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Evidence against T-cell development in the adult human intestinal mucosa based upon lack of terminal deoxynucleotidyltransferase expression

M.-E. TAPLIN,* M. E. FRANTZ,* C. CANNING,† J. RITZ,† R. S. BLUMBERG‡ & S. P. BALK*
Hematology-Oncology Division, Beth Israel Hospital, †Division of Tumor Immunology, Dana Farber Cancer Institute, and the ‡Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

SUMMARY

Several lines of evidence indicate that a subset of murine intestinal intraepithelial lymphocytes (iIEL), particularly those which express the CD8αα homodimer, mature extrathymically. This study confirms that a small fraction of adult human iIEL also express the CD8αα homodimer and demonstrates that most of these cells in the small intestine are T cells using the αβ T-cell receptor (TCR). Whether these cells or other subsets of adult human iIEL mature extrathymically in the intestine was assessed by measuring the expression of terminal deoxynucleotidyltransferase (TdT), an enzyme expressed exclusively by immature lymphocytes. Very low levels of TdT message could be detected by polymerase chain reaction (PCR) amplification in some iIEL samples. The level of TdT expression was assayed by competitive PCR amplification and compared with thymocytes and peripheral blood lymphocytes. These measurements indicated that the number of immature T cells expressing TdT in the intestinal epithelium was less than one cell per 10^7 lymphocytes. This demonstrates that there are few if any TdT expressing immature T cells in the adult human intestinal mucosa and indicates, therefore, that T-cell development in the intestinal mucosa does not contribute significantly to the T-cell repertoire of the adult human intestine.

INTRODUCTION

Lymphocytes that reside in the epithelium of the intestine, intestinal intraepithelial lymphocytes (iIEL), have many properties that distinguish them from the majority of lymphocytes in the peripheral blood and primary lymphoid tissues. Human iIEL are predominantly $CD8^+CD45RO^+$ T cells,[1–5] a phenotype that is infrequent in the peripheral blood and is indicative of previously activated cytolytic T cells. These cells are further differentiated by the T-cell antigen receptors (TCR) they express. Most human iIEL use the αβ TCR, similarly to T cells in the peripheral blood.[4–6] Sequence analysis of iIEL TCR has shown, however, that these cells are oligoclonal.[7–10] This indicates that most iIEL are directed at a very limited number of intestinal antigens, rather than the very diverse array of antigens that can be recognized by polyclonal T cells in the peripheral blood.

Most T cells develop in the thymus, where TCR capable of recognizing a vast number of antigens are generated. However, extrathymic maturation appears to be a major developmental pathway for iIEL in the mouse. Initial observations in athymic mice suggested that iIEL expressing the γδ TCR matured extrathymically,[11–16] while subsequent studies indicated that murine iIEL expressing the CD8αα homodimer and the αβ or γδ TCR mature extrathymically.[17–20] Evidence that the intestine can support the extrathymic development of T cells has been provided by transplantation of fetal intestine into thymectomized mice.[21] The presence of immature iIEL in the normal intestine has been suggested by the ability of iIEL to preferentially reconstitute the intestinal epithelium of SCID mice.[22] Finally, direct evidence for T-cell maturation in normal murine intestine has been provided by the demonstration that a small population of murine iIEL contains transcripts encoding the recombinase activating gene-1 (RAG-1), based upon polymerase chain reaction (PCR) amplification[17] and *in situ* hybridization.[23] In contrast to murine iIEL, extrathymic maturation does not appear to be a significant pathway for iIEL development in chickens.[24]

Extrathymic development, if it occurs in the human intestine, could be an important mechanism for generating the extremely restricted TCR repertoire of iIEL, through positive selection for TCR capable of recognizing a limited number of intestinal antigens. Moreover, the intestine is a potentially important site for the development of peripheral blood T cells in adults, after the thymus has involuted. Proteins that appear to be expressed exclusively in immature lymphocytes include RAG-1, RAG-2 and terminal deoxynucleotidyl transferase (TdT). Previous DNA sequencing analyses of αβ TCR from human iIEL has demonstrated extensive N-region

