The V_H repertoire and clonal diversification of B cells in inflammatory myopathies

Donna McIntyre[1*], Neta S. Zuckerman[2*], Max Field[3], Ramit Mehr[2**], David I. Stott[1**]

[1]Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow Biomedical Research Centre, Glasgow, Scotland, United Kingdom
[2] The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel
[3]Wolfson Medical School Building, University of Glasgow, Glasgow, Scotland, United Kingdom

* Equal contribution to manuscript
**Equal supervision of the work

Corresponding Author

Prof. David I. Stott
Institute of Infection, Immunity and Inflammation
University of Glasgow
Glasgow Biomedical Research Centre
Glasgow
G12 8TA
United Kingdom

Tel: +44 141 330 5206
David.I.Stott@glasgow.ac.uk

Abbreviations

AID, Activation-induced cytidine deaminase; DM, Dermatomyositis; FDC, Follicular Dendritic Cell; FWR, Framework Region; IBM, Inclusion Body Myositis; IM, Inflammatory Myopathies; MG, Myasthenia gravis; OCT, Optimum cutting temperature embedding medium; PB, Peripheral Blood; PM, Polymyositis; RA, Rheumatoid arthritis; RS, Replacement and Silent mutations; SHM, Somatic Hypermutation; SS, Sjögren's syndrome.

Keywords

B cell, V-gene repertoire, Somatic hypermutation, Clonal diversification, Myositis

Summary

The contribution of antigen-driven B cell adaptive immune responses within the inflamed muscle of inflammatory myopathies is largely unknown. In this study we investigated the immunoglobulin V_H gene repertoire, somatic hypermutation, clonal diversification, and selection of infiltrating B cells in muscle biopsies from inflammatory myopathy patients (dermatomyositis and polymyositis), to determine whether B cells and/or plasma cells contribute to the associated pathologies of these diseases. The data reveal that Ig V_H gene repertoires of muscle-infiltrating B cells deviate from the normal V_H gene repertoire in individual patients, and differ between different types of inflammatory myopathies. Analysis of somatic mutations revealed clonal diversification of muscle-infiltrating B cells and evidence for a chronic B cell response within the inflamed muscle. We conclude that muscle-infiltrating B cells undergo selection, somatic hypermutation and clonal diversification in situ during antigen-driven immune responses in patients with inflammatory myopathies, providing insight into the contribution of B cells to the pathological mechanisms of these disorders.

Introduction

Inflammatory myopathies (IMs) are a group of autoimmune diseases, associated with inflammatory cell infiltration to the muscle and muscle weakness; this can vary from mild to severe or, in some cases, absence of muscle weakness (amyopathic DM). IMs are composed of three principal subsets: dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM), each with distinct clinical and pathological features. In DM, inflammation is predominately perivascular and/or perimysial around the muscle fascicles [1;2] with infiltrations consisting largely of B cells and $CD4^+$ T cells, indicating a humoral immune response [3;4]. PM & IBM are characterised by scattered necrotic and regenerating muscle fibres and endomysial inflammation with invasion and destruction of non-necrotic muscle fibres [1;2]. Inflammatory infiltrates in both disorders mostly consist of $CD8^+$ T cells and macrophages [3;4] which invade MHC class I antigen expressing muscle fibres [5] leading to fibre necrosis. In contrast to DM, B cells are few or absent within the inflammatory infiltrates of PM and IBM. However, various autoantibodies are associated with DM and PM, and in some cases IBM [6-8], suggesting that a loss of B cell tolerance may contribute to disease pathogenesis in all IMs.

Generation of B cells expressing high affinity antibodies classically occurs within germinal centres (GCs) in secondary lymphoid organs, where they are induced to proliferate and undergo affinity maturation through the process of somatic hypermutation (SHM), further diversifying their Ig repertoire and generating clonally expanded cells. Selection of cells with the highest-affinity receptors leads to the formation of memory B cells and plasma cells, reviewed in [9]. Such GC-type reactions with biased Ig V-gene repertoires of the infiltrating B cells have also been found in the target tissues of various autoimmune and inflammatory disorders [10;11].

The contribution of ectopic GC-type reactions within inflamed muscle of the IMs is still largely unknown. We propose that B cells infiltrating the muscle tissues of myositis patients are actively proliferating in response to antigen in situ with SHM and affinity selection of their antibody receptors, resulting in aberrant Ig V-gene repertoires. An antigen-driven B cell response bears the signature of clones of B cells expressing clonally related sets of rearranged Ig V-genes. The members of each B cell clone use the same germline V_H-gene with the same V-D-J junctional sequences and both shared and unshared somatic mutations with selection for replacement mutations, especially in the complementarity determining regions (CDRs) [11]. We have investigated this by identifying muscle-infiltrating B cells and plasma cells, examining selection for their expressed Ig V-genes, analysing selection for replacement mutations, and by comprehensive analysis of their clonally-related Ig V-gene sequences using IgTree© [12]. The results clearly emphasise the role of B cells within IM disorders.

Results

Identification of muscle-infiltrating B cells in inflammatory myopathies

B cells ($CD20^+$) and plasma cells were identified in the inflamed muscle of 12 from 17 myositis patients (2/4 DM, 9/11 PM and 1/2 IBM) and absent in the normal control muscle ((Supporting Information Table 1) (Figure 1A & C), also briefly reported in [11]). Muscle-infiltrating B cells were present in loose to dense cellular aggregations in the appropriate perivascular/perimysial or endomysial locations corresponding with the myositis subset but no classical GC structures were observed. The largest infiltration of $CD20^+$ B cells was observed in the muscle samples from DM patients, with low numbers in the PM and IBM samples; each sample contained significant numbers of plasma cells. FDCs were rare in all samples, and were only observed in small proportions in 3 PM patients and the 1 IBM patient (Figure 1B). $CD3^+$, $CD4^+$ and $CD8^+$ cells were mostly found to mirror previous observations [3;4] (not shown). No non-specific staining was observed with isotype controls.

Proliferating ($Ki67^+$) cells were also observed in a number of samples (Figure 1D); double immunofluorescent staining revealed proliferating $CD20^+$ B cells (Figure 1E and [11]), as well as $CD4^+$ and $CD8^+$ cells (not shown).

