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Elements of Immune Fitness

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Abstract of the thesis

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Prvky imunitního způsobilosti

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 myší 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 myší 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 myší, přestože mají omezený repertoár TCR receptor > 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 myší, 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 inkopatibilní kožní štěpy srovnatelně s konvenčním kmenem myší a mají zachovanou schopnost aktivace T lymfocytů i opožděné hypersenzitivní reakcí. Myši QM jsou také odolné vůči infekci *Pneumocystis murina* v míře srovnatelné s konvenčním kmenem myší. Lze tedy uzavřít, že buňkami zprostředkovaná imunita u myší $JH^{-/-}$ a QM myší 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 vyzrávání protilátek. Protože odstranění nebo manipulace s thymem nesnižuje frekvenci mutací v exonech kódujících variabilní část molekuly protilátek specifických pro antigen, uzavíráme, že thymus kontroluje afinitní vyzrávání 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 vyzrávání

Elements of Immune Fitness

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: repertoir, TCR, BCR, thymus, thymectomy, B cells, affinity maturation

BACKGROUNDS

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. In models of contracted lymphocyte diversity ((Thompson and Neiman 1987; Yoshikawa et al. 2002) we tested this idea. I tested the fitness of cell mediated immunity in mice with contracted T cell diversity and I also tested the impact of contracted T cell receptor diversity on the generation of B cell responses to model antigens (Tuaillon 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.

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. In this work, we tested this prediction.

OBJECTIVE 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 murina* (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 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.

MATERIALS AND METHODS

STRAINS OF MICE

C57BL/6 mice were purchased from The Jackson Laboratory. B cell-deficient strains of mice used included $JH^{-/-}$ mice, obtained by gene-targeted deletion of the JH segments, and QuasiMonoclonal $(QM)^3$ mice, generated by gene-targeted replacement of the endogenous JH elements with a VDJ rearranged region from a 4-hydroxy-3-nitrophenylacetate-specific hybridoma. The $JH^{-/-}$ mice lack mature B cells and Ig. QM mice have 80 % of B cells that are 4-hydroxy-3-nitrophenylacetate specific. $JH^{-/-}$ 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 25 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 Vβ DIVERSITY

Generation of diversity standards. Diversity standards were prepared by generating oligonucleotide mixtures of known diversity, as previously described

Generation of lymphocyte receptor-specific cRNA. First strand cDNA was obtained by reverse transcription with a mouse TCR Cβ reverse primer, T7 plus Cβ (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGCTTGGGTGGAGTCACAT TTCTC-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 Vβ

TCR Vβ gene sequencing. Total RNA was isolated from splenocytes of C57BL/6, QM, and $JH^{-/-}$ mice with an RNeasy mini kit (Qiagen)

FLUORESCENCE ACTIVATED CELL SORTING ANALYSIS

Splenocytes were obtained by pushing minced spleen tissue through a 0.70-µm mesh followed by hemolysis in an NH_4Cl lysis buffer. The total number of splenocytes was determined using a Neubauer chamber. Cells were stained with one, two or three mAbs (all the Abs were from BD Pharmingen). 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-µg PADRE peptide aK(X)VAAWTLKAAa, where "a" is alanine and X is cyclohexyl alanine 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. and matured by incubation with LPS (from *Escherichia coli* 0111:B4; Sigma-Aldrich) at 5 µg/ml overnight in the presence of PADRE (35 µg/ml) for 5 days. Data represent the mean counts per minute of three wells ± SE in one representative experiment.

DELAYED-TYPE HYPERSENSITIVITY ASSAY

Mice were primed by the injection of 100 µg of OVA subcutaneously and challenged by intradermal injection of 20 µg 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 $JH^{-/-}$) ware cultured in a 96-well plate coated with anti-CD3 (clone H57-597) (in three different concentrations: 0.2, 1, and 10 µg/ml) in the presence of anti-CD28 at 10 µg/ml for 48 h. Alternatively, T cells were cultured with Con A (BD Biosciences) in three different concentrations (ConA to medium): ~1/20, 1/10, and 1/5 in the presence of anti-CD28 at 10 µg/ml for 48 h. Proliferation was measured by ^3H thymidine incorporation. Data represent the mean counts per minute of three wells ± SE in one representative experiment.

SKIN GRAFTS

Skin grafts were performed according to a modified technique of Billingham et al. 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.

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 12 h for the first 48 h, 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. 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. 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. Complementary determining regions were determined according to Kabat et al.

Skin Grafts: Skin grafts were performed according to a modified technique of Billingham et al. Secondary transplants were 30 days after the primary graft was shed.

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.

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. 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 IgG_1 was used as a standard for quantification.

ELISPOT: Done according to standard procedures in the laboratory. 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 % CO_2 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.

