR3 regions of genes containing $V\beta$ 13-3 (IGMT designation for $V\beta$ 8.1) in splenocytes obtained from C57BL/6 (Table 3), QM (Table 4), or $JH^{-/-}$ mice (Table 5). Tables 3, 4, and 5 show fewer repeat sequences in QM or $JH^{-/-}$ splenocytes compared with those of wild-type mice. Of 46 C57BL/6 sequences bearing $V\beta$ 13-3, 29 had different CDR3 regions, the lengths of which averaged 11.5 codons. Of 45 QM sequences bearing $V\beta$ 13-3, 43 had different CDR3 regions, the lengths of which averaged 11.5 codons. Of 67 $JH^{-/-}$ sequences bearing $V\beta$ 13-3, 54 had different CDR3 regions, the lengths of which averaged 11.9 codons. The CDR3 lengths of sequences containing the TCR $V\beta$ 13-3 obtained from mice of all strains showed a Gaussian distribution, as one might expect if differences in repertoire diversity were balanced. In further support of an equilibrated contraction without oligoclonal expansions, Table 4 shows that the $V\beta$ sequences obtained from all of the mice used diverse $J\beta$ segments. These results demonstrated that B cell-deficient mice have balanced contraction of TCR diversity and suggest that it is the gaps and oligoclonal expansions and not the repertoire contraction in itself that cause disease in immunodeficiencies such as AIDS and DiGeorge.

Increased "memory-like" $CD4^+$ and regulatory T cells in mice with contracted TCR repertoire

The normal kinetics of skin graft rejection in mice with profound contraction of the TCR repertoire suggested that the T cell compartment had "compensated" in some way. We hypothesized that such compensation might occur if T cells had proliferated to maintain the dimensions of the T cell compartment and, as a result, acquired "memory-like" functions (Mackall et al. 1997; Tanchot et al. 1997). To explore this possibility, we enumerated "memory-like" T cells in unmanipulated QM, $JH^{-/-}$, or C57BL/6 mice based on phenotype (Berard and Tough 2002; Berg et al. 1991; Goldrath et al. 2000; Sallusto et al. 1999). Figure. 9 shows that QM mice had 3-fold and $JH^{-/-}$ 2-fold more "memory-like" $CD4^+$ T cells

(CD4⁺/CD44^{high}/CD62L⁻) than C57BL/6 mice ($p < 0.05$) but similar numbers of "memory-like" CD8⁺ T cells. These results suggest that the T cell compartment compensates for the contraction of TCR diversity by homeostatic proliferation. Because thymic dysfunction or thymectomy performed in the second to the third day of life impairs the production of natural T regulatory T cells (Kim and Rudensky 2006), we questioned whether B cell-deficient mice might have impaired production of T regulatory cells, thus enhancing cell-mediated immunity. To address this question, we determined the number of T regulatory cells in QM and JH^{-/-} mice. The relative number of Forkhead box P3-positive T cells (a marker of T regulatory cells) (Fontenot et al. 2003) in the spleen was 1.9 % in JH^{-/-} mice, 1.8 % in QM mice, and 1 % in wild-type mice. Our results thus indicate that the maintenance of cell-mediated immunity cannot be ascribed to loss of natural regulatory T cells. Whether a 2-fold relative increase in T regulatory cells may modify or control T cell responses in mice with contracted T cell repertoires is not clear.

Specific Aim 4 and 5

Removal of the thymus of mature mice causes a persistent decrease in the number of CD4+ or CD8+ T cells without contracting T cell receptor diversity

To explore the role of the thymus in B cell memory responses we removed the thymus of mice at 5 weeks of age reasoning that at this age mice already have an established T cell compartment and competent cellular immunity (Miller 1965). Removal of the thymus at 5 weeks of age completely abrogated recent thymic emigrants because mice lacking the thymus (in the manuscript referred to as athymic mice) lacked any measurable T cell receptor excision circles (TRECs) at 5 and 10 weeks following thymectomy (Figure 10). Consistent with absent thymic function, athymic mice had reduced CD4+, CD25+, Foxp3 cells, at 10 weeks of age (Figure 11).

Mice from which the thymus had been removed (athymic mice) at 5 weeks of age had fewer T cells in the spleen 5 and 10 weeks after the surgery, compared to control mice. Figures 12A, 12B and Table 6 show that thymectomy caused a persistent 2.8 or 3.0 fold decrease in the number of CD4+ or CD8+ T cells respectively, 10 weeks after surgery. Sham operation of the thymus also decreased the number of CD4+ or CD8+ T cells 10 weeks after surgery, albeit less profoundly than removal of the thymus (Figures 12 A, 12 B and Table 6). Results from other laboratories are consistent with ours showing close to 2 fold reduction in the number of CD4 T cells in the spleen. Thus, Gagnerault et al. (Gagnerault et al. 2009) found a 2 fold reduction in the number of CD4 T cells in the spleen following thymectomy in 3-week-old mice; and Bourgeois et al. (Bourgeois et al. 2008) found a reduction of almost 2 fold in the number of peripheral CD4-positive T cells 15 weeks after interrupting thymic output in a model of chemical thymectomy.

To determine whether the removal of the thymus caused compensatory proliferation, we enumerated CD4+ or CD8+ cells with a memory-like phenotype (CD62L-negative and with high expression of CD44). Figures 12C, 12D and Table 6 show that athymic mice and controls had similar numbers of CD4+ or CD8+ T cells with a “memory” like phenotype in the spleen. However, the proportion of CD4+ memory-like T cells was significantly increased in athymic mice (14 %) compared to sham-operated (11 %) or control (9 %) mice. Likewise, the proportion

of CD8⁺ memory-like T cells was increased in athymic mice (45 %) versus sham-operated (25 %) or non-manipulated control mice (19 %). Since the absolute number of “memory-like” T cells is similar in athymic and control mice, the increased proportion of “memory-like” T cells brought about by removal of the thymus probably reflects the decrease in the absolute number of naïve T cells rather than compensatory proliferation of T cells brought about by removal of the thymus. The apparent lack of compensatory proliferation in athymic mice might partly reflect a decrease in IL-7 which is produced by thymic epithelial cells (Alves et al. 2009).

Because cellular immunity depends in part on the diversity of T cell receptors we analyzed TCR diversity in athymic mice and in controls 10 weeks after surgery. We used a novel approach to quantify TCR beta transcript diversity using a real-time polymerase chain reaction (PCR)-based method (Wettstein, Strausbauch, Therneau and Borson 2008). Briefly, the method amplifies TCR V beta (β) transcripts using combinations of primers specific for a total of 240 V β -J β combinations. Cycle threshold (Ct) values were determined for each V β -J β combination for each RNA template and mean Ct values were calculated. Results shown in Table 7 indicate that Ct values did not significantly differ in control (17.8), sham-operated (17.9) or athymic mice (18.7) suggesting that removal of the thymus or sham operation did not cause significant decrease in TCR diversity or oligoclonal expansions. These results were supported by Shannon entropy calculated for each V β -J β matrix in each set of mice (Shannon and Warren 1949) (Table 7). An estimate of entropy (H) was calculated by the equation $H = -\sum (p \log_2 p) / \log_2 (1/240)$ where p was the probability of abundance calculated for each V β -J β combination by the equation $p = 2^{-y/\sum 2^{-y}}$ where y was the Ct value for each V β -J β primer pair and p=0 when Ct > 40 cycles. Entropy ranges from zero to one with one representing maximal diversity. Control mice had an average entropy of 0.85, sham-operated mice had an average entropy of 0.84 and athymic mice had an average entropy of 0.85. These results agree with values reported for wild-type repertoires (0.88 on average) and contrast with values obtained in SCID-nude mice (0.76) (Dr. Wettstein, personal communication).

Thymectomy does not impair T cell memory

To determine whether and how removal of the thymus might impair memory T cell responses we used delayed type hypersensitivity (DTH) to ovalbumin as an index. Figure 13A shows that

challenge of athymic mice, produced larger foot-pad swelling than challenge of control mice, indicating that removal of the thymus did not impair and may instead enhance memory T cell responses. To determine whether removal of the thymus impairs primary T cell responses and test whether memory T cell responses are enhanced in athymic mice, we tested the rate of rejection of male to female skin grafts. Figure 13B shows that removal of the thymus slows the kinetics of skin graft rejection in athymic female recipients to male antigens since the median survival time of male skin grafts was 37 days in athymic mice and only 25 days in sham-operated and control mice, respectively. This result suggests that primary T cell responses were impaired. However T cell memory responses were intact as second set grafts were rejected with accelerated kinetics by all recipients, including those lacking the thymus. The results demonstrated that generation of T cell memory does not require an intact thymus.

Removal of the thymus in adult mice does not impair primary or secondary antibody responses but increases long-lived antibody secreting cells in the bone marrow

Manifest B cell memory requires antigen specific antibody production at times remote from primary antigen stimulation. At least some B cells engaged in a primary response must survive and some must have the ability to respond upon re-exposure. These antibody responses require T cell help (Elgueta et al. 2010). Whether the thymus is necessary to generate B cell memory responses beyond generating a diverse T cell repertoire is not known. To answer that question we tested B cell memory in mice from which the thymus had been removed or manipulated without removal 5 weeks before.

To exclude the possibility that thymectomy imposed a B cell autonomous defect independent of T cells we asked whether athymic mice mounted antibody responses to NP-Ficoll, a T-independent antigen. Figures 14A and 3B show comparable concentrations of NP-specific IgM and IgG3, 21 days following immunization, in athymic mice (230 $\mu\text{g/ml}$ IgM and 64 $\mu\text{g/ml}$ IgG3, on average) and sham-operated mice (167 $\mu\text{g/ml}$ IgM and 86 $\mu\text{g/ml}$ IgG3, on average). Hence, thymectomy did not perturb T-independent antibody production. Indeed B cells developed normally in mice lacking the thymus compared to sham-operated mice (Figure 15). Figure 15 shows that the average number of mature CD19-positive B cells is comparable in mice

lacking the thymus, sham-operated or control mice at 5 weeks and at 10 weeks of age, respectively. There were no population unbalances, as the number of marginal zone B cells (CD19⁺ and CD21⁺) or follicular (CD19⁺, CD21⁺ and CD23⁺) B cells was comparable in athymic, sham-operated and control mice.

A hallmark of B cell memory is the rapid production of high affinity antibodies upon re-exposure (Elgueta, de Vries and Noelle 2010). These properties reflect the survival of fully differentiated antigen specific B cells and plasma cells. To determine whether B cell memory responses were impaired in mature athymic mice, we studied responses to immunization with 4-hydroxy-3-nitrophenyl acetyl (NP), conjugated to ovalbumin. Figures 16A and 16B show that athymic mice produced as much NP-specific IgM or IgG1 as sham-operated mice indicating that removal of the thymus did not impair antigen-specific antibody primary or secondary antibody responses to vaccination with proteins. Consistent with that conclusion we found that the number of antibody secreting cells present in the bone marrow 6 months after immunization was maintained in sham-operated mice and increased by 2 fold in athymic mice compared to non-manipulated controls (Figure 17). In fact, since the number of ASC in athymic mice was significantly increased compared to the number of ASC in control or sham-operated mice, our results suggest that the thymus in the adult may inhibit either the differentiation or the maintenance of long-lived antibody secreting cells in the bone marrow.

Removal or manipulation of the thymus impairs the generation of Ig heavy chains associated with high affinity to NP

The most significant function associated with antibody recall responses is selection of cells bearing receptors with increased affinity for the antigen. To determine if affinity maturation requires the integrity of the thymus in the adult, we sampled antibody heavy chain variable region nucleotide and protein sequences of IgG1-positive B cells obtained from mice that had their thymus removed, manipulated (sham operation) or of non-manipulated controls, 10 days following booster immunization. Sequences were obtained from cloned PCR gene products amplified with VH186.2-specific primers (NP selects antibodies encoding the VH186.2 canonical germline sequence rearranged to DFL16.1 and JH2 (Bothwell et al. 1981)) and C1 reverse primers in a nested PCR reaction and with Pfu proof-reading polymerase. Two

sequences were obtained per clone and a consensus was generated. To determine if selection of antigen responsive B cells was perturbed in athymic or sham-operated mice we first determined the frequency of the VH186.2, DFL16.1 and JH2 joins in all the unique VH186.2 encoding HC sequences obtained for each group of mice. Out of 76 sequences encoding VH186.2 exons obtained from athymic mice, 19 had different joins (25 %) and 12 of used DFL16.1 and JH2 (63 %). In a total of 70 sequences encoding VH186.2 exons obtained from sham-operated mice, 37 had different joins (53 %), and 20 used DFL16.1 and JH2 (54 %). In 48 sequences encoding VH186.2 exons obtained from control mice, we found 17 different joins (35 %) and 11 used DFL16.1 and JH2 (65 %). These results suggested that removal of the thymus decreased, while sham operation increased, clonal diversity of NP responding B cells in comparison to controls even-though the majority of clones encoding the VH186.2 gene segment also encoded DFL16.1 and JH2 in all the three groups of mice.

Next we compared the amino-acid sequences of CDR3 regions encoded by each unique join. NP-binding antibodies often encode Tyr or Gly at position 95 (Takahashi et al. 1998). While 94 % CDR3 joins sequenced from control mice had Y or G at position 95 only 68 % of the unique CDR3 joins obtained from sham-operated mice had Y or G at position 95 and 84 % that of the unique CDR3 joins sequenced from athymic mice had Y or G at position 95. These results suggest that removal of the thymus and sham operation disturbs selection of NP-reactive clones. These results are consistent with defective selection of NP-specific antibodies in sham-operated and athymic mice.