Ig V-gene repertoires of muscle-infiltrating B cells in DM and PM differ from control repertoires

148 independent rearranged Ig V-genes from micro-dissected populations of muscle-infiltrating cells (93-DM; 55-PM) were amplified and sequenced to determine whether there is selection for specific Ig V_H, D and J_H exons. Ig V-gene repertoires were established for each patient (2 DM and 9 PM) followed by a pooled analysis from patients in each myositis DM/PM subset. The results suggest selection of particular Ig V-genes in both a patient-specific and, in some

instances, disease-specific manner relative to the normal peripheral blood (PB) B cell [13] and germline repertoires (Figure 2A-C). Over 600 sequences were initially analysed; following the removal of duplicate sequences (with the same V_H, D, J_H, CDR3 sequence and point mutations) and those which gave unreadable sequence readouts. Some of these results have been briefly described elsewhere [11]. Most of the significant deviations from the normal V_H gene repertoire were observed in DM patients (Figure 2A). Reductions in the use of V_H1 and V_H4 gene segments and increases in the use of V_H2 gene segments were observed compared with control PB B cell repertoires, while the use of V_H3 gene segments was significantly increased in the pooled DM repertoire compared with both the germline and control PB B cell repertoires. Over-representation of V_H3 gene segments was also observed in each of the individual DM patients, and in one PM patient (M23). In contrast, the pooled PM patient repertoire revealed increased use of V_H5 gene segments compared with DM. Analysis of individual PM patients suggested selection for and against particular gene segments in a patient-specific manner. Patient M3 showed significant over-representation of V_H1 gene segments, while patient M5 showed significant reduction of V_H1 usage in favour of V_H5, compared with that of control repertoires. Significant differences from normal PB B cell repertoires were also observed in the D and J_H exons (Figure 2B & C). Representation of the D6 gene segments was increased in DM patients, while that of J_H1 was decreased. In PM patients, the use of J_H2 and J_H5 exons was increased when compared with the control PB B cell repertoire. Significant differences between DM and PM subsets were also observed in the use of J_H2, J_H5 and J_H6 exons. The use of individual exons within each family was studied (not shown). DM patients had a preference for V_H3-11, V_H3-15, V_H3-30 and V_H3-9, and against V_H4-34, whereas PM patients had increased frequencies of V_H3-30 and V_H5-51 gene segments in muscle-infiltrating B cells. Despite numerous attempts, only two different rearranged V-gene sequences were isolated from the IBM sample, therefore these were not included in the statistical analysis.

Abundant hypermutation in Ig V-genes expressed by muscle-infiltrating B cells in DM and PM patients

To examine SHM and clonal expansion within the muscle infiltrating repertoire, the numbers, locations and distributions of single-point mutations in each rearranged gene were established and are presented as pooled data according to the myositis DM/PM subset (Figures 2D and 2E). The frequency of mutations suggests infiltration of distinctive subsets of B cells into inflamed muscle in DM and PM patients (Figure 2D). In DM patients, almost 50% of sequences contained 0-2 mutations in the V_H region, suggesting that there is a large population of naïve cells in this subset compared with that in PM patients, where the majority of cells contained a large number of mutations.

As expected, the majority of single point mutations were found within the CDRs (Figure 2E) and the percentage of mutations within CDR1 was similar for DM and PM patients, whereas CDR2 in PM was more heavily mutated. Due to difficulties in ascertaining mutations within CDR3, mutational analysis was not conducted for this part of the gene.

Clonal expansion of muscle-infiltrating B cells in inflammatory myopathies

Lineage trees, demonstrating clonal expansion of the infiltrating B cells in situ, were constructed from the 38 clonally-related Ig V-gene sequences isolated from 2 DM and 3 PM patients (with the exception of one clone from patient M1) using IgTree© [12] (Figures 3A-C), with the gene features of all clones summarised in Supporting Information Table 2. M16 clones A & B represent B cell clonal expansions isolated from different cellular aggregations from the same tissue section. There was no evidence to suggest the migration of clones between different cellular aggregations, suggesting that the clones were generated independently and maintained within each cell cluster.

As controls, we used lineage trees of 10 control clones from GCs in normal spleens and Peyer's patches [14], as there are no B cells in normal muscles, and lineage trees and single sequences which are not clonally-related from PB B cells of healthy controls. All but one (M16 clone B) of the myositis lineage trees had long trunks and were less branched compared to the normal GC and PB trees. The long trunks imply that the founder cells are either memory B cells which were activated elsewhere, or that the disease has been ongoing for a long period of time.

In addition to the qualitative assessments, lineage trees from myositis patients were also quantitatively measured to evaluate the extent of clonal diversification, mutation and antigen-driven selection in comparison to normal GC and PB trees (Figure 3D). Lineage tree properties demonstrated several significant differences between myositis and normal controls, as follows. The myositis trees had significantly longer minimal root-to-leaf paths (PL-min) and root-to-fork distances (DRSN-min) compared to both groups of normal control trees, due to the significantly longer trunk lengths. These results suggest that the response has been ongoing for a long time, as befits a chronic inflammatory reaction which included the observed B cell clones. In addition, the myositis clones had a significantly lower average outgoing degree (OD-avg, i.e., the average number of children per node) compared with that of the normal controls. This may imply, according to correlations with B cell response dynamic parameters [15], a higher mutation rate in the myositis clones, compared with that of the normal controls. However, this interpretation should be taken with caution, as the differences observed in OD may also be due to a lower sampling size in the myositis data group.

Comprehensive Ig V-gene mutational analyses show deviations from normal responses

To further enhance our understanding of the antigen-driven SHM and affinity maturation processes ongoing within the muscle, several types of mutational analyses were performed on the myositis and control sequences. All were performed per-tree, and thus avoid mutation over-counting. We first examined the SHM spectrum in myositis in comparison with normal GC and PB sequences (Figure 4). The mutation spectra seen in the myositis sequences were significantly different from those of the PB normal control sequences (Figure 4A). A/T nucleotides were mutated with lower frequencies than those of C/G nucleotides, and the frequencies of mutations from A and T were similar, in contrast to the normally observed mutation bias from A nucleotides. C/G nucleotides were also mutated with the same frequency, similarly to what is normally observed. The myositis sequences also exhibited a slight transversion bias, which was significantly different from the transition bias observed in both normal controls (Figure 4B) and in additional studies [16;17]. In mutations from A/T, the myositis targeting motifs were similar to those of the normal controls and the motifs reported in the literature. However, in mutations from C/G, the myositis sequences failed to show the normal targeting motifs in position +1 in mutations from C and in positions -1 and +2 in mutations from G. In addition, the different motifs around C/G mutations did not exhibit reverse complementarity, implying that the activation-induced cytidine deaminase (AID) machinery does not act equally on both strands in this case (Figure 4C). There may be several reasons for these deviations, as discussed below.

Next, we analysed R and S mutations within the FWR and CDRs of the clonally-related myositis sequences, comparing the myositis clones with normal GC and PB clones as constructed by IgTree© [12]. In the FWRs, the myositis and normal control groups showed significant positive selection for R mutations ($P=0.03$ and $2x10^{-7}$, respectively). However, most of these mutations were either conservative or neutral, as demonstrated by the amino acid (aa)

substitution analysis (see below). Thus these results indicate that there is no real selective drive towards a change in the FWR structure in the clonally-related sequences. In the CDRs the myositis group showed a weak (not significant) positive selection for R mutations, as expected if these clones, at the time of observation, were still undergoing affinity maturation and selection for the Ag.

Finally, aa substitutions were enumerated for the myositis and the normal control groups. Each mutation was enumerated in the FWRs and CDRs separately (Figure 5). The average frequency of aa substitutions in the myositis group ("all regions", referring to the combined CDRs and FWRs) was significantly higher than that of the normal GC (P-value = 0.013) and significantly lower than that of the normal PB (P-value = 4.63E-10) groups, due to the different number of mutations in each group. Most R mutations in all data groups were found in the FWRs (>70% in all groups, out of total R mutations) rather than CDRs. However, most of these were either conservative or neutral (71%, 80% and 83% in the myositis, normal GC and normal PB respectively, out of the total R mutations in the FWRs). Indeed, it was shown [18] that Ig V-genes have evolved in such a way that FWR codon usage is biased to result in a minimum of non-conservative aa replacements. In addition, most mutations were clustered around the diagonal, showing a conservation of hydrophobicity, which is important for conservation of protein structure, as expected in the FWRs.

