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Affinity maturation of antibodies requires integrity of the adult thymus

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The generation of B-cell responses to proteins requires a functional thymus to produce $CD4^+$ T cells which helps 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 the 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 the selection of B cells with high-affinity antibodies.

Key words: Affinity maturation · Antibody-secreting cells · Immunoglobulin · T-cell repertoire Thymus

Supporting Information available online

Introduction

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 produced by plasma cells generated from memory B cells upon re-exposure to the antigen and by persisting antigen-specific antibodies secreted by long-lived plasma cells in the bone marrow [1].

The generation of B-cell memory requires T cells. Thus, removal of the thymus in newborn mice (during the first 16 hr of life) causes severe cellular immunity defects [2] and abolishes antibody responses to protein antigens [3]. 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 [4–6]. Whether or not removal of the thymus in mature individuals perturbs B-cell memory is not known.

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T cells promote B-cell responses to protein antigens by directly interacting with B cells. Thus, deficiencies in 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 [7–9]. 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 the removal of the thymus and depletion of mature T cells before cardiac transplantation in infancy do not develop hyperimmunoglobulin M (IgM) syndrome and/or hypogammaglobulinemia, 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 [10]. Here, we report that selection of affinity mature antibodies generated in response to protein antigens requires the integrity of the thymus.

Results

Thymectomy in mature mice decreases the number of T cells without contracting TCR 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 [4]. 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 (Supporting Information Fig. 1). Consistent with absent thymic function, athymic mice had reduced $CD4^+$, $CD25^+$, $Foxp3^+$ cells, at 10 weeks of age (Supporting Information Fig. 3).

Mice from which the thymus had been removed at 5 weeks of age had fewer T cells in the spleen 5 and 10 weeks after the surgery, compared with the control mice. Figure 1A and B and Table 1 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 (Fig. 1A and B and Table 1). Results from other laboratories are consistent with ours showing a reduction in the number of $CD4^+$ T cells in the spleen following thymectomy. Thus, Gagnerault et al. [11] 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. [12] found a reduction of almost 2-fold in the number of peripheral $CD4^+$ T cells 15 weeks after interrupting thymic output in a model of chemical thymectomy.

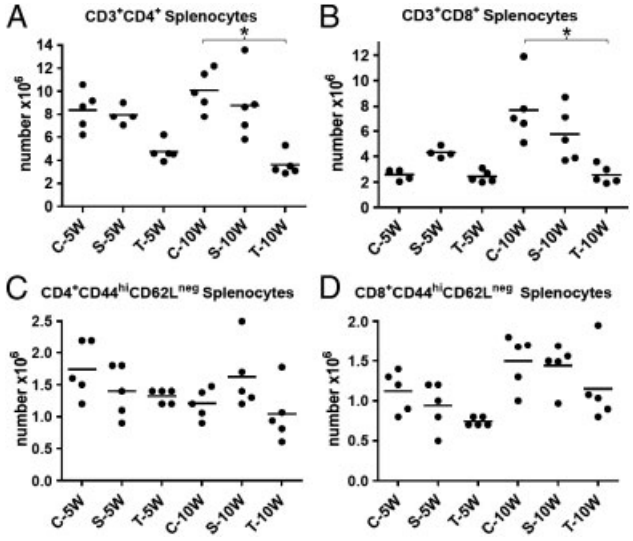

Figure 1. Number of lymphocytes in spleens harvested from mice lacking the thymus, sham-operated or non-manipulated controls at 5 and at 10 weeks of age. Thymectomies were performed 2 days before the mice turned 5 weeks old. Numbers of lymphocytes were calculated by multiplying the respective percentage as defined in a flow cytometry analysis, with specific monoclonal antibodies, by the total number of white blood cells (WBCs) in athymic (T), sham-operated (S) or unmanipulated control (C) mice at 5 and 10 weeks (W) after surgery. The number of (A) $CD4^+$ splenocytes and (B) $CD8^+$ splenocytes are shown. (C, D) The number of $CD4^+$or $CD8^+$ "memory-like" ($CD44^{hi}$/$CD62L^-$) T cells identified by flow cytometry analysis. Bars represent the average of each distribution. *$p<0.05$, paired two-tailed *t*-test. Five mice were analyzed per data point.

To determine whether the removal of the thymus caused compensatory proliferation, we enumerated $CD4^+$ or $CD8^+$ T cells with a "memory-like" phenotype ($CD62L^-$ and with high expression of CD44). Figure 1C and D and Table 1 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 with sham-operated (11%) or control (9%) mice. Similarly, 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 the 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 the 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 [13].