Because defective selection of NP-specific antibodies could result from defective somatic hypermutation we measured the mutation frequency of the unique VH gene segments obtained from athymic, sham-operated or control mice in relation to the VH186.2 germline sequence. The VH mutation frequencies were 2.6 %, 3 % and 2.3 % in athymic, sham-operated and control mice, respectively, suggesting that manipulation or removal of the thymus in the adult did not impair somatic hypermutation, *per se*. However, the frequency of mutation in the CDR1 region of VH186.2 encoding antibodies obtained from control mice was 13.6 % and consisted of very focused changes at mostly 3 positions (Figure 18A), but the frequency of mutation in the CDR1 region of antibodies obtained from athymic and sham-operated mice was only 8.5 % and 7.2 %, respectively, and less focused (Figures 18B and 18C). Decreased frequencies of mutations in the

CDR1 regions of the VH186.2 exons in athymic or sham-operated mice compared to CDR1 sequences obtained from non-manipulated mice suggested a defect in the selection of antigen-specific antibodies. In fact, the fraction of sequences containing the W33L NP-affinity enhancing mutation was decreased in athymic mice (87 %, figure 19A) and in sham-operated mice (21 %, figure 19B), compared to that fraction (98 %) in sequences obtained from control mice in which all sequences except for one contained the W33L mutation (Figure 18C). Contingency analysis (Chi-square test) revealed the reduction in the number of W33L mutations in athymic or sham-operated mice relative to control mice to be significant ($p < 0.05$, $p < 0.0001$, respectively). Remarkably, manipulation of the thymus caused a significant reduction in the number of the W33L mutations compared to that number in athymic mice ($p < 0.0001$), suggesting that manipulation of the thymus without its removal compromises affinity maturation more seriously than its removal. Because the W33L mutation in the VH186.2 exon by itself causes a 10 fold increase on affinity to NP (Allen et al. 1988) the reduction in the frequency of the W33L mutation in athymic and in sham-operated mice indicates that the integrity of the thymus is necessary for the production of high affinity antibodies (Table 8).

DISCUSSION

Specific Aim 1, 2 and 3

Cell-mediated immunity is thought to depend on the number and the diversity of T cells available to respond to an antigenic challenge. Consistent with this concept, decreased numbers of T cells and/or contractions of the T cell repertoire are viewed as causing immunodeficiency (O'Keefe et al. 2004). Although decreases in the numbers of T cells are clearly associated with immunodeficiency, we found that diversity of T cells does not necessarily predict the capacity to mount effective cellular immunity. Rather, our results indicate that extensive and homogeneous contractions of the TCR repertoire (by over 100-fold) do not preclude normal T cell-mediated responses as measured by skin graft rejection, delayed-type hypersensitivity, and host defense.

The finding that individuals with dramatically contracted diversity of the T cell repertoire (but normal numbers of T cells) can have relatively normal cell-mediated immunity is not without precedent. We (Ogle, West, Driscoll, Strome, Razonable, Paya, Cascalho and Platt 2006) have found that human subjects who undergo cardiac transplantation in infancy have profound contraction of the TCR repertoire, with diversity of $V\beta$ being as low as 10^4 (compared with 10^6 in age matched controls), but do not suffer a heightened risk of disseminated viral infections or of infections with opportunistic organisms or tumors compared with other transplant recipients. In the course of cardiac transplantation (or nontransplant cardiac surgery) early in life, the thymus is removed and the recipient is treated with Thymoglobulin or anti-CD3 to deplete mature T cells. In addition, those patients subjected to thymectomy for nonimmune disease and those who are elderly (Goronzy and Weyand 2005; Ogle, West, Driscoll, Strome, Razonable, Paya, Cascalho and Platt 2006) do not suffer from the opportunistic infections seen in subjects with DiGeorge syndrome or AIDS, conditions associated with defects in TCR diversity (Killian et al. 2004). Clearly, a narrow but otherwise unperturbed repertoire of T cells can provide enough host defense for an ostensibly normal life. Adaptation of the T cell compartment following lymphopenia may in part be contributed by the adoption of "memory-like properties" (Goldrath, Bogatzki and Bevan 2000). We would postulate that subjects with DiGeorge syndrome or AIDS have impaired host defense not because of narrowing of the repertoire of T cells but rather

because of nonbalanced lacunae in the TCR repertoire or defects in T cell function that preclude adaptation to the depletion of T cells.

The work reported here takes advantage of mice in which contractions of the T cell repertoire are secondary to B cell deficiency, lack of B cells, or B cell oligoclonality. To exclude the possibility that our tests of T cell function reflected directly the properties of the B cell compartment, we measured T cell functions that are thought to be independent of B cells or Ig — the rejection of skin transplants (Cascalho and Platt 2001; Parker, Saadi, Lin, Holzkecht, Bustos and Platt 1996). Our results showing that QM mice reject MHC-incompatible skin as quickly as wild-type mice indicate that the T cells and the lymphoid tissue in these mice are functionally normal, although, because of the high frequency of alloreactive T cells (~3 % in C57BL/6 mice) (Huseby et al. 2005), these responses indicate little about the impact of repertoire contractions. In contrast, responses to conventional peptide Ags such as minor histocompatibility Ags or OVA, which are thought to depend on responses by relatively rare T cells expressing an Ag-specific TCR (Kanagawa, Louis and Cerottini 1982; Lindahl and Wilson 1977; Suchin, Langmuir, Palmer, Sayegh, Wells and Turka 2001), were normal in QM mice. These results indicated that that a balanced TCR repertoire contraction of at least 90 % does not impair cell-mediated immunity.

Our results showing that severe contractions of the TCR repertoire do not cause increased *Pneumocystis* load in the lungs of QM compared with wild-type mice are consistent with maintained host defense. Because the QM mice have very reduced B cell diversity, a case could be made for the lack of specific Abs in the outcome of infection. However, B cell and Ab deficiency is thought to cause increased susceptibility to infections by these organisms rather than relative resistance. In an exemplary study, Marcotte et al. (Marcotte et al. 1996) reported an outbreak of *Pneumocystis carinii* in μ MT mice that have very few B cells owing to a gene-targeted deletion of the membrane exon of IgM (Kitamura et al. 1991). The authors interpreted these results to indicate that B cells and Abs are important to the clearance of *P. carinii*. Because QM mice can produce Abs against a variety of pathogens (Lopez-Macias et al. 1999) and did produce *Pneumocystis*-specific Abs (results not shown), our studies were not limited by deficient humoral responses.

Our studies raise the possibility that normal host defense might be restored in some conditions by promoting the "homeostatic" proliferation of certain clones even if that adaptation does not reconstitute repertoire diversity. The T cells resulting from homeostatic proliferation exhibit the phenotype and some functions of bona fide memory T cells (Bourgeois et al. 2005; Cho et al. 2000) and might account for the robust cellular immune responses and host defense in QM mice and in subjects thymectomized in infancy (Ogle, West, Driscoll, Strome, Razonable, Paya, Cascalho and Platt 2006).

It is not necessary to invoke memory as the only mechanism of compensation. In favor of changes other than memory is the finding that the memory T cells resulting from homeostatic proliferation may not survive as long as bona fide memory T cells generated following specific Ag stimulation (Bourgeois, Kassiotis and Stockinger 2005) and, thus, be most important when the generation of the latter is compromised (Bourgeois, Kassiotis and Stockinger 2005). Memory-like T cells may thus work best in infancy before Ag-specific memory T cells have a chance to develop (Bourgeois, Kassiotis and Stockinger 2005) and in conditions of B cell deficiency that may cause defective Ag-specific T cell memory formation. The normal functioning of the T cell compartment in humans and mice with contracted T cell repertoires might also reflect the cross-reactivity of TCR or a lesser dependence on peptide specificity than is commonly thought. Because memory T cells are functionally more cross-reactive, one may not be able distinguish the two. Regardless of what mechanism is eventually proved, our findings may inspire the design of therapies aiming at the reconstitution of cellular immunity.

Specific aim 4 and 5

Our results show that the thymus contributes to priming T cell responses (as expected), and to affinity maturation of antibodies. Surprisingly, in spite of compromised selection of B cells bearing high affinity B cell receptors, production of long lived antibody secreting cells is not defective in athymic or sham-operated mice. In fact, our results suggest the possibility that the thymus may inhibit the generation and/or maintenance of long-lived antibody secreting cells. Since removal of the thymus did not critically contract the T cell receptor diversity or decrease the number of T cells in a substantive manner, these results suggest that affinity maturation of antibodies is critically dependent on the integrity of the thymus.

Recent studies support the idea that T cell help and the B cell receptor (BCR) strength determine B cell fate in response to T-dependent antigen activation. Thus Paus et al. (Paus et al. 2006) and Phan et al. (Phan et al. 2006) suggested that high BCR affinity for antigen dictates differentiation to extra-follicular antibody secreting cells causing primary antibody responses. O'Connor et al. (O'Connor et al. 2006) proposed that B cells with a low affinity BCR typically undergo somatic hypermutation, while B cells with BCR with moderate affinity for antigen produced mostly long-lived antibody secreting cells. BCR affinity and T cell help are interdependent since B cells present antigens to T cells following Ig-dependent internalization (Lanzavecchia 1990). Thus B cells that have a competitive advantage to bind antigen owing to higher affinity receptors may also better compete for limiting T cell "help" which in turn may determine their fate. The interdependence between BCR affinity and B cell antigen presentation to T cells has made it difficult to dissociate the contributions of each to B cell selection and differentiation. In a "tour de force", Victora et al. (Elgueta, de Vries and Noelle 2010) showed that enhancing antigen B cell presentation without engaging the BCR promoted migration of B cells from the light zone to the dark zone of the germinal center, clonal expansion and plasmablast differentiation. These authors concluded that T cell help limits expansion and differentiation of B cells in the germinal center independently of BCR engagement. However, enhancing B cell antigen presentation by germinal center B cells did not induce antibody affinity maturation, suggesting that the combined signals provided by BCR ligation and engagement of T cells determine the B cell fate.

Our results showing normal or enhanced antibody responses to protein antigens suggested that T cell help in athymic or sham-operated mice is adequate to activate and promote differentiation of antibody secreting cells short- and long-term. However since removal or manipulation of the thymus compromised affinity maturation of antibodies our results suggest that disruption of thymic integrity selectively impairs affinity maturation of antibodies much in the same way as enhancing antigen presentation independently of the BCR as reported by Victora et al.(Victora et al. 2010). Because removal of the thymus interrupts the flux of new T cells we considered the possibility that the availability of cognate help may be reduced to a greater extent than non-cognate help, enhancing BCR-independent antigen presentation which in turn would impair selection of high affinity B cells. We propose that absence of optimal cognate T cell help owing to interruption of thymic emigration or following manipulation of the thymus abrogates competition for B cells expressing B cell receptors with high affinity for antigen randomizing differentiation and apoptosis. Other functions of the thymus such as production of IL7 or production of regulatory T cells could also contribute to the regulation of immunity. We observed that removal of the thymus impairs production or maintenance of T regulatory cells (Figure 11). Whether or not decreased production of T regulatory cells in athymic mice contributes to defective affinity maturation of antibodies in these mice is not known and this question will be an interesting one to resolve.

Our findings concur with those of Ahuja et al. (Ahuja et al. 2008) who proposed that the long-lived antibody secreting cell compartment is maintained independently of the memory B cell compartment because it does not decline when memory B cells are abrogated. Our results indicate that differentiation of long-lived antibody secreting cells occurs independently of affinity maturation that normally accompanies B cell memory responses. Our work suggests that strategies to immunize individuals with congenital or acquired thymic defects (such as following cardiac transplantation or cardiac surgery in infancy), or with contracted T cell repertoires (such as in aging or after T cell depletion to treat cancer) would benefit from new vaccine designs including surrogates of cognate T cell help.

FIGURES

FIGURE 1.

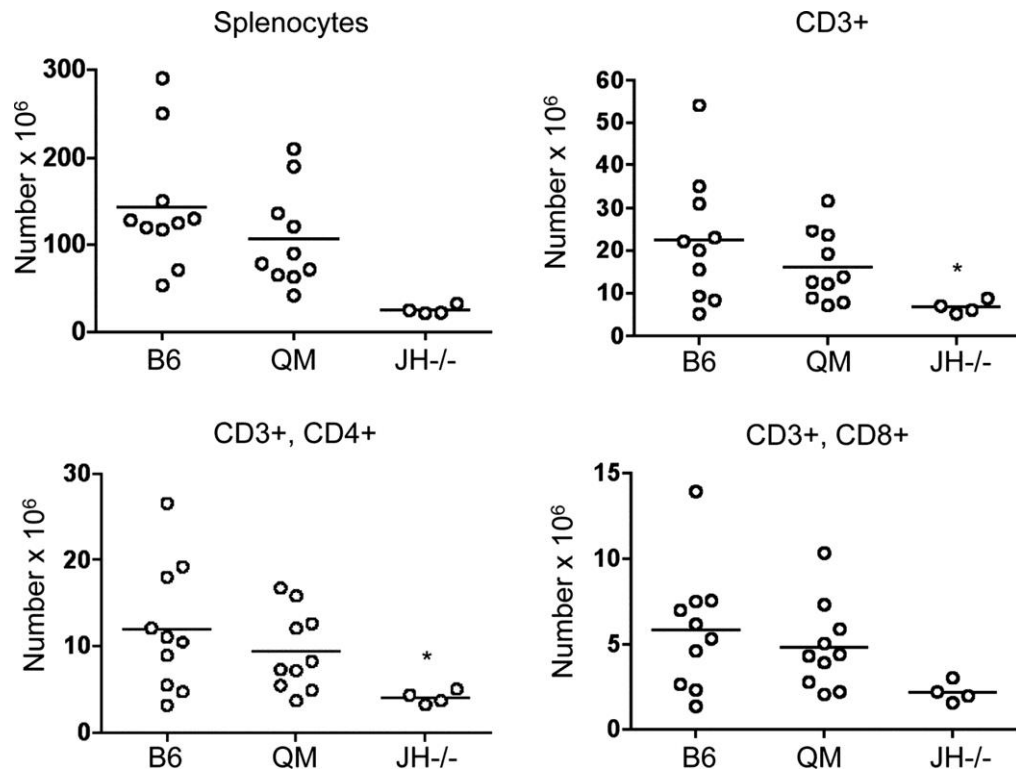


Figure 1. Numbers and TCR V β diversity of splenocytes in C57BL/6, QM, and JH^{-/-} mice. The numbers of splenocytes were calculated by multiplying the respective percentage of the total events as defined in the flow cytometry dot plot analysis with specific CD4, CD8, and CD19 mAbs by the total number of white blood cells obtained by counting using a Neubauer counting chamber. The number of total splenocytes (average \pm SD) was $1.4 \times 10^8 \pm 7.3 \times 10^7$ in C57BL/6, $1.1 \times 10^8 \pm 5.6 \times 10^7$ in QM, and $2.7 \times 10^7 \pm 4.3 \times 10^7$ in JH^{-/-} mice. The number of CD3⁺ splenocytes (average \pm SD) was $2.2 \times 10^7 \pm 1.5 \times 10^7$ in C57BL/6, $1.6 \times 10^7 \pm 8.3 \times 10^7$ in QM, and $6.7 \times 10^6 \pm 1.5 \times 10^6$ in JH^{-/-} mice. The number of CD4⁺ splenocytes (average \pm SD) was $1.2 \times 10^7 \pm 7.4 \times 10^6$ in C57BL/6, $9.4 \times 10^6 \pm 4.6 \times 10^6$ in QM, and $4.1 \times 10^6 \pm 7.6 \times 10^5$ in JH^{-/-} mice. The number of CD8⁺ splenocytes (average \pm SD) was $5.8 \times 10^6 \pm 3.6 \times 10^6$ in C57BL/6, $4.8 \times 10^6 \pm 2.5 \times 10^6$ in QM, and $2.2 \times 10^6 \pm 6.1 \times 10^5$ in JH^{-/-} mice. Asterisks mark statistically significant differences compared to wild type. Mice were between 13 and 15 wk of age.

