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Regional and global changes in TCRαβ T cell repertoires in the gut are dependent upon the complexity of the enteric microflora

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ABSTRACT

The repertoire of gut associated T cells is shaped by exposure to microbes, including the natural enteric microflora. Previous studies compared the repertoire of gut associated T cell populations in germ free (GF) and conventional mammals often focussing on intra-epithelial lymphocyte compartments. Using GF, conventional and monocolonised (gnotobiotic) chickens and chicken TCRβ-repertoire analysis techniques, we determined the influence of microbial status on global and regional enteric TCRβ repertoires. The gut of conventionally reared chickens exhibited non-Gaussian distributions of CDR3-lengths with some shared over-represented peaks in neighbouring gut segments. Sequence analysis revealed local clonal over-representation. Germ-free chickens exhibited a polyclonal, non-selected population of T cells in the spleen and in the gut. In contrast, gnotobiotic chickens exhibited a biased repertoire with shared clones evident throughout the gut. These data indicate the dramatic influence of enteric microflora complexity on the profile of TCRβ repertoire in the gut at local and global levels.

1. Introduction

The healthy adult intestine is colonised by complex microbial flora which has an intricate relationship with the gut associated immune system. Normal development of enteric immune compartments is dependent on microbial colonisation of the gut of the young animal; responses are induced and tightly regulated in the form of oral tolerance. The enteric adaptive immune system comprises different populations of cells organised in discrete compartments including those of the intra-epithelial (IEL) and *lamina propria* (LP). The IEL are dominated by T cell subsets and the LP by a combination of T and B cells which respond to microbial flora and protect the gut against invading pathogens. T cells represent a major component of the enteric immune cell populations and microbial colonization drives expansion and altered phenotypic composition of TCRαβ IEL populations [1–3]. These changes are associated with alteration in the complexity of T cell receptor (TCR) repertoire based upon clonal dynamics (expansion, deletion or movement) of T cell populations.

Initial T cell seeding of the gut occurs either *in utero* early in gestation (e.g. humans), just prior to birth (rodents) or *in ovo* just prior to hatch (chickens). In mammals, the initial polyclonal repertoire of TCRαβ T cells alters according to age, becoming increasingly oligoclonal [3–8]. Support for microbial flora as a driver for oligoclonal expansion of the repertoire is evident from comparisons in rats between those reared germ-free (GF), conventional [9] and GF rats "conventionalised" by administration of microbial flora [10]. In mammals, non-microbial factors may also influence the biology of T cells including maternally derived prolactin and other constituents of milk [11]. Weaning associates with changes in the TCRβ repertoire of normal mammals [3] and has effects on both microbial status and the availability of maternally derived signals. To explore the phylogenetic conservation of gut T cell repertoire changes associated with microbial colonisation, and to separate the effects of ongoing maternal versus environmental cues, we examined TCRαβ T cell repertoires in the gut of germ-free, conventional and monocolonised (gnotobiotic) chickens.

Chicken T cell biology has many parallels with those of mammals (reviewed in [12]) but some specific characteristics

Abbreviations: TCR, T cell receptor; GF, germ free; CDR3, complementary determining region 3.

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facilitate the use of chickens in the study of TCRαβ T cell repertoire. Perhaps the most important is that the chicken has just two families of TCRVβ gene segments [13,14] allowing global assessment of all TCRβ rearrangements in fewer assays than required for mammals which, for practical reasons, often leads to focused analysis on a small subset of the available TCRVβ. The chicken MHC is also less complex than with mammals [15], a feature that may restrict the diversity of peptides presented to T cells. In mammals, some TCRVβ-expressing T cells preferentially locate to the intestine and this is also seen with chickens which exhibit a dramatic bias towards TCRVβ1+ T cells in the intestine [16–18]. Depletion of TCRVβ1+ T cells leads to reduced IgA production in the gut [19]. The changing susceptibility of chickens to a variety of enteric diseases according to age, as seen with *Salmonella enterica* serovar Typhimurium [20] and the need to protect commercially valuable stock (with vaccines) from a young age indicates the practical importance of understanding developmental and microbiological influences on gut immune function. Hence, there is substantial literature documenting age-related changes of the chicken intestinal immune system in terms of morphology, cell populations, cytokine production and the ability to respond to vaccines, pathogens and model antigens, reviewed in [21–23]. Changes in gross anatomy and cellular subsets in germ-free chickens indicate the broad effect of interactions with the enteric microflora, including alterations in lymphocyte cell numbers [24]. T cell populations are larger in conventional birds and without TCR repertoire analysis it is difficult to determine whether these are local TCR-mediated clonal expansions, infiltration of cells from other sites without cell division or a combination of the two. Limited sequence-based analysis of TCRβ repertoire from the small intestinal IEL of a single 20-day-old conventional-reared chicken indicated some clonal over-representation of specific TCR rearrangements within a clonally diverse background population [25].

Previous analysis of the chicken TCRβ genomic region identified six Vβ1, four Vβ2, one Dβ and one Cβ segments [14,26,27] and four Jβ [17,28]. Dunon et al. [17] reported 17 different TCRVβ1 sequences derived from multiple individuals which may indicate genetic polymorphism in this region. The sequences of the two Vβ subfamilies have low homology, but each Vβ subfamily contains several members with highly homologous sequences. In addition, one of the Vβ2 gene is located 3′ to the Cβ gene and in reverse transcriptional orientation relative to the other Vβ genes. During T cell development the TCRβ locus undergoes VDJ gene rearrangement with N and P nucleotide junctional modifications contributing to the diversity of the rearranged product [14,27].

Using spectratype and sequencing approaches to assess TCRβ repertoire, we determined the effect of microbial status on the clonal distribution of TCRαβ+ T cells in the chicken intestine. Similar changes in T cell repertoire were observed with TCRVβ1+ and Vβ2+ T cells in different regions of the small and large intestine from germ-free, conventional and monocolonised chickens. The clonal composition of T cells from different parts of the gut was dependent on microbial status indicating the effect of enteric flora on T cell biology. These data have implications in our understanding of vertebrate gut immune function and specifically in the gut health of poultry.

2. Materials and methods

2.1. Experimental chickens

Specific-pathogen-free Rhode Island Red (RIR) chickens were supplied by the Poultry Production Unit of the Institute for Animal Health (IAH), Compton Laboratory. Birds were reared with *ad libitum* access to water and a vegetable-based protein diet (Special Diet Services, Witham, UK). Birds were wing-banded to allow identification of individuals.

Germ-free (GF) RIR chickens were produced at the Special Animal Production Unit of Institute for Animal Health based upon previously described methods [29,30] with disinfection of eggs shortly after lay using 1% Ambicide (2 min at 38 °C) followed by 1% Peracetic acid upon transfer to a sterile incubator. Hatched chicks were maintained in flexible plastic isolators and given sterile-water and sterile, irradiated food. The GF status was monitored at regular intervals by aerobic and anaerobic culture of cloacal swabs and of caecal samples obtained at post-mortem. Gnotobiotic chickens were the result of adventitious contamination with *Bacillus* spp. and detected at the first sample point after hatch. All birds were used at 3 weeks of age.

2.2. IEL preparation

IEL were isolated using a modified protocol as described previously [31]. Briefly, the small intestine was removed at post-mortem, excised of all Peyer's patches, cut into 1-cm pieces and washed before incubation in 1 mM DTE (Sigma–Aldrich) in PBS at 37 °C in a gently shaking water bath for 20 min. After 30 s of vigorous shaking, the supernatant was collected and cells recovered by centrifugation at 450 × *g* for 10 min at 4 °C. This procedure was repeated twice. IEL were purified using a discontinuous gradient consisting of 45 and 70% Percoll (GE healthcare). Gradients were centrifuged at 400 × *g*, for 30 min at RT and IEL were collected at the 45/70% interface and washed using PBS/2% Fetal Calf Serum (FCS) and centrifugation at 450 × *g* for 10 min. IEL were then analysed as required.