RESULTS

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 (Joao et al. 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 (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. 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 µMT mice.

We next tested whether mice with B cell defects and low diversity of thymocytes also have decreased diversity in the periphery. TCR Vβ diversity was measured according to Ogle et al. (Ogle et al. 2003). In this assay, T cell diversity is proportional to the number of hybridization hits of TCR Vβ cRNA on a gene chip and quantified by comparison to a standard curve obtained with DNA oligomers of known diversity. TCR Vβ diversity of $JH^{-/-}$ splenocytes was 1.1 x 103 per 10 µg of RNA, whereas TCR Vβ diversity of C57BL/6 splenocytes was 1.3 x 106 per 10 µg of RNA ($p < 0.05$). TCR Vβ diversity of QM splenocytes was intermediate, 8.8 x 104 per 10 µg of RNA ($p < 0.05$, compared with C57BL/6). In addition to the specificity of the primers, we assured that the assay detected TCR Vβ 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 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. Our results shows, T cells from $JH^{-/-}$ and QM mice proliferated in response to anti-CD3 or Con A, 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 µg 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 µg/ml PADRE peptide. Our results also 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.

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 (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 or T cell signaling (Lakkis et al. 2000) and, therefore, the assay would help exclude these problems. Table I 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.

Impact of TCR repertoire contraction in host defense

To determine whether a normal spectratyping profile reflects a balanced contraction of the repertoire, we sequenced the TCR CDR3 regions of genes containing Vβ13-3 (IGMT designation for Vβ8.1) in splenocytes obtained from C57BL/6 , QM , or $JH^{-/-}$ mice, It show fewer repeat sequences in QM or $JH^{-/-}$ splenocytes compared with those of wild-type mice. Of 46 C57BL/6 sequences bearing Vβ13-3, 29 had different CDR3 regions, the lengths of which averaged 11.5 codons. Of 45 QM sequences bearing Vβ13-3, 43 had different CDR3 regions, the lengths of which averaged 11.5 codons. Of 67 $JH^{-/-}$ sequences bearing Vβ13-3, 54 had different CDR3 regions, the lengths of which averaged 11.9 codons. The CDR3 lengths of sequences containing the TCR Vβ13-3 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. The results shows that the Vβ sequences obtained from all of the mice used diverse Jβ segments. These results demonstrated that B cell-deficient mice have balanced contraction of TCR diversity and suggest that it is the gaps, 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 (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 (Sallusto et al. 1999). We found that QM mice had 3-fold and $JH^{-/-}$ 2-fold more "memory-like" CD4+ T cells (CD4+/CD44high/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, 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) 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.

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. Consistent with absent thymic function, athymic mice had reduced CD4+, CD25+, Foxp3 cells, at 10 weeks of age.

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 et al. 2008). Briefly, the method amplifies TCR V beta (β) transcripts using combinations of primers specific for a total of 240 Vβ-Jβ combinations. Cycle threshold (Ct) values were determined for each Vβ-Jβ combination for each RNA template and mean Ct values were calculated. Results shown in table 2 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 Vβ-Jβ matrix in each set of mice (Shannon and Warren 1949). An estimate of entropy (H) was calculated by the equation $H=\Sigma\ (p \log 2\ p)/\log 2\ (1/240)$ where p was the probability of abundance calculated for each Vβ-Jβ combination by the equation $p=2-y/\Sigma 2-y$ where y was the Ct value for each Vβ-Jβ primer pair and p=0 when Ct > 40 cycles. Entropy ranges from zero to one with one representing maximal diversity. Control mice had average entropy of 0.85, sham-operated mice

had average entropy of 0.84 and athymic mice had 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. In our results it 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. We found 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.

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. 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. 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 aminoacid 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, 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. 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 %) and in sham-operated mice (21 %), compared to that fraction (98 %) in sequences obtained from control mice in which all sequences except for one contained the W33L mutation. 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.

DISCUSSION

In summery 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.

CONCLUSION

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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2009 **Thoracoscopic thoracic duct ligation in a child with plastic bronchitis.** M.O. Abuattieh, Abdalla E Zarroug.

2013 **Efficacy of aflibercept in neovascular AMD in bevacizumab treated eyes with persistent pigment epithelium detachment (PED).** M.O.Abuattieh, P.K.Rao, R.S.Apte.

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PRESENTATIONS

2003 **Conference sponsored by Division of Nephrology and Hypertension**, Department of Internal Medicine, Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN

2004 **National Student-Physician Scientific Conference**, 3rd place, Hradec Kralove, Czech Republic

2006 **Annual Meeting, The American Association of Immunologists**, Boston, MA

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2007 **American Transplant Congress**, San Francisco, CA

2008 **Experimental Biology 2008,** San Diego, CA