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Correspondence: Dr. S. Balk, Division of Hematology-Oncology, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215, USA.

diversity,[7–10] indicating that the precursors to these cells expressed TdT.[25,26] The expression of TdT by human iIEL was examined, therefore, to determine whether there was T-cell maturation occurring in the adult human intestine. A sensitive competitive PCR amplification method failed to detect significant levels of TdT in any iIEL sample, indicating that ongoing T-cell development involving TdT does not occur in the adult human intestinal epithelium.

MATERIALS AND METHODS

Cell isolation and cDNA synthesis

Intestinal IEL were isolated from surgical samples of grossly normal small or large intestine.[27] All donors of intestinal cells were adults over the age of 30. Fresh strips of mucosa were washed in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS) with 1 mM DTT to remove mucus, followed by incubation in 0·75 mM EDTA with gentle stirring to release iIEL and epithelial cells. In most preparations the iIEL were then purified on a discontinuous Percoll gradient (30–40–60%), collecting the cells at the 30–40% interphase. In other experiments (one case from small intestine and three from colon), to avoid possible loss of immature lymphocytes, RNA was extracted directly from the mixed iIEL and epithelial cell preparations. Finally, lamina propria lymphocytes (LPL) were prepared by digesting the EDTA-extracted tissue with collagenase and DNase followed by scraping with a wire mesh.[27] Thymocytes were obtained from children undergoing cardiac surgery and one such preparation was used as a standard in these experiments. TdT^+ lymphoblasts were obtained from the peripheral blood of a patient with acute lymphoblastic leukaemia (ALL). RNA extraction and cDNA synthesis were carried out as described previously.[7]

PCR amplification for β_2-microglobulin

Control amplifications of β_2-microglobulin transcripts were carried out to estimate the amount of cDNA in each sample. Serial dilutions of cDNA were amplified by PCR with a β_2-microglobulin sense primer spanning the junction between exons 1 and 2 (ATCCAGCGTACTCCAAAGATTCAG) and an antisense primer in exon 4 (AAATTGAAAGTTAACTTATGCACGC).[28] Each 20-μl reaction contained cDNA, 100 ng of each primer, 100 μg/ml bovine serum albumin (BSA), 0·2 mM dNTPs, 1·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 9·0 (at 25°) and 1 U Taq DNA polymerase. PCR amplifications were done for 25–30 cycles at 94° for 20 seconds, 55° for 30 seconds and 72° for 60 seconds, followed by a 7-min extension at 72°. The PCR products were separated on agarose gels, Southern blotted with a β_2-microglobulin probe and quantified using a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

PCR amplification for TdT

cDNA was amplified in 20-μl reactions containing 50 ng of each TdT primer (AGACTCCACCAATTGCTGTACA, sense in exon 3 and TCAGAGTTCTGAAACCCATCCT, antisense in exon 6)[29] and other reagents as above. The amplifications were carried out for 25–30 cycles at 94° for 20 seconds, 55° for 30 seconds and 72° for 60 seconds, followed by a 7-min extension at 72°. In some experiments the PCR products were labelled by adding 0·5 μl of $\alpha[^{32}P]$dCTP (10 mCi/ml, 3000 Ci/mM).

Competitive PCR amplification for TdT

The *Eco*R1 site in exon 4 of a TdT cDNA[29] (kindly provided by Dr M. S. Coleman, University of North Carolina) was filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I and religated to generate a competitive template that was four bases longer than wild type and missing the *Eco*R1 site. Serial dilutions of this template were then mixed with constant amounts of cDNA and amplified in 20-μl reactions with $\alpha[^{32}P]$dCTP, as described above. The products were analysed on 6% DNA sequencing gels.