2.3. Production of soluble Bacillus lysate antigen

The contaminating *Bacillus* spp. in the gnotobiotic birds was isolated and used to inoculate 250 ml Erlenmyer flasks containing 100 ml LB medium and incubated overnight at 37 °C in an orbital incubator (150 r.p.m). Bacterial cells were pelleted by centrifugation at 4080 × *g* for 25 min at 4 °C and washed twice with an equal volume of PBS followed by resuspension in 20 ml PBS. The bacterial suspension was subjected to three freeze–thaw cycles in liquid nitrogen before sonication (9× 20 s bursts with 1 min cooling between bursts) in 10 ml volumes on ice at an amplitude of 15 μm using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). The suspension was filtered through a 0.2 μm filter and subjected to ultracentrifugation (30,000 g, 20 min, 4 °C); the supernatant was retained as a soluble antigen preparation. Protein concentrations were determined using the Bradford protein determination kit (Merck, Poole, UK) standardised to rabbit gamma globulin and aliquots frozen (−20 °C) until used.

2.4. T cell proliferation assays

Single cell suspensions of splenocytes were prepared by physical disruption of the spleen through a Falcon cell strainer (BD Biosciences, Oxford, UK) in RPMI 1640 (GibcoBRL, Paisley, UK) supplemented with 5×10^{-5} M β-Mercaptoethanol, 100 U/ml penicillin, streptomycin (1 μg/ml) and 5% FCS. The majority of red blood cells (RBC) were removed by centrifugation at 35 × *g* for 10 min. The supernatant (retaining the lymphocytes and accessory cells with <10% RBC contamination) was adjusted to a concentration of 10^7 cells/ml in RPMI 1640 containing 5% FCS and added to U-bottom microtitre plates (100 μl/well). Where required, *Bacillus* lysate (10 μg/ml) or PHA (20 μg/ml) in RPMI 1640 containing 5% FCS was added to the cell suspension. After incubation at 41 °C in an atmosphere of 5% CO_2 for 72 h, the cultures were pulsed with 1 μCi ^{3}H-thymidine (Amersham, UK) per well for the last 18 h.

Plates were harvested onto a Tomtec Mach IIIM cell harvester (Receptor Technologies, Banbury, UK) and incorporation of ^{3}H-thymidine determined on a 1450 microbeta Trilux scintillation counter (PerkinElmer, UK).

2.5. RNA isolation

Tissue samples were stored in RNAlater (Qiagen Ltd., Crawley, United Kingdom) at −20 °C before disruption by homogenization (Mini-bead beater; Biospec Products, Bartlesville, Okla). Isolated cell subsets or cultured cells were disrupted directly in RLT buffer (Qiagen Ltd.) and frozen at −20 °C until RNA extraction. RNA was extracted with the RNeasy Mini kit (Qiagen Ltd.) according to the manufacturer's instructions. Contaminating DNA was digested on column with RNase-free DNase 1 (Qiagen Ltd.) for 15 min at room temperature. The RNA was eluted with 50 μl RNase-free water and stored at −80 °C.

2.6. Reverse transcription

Reverse transcription reactions were performed using the iScript Reverse Transcription system (iScript™ Select cDNA synthesis Kit, Bio-Rad, USA) according to manufacturer's instructions, using ∼2 μg isolated RNA from each sample and oligo(dT) primers. Twenty microliters of cDNA was obtained for each sample and stored at −20 °C.

2.6.1. Polymerase chain reaction (PCR)

PCRs were performed according to standard protocols. Briefly, cDNA (2 μl) was incubated with 200 μm dNTP, 1.5 mM $MgCl_2$, 1× reaction buffer [50 mM KCl, 20 mM Tris–HCl (pH 8.4)], 2 U Platinum Taq DNA polymerase (Invitrogen), 1 μl of each primer at 10 μM working concentration, in a 50 μl final reaction volume. The forward primers used for Vβ1 and Vβ2 were 5′-ACAGGTC-GACCTGGGAGAC TCT CTGACTCTGAACTG-3′ and 5′-CACGGTCGAC-GATGAGAACGCTACCCTGAGATGC-3′ respectively. While a common Cβ reverse primer used to amplify both gene families was 5′-ACAGGTCGACGTACCAAAGCATCATCCCCATCACAA-3′ [17].

PCR conditions were as follows, one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 40 s and 72 °C for 1 min, followed by one cycle at 72 °C for 10 min using a G-storm thermocycler (Gene Technologies, Essex, UK) or Eppendorf mastercycler (Eppendorf, Hamburg, Germany). The amplified products were analysed by electrophoresis through 1% agarose (Sigma–Aldrich Ltd., Poole, UK) gels in 1× Tris–borate–EDTA buffer at 50 mA for 1 h, and products were visualized by staining with ethidium bromide (Bio-Rad, Ltd.) or GelRed nucleic acid stain (Cambridge BioSciences, UK).

PCR products were purified using QIAquick PCR purification kit (Qiagen Ltd.) according to manufacturer's instructions. DNA was eluted in 50 μl nuclease free water and stored at −20 °C.

2.7. Cloning and sequencing of PCR products

To determine the sequence of expressed Vβ-chain, PCR products amplified with a set of Vβ and Cβ primers were subcloned directly into the pCR®4-TOPO vector (Invitrogen) and used to transform competent TOP10 *Escherichia coli* (Invitrogen) according to the manufacturer's instructions. After incubation on selective LB agar plates containing 100 μg/ml Ampicillin (Sigma) single bacterial colonies were picked and screened for inserts of the correct size by PCR followed by agarose gel electrophoresis. Positive colonies were used to prepare Miniprep DNA using Qiagen Miniprep kit (Qiagen Ltd.) and subsequently sequenced with plasmid-specific (M13 forward; 5′-GTAAAACGACGGCCAG-3′ or M13 reverse; 5′-CAGGAAACAGCTATGAC-3′) or Cβ specific reverse primer (5′-TGTGGCCTTCTTCTTCTCTTG-3′) to define the identity of the TCRVβ CDR3 fragments. Alternatively, the plasmid insert amplified by PCR was purified using QIAquick PCR purification kit (Qiagen Ltd.) according to manufacturer's instructions and sequenced directly using a nested Cβ specific reverse primer (above). Sequencing was carried out using capillary electrophoresis on the CEQ8000 sequencer according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA).

2.8. Spectratyping

To determine the CDR3-lengths of the amplified PCR products by spectratype analysis, a run-off reaction was performed as follows. Five microliters of the purified PCR was incubated with 200 μm dNTP, 1 mM $MgCl_2$, 1× reaction buffer [50 mM KCl, 20 mM Tris–HCl (pH 8.4)], 0.5 U Taq DNA polymerase (Invitrogen), 1 μl of a WellRED dye D4 (Sigma) labelled nested Cβ specific reverse primer (5′-TCA TCT GTC CCC ACT CCT TC-3′) at 4 μM working concentration in a 20 μl final reaction volume.

The reaction conditions were as follows: one cycle 95 °C for 2 min, followed by 4 cycles of 95 °C for 2 min, 57 °C for 2 min and 72 °C for 20 min using a G-storm thermocycler (Gene Technologies, Essex, UK) or Eppendorf mastercycler (Eppendorf, Hamburg, Germany). The run-off reaction products were diluted 5× with nuclease free water and 1 μl of the diluted product was mixed with 40 μl sample loading dye (Beckman Coulter, Fullerton, CA) containing 0.25 μl DNA size standard kit-600 (Beckman Coulter, Fullerton, CA). The samples were transferred into a 96 well plate, overlaid with a drop of mineral oil and immediately loaded into a capillary sequencing machine (CEQ8000 Genetic Analysis System, Beckman Coulter) for fragment analysis. For optimal results, samples were analysed using a modified fragment analysis program (Frag-4) by increasing separation time to 75 min. The data were compiled in CEQ8000 analysis module and for each sample the range of base pair lengths of products were identified and displayed as spectratype profiles. Peak size data were extracted from the fragment analysis software and transferred into Microsoft®Excel.