Identification of antigen-specific muscle-infiltrating B cells in inflammatory myopathies

The identification of antigen-specific cells was briefly explored towards the conclusion of this study, to elucidate the identity of antigen(s) as potential candidates for stimulating the response. Antigen-specific cells were initially identified using light microscopy (not shown) with antigen-specific B cells and plasma cells identified using double immunofluorescence microscopy on

biopsies from 5 patients whose sera contained antibodies against Jo-1 (a myositis-specific antigen), nuclear antigens Sm/RNP, Scl-70 and DNA, singly or in combination with another antigen. Both antigen-specific B cells and plasma cells were observed within the infiltrating cell populations (Supporting Information Fig.1), although there was only a limited number of antigen-specific cells in relation to the total number of infiltrating B cells and plasma cells, suggesting that alternate or modified versions of antigens may also be responsible for stimulating the B cell response within IMs. The phenotype of antigen-specific cells (B cell or plasma cell) was uniform throughout the sections, with the exception of one sample with specificity towards Scl-70. Jo-1 specific B cells and plasma cells have been described briefly elsewhere [110]. Antigen-specific cells were isolated using laser capture micro-dissection followed by single cell V-gene amplification or amplification of V-genes from a small number of cells with the same antigen-specificity. Within the constraints of the study only a limited number of sequences were isolated and sequenced; therefore, no definitive conclusions can be drawn concerning the V-gene repertoire and mutation patterns used by autoantigen-specific cells.

Discussion

Of paramount importance in the understanding of autoimmune inflammatory disorders is whether highly specific, antigen-driven adaptive immune B cell responses occur within the target tissues. We have addressed the hypothesis that B cells infiltrating the muscle tissues of myositis patients are actively proliferating in response to antigen in situ, resulting in Ig V-gene repertoires deviating from normal control values [13], with SHM and affinity selection of their antibody receptors. The work presented here supports this hypothesis; oligoclonal expansion of B cells was observed; analysis of Ig V-gene repertoire and mutational patterns suggest selection for particular Ig V-genes and an antigen-driven response. Taken together, these results indicate that antigen-driven, B cell responses occur within the target tissues of myositis patients, and provide evidence that B cells play an active and significant role in pathogenesis at the sites of inflammation of these diseases, despite the absence of ectopic GC architecture within the target tissue.

Our study supports previous observations of inflammatory infiltrates in different myositis subsets [3;4;19;20] and demonstrates the active participation of plasma cells and proliferating B cells. In addition, the identification of an abundance of Ig transcripts in IBM and PM patients and other studies demonstrating B cell maturation in situ [20-24] implicate a significant role for these antibody-secreting cells in immune responses in the inflamed muscle. Unlike our and other groups' studies of infiltrating lymphocytes within the target tissues of other autoimmune disorders [10;11], no characteristic GC structures were observed within the inflamed muscle tissue, similar to other studies of IMs [3;4;20-22]. Although FDCs are expected to be present in ectopic GCs for antigen presentation and activation of high affinity B cells, FDCs were observed in only 4 of the 12 myositis patients diagnosed with PM or IBM, and none in DM where B cells are traditionally thought to play a greater role, as also observed in a recent study

of IMs [22]. The evidence of antigen-driven reactions suggests that additional cell types substitute for the antigen presenting role of FDCs in muscle inflammatory reactions, or that these interactions occur elsewhere.

We have identified both patient-specific and, in the case of V_H5, J_H2, J_H5 & J_H6 gene segments, disease-specific selection for particular rearranged Ig V-gene families. Affinity maturation of muscle-infiltrating B cells was supported by identification of selection for replacement mutations in clonally-related sequences from myositis patients compared with normal controls. Taken together, our comprehensive lineage tree and mutation analyses provide evidence for vigorous B cell antigen receptor diversification in myositis patients, with antigen-driven selection acting to change the CDRs, as expected in normal immune responses. However, the mutation spectra differed in several aspects from those observed in controls. The SHM mechanism may be over-exhausted due to chronic activation, as was also suggested by the higher mutation rates (according to OD-avg) found in the myositis clones, and thus may be responsible for the altered mutation spectra and targeting motifs. Alternatively, different micro-environmental signals may lead to subtle differences in the expression of the enzymes that participate in SHM, as suggested in our previous study [17]. Indeed, the myositis lineage trees had long trunks, suggesting either that they started diversifying elsewhere and continued diversifying in the inflamed muscle, or that the diversification observed here started in the muscle long before the biopsies were taken, such that only its latest stages are observed. Alternatively this could indicate re-stimulation of infiltrating memory B cells. Overall, these results imply a chronic inflammatory reaction of the infiltrating B cells in inflamed muscle from myositis patients.

Bradshaw et al. [21] recently demonstrated antigen-specific B cell responses occurring in muscle of IM patients. In contrast to our study, very few $CD19^+$ or $CD20^+$ cells were observed, with the majority of B-derived cells being $CD138^+$ plasma cells which had class switched to either IgG or IgA. Salajegheh et al. [22] also isolated plasma cell Ig sequences derived from a common B cell precursor and demonstrated increased gene expression of B cell activating factor (BAFF), a cytokine crucial for B cell maturation and survival; all occurring outside the confines of ectopic GC structures. This study confirms and extends this earlier work [21;22], and strongly suggests the participation of antigen-specific B cell immune responses in the inflammatory response within the muscle. In parallel with other autoimmune diseases, we observed a bias in the Ig V_H, D & J_H gene repertoires along with the identification of V_H-gene segments which were clonally expanded in immune reactions within the target tissues, providing insights into the usage of preferred V_H gene families, clonal expansion of B cells, loss of B cell tolerance and gene dysregulation in response to antigen. Alterations in mutation spectra and targeting motifs observed in myositis sequences in this study were also observed in our previous study of the ectopic GC response in SS patients [16]. However, whereas studies in other autoimmune diseases demonstrated ongoing B cell responses within the context of ectopic GCs (reviewed in [11]), we observed the hallmarks of antigen-driven B cell responses in situ in the absence of histologically identifiable GCs in the IMs, and deviations in the mutation spectra and targeting motifs. Autoantigen-specific B cells and plasma cells have been identified in and around ectopic GCs in MG [25], SS [26;27], and RA patients [28]; we also identified autoantigen-specific B cells within the muscle-infiltrating cell population, despite the absence of ectopic GC architecture. Extrafollicular B cell responses with functionally active AID expression have been identified in autoimmune target tissues [28]. Identification of clonally related sets of Ig V-genes in the absence of GCs demonstrates that antigen-driven B cell

responses also take place in the myopathies and not solely within the periphery prior to infiltration into the muscle tissues.