FIGURE 2.

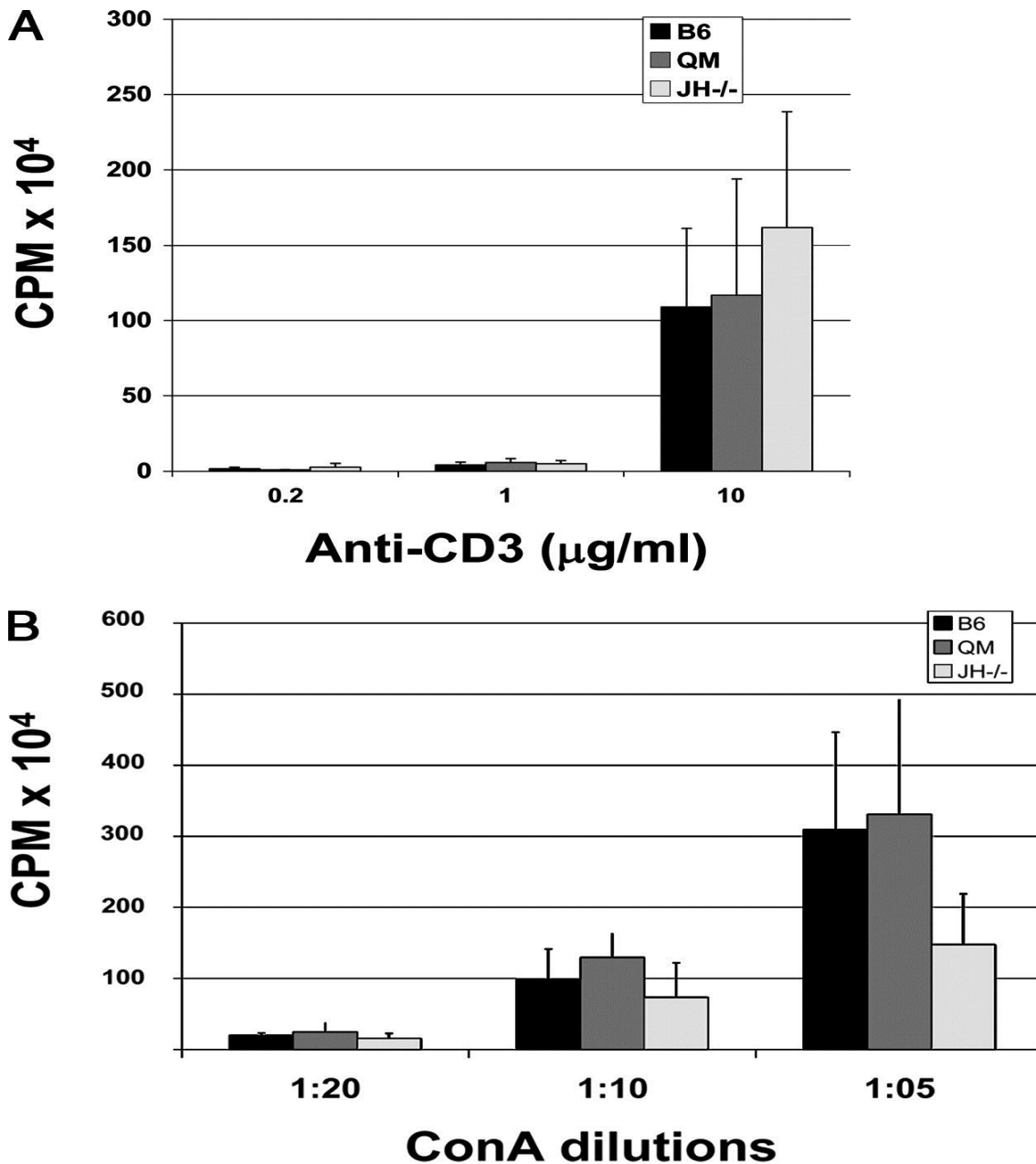


Figure 2. T cell proliferation assays. CD4^+ T cells isolated from unmanipulated C57BL/6 (B6), QM, or $\text{JH}^{-/-}$ mice were cultured on plates coated with anti-CD3 Ab in the concentrations indicated (*x*-axis) and with soluble anti-CD28 Ab (10 $\mu\text{g/ml}$) (A) or with Con A diluted as indicated (*x*-axis) (B). Proliferation measured at 72 h of culture by (^3H) thymidine incorporation is depicted in counts per minute in the *y*-axis.

FIGURE 3.

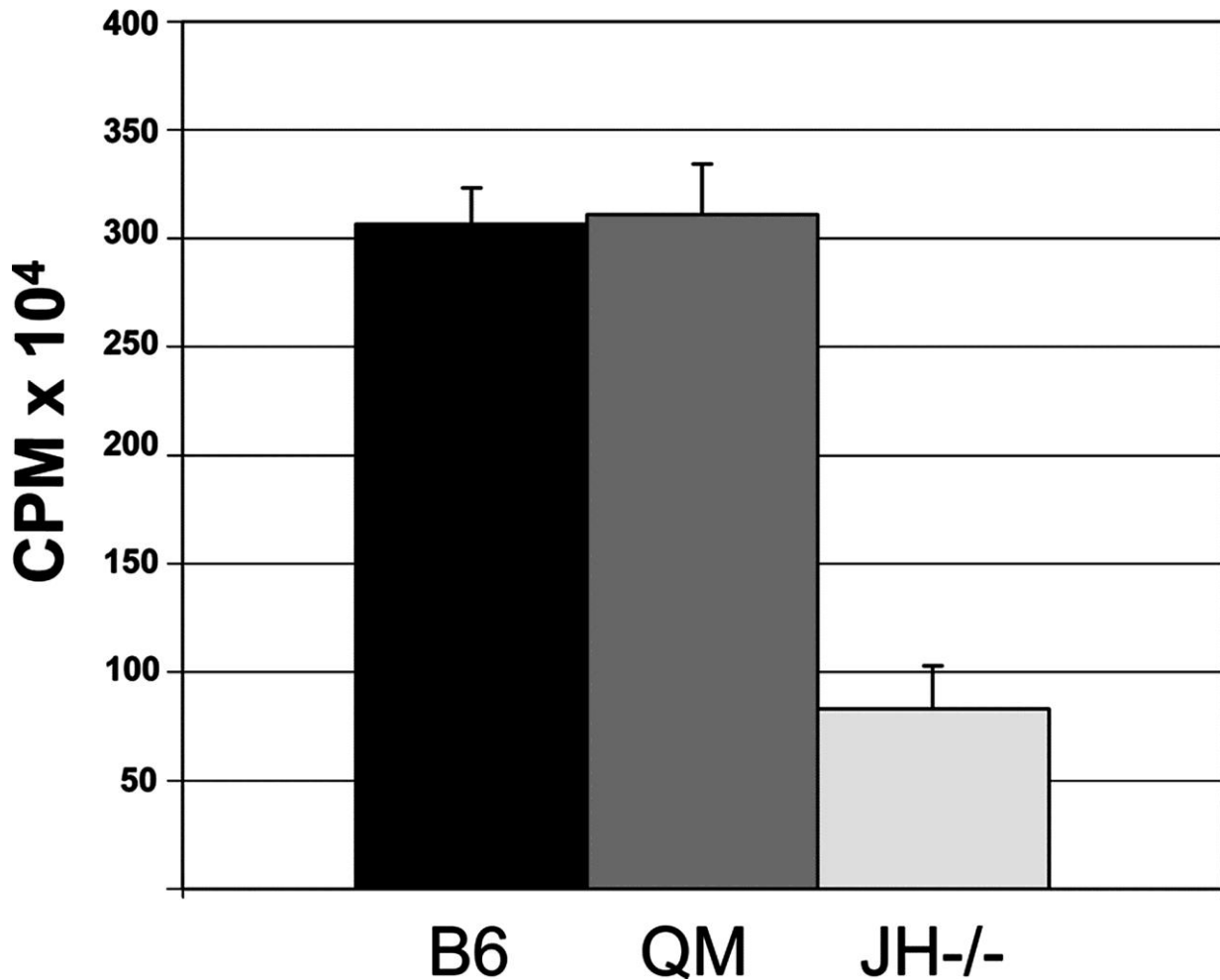


Figure 3. Proliferation assay of in vivo primed T cells. Mice were injected with a 140- μ g PADRE peptide. Fourteen days later, CD4⁺ T cells purified from the spleen were cocultured with mature dendritic cells in the presence of 35 μ g/ml PADRE peptide. QM and B6 T cells mounted robust proliferation to the PADRE peptide, JH^{-/-} T cells had reduced proliferation (26 % of QM or 27 % of B6). Proliferation measured at 72 h of culture by (³H) thymidine incorporation is depicted in counts per minute in the y-axis.

FIGURE 4.

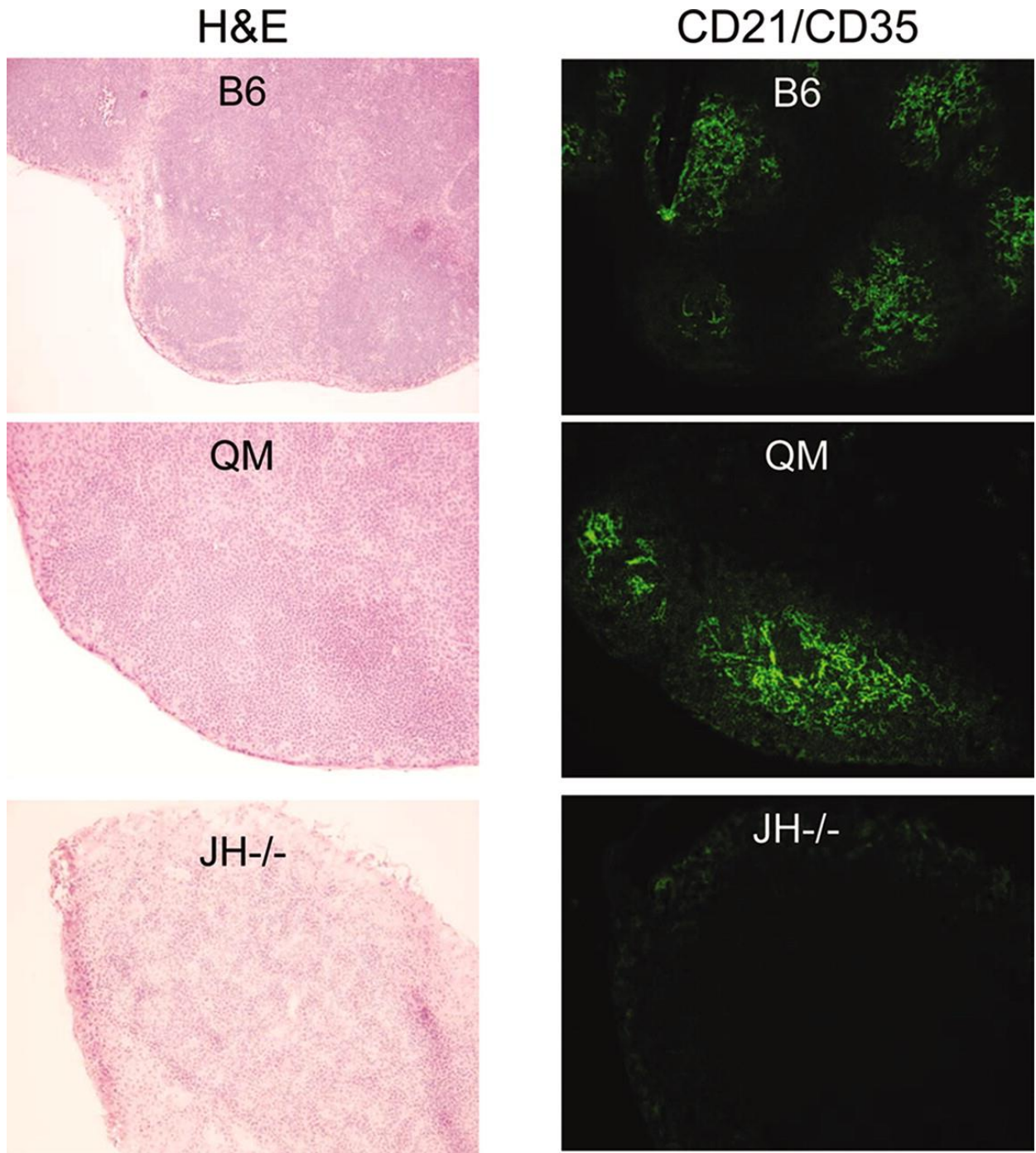


Figure 4: Follicular dendritic cell network in lymph nodes. Follicular dendritic cells were stained bright fluorescent green and were detected in cryostat sections of lymph nodes stained with mAb directed against murine CD21/CD35. H & E-stained sections are shown. Photographs are representative of three to four different mice per genotype analyzed.

FIGURE 5.