2.9. Hierarchical cluster analysis

To standardise the spectratype data and statistically infer relationships between different spectratype profiles the following procedure was used. Briefly, the range of base pair lengths (''est frag size [nt]'') of products was identified for Vβ1 and Vβ2, quality control filters applied and the peaks aligned. Then the ''reading frame'' of the product sizes for each data row, profile and the whole dataset was determined assuming that all theoretical sizes in range should be exactly 3nt apart. This involved selecting the top 5 products according to their ''pk area (rfu × mm)'' values and assigning their theoretical sizes to be the nearest integer and using these sizes to identify the reading frame of the products. The frame of the entire profile was then obtained based on voting of the frames of these 5 products and the most frequently occurring reading frame was set to be the frame for the whole dataset. The product amount values were then converted into percentages of total signal. Therefore, at this point, peaks of the same size from different profiles indicate PCR products with the same length, and could therefore be compared in the model. For every profile which had missing values replaced, all of its values were rescaled so that they sum to 100%. The product size was regarded as a random variable and the profile represented a collection of such independent and identically distributed variables. Taking each profile as a distribution, we used a modified Kullback–Leibler divergence as the quantitative to compare two profiles as follows.

Each profile was regarded as a distribution of discrete random variable, so for two profiles P and Q, the dissimilarity (or distance) between them is:

$$D(P,Q) = 0.5 \times \sum \left[(p_i - q_i) \times \log \frac{p_i}{q_i} \right]$$

So that $D(P, P) = 0$.

Unlike the standard Kullback–Leibler divergence, it is symmetric:

$$D(P,Q) = D(Q,P),$$

and satisfies the triangle inequality most of the time:

$$D(P,Q) \leq D(P,R) + D(R,Q).$$

For unsupervised clustering, this distance between every pair of profiles was calculated to build the distance matrix. Traditional Hierarchical Clustering in *R* was applied to generate the clusters based on this distance matrix. Several Perl (version 5.8.5) and R (R version 2.7.2, R foundation for statistical computing, Vienna, Austria) scripts were used in this pipeline.

2.10. Statistical analysis of proliferation data

Statistical significance was evaluated with unpaired, two-tailed Student's *t*-test on Graphpad software. Differences between experimental samples were considered significant for $p < 0.05$.

3. Results

3.1. Chickens have a minimal TCRβ gene set

cDNA sequences of chicken Vβ1 and Vβ2 [14,26] were used to BLAST search the Ensembl chicken genome assembly (version 52, http://www.ensembl.org/Gallus_gallus). Similarly, the published genomic DNA sequence (acc. no. AB092341) was used to locate Jβ, Dβ and Cβ genes [28] with the TCRβ locus identified on chromosome 1. Previous studies reported identification of six Vβ1 gene segments in the genome [26] although 17 different Vβ1 sequences were detected by RTPCR from multiple birds [17]. Our analysis of the genomic sequence identified nine Vβ1 gene segments at the 5′ end of Vβ coding region. Four Vβ2 gene segments were identified and, as reported previously, three of these were located 5′ of D-J-Cβ region while the fourth was located 3′ to the Cβ in reverse transcriptional orientation relative to the other gene segments in the TCRβ locus [13]. We confirmed the location of four Jβ, one Dβ and one Cβ [17,28]. All gene fragments were located on the genomic sequence with appropriately positioned recombination signal sequences and distributed in a region of approximately 185 kb (Fig. 1A). No additional TCRβ-loci, psuedogenes or unassigned intact V, D, J or C fragments were detected in our BLAST searches of the *Gallus gallus* genome assembly. The Vβ segments grouped into two clear families with

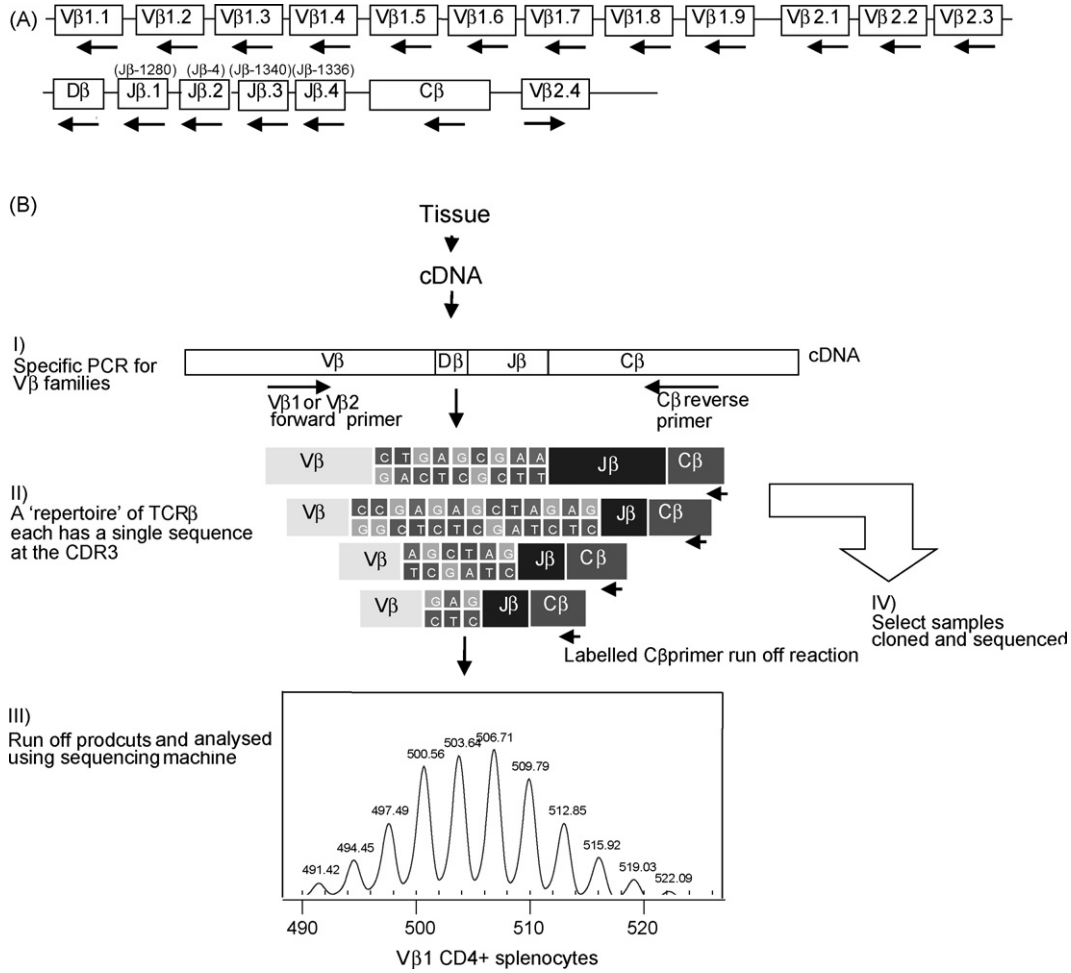

Fig. 1. (A) Genomic organisation of the chicken TCRβ locus highlighting the location of Vβ1, Vβ2, Dβ, Jβ, and Cβ gene segments. Open boxes indicate gene segments; arrows indicate transcriptional orientation. Multiple gene copies within a family are numbered according to their position in the locus as proposed by IMGT. (B) Schematic of chicken TCRβ repertoire analysis. (I) Depicts location of primers used to generate products for spectratyping. (II) Depicts the consequence of random addition/deletion of nucleotides in generating PCR products of varying length. (III). Depicts a non-biased TCR spectratype profile derived from CD4+ splenocytes. (IV). Depicts cloning and sequencing of selected PCR products to support spectratype data.