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 β transcript diversity using a real-time polymerase chain reaction (PCR)-based method [14]. Briefly, the method

Table 1. Numbers of lymphocytes in the spleen of athymic (T), sham-operated (S) or non-manipulated (C) control mice

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

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. The 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 [15] (Table 2). An estimate of entropy (H) was calculated by the equation $H = \Sigma\ (p \log 2p)/\log 2(1/240)$ where p was the probability of abundance calculated for each Vβ-Jβ combination by the equation $p = 2{-}y/\Sigma 2y$, where y was the Ct value for each Vβ-Jβ primer pair and $p = 0$ when Ct > 40 cycles. Entropy ranges from 0 to 1, with 1 representing maximal diversity. Control mice had 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 WT 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 2A shows that challenge of athymic mice produced greater footpad 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

Table 2. Cycle threshold values (Ct values) were estimated for all Vβ-Jβ combinations for each RNA template and mean Ct values were calculated. Ninety-five percent confidence intervals (CI) are shown[a)]

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

[a)] Diversities of expressed Vβ-Jβ pairs were calculated with Shannon entropy [15]. An estimate of scaled entropy (H) was calculated for each Vβ-Jβ matrix by the equation $H = \Sigma(p \log 2p)/\log 2(1/240)$, 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 the Vβ-Jβ 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.

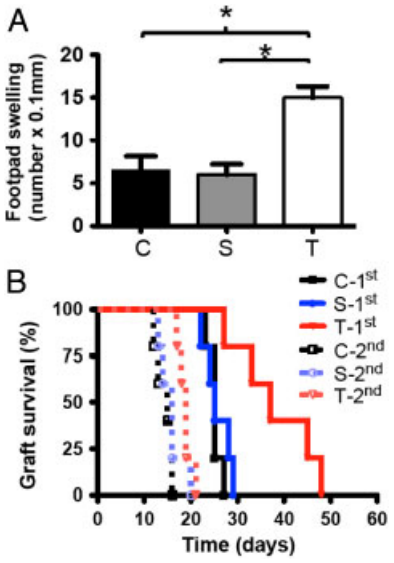

Figure 2. Primary cellular immune responses are delayed in mice lacking the thymus but T-cell memory responses are maintained. (A) DTH responses in control (C), sham-operated (S) or athymic (T) mice to intradermic injection of 20 μg ovalbumin were examined in the footpad of mice 6 days after priming by subcutaneous injection with 100 μg of ovalbumin (priming) or PBS (control). Footpad swelling measured in millimeters is indicated on the *y*-axis. Data are shown as mean ± SEM of $n = 5$. $^*p<0.05$, 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. Grafts were considered rejected when 90% or more of the graft lacked any viable signs in the 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 8–12 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. The results reflect transplants done in five mice of each group.

to female skin grafts. Figure 2B 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 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 [16]. Whether the thymus is necessary to generate B-cell memory responses beyond generating

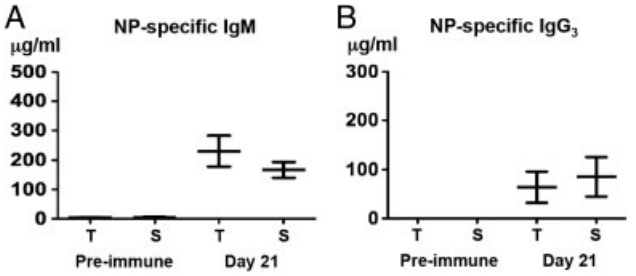

Figure 3. T-independent antibody responses maintained in athymic mice. T-independent antibody responses to NP-Ficoll in athymic mice (T) or in sham-operated (S) mice. The concentrations of (A) NP-specific IgM or (B) NP-specific IgG3 prior to and 21 days after immunization are shown. Mice lacking the thymus and sham-operated mice had on average 4.0 and 5.0 μg/mL NP-specific IgM respectively, and non-detectable NP-specific IgG3, prior to immunization. Athymic and sham-operated mice had 230 and 167 μg/mL NP-specific IgM, respectively. on average, 21 days after immunization, Mice lacking the thymus and sham-operated mice had on average 64 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. Data reflect mean ± SE, $n = 6$.