Three-step competitive PCR amplification for TdT

Equivalent amounts of cDNA, based upon β_2-microglobulin expression, were amplified with TdT primers in 20-μl reactions for 15 cycles in the absence of competitive template, using the exon 3 and 6 primers and conditions described above for the amplification of TdT (this initial limited amplification allowed the use of a larger aliquot of cDNA). cDNA from thymus and ALL were used at 300-fold lower concentrations. A series of 20-μl PCR reactions was then set up with each containing 1 μl of the 15-cycle amplified cDNA and serial five-fold dilutions of the competitive template. These were amplified for 30 cycles, again using the same primers and conditions as described above for the amplification of TdT. Finally, 1 μl from each sample was reamplified with an internal sense TdT primer (GTCAGAGAAGAACCACTTTAAACA, exon 3) and the same exon 6 antisense primer above for 22 cycles. This final amplification step was necessary to detect low levels of TdT by ethidium bromide staining. To distinguish the competitive template, each reaction was then digested with *Eco*R1 and analysed on agarose gels by ethidium bromide staining.

Immunofluorescence

Fresh iIEL were analysed by direct or indirect immunofluorescence with antibodies against CD3 [anti-Leu-4–phycoerythrin (PE); Becton Dickinson, Mountain View, CA], TCR$\alpha\beta$ [WT31-fluorescein isothiocyanate (FITC), Becton Dickinson], CD4 (OKT4), CD8α (anti-Leu-2a–PE; Becton Dickinson; or DK25–FITC; Dako Corporation, Carpinteria, CA) and CD8β (2ST8.5H7).[30] The CD8β antibody was directly conjugated to Allophycocyanin (APC) through the use of a heterobifunctional cross-linker.[31] Three-colour immunofluorescence was carried out using the directly conjugated antibodies described above. $CD8\beta^-$ cells were identified by comparison with an isotype-matched, APC-conjugated antibody. IEL T-cell clones derived as described previously[7] were analysed similarly.

RESULTS

Assessment of TdT expression by intestinal lymphocytes

Immunohistochemical studies performed initially to detect TdT-positive cells in the intestine were negative (data not shown), demonstrating that immature T cells expressing TdT were not present at a high frequency. To detect TdT that might be expressed by a small minority of cells, iIEL cDNA was synthesized and amplified by PCR with primers specific for TdT. The primers were chosen to be in different exons so that only spliced mRNA, rather than contaminating genomic DNA, would be detected. Figure 1 shows that TdT transcripts could be detected by PCR amplification in some peripheral blood

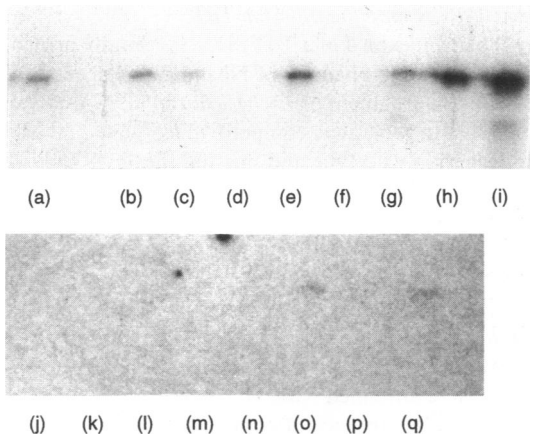

Figure 1. PCR amplification of TdT. cDNA was amplified with TdT primers in exons 3 and 6 in the presence of $\alpha[^{32}P]$dCTP for 35 cycles. The PCR products were analysed immediately on a 6% DNA sequencing gel. Lane a, thymocyte cDNA; lanes b–i, PBMC cDNA; lanes j–q, iIEL cDNA. Lanes a–i were exposed for 1 day while j–q were exposed for 6 days, both with an intensifying screen.

mononuclear cell (PBMC) samples (lanes b–i). These probably derived from small numbers of circulating immature lymphocytes. TdT message was also detected in some iIEL samples, but required a long exposure and appeared to be less abundant than in the PBMC (Fig. 1, lanes j–q).