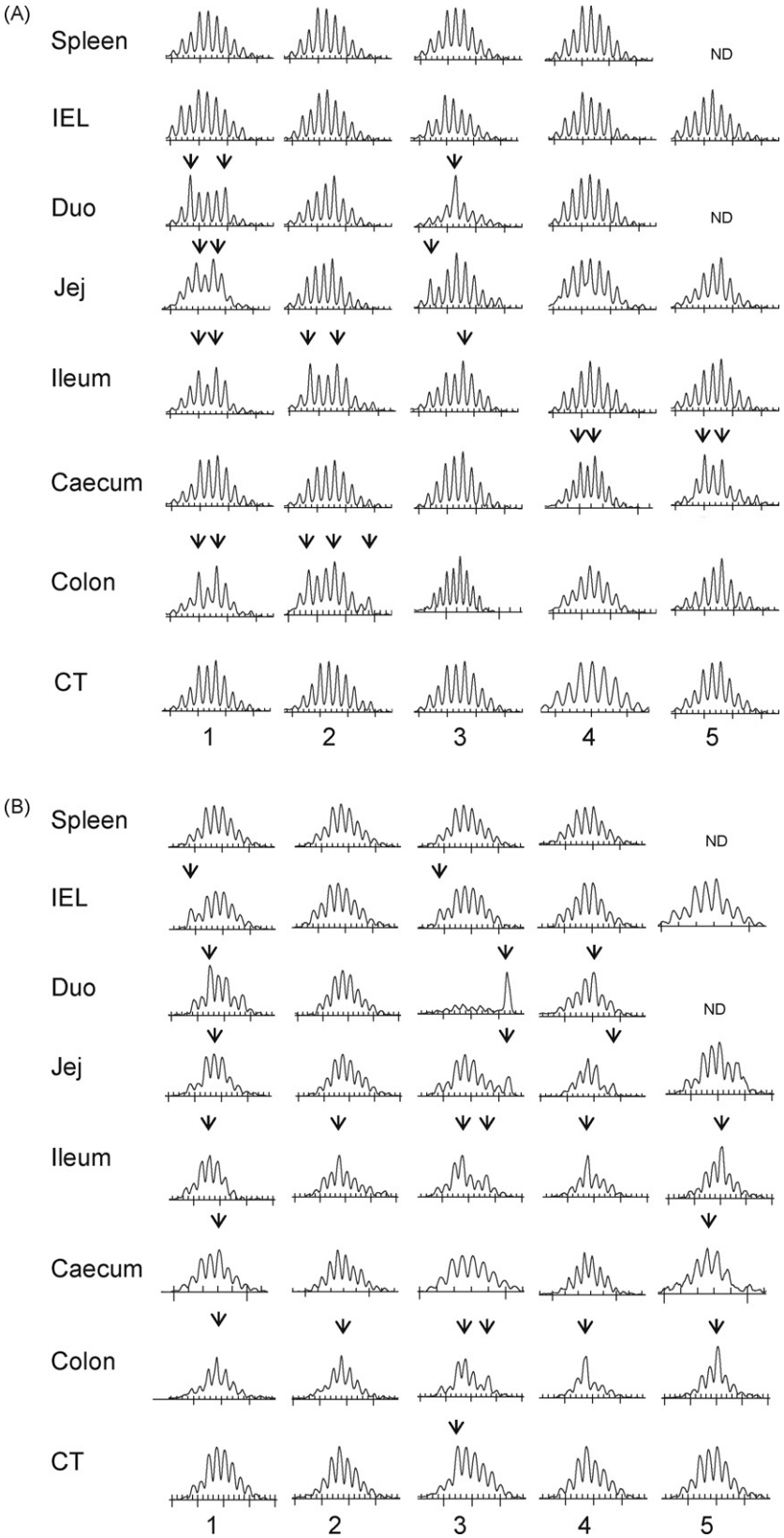

Fig. 2. Complex, biased TCR repertoires are derived from intestinal segments of conventionally reared birds. Spectratypes of TCR Vβ1 transcripts (A) and Vβ2 transcripts (B) in spleen and intestinal tissues from five 3 weeks old chickens (1–5). The spleen spectratypes show a polyclonal repertoire with Gaussian distribution while skewed distributions are evident in several intestinal regions with both Vβ1 and Vβ2. A selection of peaks representing CDR3-length bias are indicated (↓) with some being shared between intestinal segments. IEL, intra-epithelial lymphocyte; Duo, duodenum; Jej, jejunum; CT, caecal tonsil; ND, no data.

amino-acid identity of >85% within families and >21% amino-acid identity (>45% similarity) between Vβ1 and Vβ2 family members. However, within each family, groups of sequences were detected with differences evident in CDR1 and CDR2 encoding regions. For example, within the Vβ2 family there were two subgroups with 90% identity between Vβ2.1 and Vβ2.3 and 95% for Vβ2.2 and Vβ2.4.

The "minimal" TCRβ locus of chickens with two families of Vβ suggested that global TCRVβ repertoire analysis would be achieved more easily than with mammals (with much larger numbers of Vβ families). The repertoire analysis procedure is depicted in Fig. 1B; briefly, RT-PCR was achieved using Vβ1 and Vβ2 specific forward primers in combination with a common Cβ reverse primer to create template for CDR3-length profiling (spectratype analysis) and sequencing. Purified RT-PCR products were subjected to a "run-off" reaction with a labelled nested Cβ specific primer. The labelled products were subjected to fragment analysis with a capillary sequencing machine (CEQ8000 Genetic Analysis System, Beckman Coulter) and data compiled in CEQ8000 analysis module and the range of base pair lengths of products identified. As expected for a spleen-derived polyclonal T cell population, normally distributed spectral peaks were obtained for purified $CD4^+$ T cells from the spleen of a 3-week-old RIR chicken (Fig. 1B) and $CD8^+$ T cells (data not shown). Typically, the spectratype distribution of CDR3-lengths in the spleen cell populations consisted of approximately nine distinguishable peaks, each separated by three base pairs and with read sizes that indicate in-frame sequences (confirmed by sequence data). For further processing, peak size data were extracted from the fragment analysis software and transferred into Microsoft® Excel and used for cluster hierarchical analysis to visualise relationships between the CDR3-length spectra of test samples. Selected samples were processed for clonal sequence analysis.

3.2. Conventionally reared birds display complex TCRβ repertoires with regional oligoclonality in the intestine

Spectratype analysis of five 3-week-old conventionally reared RIR was carried out to study the distribution of the global repertoire of TCR Vβ transcripts in the spleen and regions of the gut. The splenocyte-derived spectratype profiles exhibited a Gaussian-like CDR3-length distribution (with ~9 detectable peaks) consistent with the interpretation of polyclonal repertoire in both TCRVβ1 and TCRVβ2 expressing T cells (Fig. 2A and B). Similarly, many of the samples obtained from different regions of the small (duodenum, jejunum and ileum) and large intestine (caecum, colon and caecal tonsil) or from whole small intestinal IEL exhibited a complex CDR3-length profile. Despite the clonal complexity seen with the intestinal samples, some CDR3-lengths were over-represented in all regions of the gut except for the caecal tonsil (indicated by arrows in Fig. 2; A for Vβ1 and B for Vβ2). The analysis of caecal tonsil failed to reveal any bias in the CDR3-length profiles indicating complex polyclonal repertoires in this anatomically distinct site (Fig. 2A and B). Isolation of IEL from the whole small intestine also exhibited polyclonal CDR3-length profiles, which did not match the individual segments of the small intestine. This may be the result of regional differences in profiles being lost by analysis of IEL from the whole small intestine or that the observed bias was derived from non-IEL (e.g. lamina propria lymphocytes) or related to the IEL isolation procedure. With the intestinal segments, different birds exhibited different CDR3-length biases but within an individual some adjacent regions produced profiles that had over-representation of peaks with similar sequence size values. For example, some shared peaks in the jejunum/ileum of Vβ1-profiles in bird 1(Fig. 2A) and duodenum/jejunum regions of Vβ2-profiles in bird 3 (Fig. 2B). Overall, the profiles of gut segments consistently exhibited biased CDR3-length profiles but these were usually different between segments within a single bird or at any site between birds.

The CDR3-length distributions were compared by cluster analysis to explore the relationship between different samples. The cluster analysis dendrogram (Fig. 3A for Vβ1 and B for Vβ2) revealed no clear grouping of sample profiles for Vβ1 or Vβ2 TCRs either between or within birds. The diversity detected with Vβ2 was generally greater than that seen with Vβ1, as judged by the higher cluster difference score (Fig. 3B). Despite the complexity in CDR3 profiles, some neighbouring regions from the same bird clustered together in this unsupervised analysis supporting the observations made from direct examination of the CDR3-length profiles.