Mutation analysis of clonally-related sequences revealed significant differences between myositis and normal control sequences. The spectrum and targeting motifs of nucleotide substitutions is largely dependent on the enzymes and pathways that repair the U:G lesions created by AID. The lower number of mutations from A/T nucleotides compared to C/G nucleotides observed in the myositis data may result from a deficiency in the low fidelity polymerase-eta, which is the main contributor to mutations from A/T nucleotides [29-33]. Polymerase-eta deficient mice gain the ADK/MHT motif (where D = A/G/T; H = A/C/T; K = G/T; M = A/C) [29], also found in our myositis group and in paucity in both controls, especially in mutations from T, where C was in excess in position -1 and -2. The myositis sequences also demonstrated a transversion bias. Polymerase-eta has been shown to favour transition mutations [29], and thus its absence or malfunction may lead to fewer transition mutations. Previous studies have shown that polymerase-kappa, a low fidelity polymerase with a bias toward transversion mutations, is recruited in the absence of polymerase-eta. The myositis sequences also failed to show the normal targeting motifs in mutations from C/G in several positions, which may indicate additional defects in the first phase of the SHM mechanism, which is responsible for mutations from C/G nucleotides. Indeed, disruptions in the activity of uracil DNA glycosylase (UNG), which is responsible for the excision of the U incorporated by AID, were previously shown to block transversion mutations from C/G nucleotides [29]. Recently, UNG has been found to be upregulated in MG patients [34;35], and a transversion bias was demonstrated in patients with MG [17], similar to the findings in the myositis sequences. These results may reflect the ectopic microenvironment in which these B cells undergo SHM; the lack of regulation outside GCs [36] may induce different types or quantities

of signals in the mutating B cells. These signals may activate different enzymes and pathways, which, in turn, may contribute to deviation of the mutation spectra and targeting motifs from what is normally observed. Alternatively, the altered spectra may result from over-exhaustion of the SHM mechanism, possibly caused by the chronic nature of the response.

The significance of B cells in autoimmune diseases, and the potential of these cells as therapeutic targets, is further substantiated by B cell depletion therapies, which have emerged as influential therapeutic strategies for several autoimmune disorders, including myositis [37-41].

To conclude, we confirmed the hypothesis that antigen-driven B cell responses occur within the target tissues of myositis patients, and demonstrated deviations in the Ig V-gene repertoire from normal control values. Comprehensive mutational analysis revealed that antigen-driven SHM and clonal diversification of B cells is taking place within the muscle tissues. The responding B cells are proliferating and mutating outside the confines of ectopic GC structures commonly found in the target tissues of autoimmune disorders. To understand these intramuscular, antigen-specific, B cell reactions more fully, future studies need to address the nature of the stimulating antigen(s), the specificity and pathogenicity of the secreted antibodies and to establish the pathological mechanisms of these antibodies within the myopathies, leading to new and improved therapies for these diseases.

Materials and methods

Patients and samples

Archival muscle biopsies were obtained from the Pathology Department, Western Infirmary, Glasgow after informed consent, with approval of the area Research Ethics Committee (Supporting Information Table 1). Biopsies were embedded in OCT and either stored in liquid nitrogen or at -70°C. Serial frozen sections (8 μm) were cut and mounted on positively charged slides (VWR, UK), air dried, fixed in acetone for 10 minutes and stored at -70°C.

Immunohistochemical staining of tissue sections

Serial frozen sections were stained for cellular infiltrates at intervals of 40 – 80 μm, as previously described [25], using mouse monoclonal antibodies to CD20 (1:50), CD3 (1:100), CD4 (1:10), CD8 (1:50), Follicular Dendritic cell (FDC) clone CNA.42 (1:100), plasma cell (1:200) clone VS38c and Ki67 (1:100) (Dako) as well as control isotype antibodies.

Double immunofluorescence staining for identification of proliferating cells

Double immunofluorescent staining on frozen serial sections was conducted using the Mouse on Mouse (M.O.M) Immunodetection Immunofluorescent Kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. For identification of proliferating cells frozen sections were stained for CD20 (1:100), CD3 (1:100), CD4 (1:50), CD8 (1:100) and Ki67 (1:100) (Dako) using mouse monoclonal antibodies as well as control isotype antibodies. Sections were mounted in VECTASHIELD hardset mounting medium (Vector Laboratories) and immunofluorescence was visualised using a Zeiss Axiovert microscope with a Zeiss Plan-NEOFLUAR 63x lens (Carl Zeiss Ltd., Welwyn Garden City, UK) and a digital CCD camera (Hamamatsu, Tokyo, Japan) with Openlab Deconvolution software (Improvision). For each view, 5 images were taken at 0.5 micron intervals.

Double immunofluorescence staining of antigen-specific cells

Identification and phenotyping of antigen-specific cells was performed as described aboveusing 2 μg/ml biotinylated recombinant proteins, including Jo-1 (A kind gift from Prof. Paul Plotz, NIH, Bethesda, MD, USA); Calf thymus DNA (Sigma); Sm/RNP & Scl-70 (The Binding Site, UK), with either a mouse monoclonal antibody to CD20 (1:100) or to plasma cells (1:100) (Dako).

Micro-dissection and DNA extraction

To establish the antibody repertoire, areas containing B cell and plasma cell aggregations were micro-dissected using sterile blood lancets under a Nikon Diaphot inverted microscope (Melville, NY), and DNA was released by proteinase K digestion using QIAGEN DNA Micro Kit (QIAGEN, Sussex, UK), according to the manufacturer's instructions. The DNA preparation was used as a template for subsequent primary PCR reactions.

Amplification, cloning and sequencing of rearranged immunoglobulin V_H genes

Immunoglobulin genes from infiltrating plasma and B cells were amplified using a nested PCR system as previously described [25]. All amplification reactions were prepared in a Captair™Bio Biocap™RNA/DNA hood (Erlab, France) and were conducted on a T-professional Thermocycler (Biometra, Tampa, FL) using the Expand High Fidelity PCR System with 2.6U DNA polymerase (Roche, Mannheim, Germany). For primary amplification reactions, 10 pmole of V_H leader primer mixture and 10 pmole of universal J_H primer were used with an annealing temperature of 50°C. Amplified DNA bands, approximately 400 base pairs, were excised using QIAquick gel extraction columns (QIAGEN, Sussex, UK) according to the manufacturer's instructions then ligated with TA cloning vector PcRII and transformed into

TOP10F' cells (Invitrogen) and cloned. Plasmid DNA was prepared using QIAprep mini-prep kits (QIAGEN, Sussex, UK) according to the manufacturer's instructions and sequenced. DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Repertoire of rearranged V_H genes expressed on muscle infiltrating B cells from DM and PM patients

To establish the Ig V-gene repertoire and SHM characteristics of the muscle infiltrating plasma and B cells, and to identify clonally related sets of sequences, sequences were analysed using Chromas Lite software and the best matching corresponding human germline genes were identified by searching the IMGT database using JOINSOLVER (http://joinsolver.niams.nih.gov) and IMGT/V-QUEST software (http://imgt.cines.fr) [42;43]. The full repertoire of Ig genes expressed on muscle infiltrating B cells and/or plasma cells was established for each patient; data were then pooled and presented according to the myositis subset (DM/PM). In order to assign the best matching D gene segment with 95% probability, methods were conducted as described [43]. D genes were statistically assigned with 95% probability in 59% and 42% of DM and PM repertoires respectively. Clonally related sequences were identified on the basis of identical VDJ gene rearrangements, CDR3 regions and junctional sequences and the number of base changes regarded as significant was based on the PCR error rate for the polymerase, previously determined, to be less than one base per 4 V_H gene segments [25].