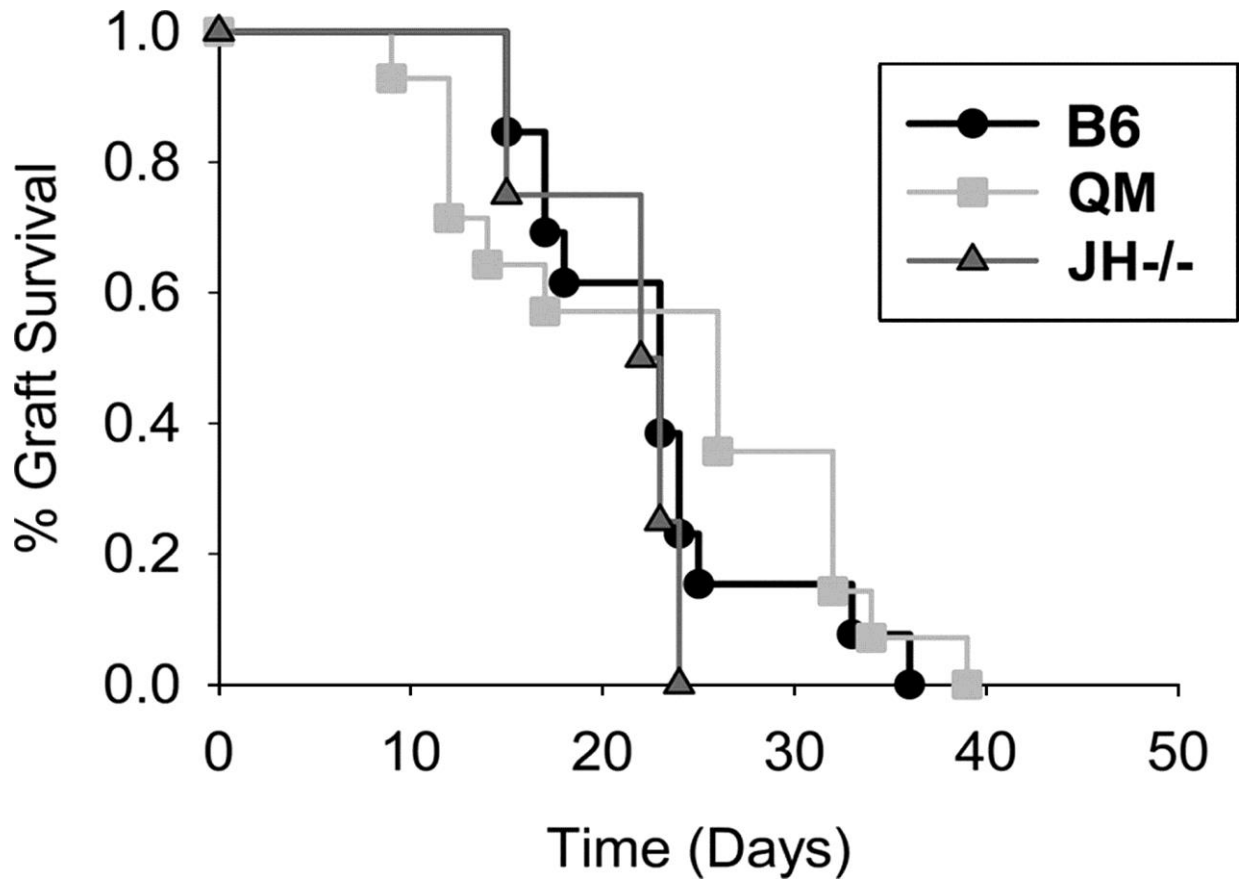


Figure 5: Kaplan Meier survival curves for H-Y-incompatible skin grafts in C57BL/6 (B6), QM, and JH^{-/-} mice. The *x*-axis depicts days following surgery and the *y*-axis depicts the skin graft survival fraction values. Grafts were considered rejected when 90 % or more of the graft lacked any viable signs, i.e. hair, pigment, and scale pattern. The median time of rejection was 23 days in C57BL/6, 26 days in QM, and 23 days in JH^{-/-} mice. Statistical analysis was done with a log-rank test.

FIGURE 6.

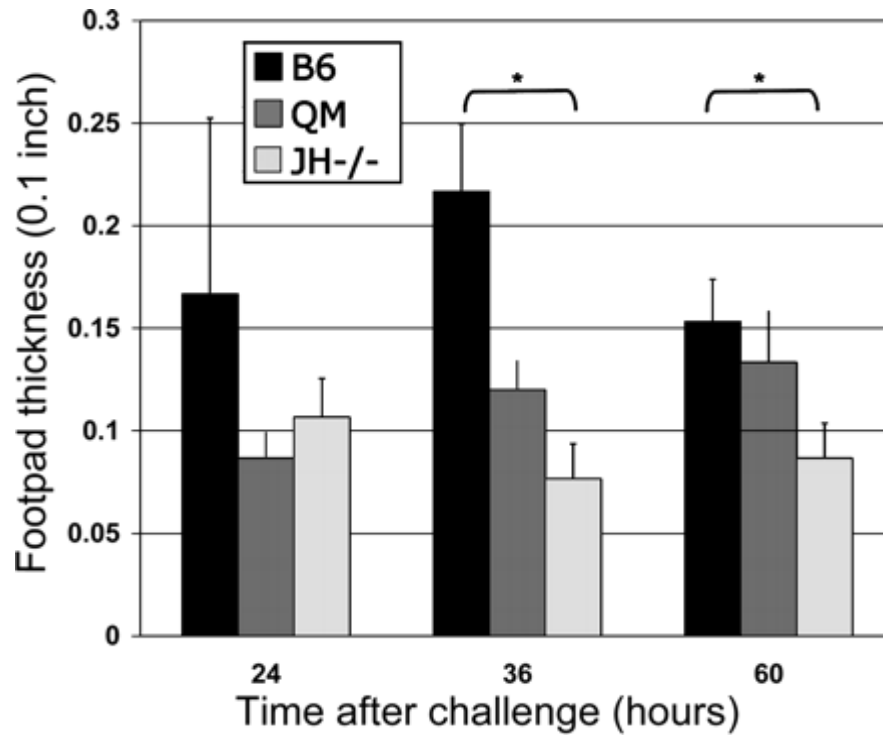


Figure 6: Delayed-type hypersensitivity assay. We compared delayed-type hypersensitivity responses in QM and C57BL/6 (B6) mice. Delayed-type hypersensitivity responses to OVA were examined at 24, 36, and 60 h after challenge with 20 μ g of OVA (intradermally) in the footpads of mice primed 6 days earlier with 100 μ g of OVA (s.c). The bars represent the mean footpad thickness measured in primed mice minus the mean footpad thickness measured in mice injected with PBS and the SD values of the mean; asterisks indicate statistically significant differences (*, $p < 0.05$).

FIGURE 7.

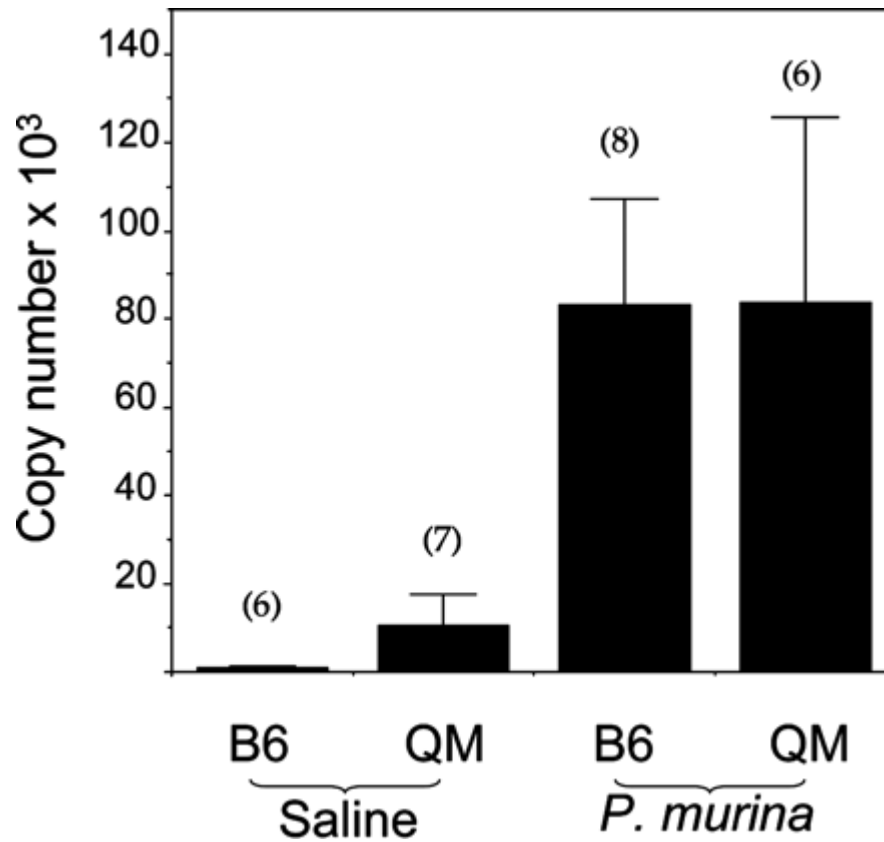


Figure 7: *P. murina* organismal burden. *Pneumocystis murina* was isolated from the lungs of heavily infected, SCID mice as previously described (Keely, Fischer, Cushion and Stringer 2004). Inoculation was as described by Shellito et al. (Shellito, Suzara, Blumenfeld, Beck, Steger and Ermak 1990). The number of *P. murina* genomes was determined by real time RT-PCR in lung tissue of mice infected 2 wk earlier or of mice treated with PBS. Quantitative RT-PCR to enumerate *Pneumocystis* was performed using the Bio-Rad iCycler system, SYBR Green detection software, and primers targeting the *Pneumocystis* large mitochondrial subunit (Shellito, Suzara, Blumenfeld, Beck, Steger and Ermak 1990). Amplifications of unknown samples were compared with plasmid standards containing mouse *Pneumocystis*-specific mitochondrial DNA. The bars represent the SD values of the mean and the numbers above refer to the number of mice examined per group.

FIGURE 8.

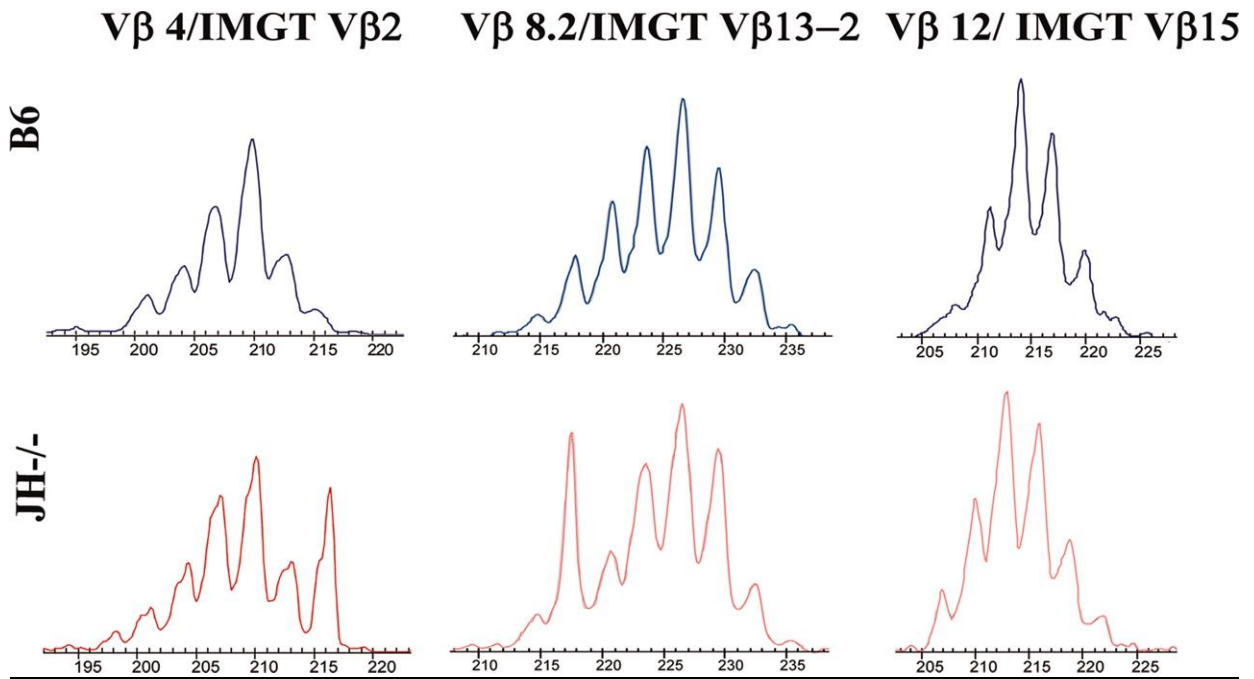


Figure 8: Spectratyping analysis of the V β repertoire of C57BL/6 and JH^{-/-} splenocytes. Shown are the spectra of CDR3 length distribution corresponding to the families V β 4, V β 8.2, and V β 12, which differed the most in C57BL/6 and JH^{-/-} mice.

FIGURE 9.

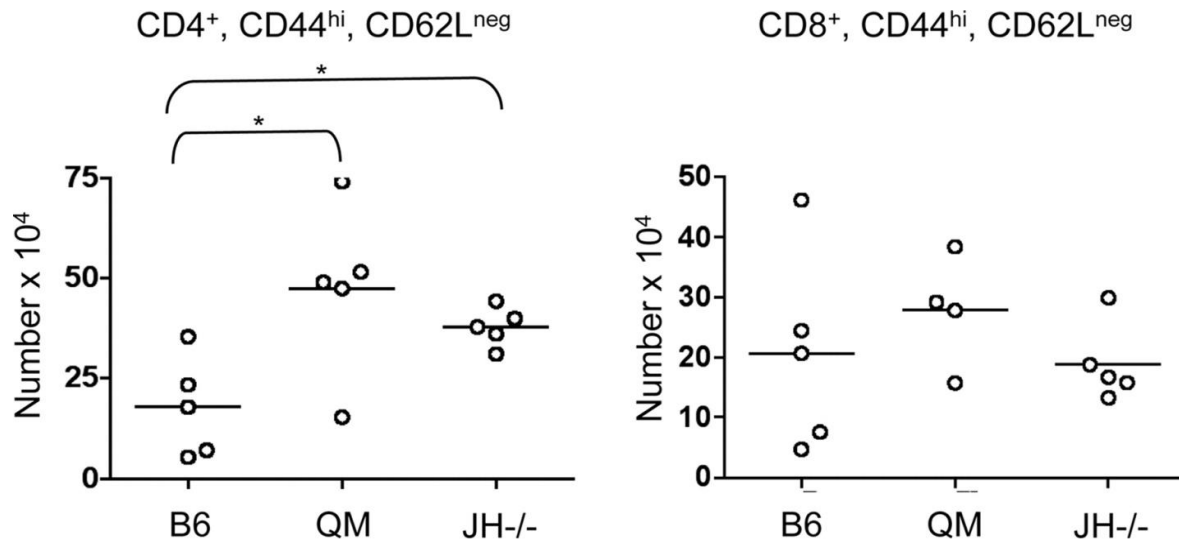


Figure 9: Numbers of "memory-like" CD4-positive or CD8-positive T cells in the spleens of C57BL/6 (B6), QM or JH^{-/-} mice. Memory CD4⁺ or CD8⁺ T cells were defined as CD4⁺/CD44^{high}/CD62L⁻ by FACS analysis. In the graph the height of the bar represents the average of each distribution. All of the mice were between 13 and 15 wk of age. Significant differences are noted with asterisks.