RT-PCR products from Vβ2 specific amplification of duodenal, jejunal and ileal samples of the conventionally reared bird 3 were selected for CDR3-sequence analysis. Sequences for between 10 and 15 CDR3 were obtained from each of the three small intestinal sites (Fig. 4). Translation of the sequences confirmed in-frame status and identified broad usage of Jβ segments with a slight under-representation of Jβ4 (4/34 unique sequences compared with 7–13 for the other Jβ fragments). Many of the CDR3 were only

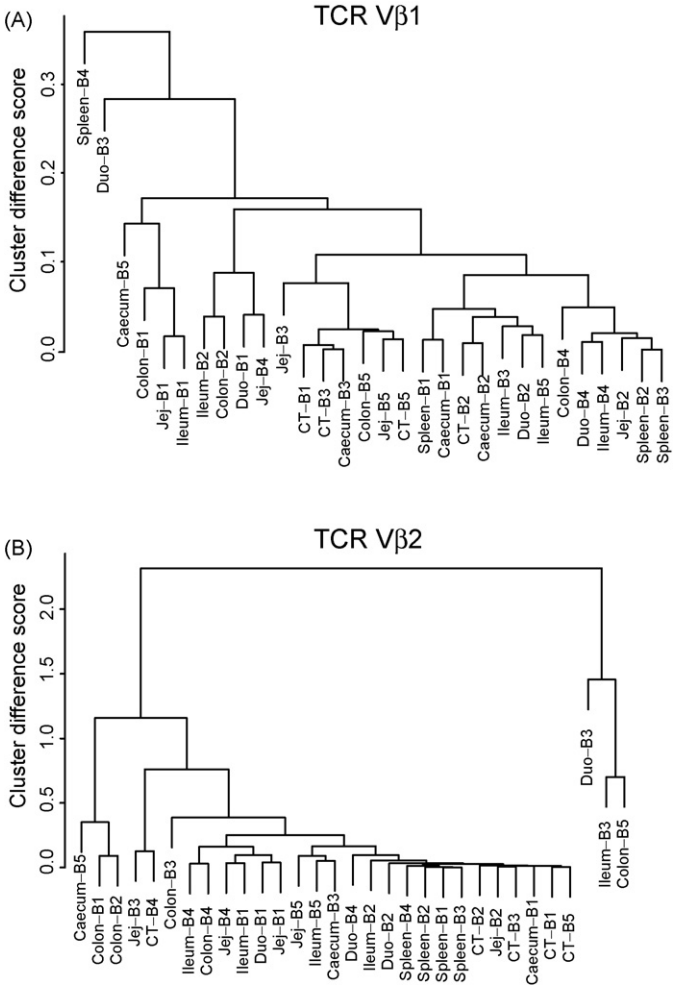

Fig. 3. Dendogram display of hierarchical cluster analysis of all specratype data from conventionally reared RIR chickens. (A) Vβ1 and (B) Vβ2. The dissimilarity (or distance) matrix of individual TCR Vβ spectratype profiles was created by Perl scripts based on a modified Kulback–Leibler divergence. R package was applied to generate the clusters based on the distance matrix. The distance between samples is displayed as cluster difference score on *y*-axis. Spectratypes clustered in a complex pattern with some grouping of samples from different segments of the gut within birds. These were more prominent with Vβ2 compared with Vβ1 profiles.

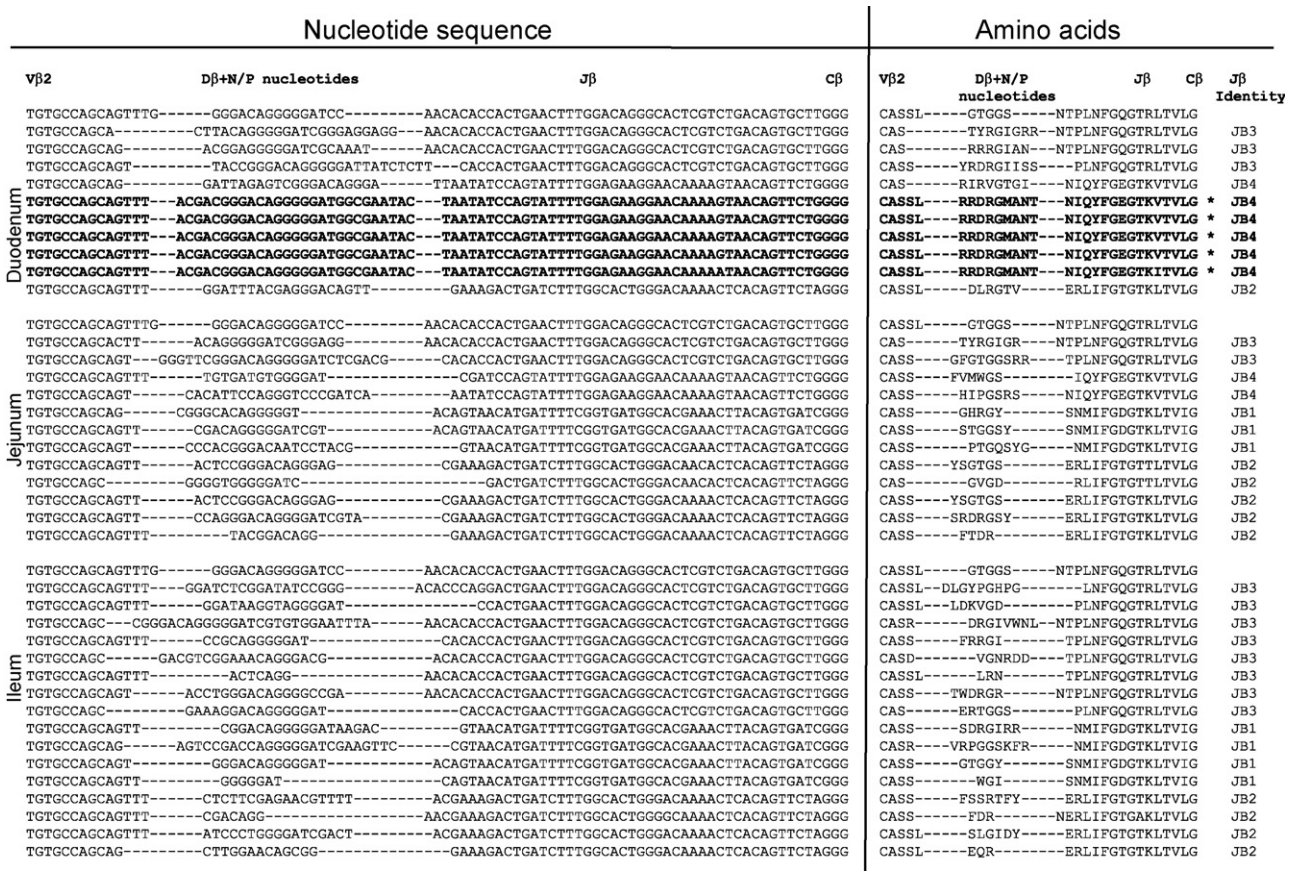

Fig. 4. TCRVβ2 CDR3-sequence identity from the small intestine of a conventionally reared chicken. TCRVβ2 to Cβ PCR products cloned and sequenced from single colonies of transformed *E. coli*. Sequences derived from the duodenum, jejunum and ileum of chicken 3 (Fig. 2). The nucleotide sequences of the 3′ end of Vβ, whole Dβ (with N and P nucleotide modifications), whole Jβ and the 5′ end of Cβ (left column) and translated amino acid sequences (right column) are depicted. A repeated sequence in the duodenum is highlighted in bold and marked *. The identity of Jβ usage is indicated to the right of the AA sequence.

represented as singlets in the analysis with one sequence over-represented in the duodenum at a level of 50% of the sequences, confirming the clonal basis for the biased profile seen in Fig. 2. The regional nature of this clone was such that it was not detected in the sequences obtained from the jejunum or ileum of this bird, which were polyclonal at the level of sequencing performed (although there was a bias in the jejunal spectratype of this bird the proportion of sequences at this CDR3 size would be below the detection level of our sequence analysis).