Quantitative analysis of TdT expression

To measure the level of TdT expression by lymphocytes in the intestine, a TdT template for competitive PCR amplification was constructed. This template generated a PCR product that was four base pairs larger than the wild type transcript and had a deleted *Eco*R1 restriction site at nucleotides 638–643. Serial three-fold dilutions of this competitive template were mixed with a constant amount of cDNA. The samples were then amplified by PCR for 40 cycles, using TdT primers in exons 3 and 6. Equivalent amounts of cDNA were used for each PBMC and iIEL sample, based on control PCR amplifications with primers specific for β_2-microglobulin. Thymocyte cDNA, which should contain high levels of TdT, was used at a 100-fold lower concentration. The PCR amplifications were carried out in the presence of $\alpha[^{32}P]$dCTP and the products were then analysed on DNA sequencing gels.

The thymocyte TdT cDNA and competitive template were equal at a 243-fold dilution (Fig. 2, thymus). The PBMC samples, which contained approximately 100-fold more cDNA, were equal at dilutions ranging from 729-fold (approximately 300-fold less than thymus; Fig. 2, PBMC) to over 50 000-fold (data not shown). These results were consistent with the presence of a small but variable number of immature lymphocytes circulating in the peripheral blood. In contrast, TdT message from iIEL was not detectable at the highest dilution of the competitive template in any of the iIEL samples from Fig. 1 (Fig. 2, IEL, and data not shown). Further dilutions of the competitive TdT template were not reproducibly detected after 40 cycles of amplification.

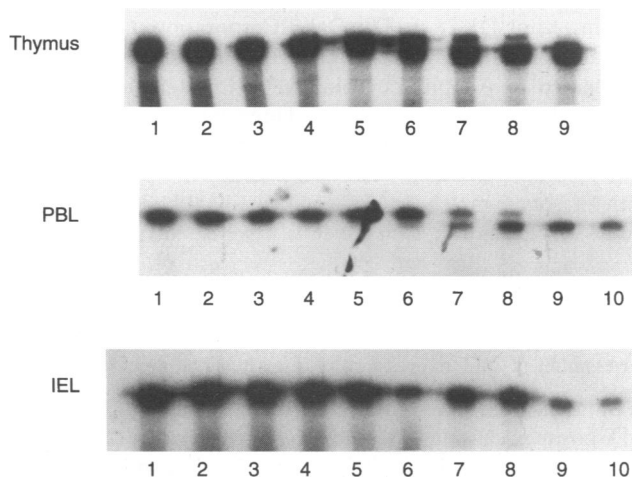

Figure 2. Competitive PCR amplification of TdT. cDNA was amplified for 40 cycles and analysed immediately on a 6% DNA sequencing gel. Lanes 1–10 represent serial three-fold dilutions of the competitive template. The IEL cDNA was from jejunal IEL and the PBMC (PBL) cDNA was from the same patient. Approximately 100-fold less cDNA, based on β_2-microglobulin expression, was used in the thymus samples. The gel was exposed for 2 days with an intensifying screen.