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. Figure 3A and B shows comparable concentrations of NP-specific IgM and IgG3 21 days following immunization in athymic mice (230 μg/mL IgM and 64 μg/mL IgG3, on average) and sham-operated mice (167 μg/mL IgM and 86 μg/mL IgG3, on average). Hence, thymectomy did not perturb T-cell-independent antibody production. Indeed, B cells developed normally in mice lacking the thymus compared with sham-operated mice. Supporting Information Fig. 2 shows that the average number of mature $CD19^+$ 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 imbalances, 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 [16]. 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. Figure 4A and B shows 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 primary or secondary antibody responses to vaccination with proteins. Consistent with that conclusion we found that the number of antibody-secreting cells (ASCs) present in the bone marrow 6 months after immunization was maintained in sham-operated mice and increased by 2-fold in athymic

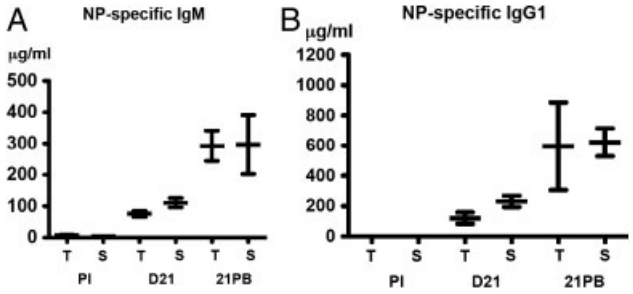

Figure 4. Removal of the thymus or sham operation maintained IgG1-specific antibody responses. (A, B) T-dependent responses to NP-ovalbumin. The concentrations of NP-specific IgM or NP-specific IgG1 prior to (PI) and 21 days after primary (D21) or booster immunization (21PB) respectively are shown. (A) Athymic mice (T) and sham-operated mice (S) had an average of 8.4 and 4.4 μg/mL NP-specific IgM, prior to immunization (PI), 76 and 111 μg/mL, 21 days after immunization (D21), 293 and 296 μg/mL 21 days after boosting (21PB) respectively. (B) Mice lacking the thymus or sham-operated mice had no detectable NP-specific IgG1 prior to immunization, but produced on average 121 and 231 μg/mL NP-specific IgG1 21 days after immunization, 596 and 622 μg/mL 21 days after boosting respectively. Data reflect mean ± SE, $n = 6$ mice per group. Paired two-tailed *t*-test analysis revealed no significant differences between athymic and sham-operated mice.

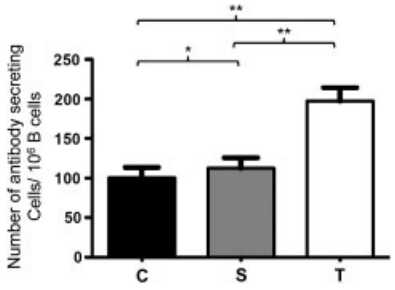

Figure 5. Number of NP-specific IgG1 antibody-secreting cells (ASCs) in the bone marrow of mice lacking the thymus or control mice, 6 months after boost immunization. Mice lacking the thymus (T) had on average 198 ASCs per 10^6 B cells while sham-operated mice (S) had an average of 113 ASCs per 10^6 B cells and, control mice (C) had an average of 100 ASCs per 10^6 B cells NP-specific IgG1 antibody-secreting cells in the bone marrow. Data are shown as mean ± SEM of $n = 4$ mice per group. The number of ASCs in athymic mice was significantly increased compared with the number of ASCs in control ($p = 0.0042$) or sham-operated mice ($p = 0.0075$) (unpaired two-tailed *t* test).

mice compared with non-manipulated controls (Fig. 5). In fact, since the number of ASCs in athymic mice was significantly increased compared with the number of ASCs 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 ASCs in the bone marrow.

Removal or manipulation of the thymus impairs affinity maturation of antibodies

The most significant function associated with antibody recall responses is the selection of cells bearing receptors with increased affinity for the antigen. To determine whether 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 [17]) and Cγ1 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 whether 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 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, the clonal diversity of NP-responding B cells in comparison with 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 [18]. 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% 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 three positions (Fig. 6A), 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 (Fig. 6B and C). Decreased frequencies of mutations in the CDR1 regions of the VH186.2 exons in athymic or sham-operated mice compared with 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