3.3. Microbial complexity shapes the gut TCRβ repertoire

Three-week-old GF chickens and age matched gnotobiotic birds (colonised with a single culturable *Bacillus* species) were analysed to identify the Vβ repertoire of gut associated T cells. As with conventionally reared chickens, the repertoire in the spleen displayed a broad CDR3-length profile consistent with that of a polyclonal repertoire with both TCRVβ1 and Vβ2. Birds reared under germ-free status exhibited broad TCRVβ1 CDR3-length repertoires in all regions of the gut except in the case of the duodenum of bird 1 (Fig. 5A). Broad repertoires were also detected with TCRVβ2 of germ-free chickens although there was some divergence from the Gaussian-like distributions seen in the spleen. Spectratype analysis of gut segments from gnotobiotic birds colonised with *Bacillus* spp. revealed clear over-representation of peaks with particular CDR3-lengths (indicated by arrows, birds 4–6 Fig. 5A and 5B) in both TCRVβ1 and TCRVβ2. In contrast to birds reared under all other conditions, many of these over-represented peaks of CDR3-length in the gut of gnotobiotics were shared in multiple sites within the same bird. The multi-site bias in CDR3-length profiles were easier to identify visually with TCRVβ2 and were also present in the total small intestinal IEL population which probably reflected the lower number of TCRVβ2 cells that populate the gut of chickens [16,17,19]. The identities of the over-represented CDR3-lengths were different between birds indicating unique bias in different individuals. These shared peaks were present in the background of a more complex CDR3-length distribution and in individual birds more than one over-represented peak was evident in more than one site (e.g. Vβ2 bird 5 Fig. 5B). The results of cluster analysis with these samples identified groupings that contained multiple gut sites from gnotobiotic birds, being particularly evident for bird 4/bird 6 in Vβ1 and bird 6 in Vβ2 (Fig. 6A for Vβ1 and B for Vβ2).

The presence of monoclonal expansion in the skewed CDR3 peaks was confirmed by sequencing of the Vβ1 and Vβ2 transcripts of duodenum, jejunum and ileum derived T cells of gnotobiotic bird 4 (Fig. 7). One repeated TCRVβ1 CDR3 sequence was detected in all three regions of the small intestine and corresponded in size to the major spectratype peak for Vβ1 seen in these tissue samples (Fig. 7A). The frequency of the over-representation is 2/13, 3/13, 2/13 respectively. Within the Vβ2 (Fig. 7B) of the same bird and corresponding regions, a repeated sequence was observed in the jejunum (2/10) and this sequence appeared in the ileum (1/14). A second repeat sequence was identified (2/14 sequences) in the ileum. The sequencing data confirmed that clonal expansion contributed to skewing of spectratype profiles and that major clones were present and within an individual, shared between different regions of the

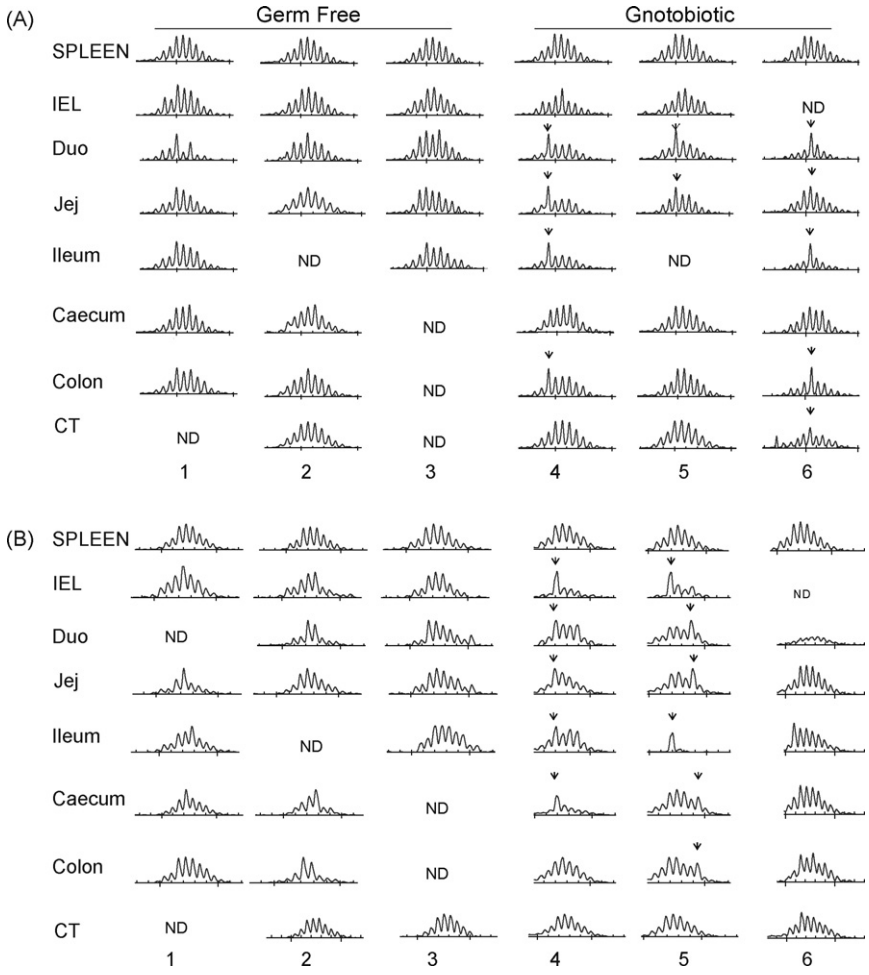

Fig. 5. Germ-free and gnotobiotic status dramatically affects the TCRVβ repertoire in the intestine. Spectratypes of TCR Vβ 1 transcripts (A) and Vβ 2 (B) in the spleen and intestinal T cells from 3 weeks old chickens reared under germ free (GF; birds 1–3) and gnotobiotic (4–6) status. In both conditions the spleen exhibited a Gaussian-like distribution of CDR3-lengths. With GF birds the broad, polyclonal distributions were evident in almost all gut samples compared with substantial over-representation of spectratype peaks seen in the gut of gnotobiotic birds, indicated by (↓). In the gnotobiotic birds the over-represented peaks were of identical size in multiple segments of the intestine of an individual bird. IEL, intra-epithelial lymphocyte; Duo, duodenum; Jej, jejunum; CT, Caecal tonsil; ND, no data.

intestine. All Jβ fragments were detected with both Vβ1 and Vβ2 with a slight under-representation in Jβ4 (10/62 unique sequences compared with 16–19 for the other Jβ).

3.4. αβ *T cells in spleen respond to antigens initially encountered in the intestine*

To test for antigen-specific reactivity a standard exogenous antigen-driven proliferation assay was performed with splenocytes and caecal tonsil cells from *Bacillus*-colonised gnotobiotic chickens. Splenocytes prepared from gnotobiotic chickens incorporated significantly greater amounts of ^{3}H-thymidine when exposed to a whole cell *Bacillus* antigen preparation, compared with cells incubated in medium with no antigen (Fig. 8). In similar assays, CT lymphocytes from the same chickens also responded to the same *Bacillus* lysate in an antigen-specific manner but the very small numbers of lymphocytes led to very low levels of incorporated ^{3}H-thymidine (data not shown). The level of ^{3}H-thymidine incorporation with antigen was much lower than that with the T cell mitogen (PHA) which supports the premise that the *Bacillus* antigen-specific T cells were present at low frequency (which is why they do not bias the spleen spectratype profiles). Nonetheless, the presence of antigen-specific T cells in the spleen of gnotobiotic birds indicates that colonisation with *Bacillus* leads to specific induction of T cell responses.

4. Discussion

Appropriate development of the vertebrate gut immune system is dependent on enteric microflora with the effects including structure, organisation, cellular recruitment and proliferation (reviewed in [32–35]). The number and repertoire of gut-resident T cells is also influenced by microbial colonisation and responses generated against microbial flora are important in maintaining gut health and a balanced relationship with the "commensal" microorganisms. Under normal circumstances, the enteric microflora are essentially non-pathogenic and may be protective against incoming pathogens by processes such as microbial competition. However, it is clear that the host responds specifically to microbes in the intestine, albeit in a highly controlled manner [36–38]. These responses can modulate the composition of the microbial flora demonstrating a complex interplay between enteric microorganisms and the host response [39,40]. In mammals, changes in the clonality of TCRαβ+ T cells has been associated with age and early postnatal events such as weaning and changes in diet that may be related to changes in the enteric microflora (reviewed in [41]). Studies comparing the TCRVβ repertoire in germ-free, conventional or conventionalised ex-germ-free rats indicated that microbial colonisation dramatically affects the gut TCRVβ repertoire [9,10,42]. Here, we report changes in the chicken gut TCRβ repertoires associated with differences in the microbial complexity