Three step competitive PCR amplification

To increase the sensitivity of this assay and measure the low

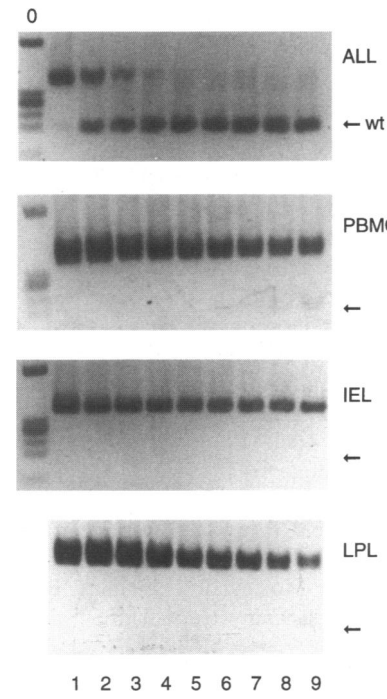

Figure 3. Three-step competitive PCR amplification analysis of TdT expression. cDNA from the peripheral blood of a patient with B cell ALL (30% TdT^+ blasts) and from PBMC, iIEL and LPL from one donor were analysed. The concentration of cDNA in the PBMC, iIEL and LPL samples, based on β_2-microglobulin expression, was approximately equal and in the ALL sample was approximately 300-fold lower. Lane 0, *Hae*III-digested ΦX174 markers; lanes 1–9, serial fivefold dilutions of the competitive template. wt, wild-type TdT.

Table 1. Competitive PCR amplification analysis of TdT expression

Donor	Approximate number of lymphoblasts per 10^7 cells		
	PBMC	iIEL	LPL
1	10	<1	2
2	10^5	<1	<1
3	10^4	<1	na
4	na	<1	na
5	na	<1	na
6	na	<1	na
7	10	na	na
8	<1	na	na

Equivalent amounts of cDNA, based upon β2-microglobulin expression, were amplified with serial fivefold dilutions of a competitive template, digested with EcoR1 and analysed by agarose gel electrophoresis and ethidium bromide staining. TdT expression in each sample was then compared with thymocytes to estimate the number of TdT positive cells. The IELs and LPLs from each donor were Percoll gradiant purified. Donors 1–4 were from the jejunem and 5 and 6 were from the colon. Samples that were not available (na) are indicated.

levels of TdT that may be present in some iIEL preparations (Fig. 1), the cDNA were amplified by PCR in three steps. Each cDNA was amplified with TdT primers for 15 cycles initially in the absence of the competitive template. These initial amplifications were carried out under identical conditions, except that 300-fold less cDNA was used for the ALL and thymus samples, which contained high levels of TdT message. The amplified cDNA was then mixed with serial fivefold dilutions of the competitive template and amplified for an additional 30 cycles. Finally, the samples were amplified with a nested TdT-specific primer for 22 cycles to generate a large amount of double-stranded DNA, which could be easily detected by ethidium bromide staining. To discriminate between the wild type and competitive templates, the PCR products were digested with *Eco*R1 and analysed by agarose gel electrophoresis.

The results of this competitive PCR amplification from one donor (donor 1) and from the peripheral blood of a patient with TdT^+ ALL (approximately 30% circulating TdT^+ blasts) are shown in Fig. 3. In the ALL sample, the wild type TdT transcript was detectable in the presence of the undiluted competitive template (lane 1) and was equivalent after a fivefold dilution of the competitive template (lane 2). In contrast, the wild type TdT in the PBMC sample was just detectable in lane 8, which represents a 78 125-fold dilution. In the iIEL sample from this donor, wild type TdT was undetectable even at the highest dilution of the competitive template (lane 9). Finally, lamina propria lymphocytes (LPL) from this donor yielded a faint band at the highest dilution.

Table 1 summarizes the data from a series of competitive PCR amplifications. The number of lymphoblasts in each sample was approximated by comparing the amount of TdT message in each sample with the amount in thymocytes. TdT expression in thymocytes was approximately 25-fold lower than in the ALL sample (normalized for β_2-microglobulin expression, data not shown), but thymocytes should more closely reflect the amount of TdT expressed by normal immature T cells. The results in Table 1 indicate that the number of immature lymphocytes in peripheral blood varies, but may be up to 1% (10^5 per 10^7 cells in donor 2). This high number of immature cells may reflect a stress-induced left shift, as the PBMC samples from donors 1–3 were obtained at the time of surgery.