FIGURE 10.

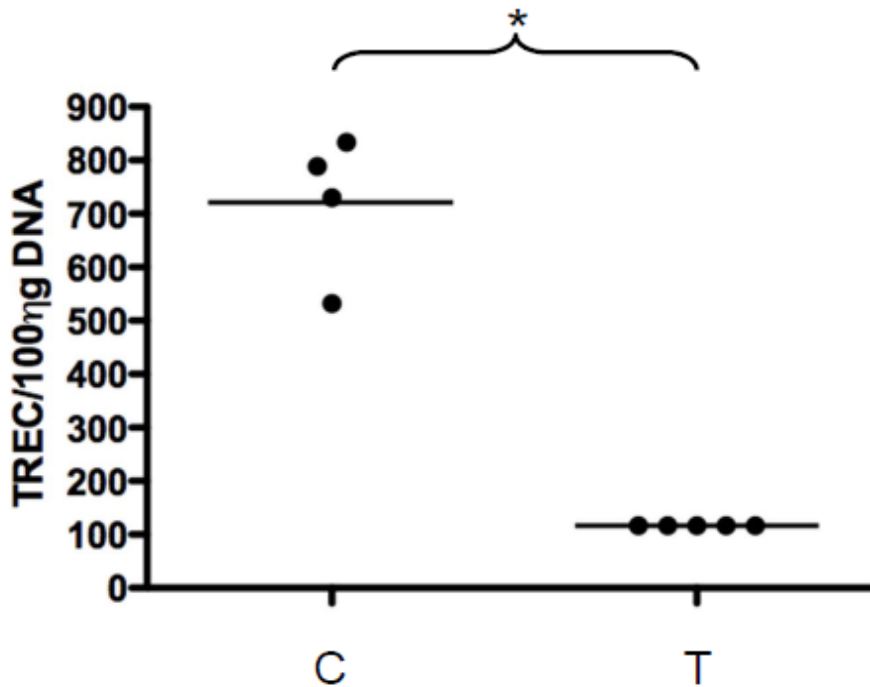


Figure 10: Measurement of T cell receptor (TCR) excision circles (TREC). Figure depicts the number of TRECs per 100 nanogram (ng) of genomic DNA in mice lacking the thymus (T), 5 weeks after surgery, and in age matched controls (C) by real time PCR. Control mice had an average of 721 TRECs per 100 ng of DNA while mice lacking the thymus had less than 117 per 100 ng DNA which is the detection limit of the assay. The two groups differed significantly by paired T test analysis ($p=0.0028$, two-tailed).

FIGURE 11.

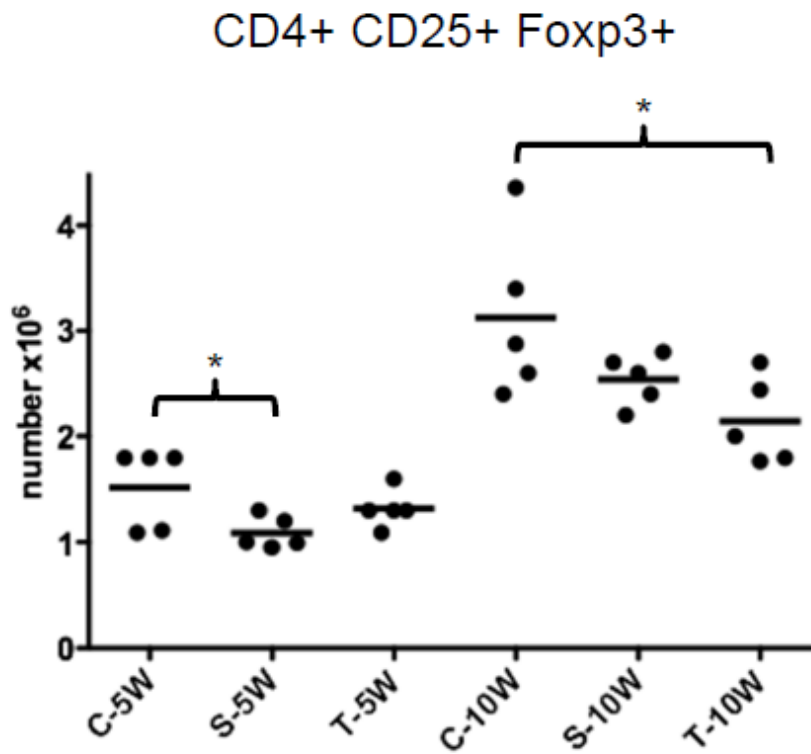


Figure 11: Number of CD4+, CD25+, Foxp3+ (Treg) cells in the spleens of athymic (T), sham-operated (S) or non-manipulated control (C) mice, 5 weeks and 10 weeks after surgery.

Numbers were calculated by multiplying the respective percentage as defined in a flow cytometry dot plot analysis with specific CD4, CD25 and Foxp3 monoclonal antibodies, by the total number of white blood cells (WBC). Groups compared by T test analysis. Statistically significant differences were denoted by an asterisk and indicate $P < 0.05$.

FIGURE 12.

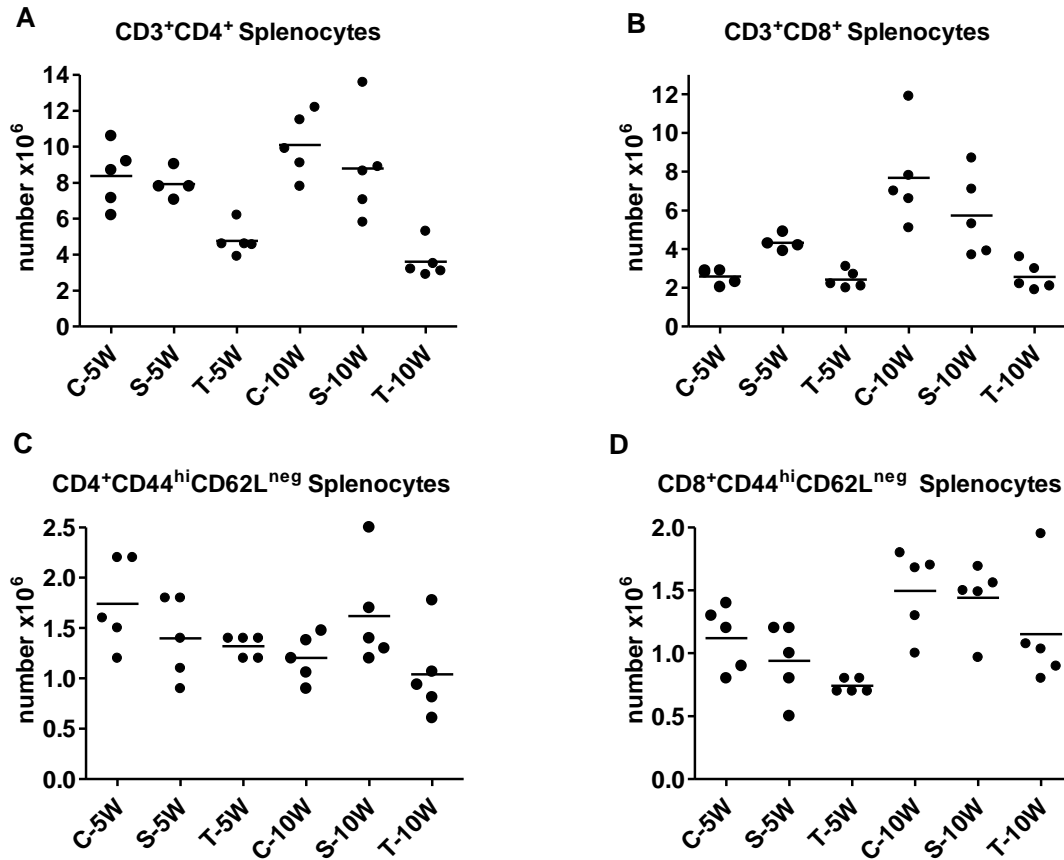


Figure 12: Number of lymphocytes in spleens harvested from mice lacking the thymus, sham-operated or non-manipulated controls at 5 and at 10 weeks of age. Thymectomies were performed 2 days before the mice turned 5 weeks old. Numbers of lymphocytes were calculated by multiplying the respective percentage as defined in a flow cytometry dot plot analysis, with specific monoclonal antibodies, by the total number of white blood cells (WBC) in athymic (T), sham-operated (S) or unmanipulated control (C) mice at 5 and 10 weeks (W) after surgery. **(A)** Number of CD4⁺ splenocytes or, **(B)** Number of CD8⁺ splenocytes. **(C, D)** Number of memory-like T cells in spleens defined as (CD4⁺ or CD8⁺)/CD44^{hi}/CD62L⁻ by FACS analysis. In the graphs, the bar represents the average of each distribution. Means were compared by a paired two-tailed T test. Statistically significant differences are denoted by an asterisk and indicate P < 0.05.

FIGURE 13.

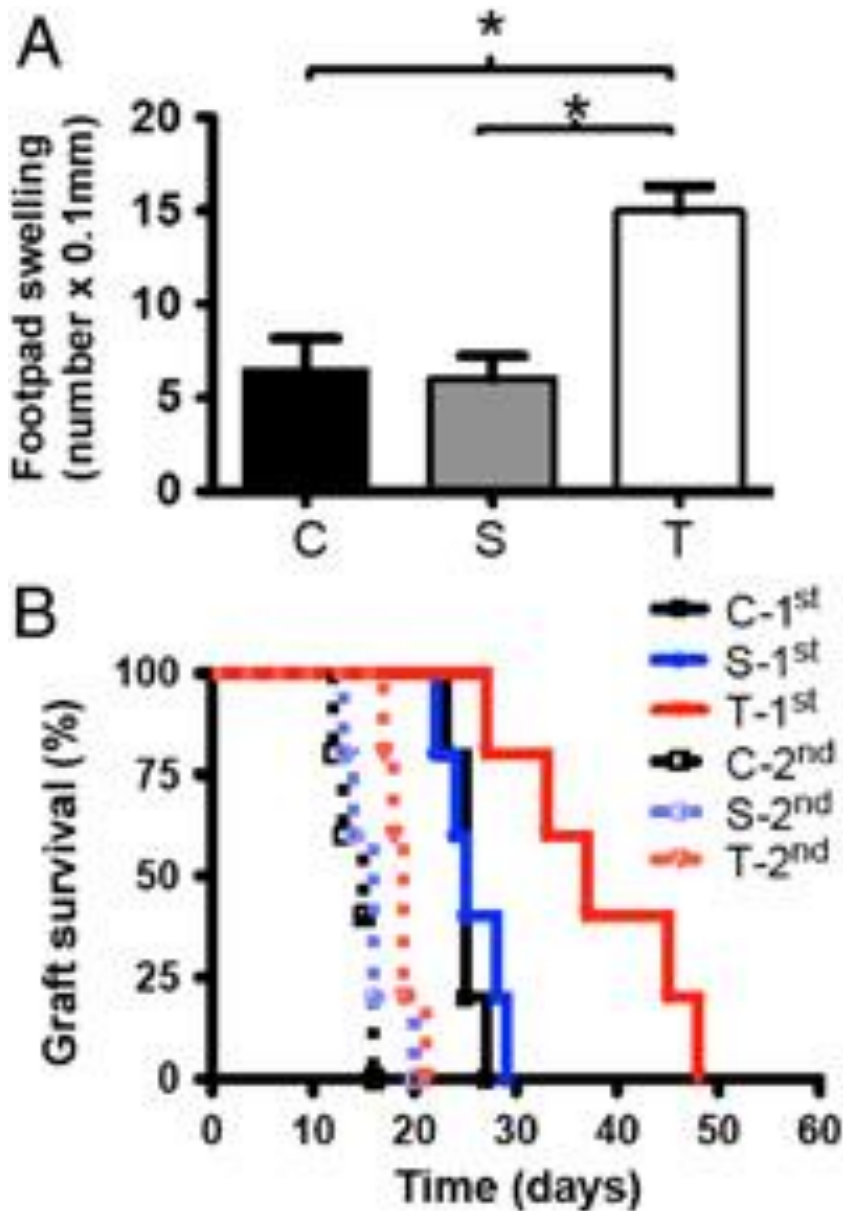


Figure 13: Primary cellular immune responses are delayed in mice lacking the thymus but T cell memory responses are maintained. Affinity maturation of antibodies requires integrity of the adult thymus (A) Delayed Type Hypersensitivity (DTH) responses to ovalbumin in control (C), sham-operated (S) or athymic (T) mice. DTH responses to intradermic injection of 20 μ g ovalbumin were examined in the footpad of mice 6 days after priming by subcutaneous injection with 100 μ g of ovalbumin (priming) or PBS (control). Footpad swelling

measured in mm is indicated on the y-axis. Mice lacking the thymus produced significantly larger swelling (15 mm, on average) in response to challenge than sham-operated mice (6.0 mm, on average) or control mice (6.5 mm, on average). Footpad swelling was compared by a paired two-tailed T test.

(B) Kaplan Meier survival curves for H-Y incompatible skin grafts in athymic (T), sham-operated (S) or control (C) mice. X-axis, days following surgery; y-axis, skin graft survival fraction. Grafts were considered rejected when 90 % or more of the graft lacked any viable signs: hair, pigment and scale pattern. The median survival time of first set grafts was 25 days in control mice, 25 days in sham-operated mice and 37 days in mice lacking the thymus. Skin graft rejection by athymic mice was significantly delayed compared to rejection in controls ($p=0.0052$, log-rank, Mantel-Cox test). Secondary transplants were done 8 to 12 weeks after rejection of the first transplant. The median survival time of initial transplants was 15 days in control mice, 16 days in sham-operated mice and 19 days in athymic mice. Secondary graft survival in athymic recipients did not significantly differ from graft survival in control or sham-operated recipients.

FIGURE 14.