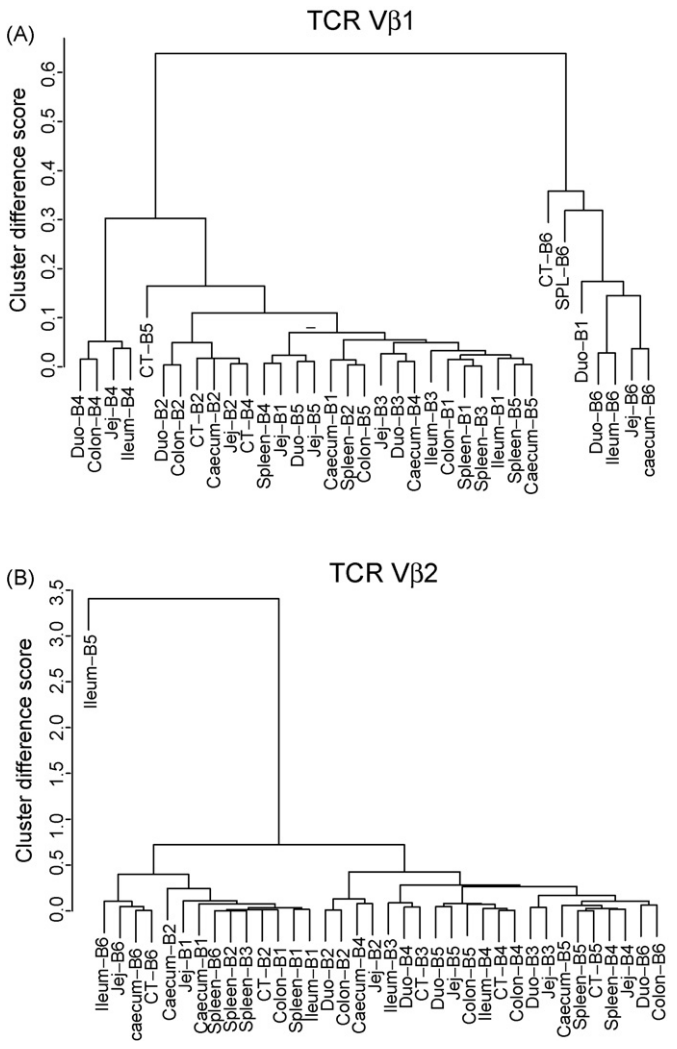

Fig. 6. Dendrogram display of hierarchical cluster analysis of the specratype data from GF and gnotobiotic TCRVβ spectratypes. (A) Vβ1 and (B) Vβ2. The analysis was carried out as described for Fig. 3. Spectratypes clustered in a complex pattern with overall grouping of samples derived from GF gut, GF or gnotobiotic spleen and separation of samples from gut segments of gnotobiotic animals. The scale of differences was more prominent with Vβ2 compared with Vβ1 profiles. In a number of cases, clustering of multiple gut sites from gnotobiotic birds was evident e.g. with Vβ1 (bird 4 and bird 6) and Vβ2 (bird 6).

of the gut flora combining examination of conventional, germ-free and gnotobiotic monocolonised birds with global TCRVβ repertoire assessment and regional dissection of the intestine.

The complexity of mammalian TCRVβ loci has necessitated extrapolation of TCR repertoire biology from examination of a small fraction of selected TCRVβ. In chickens, the TCRβ locus is much simpler than seen with mammals [26]. Analysis of the TCRβ locus in the *Gallus gallus* genome resource (http://www.ensembl.org/Gallus_gallus) revealed 9 TCRVβ1, 4 TCRVβ2, 1 Dβ, 4Jβ and 1Cβ gene segments distributed over 185 kb. The numbers of gene segments is in broad agreement with previously published data [14,17,26,28]. We propose that the Jβ segments be renamed and the other TCR gene segments given identifiers according to the system proposed by IMGT (http://imgt.cines.fr/textes/IMGTrepertoire) and indicated in Fig. 1 (with both old and new identifiers). The simplicity of the TCRβ locus may be linked with the simplicity of the "minimal essential" MHC locus in chickens [15], fewer expressed MHC may have reduced the evolutionary drive for extensive gene duplication in the TCRβ locus. The low complexity of the chicken TCRβ locus facilitated development of global TCRβ-repertoire analysis strategies where all TCRβ in an individual are considered in fewer assays than necessary for mammals. Our approach consisted of the development of spectratyping methods for TCRVβ1 and TCRVβ2 families supported by multi-sample sequence analysis of cloned PCR products. These reagents gave consistent results with different T cell subsets derived from inbred and outbred lines of birds. Other primers that may be useful for more detailed analysis of complex circumstances have been designed against the subfamilies of TCRVβ or against the Jβ regions but in our study of enteric TCRαβ repertoires these proved unnecessary.

Previous studies linking microbial status with TCRβ repertoire complexity have identified age-dependent oligoclonality in rodents and humans [3,4] often focussing on IEL or subsets of cells within the IEL (e.g. CD8αα or CD8αβ+ T cells) with analysis of a restricted subset of TCRVβ. Comparisons between germ-free rats and those colonised with a complex gut flora (either by conventional rearing or as ex-germ-free experimentally colonised animals) clearly indicate that a broad repertoire in germ-free animals becomes oligoclonal in association with enteric microflora [10,42]. We report similar findings in our "global" analysis of chicken gut associated TCRβ repertoires where oligoclonality was identified in both TCRVβ1 and TCRVβ2 of birds reared conventionally. In contrast, where birds were reared germ free the intestinal gut associated TCRVβ1 CDR3-length profiles were consistent with the interpretation of the existence of a polyclonal repertoire. With Vβ2 in germ-free birds the repertoire of CDR3-lengths deviated from a Gaussian distribution but remained relatively broad. This feature may be due to the small numbers of TCRVβ2+ T cells that home to the gut [16,17,19] and is a feature noted for TCRβ-repertoires in the IEL of germ-free mice which have very low numbers of TCRαβ+ T cells [2,3,43,44]. In all birds the spleen-derived repertoires were distributed polyclonally as were those detected in the organised gut associated lymphoid tissue known as the caecal tonsil. The oligoclonality of TCRβ repertoire in conventional birds was highly regionalised with spectratype profiles obtained from different regions of gut. Regionalisation of TCR repertoires has also been reported in the IEL populations derived from microbially colonised mammals [7,45]. Where repertoire analysis is performed on samples of IEL taken from the entire small intestine the regional oligoclonalities may be hidden. For example, in our analysis the spectratypes obtained from whole small intestinal IEL from conventional-reared birds did not reveal the biased spectratypes seen when samples were derived from duodenal, jejunal or ileal regions of the gut. Different birds reared under the same conditions exhibited very different CDR3-length profiles indicating the private nature of the oligoclonal gut repertoires as has also been described for mammals (reviewed in [41]). Sequence analysis identified at least one very large clonally expanded CDR3 in the TCRVβ2 repertoire of the duodenum (5 of 10 clones sequenced) supporting the large bias seen with this spectratype profile. Imhof et al. [25] sequenced the TCRVβ1 CDR3 from CD8αα and CD8αβ IEL derived from the entire small intestine of a conventionally reared 20-day-old HB.19 chick and in both populations were able to detect repeated CDR3 sequences including one in the CD8αα population that comprised 5 of 33 sequences. These authors concluded that a diverse repertoire existed in the IEL with some larger clones present. However, our observations of regional oligoclonality would suggest that more focussed repertoires would be evident in IEL taken from segments of the small intestine.