Comprehensive mutation analysis of clonally-related Ig V-genes from muscle infiltrating B cells

Mutations in clonally-related sets of V-genes identified from DM and PM patients (analysed collectively as myositis patients) and GC controls were enumerated and analysed in further detail using IgTree© [12] and additional programs incorporated into IgTree© [16;17;44;45] that perform analysis of mutation targeting motifs, aa replacements and RS analyses utilizing the focused binomial test of Hershberg et al. [46]. Thus, the results are subject to the tree structure created by the IgTree© program, as previously described [16;17;44;45].

Statistical analysis

The distribution of V_H, D and J_H family usage in each myositis subset, as well as individual V_H, D and J_H gene usage, was compared to normal control values described elsewhere [13] and assessed using χ^2 analysis using GraphPad Prism (GraphPad Software Inc., San Diego CA, USA), P values < 0.05 were deemed significant. Statistical analysis for the comprehensive mutational analysis of clonally related Ig V-genes was conducted as previously described [12;16;17;44;45].

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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Figure Legends

Figure 1**. B cells and plasma cells are components of the muscle-infiltrating inflammatory population**

Representative examples of serial sections of inflamed muscle tissue from IM patients stained for (A) $CD20^+$ B cells, (B) follicular dendritic cells (FDCs), (C) plasma cells (clone VS38c) and (D) proliferating $Ki67^+$ cells visualised using APAAP labelling (red) and haematoxylin (light blue), except Ki67 sections which were not stained with haematoxylin as both constitute nuclear stains. (E) Double immunohistochemical staining was performed for CD20, visualised by Fluorescein Avidin D (green), and Ki67, visualised by Texas Red Avidin D (red) to identify proliferating B cells within the infiltrating cell population. (A, B & D) Images were taken from samples from 2 DM patients (M16 (A, B); M1 (D)). (C, E) Images were taken from samples from PM patients (M5 and ME respectively). (C) Black arrows indicate the presence of plasma cells; (E) white arrows indicate $Ki67^+$, $CD20^+$ proliferating B cells. Initially sections at intervals of between 40 to 80 μm were stained for $CD20^+$ B cells and plasma cells to determine the presences of these phenotypes (n=3-7 depending on the tissue availability). Serial sections surrounding areas of B cell and/or plasma cell infiltration were used to establish the infiltration of the other cell phenotypes ($n \geq 3$). Original objective lens magnification for images A-D: 40x; E: 63x. (E) Scale bar is 15 μm.

Figure 2

Diverse V-gene family usage and abundant somatic mutations in muscle-infiltrating B cells and plasma cells in DM and PM patients

Comparison of (A) V_H, (B) D and (C) J_H gene family usage in functionally rearranged V-genes expressed by muscle-infiltrating B cells and plasma cells in DM and PM patients. DNA was released from micro-dissections ($n \geq 3$ from different cellular aggregations within the same

section and/or different sections) and amplified using a nested PCR system. Best matching germline genes were identified from aligning sequences in the IMGT database using JOINSOLVER software [43]. Gene distributions were compared, from 148 independent sequences (DM-93; PM-55) to expected values based on the germline complexity and published normal PBL control gene distributions [13]. * Represents a significant difference from the germline composition; ** represents a significant difference from normal control values, † represents a significant difference between DM and PM myositis subsets ($p<0.05$, $\chi 2$ test). (D) The number of single point somatic mutations in V-gene regions was identified by comparison with the closest matching germline V_H gene. The first 24 nucleotides of the genes were excluded from the analysis as they were encoded by the 5' V_H primer. (E) The locations of mutations observed within functionally rearranged genes were categorised as being within FWR or CDR regions. The number of mutations within these regions was expressed as a percentage of the entire length of the region, to correct for the longer lengths observed within the FWR.

Figure 3

Oligoclonal expansion of muscle-infiltrating B cells and plasma cells in DM and PM patients

Lineage trees were constructed from sequences isolated from muscle-infiltrating B cells and plasma cells in individual patients using the IgTree© [12]; sample drawings of lineage trees from (A) myositis patients, (B) normal control spleen and Peyer's Patch GCs (data included 19 clones from 5 patients [14]) and (C) normal control PB (data included 33 clones from 3 patients, [47]) are shown. The double circles represent the germline gene, empty circles are hypothetical split node intermediates and black circles are the observed sequences. The numbers by each edge represent the number of mutations that separate the corresponding nodes. Edges that are not accompanied by a number represent one mutation. (D) Comparison of lineage tree

properties between myositis patients and normal GC and PB controls. Data are shown as the mean and SD calculated on the PL-min, DLFSN-avg, DRSN-min, T, OD-avg, RootD and DASN-min measurements of the trees. There were 8 myositis (pooled DM and PM) trees, 19 normal GC trees, and 33 normal PB trees. Each tree represents one clone. PL-min: minimum path length; DLFSN-avg: average trunkless path; DRSN-min: minimum root-fork distance; T: trunk; OD-avg: average outgoing degree; RootD: minimum root-fork distance; DASN-min: minimum fork-fork distance. Statistical significance was determined by the Mann–Whitney U-test and the FDR correction for multiple comparisons.

Figure 4

SHM spectrum and targeting motifs of clonally-related sequences from myositis patients differ from those of controls. (A) Mutation frequencies. The number of mutations from each nucleotide, presented as percentage out of the total number of mutations. The numbers above each circle represent the total number of mutations analysed in each group. (B) Transition (white) versus transversion (black) mutations, presented as percentage of the total number of mutations. (C) SHM targeting motifs. Nucleotides flanking the mutated nucleotide in six positions were enumerated for each type of mutation. On the X-axis, −1, −2 and −3 denote the three positions flanking the mutation upstream; +1, +2 and +3 denote the three positions flanking the mutation downstream. Letters on the positive and negative sides of the Y-axis denote excess or paucity of the indicated nucleotide in that position, respectively. The size of each letter is proportional to the "% difference", that is, the percentage composition of each base at each position flanking a p articular mutation, minus the baseline percentage composition of the germline sequences, to show any differences particular to that mutation. The levels of significance of the frequency of the mutated nucleotide compared with its theoretical frequency ($\chi 2$ test) are indicated by asterisks: *p<0.05, **p<0.005 and ***p<0.0005. Motifs were

compared with normal targeting motifs reported in the literature [48-53], as shown at the bottom in the line marked “Reported motifs”; the mutated nucleotides are depicted in colour.

Figure 5

AA substitutions in sequences from myositis patients mostly maintain the receptor's structure. Enumeration of aa substitutions for myositis and normal GC and PB sequences. The rows (indicated on the left) represent the pre-mutation aa and the columns (indicated on the top) represent the post-mutation aa. The coloured squares represent the average frequency of a mutation in all trees in a data group – the unscaled frequencies for "all regions" and scaled by region lengths (in parentheses) for the FWRs/CDRs are given in the key. The coloured circles represent the nature of aa substitutions – conservative, neutral or altering.