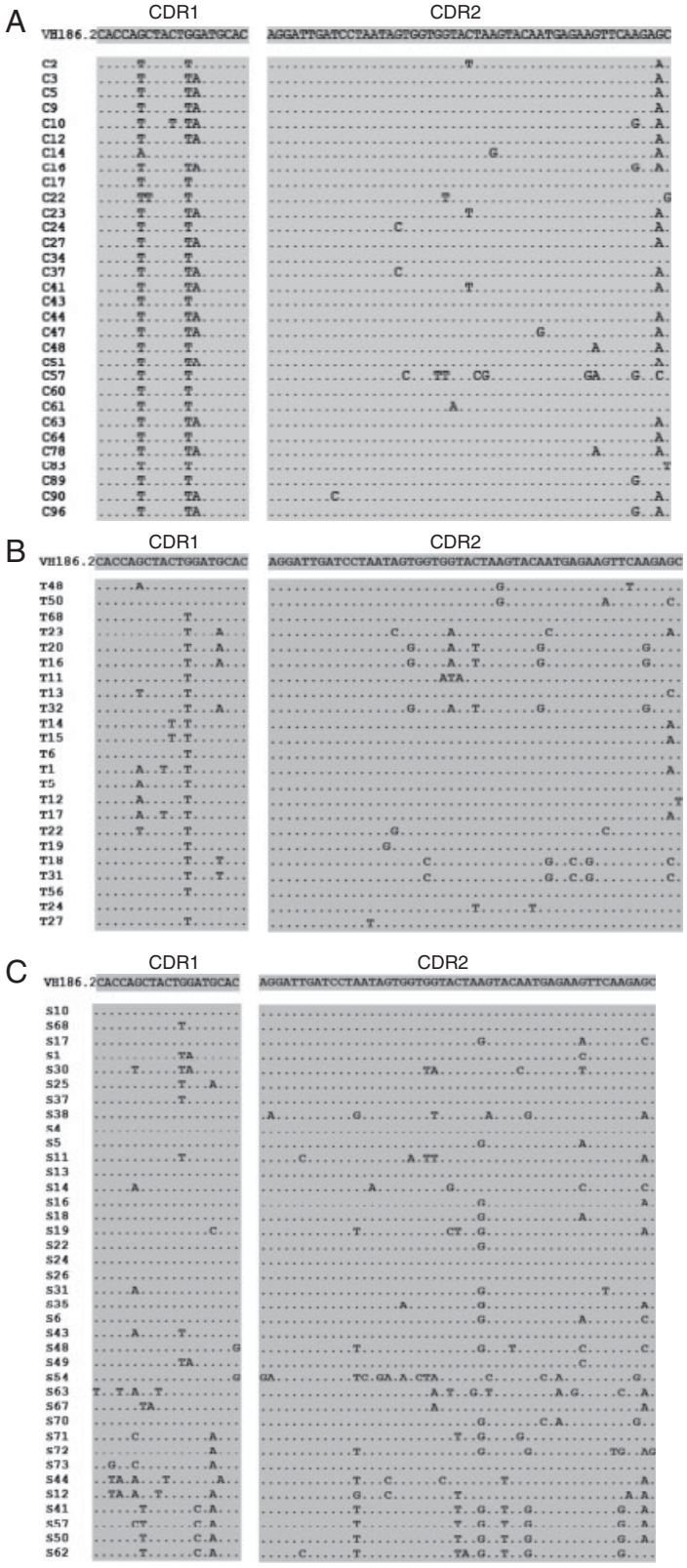

Figure 6. VH CDR1 and CDR2 DNA sequences of IgG1-B cells from mice lacking the thymus (T) sham-operated (S) or control (C) mice. Sequences were obtained from the spleens of mice 10 days following boost immunization. Shown are the CDR1 or CDR2 sequences of all the distinct VH sequences aligned to the germline VH186.2 segments. CDR1 and CDR2 regions are shadowed. (A) Sequences obtained from control mice, (B) sequences obtained from athymic mice, and (C) sequences obtained from sham-operated mice are shown.