The intestinal segments of gnotobiotic birds, adventitiously colonised with *Bacillus* spp. early post-hatch were highly informative. In these birds, over-represented peaks in the CDR3-length distributions were detected in multiple gut compartments, some of which were conserved between different segments of the

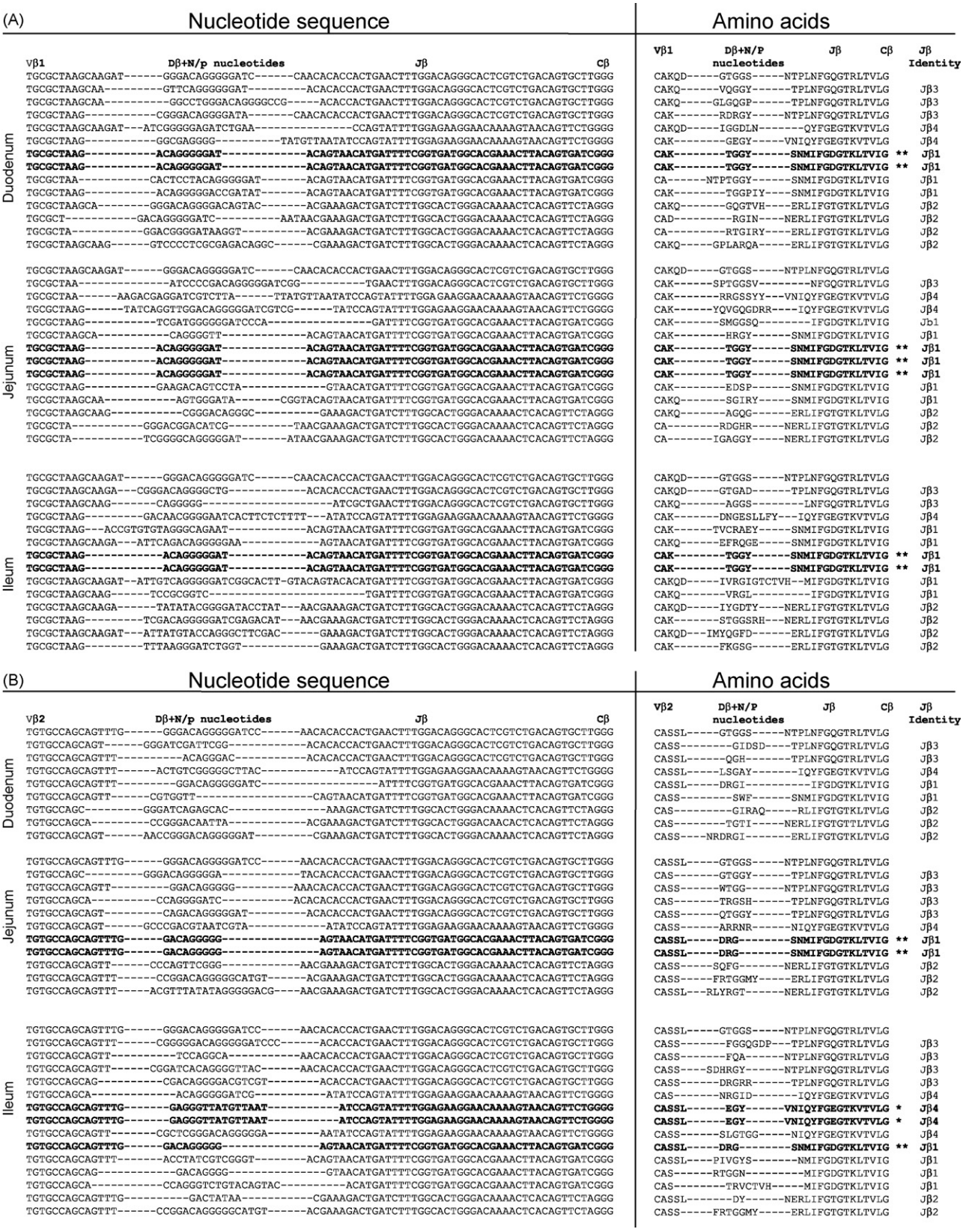

Fig. 7. TCRVβ CDR3-sequence identity from the small intestine of a gnotobiotic chicken revealed shared clones in multiple sites. Sequences obtained from CDR3 region of cloned Vβ1 (A) and Vβ2 (B) PCR products from the duodenum, jejunum and ileum of gnotobiotic chicken 4 (Fig. 5). The nucleotide sequences of the 3′ end of Vβ, whole Dβ (with N and P nucleotide modifications), whole Jβ and the 5′ end of Cβ (left column) and translated amino acid sequences (right column) are shown. Identical sequences in each case are highlighted in bold and marked, present in single site *; present in multiple sites **. The identity of Jβ is indicated to the right of the AA.

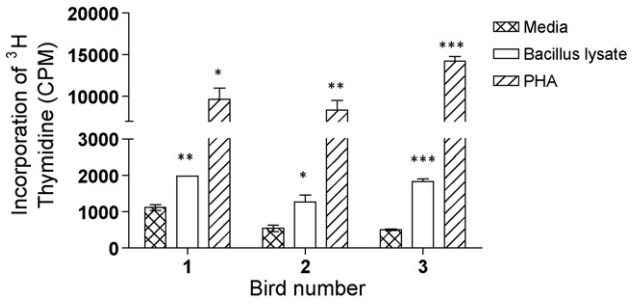

Fig. 8. Antigen-specific proliferation of splenocytes from 3 weeks old gnotobiotic chickens colonised with *Bacillus* spp. Splenocytes were cultured with or without an antigen preparation lysate derived from the *Bacillus* isolated from screening. Proliferation in response to the *Bacillus* lysate or phytohemagglutinin (PHA) was assessed by incorporation of [^{3}H]-thymidine for the last 18 h of a 72 h culture period. The error bars indicate standard errors of the mean incorporation derived from three replicates. Within birds, statistically significant differences are denoted as $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$; combining data from all three birds, antigen-specific proliferation was statistically significant $p < 0.05$.

gut (within the same bird). Overall, the level of spectratype-defined oligoclonality observed in intestinal segments was greater than that seen with conventionally reared birds with a complex microflora. To our knowledge, the influence of microbial complexity (restricted versus conventional flora) on TCR repertoire has not been addressed in any system prior to this study. The sequence data obtained from segments of the small intestine of a 3-week-old gnotobiotic bird confirmed the increased level of oligoclonality and the existence of shared TCRβ CDR3 sequences in multiple segments indicated broader over-representation and distribution of clonally derived T cells than seen with conventional-reared animals. For example, in one bird identical CDR3 sequences were readily detected in three small intestinal sites with Vβ1 and in two sites with Vβ2. This distribution of conserved TCRβ CDR3 may have been due to the wider distribution of a single gut associated microorganism due to lack of competition from other components of a naturally complex flora. Alternatively, individual T cell clones may have been present at different numbers throughout the gut of conventional and gnotobiotic birds but local competition with other expanded clones of T cells may have obscured their presence in conventionally reared birds. This notwithstanding, it was unexpected that a single bacterial species in the gut (which would present thousands of potential antigens to the immune system) stimulated a restricted set of T cell clones that were represented throughout the small intestine. Shared T cell clones were also detected by sequence analysis of different intestinal segments in mice and human [8,46]. Hence, induction of T cell expansion by enteric microflora leads to clonal expansions that may be present throughout the gut although a variety of factors including microenvironmental differences in clonal stimulation or clonal competition may lead to clustered or regional distribution of T cell clones.

Antigen-specific splenocyte proliferation was detected in the gnotobiotic birds confirming the specific recognition of antigens from the colonising *Bacillus* microorganism. The systemic response to a gut microbe may be considered unusual in the context of oral tolerance but it is clear that mammals can respond to intestinal microbes [36–38] and chickens have a short developmentally restricted window of oral tolerance (~1 week post-hatch) [22]. Increased IgY and IgA levels were evident in birds monocolonised with *E. coli* but not with GF birds treated with heat-inactivated *E coli* [47], indicating the importance of live microbes in immune induction. The over-riding influence of live, microbial-derived over food-derived antigen is also evident from the differences in TCR repertoire profile between germ-free, gnotobiotic and conventional colonised birds which all received the same diet.

The combination of spectratype and sequence data with regional analysis of the gut under different conditions of microbial stimulation revealed a complex clonal structure within the gut TCRαβ T cell populations. The pattern of geographical restriction with dispersal of some T cell clones throughout the gut suggests a dynamic relationship with the complexity of microbial flora influencing structural and clonal development of the gut immune system. Some bacterial species may induce greater changes in the TCRβ repertoire than others and it remains to be seen whether these are beneficial or detrimental to the development of gut immune function. For example, it may be good to induce T cell activation in the intestine to orchestrate effective local immune development but this may also be counterbalanced by the potential negative effects of niche competition between "good" or "irrelevant" T cell clones. With increased interest in generic improvements for gut health in humans and livestock species and the application of pre- and probiotic preparations, it is important to identify the basis for the most effective rapid generation of effective immunity in the gut. Understanding the value and potential costs of specific T cell activation in the process of microbial flora-driven gut immune maturation would facilitate the selection of suitable components of optimal probiotic preparations in human and livestock disease.

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