The V_H repertoire and clonal diversification of B cells in inflammatory myopathies

Donna McIntyre, Neta S. Zuckerman, Max Field, Ramit Mehr, David I. Stott

Supplementary Information

1) Supplementary Tables 1 and 2
2) Supplementary Results and Supplementary Figure 1
3) Supplementary Methods

Supplementary Table 1

Clinical Characteristics of IM Patients

Subject	Diagnosis	Age at biopsy/Gender	Disease Duration from Diagnosis	CK (IU/L)	Therapies at time of biopsy
M1	DM [a)]	40/F	0	142	-
M16	DM	33/F	0	1076	-
M3	PM	80/M	0	897	NSAID
M5	PM	40/F	3.5 years	1031	Pred, IVIg
M6	PM	63/M	0	919	-
M7	PM	50/M	0	8390	-
M19	PM	62/F	0	Normal	MTX, Corticosteroid
M23	PM	45/F	0	5829	-
M24	PM	59/F	0	6623	Pred, Statins
MC	PM	66/M	0	2500	Pred, Statins
ME	PM	49/F	0	NR	Pred
M17	IBM	66/F	0	2716	-
Control	N/A	37/F	N/A	NR	N/A

[a)] DM, Dermatomyositis; PM, Polymyositis; IBM, Inclusion Body Myositis; F, Female; M, Male; N/A, Non-Applicable; NR, No Record; NSAID, Non-Steroidal Anti-Inflammatory Drugs; Pred, Prednisolone; IVIg, Intravenous Immunoglobulin; MTX, Methotrexate; CK, Creatine Kinase.

Supplementary Table 2

Sequence Characteristics of Clonally-related Ig V-genes Isolated from Muscle-Infiltrating B cells [a)]

Patient (Diagnosis)	Clone	Germline Genes			Range of point mutations/V_H region [b)]	Number of Observed Clonal Variants using IgTree© [c)]	CDR3 Length (aa)
		V_H	D	J_H			
M1 (DM)	A	3-66*01	2-21*01	3*02	26-31	2	19
M16 (DM)	A	3-07*01	No D Gene Assignment	3*02	24-34	9	14
	B	3-11*01	No D Gene Assignment	6*03	0-13	5 [d)]	19
M3 (PM)	A	1-46*01	No D Gene Assignment	5*02	10-11	2	14
M5 (PM)	A	3-30*04	5-12*01	6*02	16-19	2	22
	B	3-53*01	3-22*01	4*02	18	2	16
	C	4-34*01	No D Gene Assignment	4*02	25-28	3	10
	D	5-51*01	No D Gene Assignment	3*02	12-32	4	18
M23 (PM)	A	3-33*01	No D Gene Assignment	5*02	8-18	2 [e)]	14

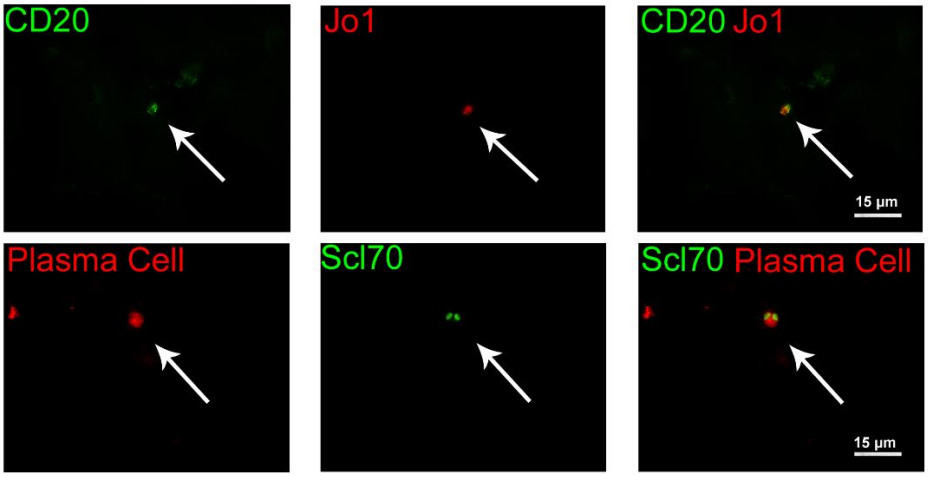
a) Clonally-related sequences were identified based on a common VDJ gene rearrangement, CDR3 and junctional composition. Best matching germline genes were identified by searching the IMGT database using the JOINSOLVER software. In some cases a D gene could not be assigned with statistical significance according to the assignment criteria [43].
b) A total of 252 mutations were analysed using IgTree©;
c) The total number of sequences analysed, including replicates, was 38;
d) Indicates that the germline gene was also isolated from the myositis B cell infiltrating population;
e) Two additional genes were also identified which exhibited the same CDR3, VD and DJ junctional sequences and a similar mutational pattern but the V_H sequences were matched with a higher score to a different gene.

Supplementary Results

Identification of antigen-specific muscle-infiltrating B cells in inflammatory myopathies

The identification of antigen-specific cells was briefly explored towards the conclusion of this study, to elucidate the identity of antigen(s) as potential candidates for stimulating the response. Antigen-specific cells were initially identified using light microscopy (not shown) with antigen-specific B cells and plasma cells identified using double immunofluorescence microscopy on biopsies from 5 patients whose sera contained antibodies against Jo-1 (a myositis-specific antigen), nuclear antigens Sm/RNP, Scl-70 and DNA, singly or in combination with another antigen. Both antigen-specific B cells and plasma cells were observed within the infiltrating cell populations (Supplementary Figure 1), although there was only a limited number of antigen-specific cells in relation to the total number of infiltrating B cells and plasma cells, suggesting that alternate or modified versions of antigens may also be responsible for stimulating the B cell response within IMs. The phenotype of antigen-specific cells (B cell or plasma cell) was uniform throughout the sections, with the exception of one sample with specificity towards Scl-70. Jo-1 specific B cells and plasma cells have been described briefly elsewhere [10]. Antigen-specific cells were isolated using laser capture micro-dissection followed by single cell V-gene amplification or amplification of V-genes from a small number of cells with the same antigen-specificity. Within the constraints of the study only a limited number of sequences were isolated and sequenced; therefore, no definitive conclusions can be drawn concerning the V-gene repertoire and mutation patterns used by autoantigen-specific cells.