In contrast to the PBMC samples, no wild type TdT was detected in any of the iIEL samples even at the highest dilution of the competitive template. This result indicates that immature T cells are present at a frequency of less than one cell per 10^7 lymphocytes, consistent with the very weak TdT bands detected by PCR in the absence of competitive template in some IEL samples (Fig. 1). TdT expression was detected in one LPL sample, but was not expressed at a level which was higher than in peripheral blood from the same donor (donor 1). Finally, TdT expression was not detected in cDNA prepared from unfractionated mixtures of iIEL and intestinal epithelial cells (one sample from small intestine and three from colon, data not shown), indicating that immature T cells were not selectively lost into the epithelial cell fraction during the iIEL purification.

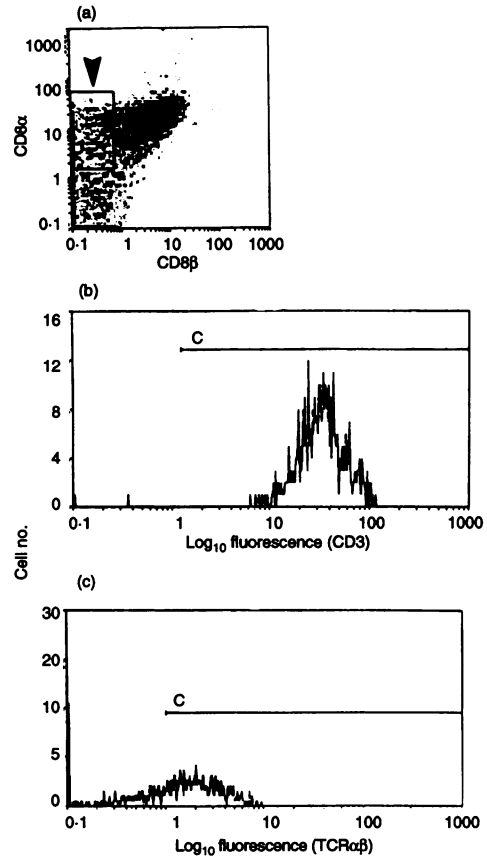

Figure 4. Three color immunofluorescence analysis of iIEL. Jejunal iIEL were stained with directly conjugated monoclonal antibodies to CD8α, CD8β, CD3 or TCRαβ. The $CD8\alpha^+\beta^-$ population shown in (a), based upon staining with an isotype-matched, APC-conjugated irrelevent antibody, was gated on (boxed with arrow) and analysed for CD3 expression with a CD3–PE conjugate (b). TCRαβ expression was analysed similarly, except using CD8α–PE and TCRαβ–FITC conjugates (c). The control cursor (labeled C) in (b) and (c) indicates positive cells based upon fluorecence which is greater than 95% of the cells stained with isoltype matched, irrelevant antibodies.

Table 2. Nucleotide sequence of a TCR$\alpha\beta^+$, CD8$\alpha^+\beta^-$ iIEL clone

Vβ	N/D/N	Jβ
leutyrphecysalaser	serphepheglya	spthrglualaphephegly
Vβ14-CTGTACTTCTGTGCCAGC	AGTTTCTTTGGGG	ACACTGAAGCTTTCTTTGGA-Jβ1.1

The 3′ end of the V region is based upon a comparison with a previously described clone. Nucleotides which may have been derived from Dβ1·1 are underlined. The deduced amino acid sequence is shown in three-letter code.

Identification of CD8$\alpha^+\beta^-$ intestinal IEL using the $\alpha\beta$ TCR

Evidence in mice has indicated that TCR$\alpha\beta$ or TCR$\gamma\delta$ iIEL that express the CD8 molecule as an $\alpha\alpha$ homodimer are the subpopulation which develop extrathymically in the intestine.[17–20] Previous studies demonstrated that a small population of human iIEL expressed the CD8$\alpha\alpha$ homodimer,[5] but the phenotype of this population has not been clearly established. Two- and three-colour immunofluorescence of human jejunal IEL was performed, therefore, to determine whether the failure to detect immature T cells in the human intestine might reflect the absence of CD8$\alpha^+\beta^-$ T cells.