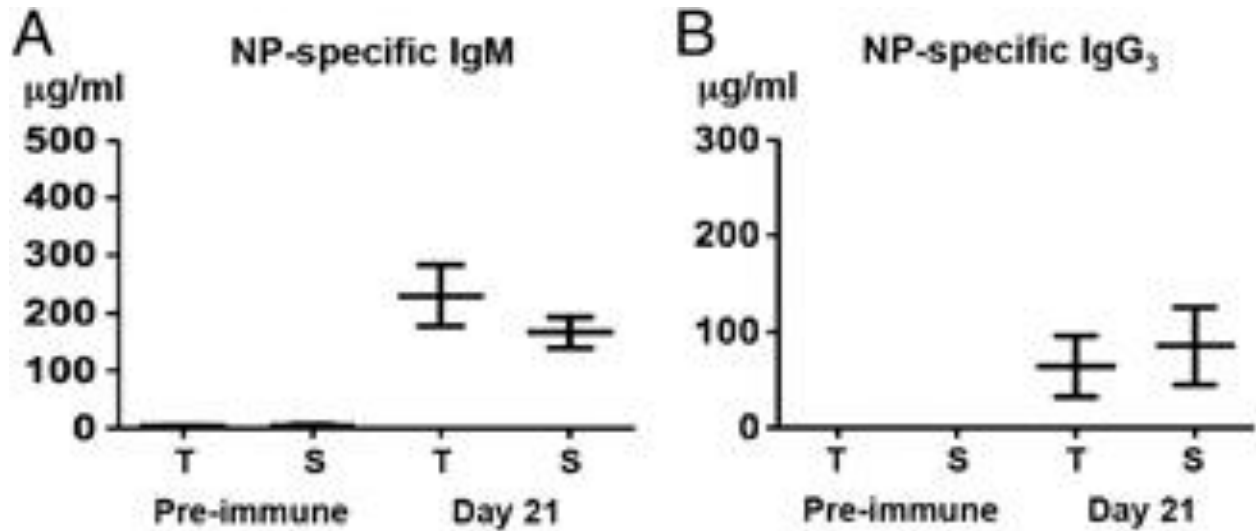


Figure 14: T-independent antibody responses maintained in athymic mice. (A, B) T-independent antibody responses to NP-Ficoll in athymic mice (T) or in sham-operated (S) mice. Figures 3A and 3B represent the concentrations of NP-specific IgM (A) or NP-specific IgG3 (B), in µg/ml (y-axis) prior to and 21 days after immunization. Mice lacking the thymus and sham-operated mice had on average 4.0 µg/ml and 5.0 µg/ml NP-specific IgM respectively, and non-detectable NP-specific IgG3, prior to immunization. Athymic and sham-operated mice had 230µg/ml and 167µg/ml NP-specific IgM, on average, 21 days after immunization, respectively. Mice lacking the thymus and sham-operated mice had on average 64µg/ml and 86 µg/ml NP-specific IgG3, 21 days after immunization, respectively. The concentrations of NP-specific IgM or IgG3 in athymic mice and in sham-operated mice did not significantly differ.

FIGURE 15.

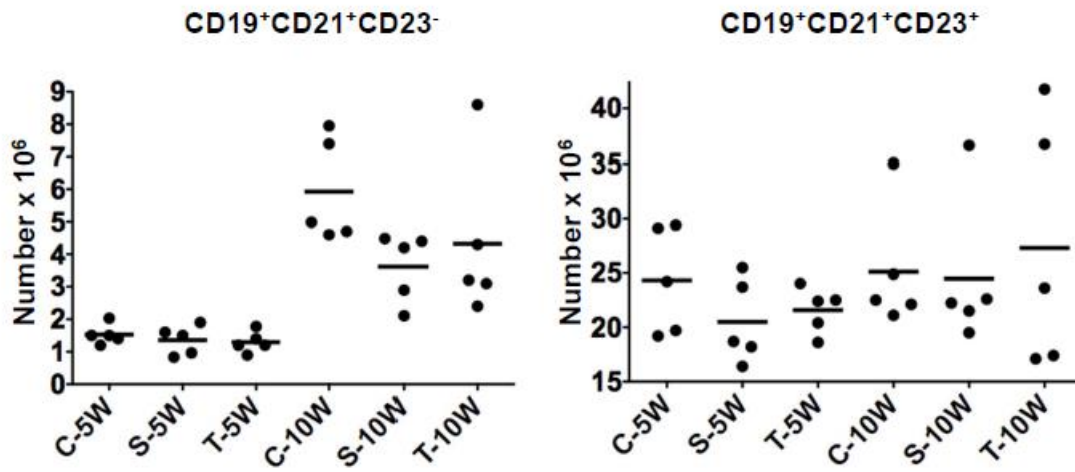


Figure 15: Number of B cells in the spleens of athymic (T), sham-operated (S) or non-manipulated control (C) mice, 5 weeks and 10 weeks after surgery.

Numbers were calculated by multiplying the respective percentage as defined in a flow cytometry dot plot analysis with specific CD19, CD21 and CD23 monoclonal antibodies, by the total number of white blood cells (WBC). No significant differences were found between groups of similar age.

FIGURE 16.

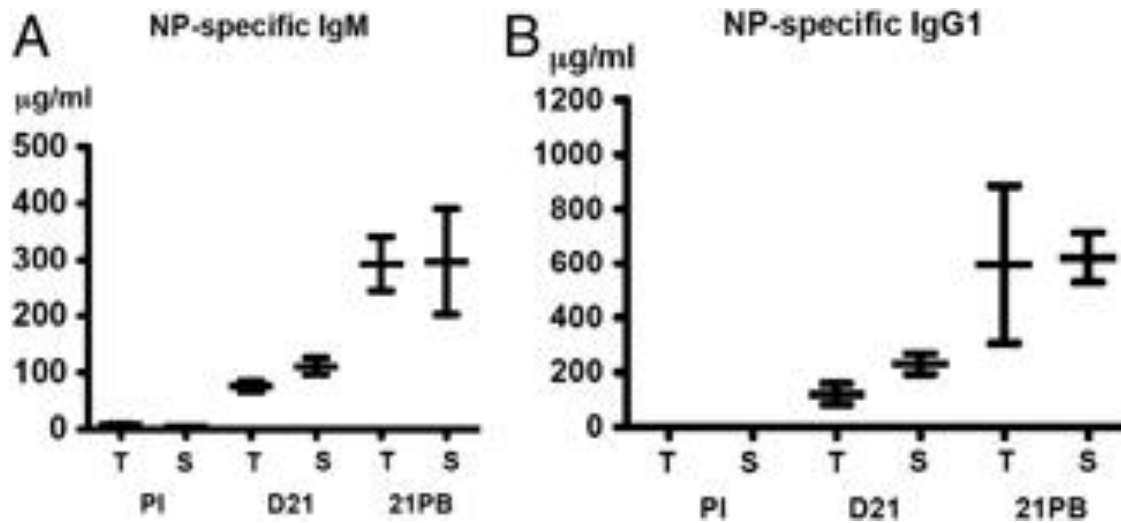


Figure 16: Removal of the thymus or sham operation maintained IgG1 specific antibody responses (A, B) T-dependent responses to NP-ovalbumin. Figures 4A and 4B represent the concentrations of NP-specific IgM or NP-specific IgG1, in µg/ml (y-axis) prior to (PI) and the 21 days after primary (D21) or booster immunization (21PB), respectively. **(A)** Athymic mice (T) and sham-operated mice (S) had an average of 8.4µg/ml and 4.4µg/ml NP-specific IgM, prior to immunization (PI), 76 µg/ml and 111µg/ml, 21 days after immunization (D21), 293 µg/ml and 296 µg/ml 21 days after boosting (21PB), respectively. There were no significant differences between athymic and sham-operated mice. **(B)** Mice lacking the thymus or sham-operated mice had no detectable NP-specific IgG1 prior to immunization, but produced on average, 121µg/ml and 231µg/ml NP-specific IgG1 21 days after immunization, 596 µg/ml and 622 µg/ml 21 days after boosting, respectively. T test analysis revealed no significant differences between athymic and sham-operated mice.

FIGURE 17.

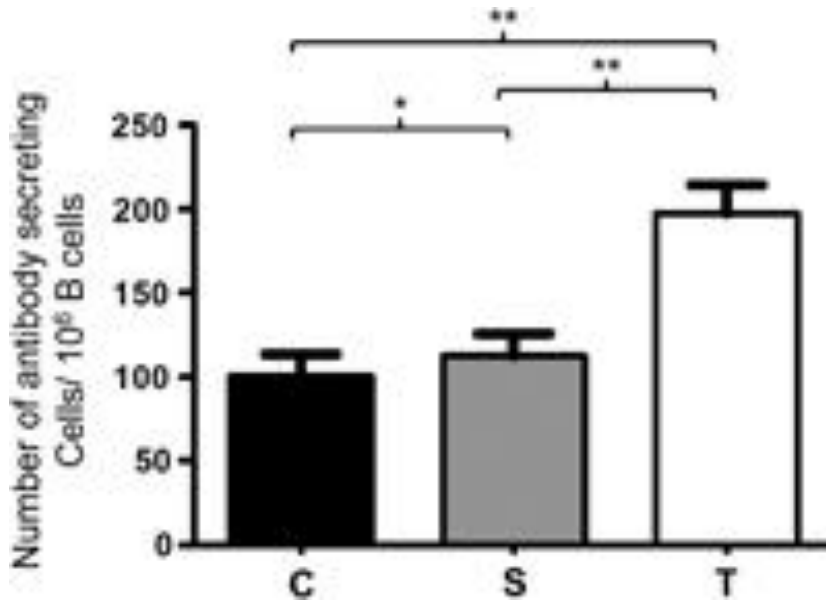


Figure 17: Number of NP-specific IgG1 antibody secreting cells (ASC) in the bone marrow of mice lacking the thymus or control mice, 6 months after boost immunization. Mice lacking the thymus (T) had on average 198 ASC per 10^6 B cells while sham-operated mice (S) had an average of 113 ASC per 10^6 B cells and, control mice (C) had an average of 100 ASC per 10^6 B cells NP-specific IgG1 antibody secreting cells in the bone marrow. The number of ASC in athymic mice was significantly increased compared to the number of ASC in control (P=0.0042) or sham-operated mice (P=0.0075) (unpaired T test). The number of ASCs was calculated from 4 mice per group.

FIGURE 18.

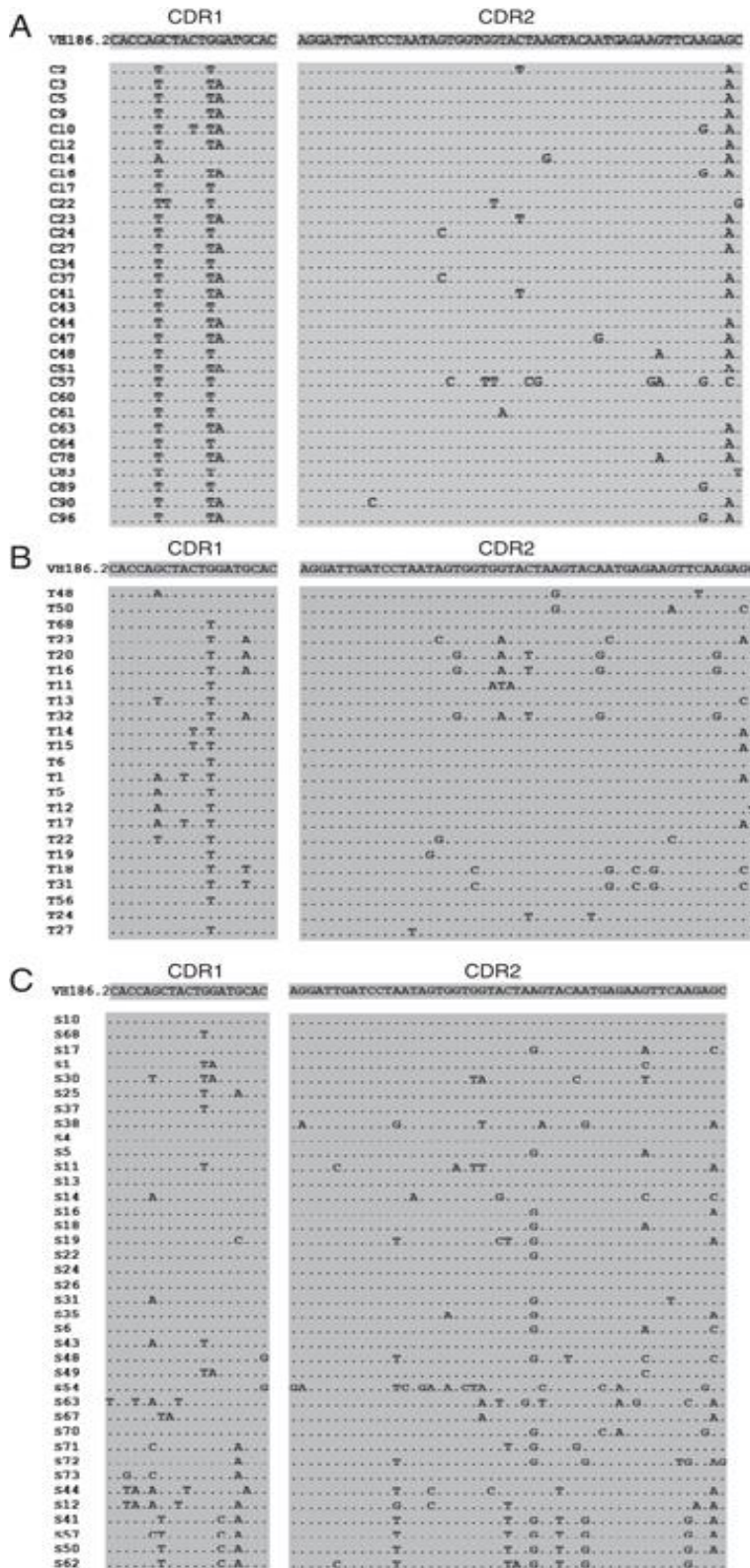


Figure 18: Heavy chain VH CDR1 and CDR 2 DNA sequences of IgG1-B cells obtained from the spleen of mice lacking the thymus (T), sham-operated (S) or control (C) mice, 10 days following boost immunization.

Figures show the CDR1 or CDR2 sequences of all the distinct VH sequences aligned to the germline VH186.2 segments. Shaded are CDR1 and CDR2 regions.

- (A) Sequences obtained from control non-manipulated mice;
- (B) Sequences obtained from athymic mice;
- (C) Sequences obtained from sham-operated mice.

FIGURE 19.