Supplementary Figure 1

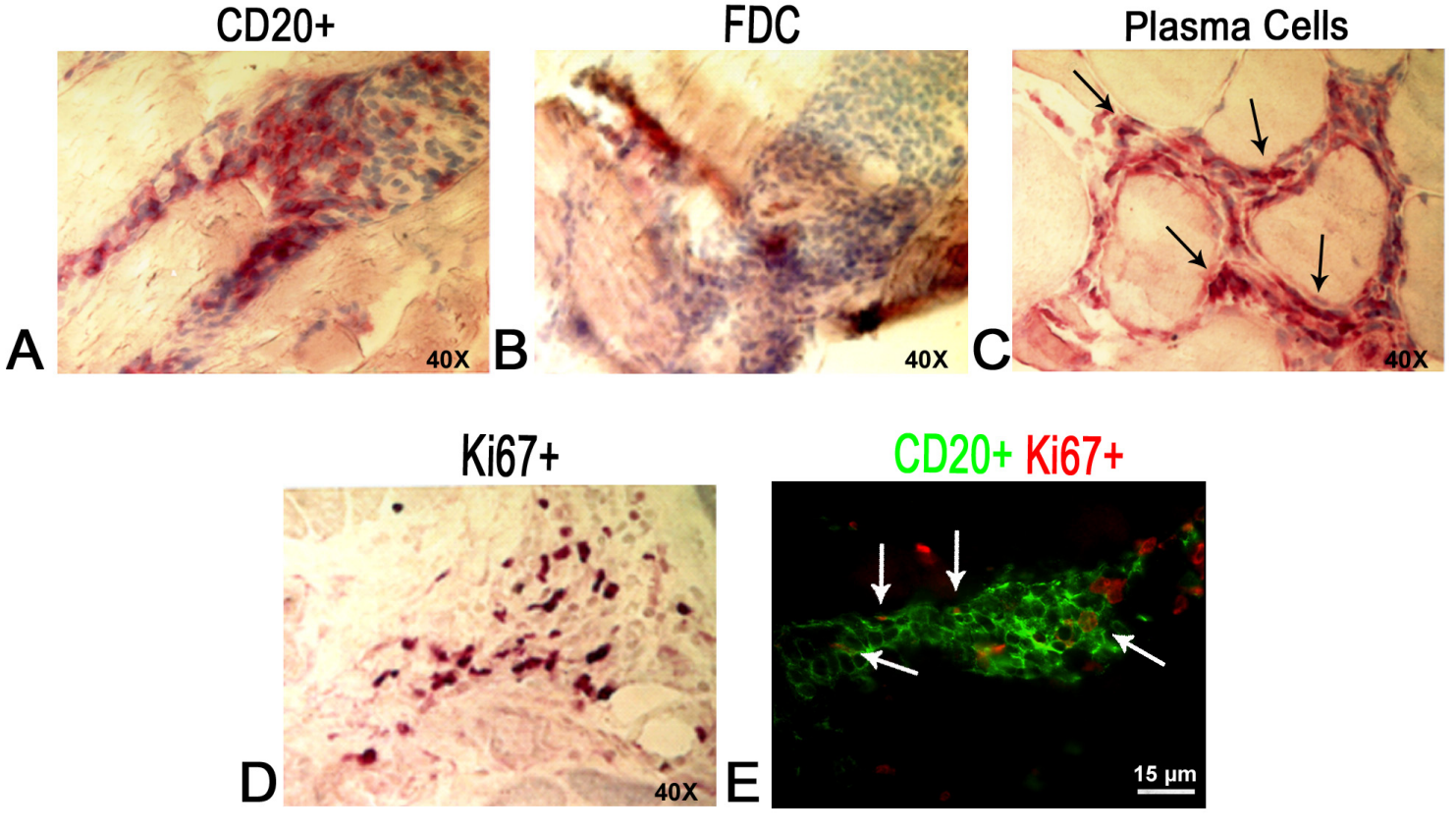


Representative examples of sections of inflamed muscle from IM patients stained for $CD20^+$ B cells and plasma cells and with biotinylated antigens to identify the phenotype of muscle-infiltrating, antigen-specific cells. Antigen-specific cells were visualised by Fluorescein Avidin D (green) and Texas Red Avidin D (red). Images were taken at 63x (objective lens); Scale bar represents 15 µm. Arrows identify antigen-specific cells within the muscle-infiltrating population. Images shown are from sections surrounding areas of B cell and/or plasma cell infiltration, as described for Figure 1, and were used to establish the infiltration and phenotype of antigen-specific cells ($n \geq 3$).

Supplementary Methods

Double immunofluorescence staining of antigen-specific cells

Identification and phenotyping of antigen-specific cells was performed as described in Materials and Methods using 2 µg/ml biotinylated recombinant proteins, including Jo-1 (A kind gift from Prof. Paul Plotz, NIH, Bethesda, MD, USA); Calf thymus DNA (Sigma); Sm/RNP & Scl-70 (The Binding Site, UK), with either a mouse monoclonal antibody to CD20 (1:100) or to plasma cells (1:100) (Dako).


CD20+
FDC
Plasma Cells
A
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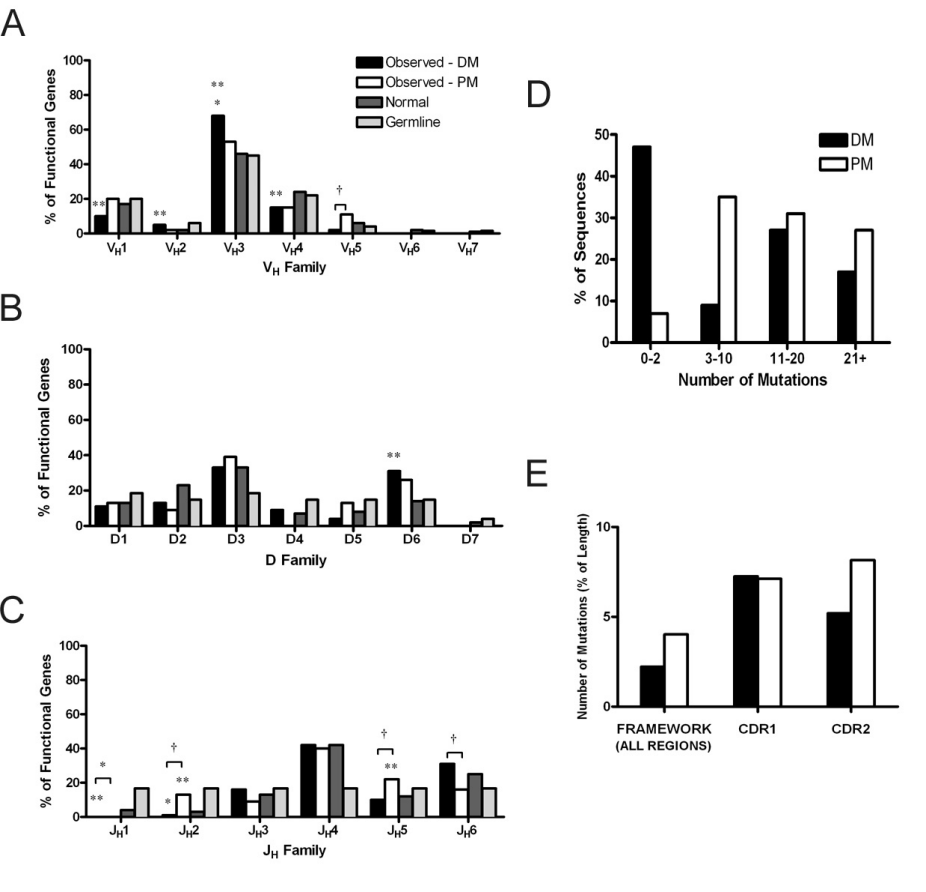
B
40X
C
40X
Ki67+
CD20+ Ki67+
D
40X
E
15 µm


A
% of Functional Genes
Observed - DM
Observed - PM
Normal
Germline
VH1
VH2
VH3
VH4
VH5
VH6
VH7
VH Family
B
% of Functional Genes
D1
D2
D3
D4
D5
D6
D7
D Family
C
% of Functional Genes
JH1
JH2
JH3
JH4
JH5
JH6
JH Family
D
% of Sequences
DM
PM
0-2
3-10
11-20
21+
Number of Mutations
E
Number of Mutations (% of Length)
FRAMEWORK (ALL REGIONS)
CDR1
CDR2