Two-colour immunofluorescence analysis of iIEL with antibodies specific for CD8α and CD8β confirmed that a small minority of jejunal iIEL, usually less than 5%, were CD8$\alpha^+\beta^-$ (Fig. 4a). Three-colour immunofluorescence gating on these cells showed that the majority of the CD8$\alpha^+\beta^-$ cells were T cells, based upon CD3 expression (Fig. 4b), and expressed the $\alpha\beta$ TCR (Fig. 4c). A previous report suggesting that these cells in human colon used the $\gamma\delta$ TCR may reflect the higher proportion of $\gamma\delta$ T cells in the colon versus the jejunem.[5]

A T-cell clone expressing the $\alpha\beta$ TCR and the CD8$\alpha\alpha$ homodimer was also isolated from normal jejunem by limiting dilution cloning.[7] The TCR β chain from this clone was isolated by PCR amplification with a series of Vβ primers[9] and sequenced to determine whether it contained N-region nucleotides. Multiple N-region nucleotides at the V–J junction were identified (Table 2), consistent with TdT expression during the development of this clone.

DISCUSSION

This study used TdT expression as a sensitive and specific marker to determine whether iIEL maturation occurs in the adult human intestinal epithelium. TdT expression could be detected by PCR in some human iIEL samples, but quantitative PCR amplification analysis indicated that the level of TdT expression was extremely low. Comparisons of TdT expression by iIEL and thymocytes indicated that the frequency of immature T cells in the intestinal epithelium was less than one cell per 10^7 lymphocytes, which was lower than the frequency in most peripheral blood samples. These results demonstrate T-cell development involving TdT does not occur in the adult intestine.

RAG-1 and RAG-2 gene expression also appear to be specific for immature lymphocytes, although their expression during class switching in mature B cells has not been entirely ruled out and the lack of established introns in both the RAG-1 and −2 genes makes it more difficult to control for amplification from contaminating genomic DNA.[32] None the less, recent results indicate that RAG-1 and −2 are expressed in adult human small intestine[33] as well as in murine intestine.[17,23] This suggests that T-cell development may occur in the intestine in the absence of TdT. However, the contribution that such T cells might make to the iIEL population is very small as previous analyses of iIEL TCR showed the presence of extensive N-region diversity.[7–10] Further studies of the TCR expressed by CD8$\alpha^+\beta^-$ iIEL will be needed to determine whether the development of some of these cells may be TdT independent. Similar studies of TdT expression and TCR used by CD8$\alpha^+\beta^-$ IEL in murine intestine would also be of interest.

T-cell development in fetal, neonatal or pediatric intestine is also not addressed in this report. Expression of the CD8$\alpha\alpha$ homodimer by a large fraction of human fetal iIEL has been reported, suggesting that iIEL in fetal intestine may be largely extrathymic.[34] In support of this suggestion, we have analysed TCR expression by fetal iIEL and found evidence of ongoing TCR gene rearrangement.[35] However, the TCR isolated from fetal iIEL have had very limited N-region diversity, consistent with low or absent expression of TdT in the fetus. As above, this limited N-region diversity suggests that these fetal iIEL do not contribute significantly to the adult iIEL repertoire.

In conclusion, this study demonstrates that T-cell development in the intestine does not make any significant contribution to the repertoire of T cells in the adult human intestinal mucosa. This indicates that the ability of the adult intestinal mucosa to respond to new antigens is dependent upon recruitment and antigen-dependent expansion of mature T cells. It seems most likely that these mature T cells are thymus derived, but extrathymic maturation at sites outside the intestine or a reservoir of naive T cells which develop in the neonatal or pediatric intestine cannot be ruled out at this time.

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