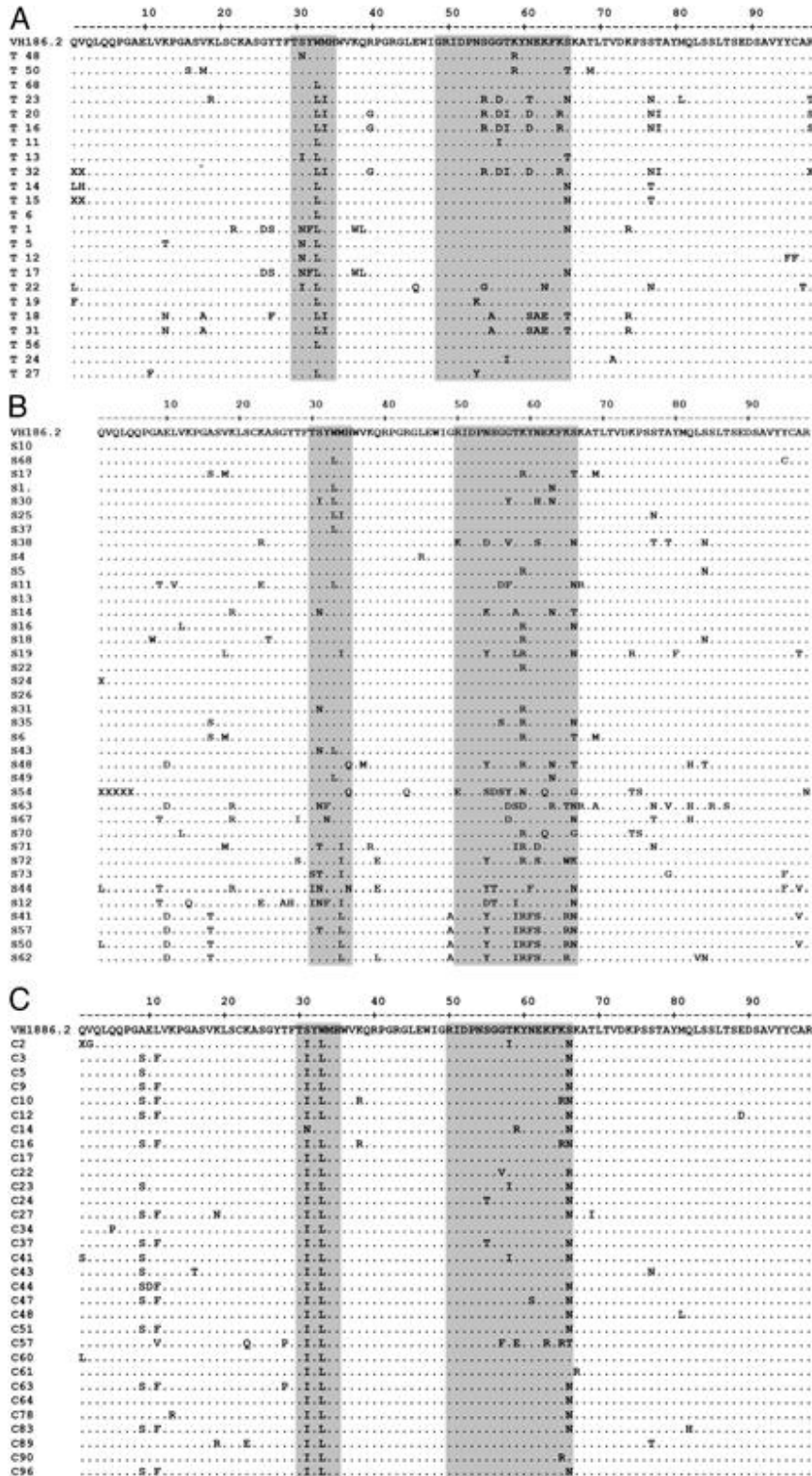


Figure 19: Heavy chain VH amino-acid sequences of IgG1-B cells obtained from the spleen of mice lacking the thymus (A), sham-operated (B) or control (C) mice, 10 days following boost immunization.

Figures show the aligned translation of all the distinct VH186.2 segments obtained from each group of mice. Shaded are CDR1 and CDR2 regions and in a darker grey shade residue #33 is indicated. Antibodies with high affinity to NP often encode a W to L mutation in this position.

TABLES

Table 1. *Median rejection times for H-Y- and MHC-incompatible mice*

	H-Y-Incompatible Mice Median Rejection Times	MHC-Incompatible Mice Median Rejection Times
C57BL/6	23 (<i>n</i> = 12; 15, 15, 17, 17, 18, 23, 23, 23, 24, 25, 33, 36)	13 (<i>n</i> = 3; 13, 13, 13)
QM	26 (<i>n</i> = 13; 12, 12, 12, 14, 17, 26, 26, 26, 32, 32, 32, 34, 39)	11 (<i>n</i> = 4; 11, 11, 12, 13)
JH ^{-/-}	23 (<i>n</i> = 15, 22, 23, 24)	Not done

Table 2. *Median rejection times for first and second transplants*

	First Transplant Median Rejection Times (days)	Second Transplant Median Rejection Times (days)
C57BL/6	17	13
C57BL/6	24	15
C57BL/6	24	15
QM	32	13
QM	32	16
QM	32	20
QM	26	15
QM	26	16
QM	26	18

Table 3. Sequences obtained by PCR amplification of cDNA obtained from one C57BL/6 Spleen. ^a Sequences were aligned using the software provided by the IMGT server (Bosc and Lefranc 2000; Giudicelli, Chaume and Lefranc 2005).

TCR V β ₁₃₋₃	V Region	N1/P1	D Region	N2/P2	J Region
C57.17	GCCAGCAGTGA		AGGGGG		AGAAGTCTTCTTT
C57.19	GCCAGCAGTG		GGACAGGGG	C	CACAGAAGTCTTCTTT
C57.6	GCCAGCAG	CGTA	GGGAC	GGC	TTCTGGAAATACGCTCTATTTT
C57.50	GCCAGCAGTGAT	CCCGGC	GGGG	A	TTCTGGAAATACGCTCTATTTT
C57.39	GCCAGC	G	TGGG		TTCTGGAAATACGCTCTATTTT
C57.22	GCCAGCAGT	C	CAGGGG	C	CGAAAGATTATTTTTC
C57.24	GCCAGCAG	AA	GGACAG	A	TAACAACCAGGCTCCGCTTTTT
C57.1	GCCAGCAGTGAT	CGGC			ACCAGGCTCCGCTTTTT
C57.29	GCCAGCAGT	CCC	GGGACAGGGGG	AAGG	TCGCCCCTCTACTTT
C57.5	GCCAGCAGTG	GGGA			TAACTATGCTGAGCAGTTCTTC
C57.36	GCCAGCAGTGATG	GGGC			TGCTGAGCAGTTCTTC
C57.37	GCCAGCAGTGATG	CG	CAGGGGGC	T	TATGCTGAGCAGTTCTTC
C57.18	GCCAGCAGTGATG	CAAG	GGGACTGGGGGG	CCCC	TAACTATGCTGAGCAGTTCTTC
C57.28	GCCAGCAGT	CC	GGACTGGGGGG	A	TAACTATGCTGAGCAGTTCTTC
C57.8	GCCAGC		GGGGGGC	T	TGCAGAAAACGCTGTATTTT
C57.34	GCCAGCAGTG		GGACAG		GTGCAGAAAACGCTGTATTTT
C57.9,14, ,15,16,25, 27,42,43,44 ,48,31,38	GCCAGCAG	CA	ACAGGGGG	GGAGAAG	ACACCTTGACTTT
C57.12	GCCAGCAGTGA	GG	CAGGGGG		CACCTTGACTTT
C57.47	GCCAGCAGTG	GGG	GTCCC	GGGAC AGGGGG	AGTCAAAAACACCTTGACTTT
C57.2,7 ,10,20,35	GCCAGCAGTGAT		CTGGGG	AGG	CAAAACACCTTGACTTT
C57.3	GCCAGCAG		GGACTGGGGG	CCT	AGTCAAAAACACCTTGACTTT

C57.21,46	GCCAGCAGTGA	AGC	GGGGGGGC	GC	AACACCTTGACTTT
C57.11	GCCAGCA	A	GGACA	TA	CAAGACACCCAGTACTTT
C57.13	GCCAGCAGTGAT	CC	GGGACA	A	CAAGACACCCAGTACTTT
C57.26	GCCAGCAGTGA	CGC	GGACA	T	CCAAGACACCCAGTACTTT
C57.4,23	GCCAGCAGTG		GGACAGGG	AGGGG	TGAACAGTACTTC
C57.49	GCCAGC		GGACAGG	TTC	CCTATGAACAGTACTTC
C57.32	GCCAGCAGT		CTGGGGGGGC	GA	GAACAGTACTTC
C57.30	GCCAGCAGTGA	CAA			CTCCTATGAACAGTACTTC

Table 4. Sequences obtained by PCR amplification of cDNA obtained from one QM spleen^a

TCR V _H 13-3	V Region	N1/P1	D Region	N2/P2	J Region
QM.6	GCCAGCAGTGAT	CC	GGGA	TTGG	ACACAGAAGTCTTCTTT
QM.16,37	GCCAGCAGTGATG		GGGAC	GG	GAACACAGAAGTCTTCTTT
QM.18	GCCAGC	GGAGGG	CAGGGG	C	CAGAAGTCTTCTTT
QM.50	GCCAGCAG		GGACAGGGGGC	CCC	CAGAAGTCTTCTTT
QM.19	GCCAGCAGTG			TTCCAGAG	GTCTTCTTT
QM.57	GCCAGCAGTGAT	TCCGGAAT			CACAGAAGTCTTCTTT
QM.58	GCCAGCA	AAG			CAAACACAGAAGTCTTCTTT
QM.8	GCCAGCAGTGATG		GGGACAGGG	C	CTCCGACTACACCTTC
QM.38	GCCAGCAGTG	GG	CAGG	CGC	ACTCCGACTACACCTTC
QM.40	GCCAGCAGT	TGG	CAGGGG	AC	CTCCGACTACACCTTC
QM.21	GCCAGCAGTGA	AG	AGGGGG		CTGGAATACGCTCTATTTT
QM.53	GCCAGCAGTGATG		GGACAGGG	CC	TTCTGGAAATACGCTCTATTTT
QM.2	GCCAGCA	AGGGA	GGGAC	C	TCCAACGAAAGATTATTTTTC
QM.9,10	GCCAGCAGTGA	CC	GGGACA	A	TTCCAACGAAAGATTATTTTTC
QM.24	GCCAGCAGTGATG		GGGGC	G	CCAACGAAAGATTATTTTTC
QM.27	GCCAGCAGTGATG		ACAGGGGGC	GGGT	CAACGAAAGATTATTTTTC
QM.29	GCCAGCAGTGA		CAGGG		ACAACCAGGCTCCGCTTTTT
QM.11	GCCAGCAGTGAT			AGACG	CAACCAGGCTCCGCTTTTT
QM.3	GCCAGCAG	GGCGACGG			CCTATAATTCGCCCTCTACTTT
QM.5	GCCAGCAGTGATG			ACAA	TTCTATAATTCGCCCTCTACTTT
QM.14	GCCAGCAGTG			CCGG	TTCTATAATTCGCCCTCTACTTT
QM.31	GCCAGCAGTGA			CA	ATAATTCGCCCTCTACTTT
QM.4	GCCAGCAGTGATG	CACCCTCT	CTGGG	TT	CTATGCTGAGCAGTTCTTC
QM.44	GCCAGCAGTG		GGGACTGGGGG	CTA	TATGCTGAGCAGTTCTTC
QM.46	GCCAGCAGTGAT	AAC	CTGGGGGGG	TCT	TATGCTGAGCAGTTCTTC
QM.7	GCCAGCAGTGAT			CTCGGG	AACTATGCTGAGCAGTTCTTC

QM.42	GCCAGCAGTGATG		CAGGGGG		CACCGGGCAGCTCTACTTT
QM.48	GCCAGCAGTG		GGACAGGGG		ACACCGGGCAGCTCTACTTT
QM.43	GCCAGCAGTGAT	T	TGGGGG		ACACCGGGCAGCTCTACTTT
QM.51	GCCAGCAGT	AC	GACTGGGGGGGC	GCC	CACCGGGCAGCTCTACTTT
QM.33	GCCAGCAGTGA		GAACAA		AAACACCGGGCAGCTCTACTTT
QM.17	GCCAGCAGTG		GGACA	CAG	GCAGAAACGCTGTATTTT
QM.13	GCCAGCAG	CCCT	GGACTGGGGG		ACACCTTGTACTTT
QM.20	GCCAGCAGTGATG		TGGGGGGG		CAAAACACCTTGTACTTT
QM.22	GCCAGCAG	GG	CTGG		AGACACCCAGTACTTT
QM.15	GCCAGCAGTGAT	C	CAGGGG	AC	GAACAGTACTTC
QM.23	GCCAGCAGTGA	GA	GGGACAGGG	AT	TATGAACAGTACTTC
QM.30	GCCAGCAGTGATG	T	ACAG		TATGAACAGTACTTC
QM.36	GCCAGCAGTGA		CAGGG	CT	TATGAACAGTACTTC
QM.1	GCCAGCAGTGAT	CCC	GACTGGGGG	AG	CTCCTATGAACAGTACTTC
QM.28	GCCAGC				TATGAACAGTACTTC
QM.39	GCCAGCAGTGATG	GGG			CCTATGAACAGTACTTC
QM.45	GCCAGCAGTG	GG			CTATGAACAGTACTTC

^a Sequences were aligned using the software provided by the IMGT server (Bosc and Lefranc 2000; Giudicelli, Chaume and Lefranc 2005).

Table 5. (A) Sequences were obtained by PCR amplification of cDNA obtained from one *JH*^{-/-} spleen^a. Sequences were aligned using the software provided by the IMGT server.