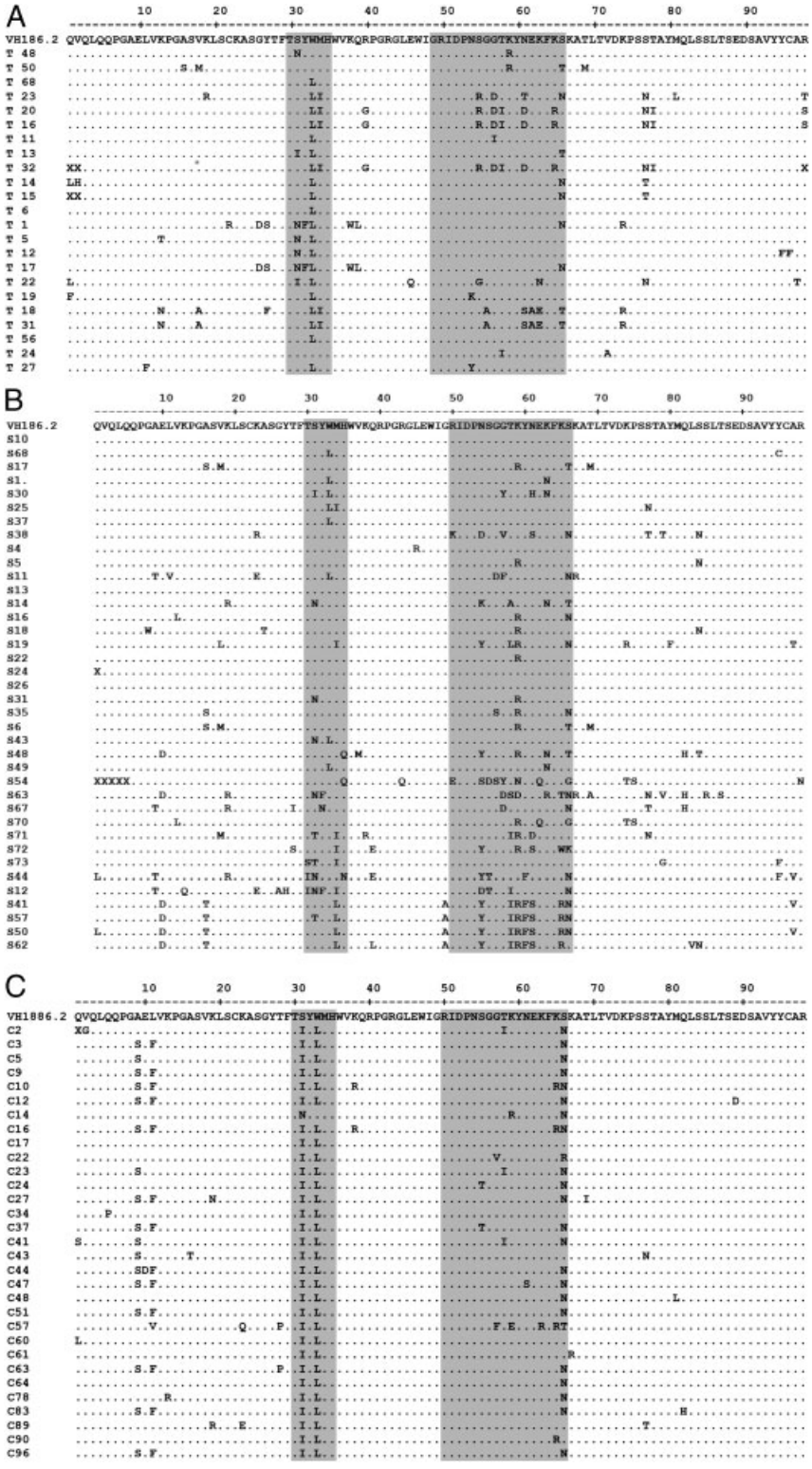

Figure 7. VH aminoacid sequences of IgG1-B cells. The aligned translation of all the distinct VH186.2 segments obtained from (A) mice lacking the thymus, (B) sham-operated mice and (C) control mice are shown. CDR1 and CDR2 regions are shadowed, and residue 33 is indicated in a darker grey shade.

NP-affinity enhancing mutation was decreased in athymic mice (87%, Fig. 7A) and in sham-operated mice (21%, Fig. 7B), compared with that fraction (98%) in sequences obtained from the control mice in which all sequences except for one contained the W33L mutation (Fig. 6C). Contingency analysis (χ^2 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 with 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 [19], 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

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 ASCs 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 ASCs. 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. [20] and Phan et al. [21] suggested that high BCR affinity for antigen dictates differentiation to extra-follicular ASCs, causing primary antibody responses. O'Connor et al. [22] proposed that B cells with a low-affinity BCR typically undergo somatic hypermutation, while B cells with BCRs of moderate affinity for antigen produced mostly long-lived ASCs. BCR affinity and T-cell help are interdependent since B cells present antigens to T cells following Ig-dependent internalization [23]. 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. [24] 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 ASCs 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 in much the same way as enhancing antigen presentation independently of the BCR as reported by Victora et al. [24]. 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 IL-7 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 (Supporting Information Fig. 3). 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. [25] who proposed that the long-lived ASC 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 ASCs 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.