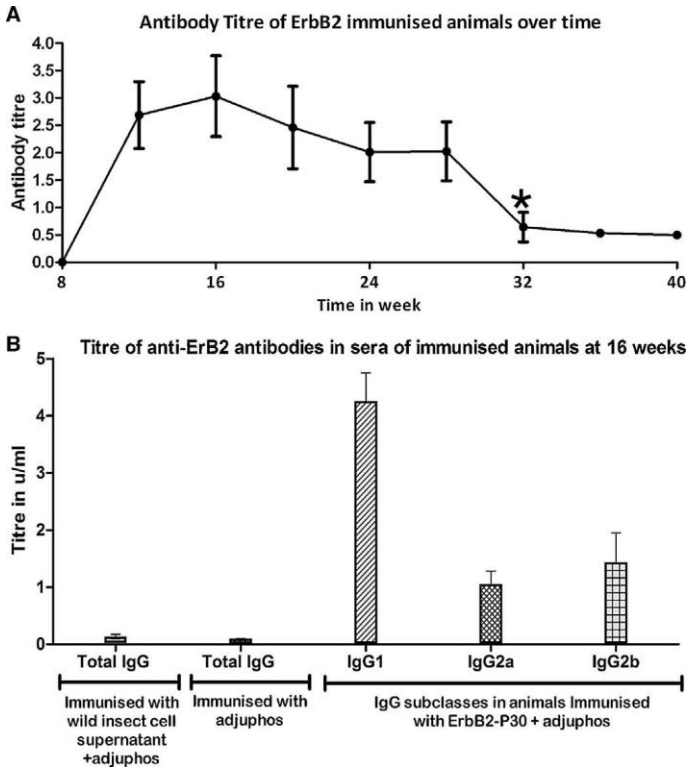


Fig. 3. Titre of anti-ErB2 antibodies in sera of immunised animals at 16 weeks. (A) All 15 of the ErbB2-P30 immunised animals showed high titres of anti-ErbB-2 antibody up to 28 weeks of age, diminishing significantly by week 32. (B) The control animals immunised with either wild type insect cell supernatant and adjuphos or adjuphos only did not show any ErbB2-P30 specific antibody response when the titre was compared to the IgG subclasses of animal immunised with ErbB2-P30. In the latter animals IgG1 was significantly higher compared with IgG2a at the age of 16 weeks when the titre was at its highest.

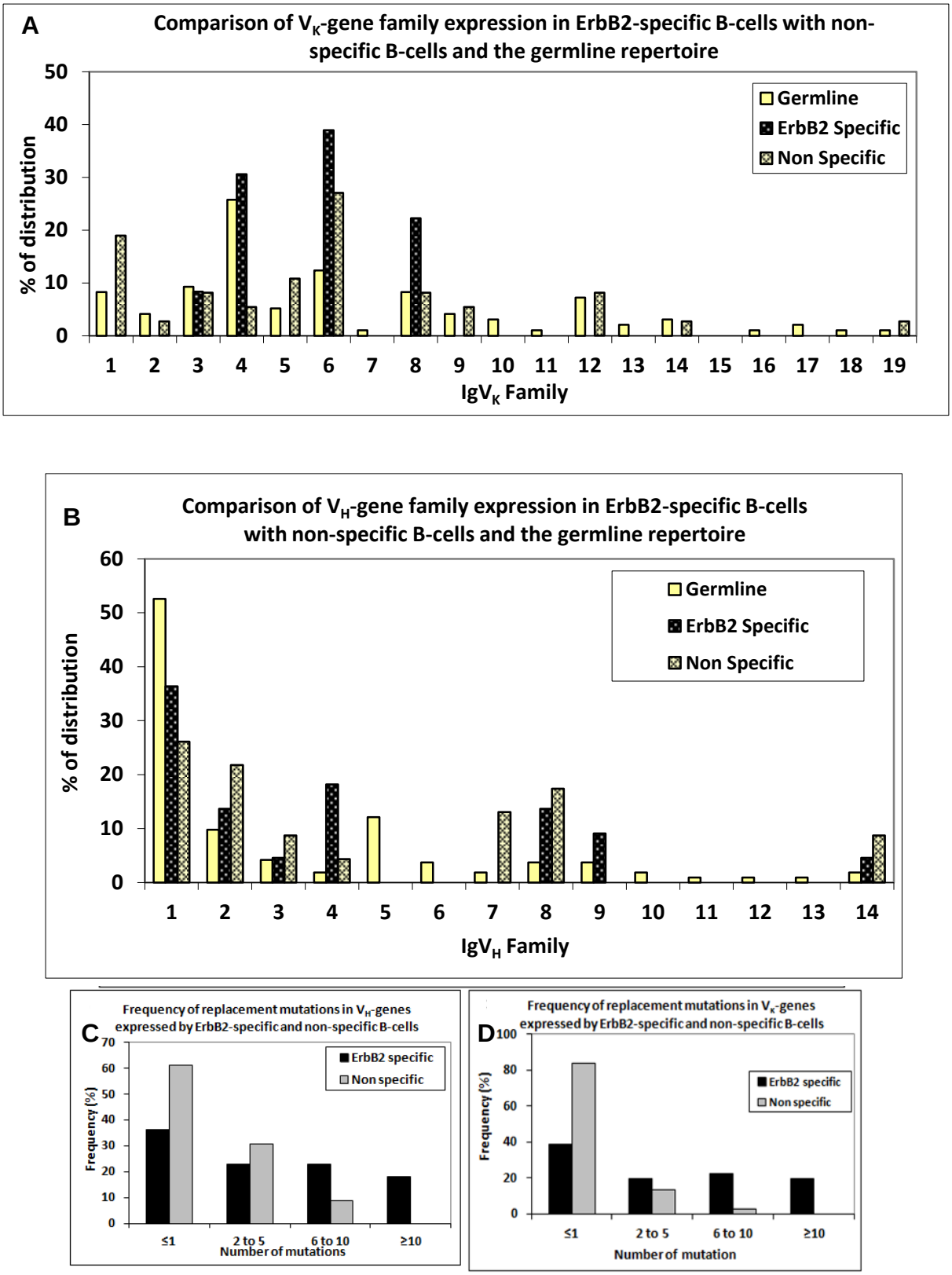


Figure 1: Expression of IgVκ and IgV_H gene families and somatic hypermutation in anti-ErbB-2 antibodies. (A & B) Relative distribution of expression of IgVκ and IgV_H families among ErbB-2 specific compared with non-specific, splenic B cells and the germline repertoire; (C & D) Comparative distribution of replacement mutations in IgV_H & $IgV_κ$ genes expressed by ErbB-2 specific and non-specific, B cells in ErbB2-P30 immunised mice. There is restricted expression of V-gene families in

ErbB-2 specific B cells compared with the number of members in germline genes and the non-specific repertoire, especially $V_{\kappa}4$, $V_{\kappa}8$ and V_{H4}. A high level of replacement mutations was observed in both V_H and V_{κ}-genes from ErbB-2 specific B cells compared with non-specific cells.