TCR V _H 13-3	V Region	N1/P1	D Region	N2/P2	J Region
JH.18	GCCAGCAGTGA		GACAGGG		GAAGTCTTCTTT
JH.63	GCCAGC	GGAGGG	CAGGGG	C	CAGAAGTCTTCTTT
JH.46	GCCAGCAGTGATG		TGGG		CAAACACAGAAGTCTTCTTT
JH.5,19	GCCAGCAGTGA		GACAGGGGGC		AACTCCGACTACACCTTC
JH.30	GCCAGCAGTGAT		CTGGGGGGGC	CGT	ACACCCAGTACTTT
JH.29	GCCAGCAGTGA	G	AGGG	T	TTCTGGAAATACGCTCTATTTT
JH.35	GCCAGCAGTG	G	GGGACA	AAA	TTCTGGAAATACGCTCTATTTT
JH.50	GCCAGCAGTG	GC	GGGACA	AA	TTCTGGAAATACGCTCTATTTT
JH.60	GCCAGCAGTGA	AT	GGACAGGGG		CTGGAAATACGCTCTATTTT
JH.43	GCCAGCAGTGA			CAA	TTCTGGAAATACGCTCTATTTT
JH.62	GCCAGCAGTGA	CAA			TTCTGGAAATACGCTCTATTTT
JH.9,10	GCCAGCA	CC	GACAGGGG	AC	CCAACGAAAGATTATTTTTC
JH.32	GCCAGCA	TCC	GGGA	TA	TTCCAACGAAAGATTATTTTTC
JH.45	GCCAGCAGTGATG	CGTGGT			TTCCAACGAAAGATTATTTTTC
JH.71	GCCAGCAGCC			CCCGGCATC	TTCCAACGAAAGATTATTTTTC
JH.51	GCCAGCAG		GGGA	AT	CAACCAGGCTCCGCTTTTT
JH.41	GCCAGCAGTGATG			CACGG	AACAACCAGGCTCCGCTTTTT
JH.6	GCCAGCAGTGATG	CTC	GGGGG	GGGTC	GCTGAGCAGTTCTTC
JH.67	GCCAGCAGTG	TCC	GGGACTGGGGGGGC		GCTGAGCAGTTCTTC
JH.3	GCCAGCAGTGA	AGAC			AACTATGCTGAGCAGTTCTTC
JH.20,22	GCCAGCAG	GT	GGGACAG	AG	ACACCGGCAGCTCTACTTT
JH.48,54	GCCAGCAGTGAT		CACCGGGC	GGT	CACCGGCAGCTCTACTTT
JH.36	GCCAGCAGTGATG		GGGGG	A	AAACACCGGCAGCTCTACTTT
JH.66,68,72	GCCAGCAG	CCTCGC	GGGGG	T	AACACCGGCAGCTCTACTTT
JH.4	GCCAGCAGTGATG	CCA			CAAACACCGGCAGCTCTACTTT

JH.47	GCCAGCAGTGAT	C	GGGGGGGC	GC	TGCAGAAACGCTGTATTTT
JH.27,55	GCCAGCAGT	CCTGAC	GGGGG	G	GTCAAAACACCTTGTACTTT
JH.42	GCCAGCAGTGATG		ACAGGGG	AG	CCTTGTACTTT
JH.59	GCCAGCAG	CC	GGGACA	AC	CAAAACACCTTGTACTTT
JH.34	GCCAGCAG	GTC	ACTGGGGGGG	GGC	CAAAACACCTTGTACTTT
JH.39	GCCAGCAGT	TT	ACTGGGGGGG	GGGGG	CAAAACACCTTGTACTTT
JH.70	GCCAGCAG		CTGGGGGGG	G	AGTCAAAACACCTTGTACTTT
JH.38	GCCAGCAGTG			GAGGT	AGTCAAAACACCTTGTACTTT
JH.25	GCCAGCAGTGA		CAGG	CCCTT	CCAAGACACCCAGTACTTT
JH.21	GCCAGCAG		GGACA	AACT	CCAAGACACCCAGTACTTT
JH.44	GCCAGCAGTGATG	TC	GGGACAGGGGG	CGG	GACACCCAGTACTTT
JH.57	GCCAGCAGTGA		CAGGGG		CCAAGACACCCAGTACTTT
JH.31	GCCAGCAGTGA		GACAGGGGG		AACTCCGACTACACCTTC
JH.37	GCCAGCAGT	AACAGGGC	CTGGGGGGG	G	ACCAAGACACCCAGTACTTT
JH.40	GCCAGCAGTGATG		GGACTGGGG	T	AACCAAGACACCCAGTACTTT
JH.56	GCCAGCAGT	C	GACTGGG	T	CAAGACACCCAGTACTTT
JH.23	GCCAGCAG		GACTGGGGGG	G	CTATGAACAGTACTTC
JH.33	GCCAGCAGT	CCC	GGGACAGGGGG		CTCCTATGAACAGTACTTC
JH.49	GCCAGCAGT	TT	GGACAGGGG	A	GAACAGTACTTC
JH.52	GCCAGCAGT	ACC	GGGACA		TCCTATGAACAGTACTTC
JH.61	GCCAGCAGTGATG		GGGGG	TC	CTCCTATGAACAGTACTTC
JH.64	GCCAGCAGTG	G	AGGG		GAACAGTACTTC
JH.65	GCCAGCAGTGATG		ACAG		CTATGAACAGTACTTC
JH.1,15	GCCAGCAGTG		GGACTGGGGG		TATGAACAGTACTTC
JH.7,8,11,12,14,16	GCCAGCAGTGA	CCTC	GGGACTGGGGG		TCCTATGAACAGTACTTC
JH.13	GCCAGCAG	A	GGGGGGC	C	CTCCTATGAACAGTACTTC
JH.24	GCCAGCAGTGATG	ACAG			CTATGAACAGTACTTC
JH.26	GCCAGCAGTGATG	CCCTTC	ACTGGGGG		CTCCTATGAACAGTACTTC
JH.28	GCCAGCAGTG	GAG	CTGGGGGGG	GCG	TGAACAGTACTTC

Table 5. (B) *The number of V β 13-3 sequences containing each J β per strain^a*

TCR β J Genes	C57BL/6	QM	JH ^{-/-}
J1-1	2	7	4
J1-2	0	3	3
J1-3	3	2	6
J1-4	1	4	5
J1-5	2	2	2
J1-6	1	4	0
J1-7	5	0	0
J2-1	0	4	3
J2-2	0	5	9
J2-3	2	6	1
J2-4	14	2	8
J2-5	3	1	8
J2-7	5	8	9

^a Sequences were aligned using the software provided by the IMGT server (Bosc and Lefranc 2000; Giudicelli, Chaume and Lefranc 2005).

Table 6. Numbers of lymphocytes in the spleen of athymic (T), sham-operated (S) or non-manipulated (C) control mice.

Number (average \pm SD)	5 Weeks			10 Weeks		
	T	S	C	T	S	C
CD4+	4.77 x 10 ⁶ \pm 0.85 x 10 ⁶	7.92 x 10 ⁶ \pm 0.82 x 10 ⁶	8.37 x 10 ⁶ \pm 1.73 x 10 ⁶	3.6 x 10 ⁶ \pm 0.97 x 10 ⁶	8.8 x 10 ⁶ \pm 2.96 x 10 ⁶	10.1 x 10 ⁶ \pm 1.78 x 10 ⁶
CD8+	2.42 x 10 ⁶ \pm 0.47 x 10 ⁶	4.32 x 10 ⁶ \pm 0.41 x 10 ⁶	2.59 x 10 ⁶ \pm 0.39 x 10 ⁶	2.52 x 10 ⁶ \pm 0.72 x 10 ⁶	5.74 x 10 ⁶ \pm 2.14 x 10 ⁶	7.68 x 10 ⁶ \pm 2.55 x 10 ⁶
CD4+ CD44 ^{hi} CD62L-	1.3 x 10 ⁶ \pm 0.1 x 10 ⁶	1.4 x 10 ⁶ \pm 0.4 x 10 ⁶	1.7 x 10 ⁶ \pm 0.45 x 10 ⁶	1.04 x 10 ⁶ \pm 0.44 x 10 ⁶	1.6 x 10 ⁶ \pm 0.53 x 10 ⁶	1.2 x 10 ⁶ \pm 0.23 x 10 ⁶
CD8+ CD44 ^{hi} CD62L-	0.74 x 10 ⁶ \pm 0.05 x 10 ⁶	0.94 x 10 ⁶ \pm 0.3 x 10 ⁶	1.1 x 10 ⁶ \pm 0.26 x 10 ⁶	1.15 x 10 ⁶ \pm 0.46 x 10 ⁶	1.44 x 10 ⁶ \pm 0.3 x 10 ⁶	1.5 x 10 ⁶ \pm 0.34 x 10 ⁶
CD19+CD21+CD23-	1.29 x 10 ⁶ \pm 0.32 x 10 ⁶	1.36 x 10 ⁶ \pm 0.45 x 10 ⁶	1.53 x 10 ⁶ \pm 0.3 x 10 ⁶	4.3 x 10 ⁶ \pm 2.5 x 10 ⁶	3.6 x 10 ⁶ \pm 01.1 x 10 ⁶	5.93 x 10 ⁶ \pm 1.6 x 10 ⁶
CD19+CD21+CD23+	21.59 x 10 ⁶ \pm 2.11 x 10 ⁶	20.50 x 10 ⁶ \pm 3.89 x 10 ⁶	24.32 x 10 ⁶ \pm 4.9 x 10 ⁶	27.31 x 10 ⁶ \pm 11.33 x 10 ⁶	24.49 x 10 ⁶ \pm 6.9 x 10 ⁶	25.12 x 10 ⁶ \pm 5.7 x 10 ⁶

Table 7. Cycle threshold values (Ct values) were estimated for all V β -J β combinations for each RNA template and mean Ct values were calculated. Ninety-five percent confidence intervals (CI) are shown. Diversities of expressed V β -J β pairs were calculated with Shannon entropy (86). An estimate of scaled entropy (H) was calculated for each V β -J β matrix by the equation $H = \sum (p \log_2 p) / \log_2 (1/240)$ where p was the probability of abundance calculated for each V β -J β combination by the equation $p = 2^{-y} / \sum 2^{-y}$ where y was the Ct value for the V β -J β primer pair and p=0 when Ct > 40 cycles. Scaled entropy ranges from zero to one with one representing maximal diversity.

T=athymic mice, S=sham-operated mice, C=non-manipulated mice

Sample	Entropy	Mean Ct	95 % CI
C-spleen #1	0.85	17.4	17.1-17.4
C-spleen #2	0.84	18.1	17.8-18.4
S-spleen #1	0.83	18.0	17.7-18.4
S-spleen #2	0.84	17.8	17.4-18.2
T-spleen #1	0.84	18.8	18.4-19.1
T-spleen #2	0.85	18.5	18.2-18.8

Table 8.

Table 8: Analysis of Vh186.2 Somatic mutation in NP-immunized mice. Expressed sequences from twice boosted Controls, sham and thymectomized mice were analyzed. The Vh186.2 Sequences were 294 bp that translate to 98 AA. We found that thymectomized mice do have somatic mutation frequency as wild type but have reduced frequency of affinity enhancing mutation at codon 33 of DR1. Also thymectomized mice showed higher frequency of repeated sequences suggesting of possible clone expansion.

Day of rejection	1st Tx	2nd Tx	Difference		1st Tx	2nd Tx	Difference		1st Tx	2nd Tx	Difference
Control 1	23	12	11	Sham 1	22	13	9	Thymectomized 1	27	19	8
Control 2	25	15	10	Sham 2	24	16	8	Thymectomized 2	33	21	12
Control 3	25	16	9	Sham 3	25	16	9	Thymectomized 3	45	17	28
Control 4	25	16	9	Sham 4	28	20	8	Thymectomized 4	48	19	29
Control 5	27	13	14	Sham 5	29	14	15	Thymectomized 5	37	18	19
Mean	25	14.4	10.6	Mean	25.6	15.8	9.8	Mean	38	18.8	19.2

Base mutation	Total Number of sequences	Repeated sequences	Total # of mutations	Mutation frequency	Proportion of (C->T or G->A) Mutation
Control	80	21	489	2.819 %	34.56 %
Thymectomized	86	62	196	2.778 %	37.24 %

AA Changes	Total Number of sequences	Repeated sequences	Total # of replacement mutations	Replacement frequency	Frequency(33)W->L
Control	77	20	336	6.02 %	80.702 %
Thymectomized	85	60	131	5.35 %	64.000 %

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SUMMARY AND CONCLUSION

In summary after our lab found that TCR diversification in the thymus depends on B cell receptor or immunoglobulin (Ig) diversity we also found that B cells help maintain the number and diversity of T cells in the peripheral T cell compartment. In this work we determined the extent to which thymus output of T cell, peripheral survival or proliferation maintain the size and diversity of the T cell compartment and how it adapts to contraction of T cell diversity.

We also found that when T cell diversity is contracted, T cells commonly exhibit a "memory-like" phenotype of unknown functional significance.

In our study we also investigated mice with severe contraction of T cell diversity and their response to pathogenic microorganisms or if they suffered increased susceptibility to auto-immunity. We described how mice respond to thymectomy and determined if thymectomy perturbed the T and B cell compartments. We also specifically studied if B cell memory is maintained following thymectomy in young mice

B cell memory antibody responses critically depend on T cell help. To determine the extent to which T cell function was maintained in thymectomized mice we performed male to female skin grafts. The median survival time of male skin grafts was 37 days in thymectomized female mice and only 25 days in sham operated and control mice. Thus, thymectomy impairs cellular immunity to minor antigens. Re-transplant 30 days after shedding of the primary graft, hastened graft rejection in all mice even though thymectomized recipients had delayed graft rejection compared to controls. Accelerated secondary graft rejection indicates efficient generation of T cell memory.

To determine whether thymectomy perturbed primed T cell responses we tested delayed type hypersensitivity (DTH) to ovalbumin in the footpad of mice. After priming by subcutaneous injection of 100 micrograms of ovalbumin in PBS, thymectomized mice mounted a significant DTH response to the challenge comparable to the responses in sham operated and control mice. Our results are consistent with the idea that thymectomy does not impair primed T cell responses.

One hallmark of memory is the production and maintenance of long-lived plasma cells capable of maintaining serum specific antibodies for very long periods of time following last exposure to the antigen. If thymectomy impairs B cell memory, the number of long lived antibody secreting cells (ASC) should be reduced at times remote from antigenic exposure. We determined the number of NP-specific antibody secreting cells in the spleen or in the bone marrow 6 months after boosting thymectomized, sham operated or control C57BL/6.

In our conclusion we found that manipulation of the thymus may perturb affinity maturation in sham operated mice.

Thymectomy impairs primary T cell responses while sustaining “normal” T cell memory and primed responses as depicted by delayed primary male to female skin graft rejection and faster secondary skin graft rejection and normal DTH responses. Surprisingly despite impairing primary T responses, thymectomy appears to enhance B cell memory. Enhanced B cell memory in the presence of certain T cell dysfunctions suggests dissociation between T cell help requirements to generate B cell memory and those required to generate primary T cell responses. We will also consider the possibility that T cell regulation of B cell memory is altered by thymectomy.

Our findings propose that the long-lived antibody secreting cell compartment is maintained independently of the memory B cell compartment because it does not decline when memory B cells are abrogated. Our results indicate that differentiation of long-lived antibody secreting cells occurs independently of affinity maturation that normally accompanies B cell memory responses. Our work suggests that strategies to immunize individuals with congenital or acquired thymic defects (such as following cardiac transplantation or cardiac surgery in infancy), or with contracted T cell repertoires (such as in aging or after T cell depletion to treat cancer) would benefit from new vaccine designs including surrogates of cognate T cell help.

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