Materials and methods

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) ip. 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

This was 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. [26] by injecting mice ip 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 ip injection of 100 μL of an emulsion of incomplete Freund's adjuvant containing 100 μg NP(25)-ovalbumin (Biosearch Technologies) and boost immunizations performed by ip 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 iv injection of 50 μg NP(25)-ovalbumin dissolved in 100 μL of PBS.

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.

Ig gene analysis

RNA was obtained from spleen cells and extracted with QIAGEN RNeasy (Qiagen, 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 Cγ1-specific primers in a nested reaction and with Pfu polymerase, followed by cloning with pCR4-TOPO (Invitrogen, Carlsbad, CA, 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 Cγ1). PCR was performed for 30 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. Nested PCR amplification forward primer: CAGGTCCAACTGCAGCAG, and reverse primer, AGTTTGGGCAG-CAGA. Sequences were aligned and analyzed using Sequencher software (Gene Codes, MI, USA) and with a software program developed by Dr. Cavalcoli 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 [27]. Complementary determining regions were determined according to Kabat et al. [28].

FACS analysis and antibodies

Splenocytes were obtained and prepared for flow cytometry analysis as in [29]. 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 CD3ε (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). Biotynilated 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.

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 h post-challenge.

Skin grafts

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

TRECS

DNA was obtained from splenocytes with a DNeasy Blood and Tissue Kit (Qiagen). 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 δRec-ψJα excision circles. Real-time PCR cycler conditions were set for 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min with 5 μM forward and reverse primers and 0.05 μL of 100 μM FAM-QSY probe. Forward primer (upstream of

ψJα segment): 5′-CAT TGC CTT TGA ACC AAG CTG-3′; Reverse primer (downstream of the δRec1 segment): 5′-TTA TGC ACA GGG TGC AGG TG-3′ according to [31]. 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 of sjTREC plasmid DNA or of C57BL/6J DNA followed by *Tfrc* gene amplification.

Statistical analysis

Performed using Prism software (Prism Software, Irvine, CA, USA). 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

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 h at room temperature. ELISA was performed according to previously described protocols [29, 32]. Plates were developed with ABST (SouthernBiotech) read at 405 nm in microplate reader Synergy 2 (BioTec Laboratories, Suffolk, UK) and analyzed using Gen 5 software version 1.04.5 (BioTek, VT, USA). The 17.2.25 IgGl was used as a standard for quantification.

ELISPOT

ELISPOT was done according to standard procedures in the laboratory [26]. 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 h 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 ASCs obtained from adoptively transferred recipients were done with splenocytes. To detect NP-specific ASCs, each well was washed and incubated with AP-conjugated goat anti-mouse IgM or IgG antibody (SouthernBiotech) for 2 h 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, Shaker Heights, OH, USA) and confirmed by direct observation.

TCR β chain diversity analysis

TCR β chain diversity analysis was done as reported [14]. Briefly, RNA was obtained from spleens using a RNeasy Protect Minikit (Qiagen). Residual DNA was removed from RNA samples using an RNase-Free DNase Set (Qiagen). cDNA was produced from 15 ng of RNA with a 20 pmol of a 5′biotynilated TCR CββJb primer and pools of 21 different TCR Vβ primers homologous to the CDR1 region providing 66 pmol of each (three pools of five and one pool of six primers), at 50°C for 32 min followed by incubation at 94°C to inactivate the reverse transcriptase. cDNA synthesis was followed by PCR amplification at 1 min at 94°C, 30 sec at 60°C and 1 min at 72°C for 25 cycles. RT-PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and biotynilated products separated with MyOne™ Streptavidin C1 Dynabeads (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's instructions. TCR Vβ diversity was determined by real-time PCR in a total of 240 individual reactions using combinations of 20 TCR Vβ and 20 TCR Jβ primers, as described [14]. Reactions were performed in a 10 mL volume containing 10 pmol of a nested TCR Vβ primer homologous to TCR Vβ CDR2, 10 pmol of a TCR Jβ primer, 1 μL of purified PCR products and 5 μL of Power SYBR Green PCR master mix (2×) (Applied Biosystems). Cycling was preceded by incubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. 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 [14] and synthesized by Invitrogen.

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Abbreviations: **ASC:** antibody-secreting cell · **DTH:** delayed-type hypersensitivity · **TREC:** T-cell receptor excision circle

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