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Antigen-specific and polyclonal CD4$^+$ lamina propria T-cell lines: phenotypic and functional characterization

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SUMMARY

Surface phenotype and function of lamina propria CD4$^+$ T cells have been evaluated. In addition, long-term, antigen-specific and polyclonal lamina propria CD4$^+$ T-cell lines have been generated and characterized. Lamina propria CD4$^+$ T cells represent approximately 30% of lamina propria lymphocytes and are responsive to a variety of T-cell mitogens, including anti-CD3, concanavalin A, phytohaemagglutinin and pokeweed mitogen. In each case, however, lamina propria T cells are less responsive to these mitogens than spleen T cells. Freshly isolated lamina propria T cells produce substantial amounts of interleukin-2 (IL-2), interleukin-4 (IL-4), gamma interferon and to a lesser extent interleukin-5 (IL-5). Antigen-specific lamina propria CD4$^+$ T-cell lines were generated by orally immunizing animals with antigen (KLH) in conjunction with cholera toxin as an oral adjuvant. Polyclonal lamina propria CD4$^+$ T-cell lines were generated from unimmunized animals using anti-CD3 as a polyclonal stimulus. Both antigen-specific and polyclonal CD4$^+$ T-cell lines were Thy-1$^+$, $\alpha\beta$ TCR$^+$ and CD8$^-$. The antigen-specific CD4$^+$ T-cell line when stimulated by anti-CD3 and PMA produces predominantly IL-2, IL-4 and gamma interferon, with very little IL-5. In contrast, the polyclonal CD4$^+$ T-cell line when similarly stimulated produces predominantly IL-4 and IL-5, with very little IL-2 and no detectable gamma interferon. In summary, lamina propria CD4$^+$ T cells have been evaluated and *in vitro* conditions have been determined for successful generation of lamina propria CD4$^+$ T-cell lines.

INTRODUCTION

The mucosal immune system represents the first line of immunological defence against a variety of pathogens which either directly involve the various mucosal surfaces of the body or gain access to the body through mucosal surfaces. In recent years interest has focused on the characteristics and function of T and B lymphocytes within mucosal surfaces, particularly those in the gut mucosa (for review, see[1]). For example, the population of intra-epithelial lymphocytes (IEL), which contains a high proportion of $\gamma\delta$ TCR-bearing T lymphocytes, has attracted significant attention recently.[2–6] In contrast, less information is available on the phenotype and function of T lymphocytes within the lamina propria.

Part of the reason for this paucity of information concerning lamina propria T cells has to do with the time-consuming and cumbersome techniques necessary for isolation of lamina propria lymphocytes (LPL). As a result, few laboratories have attempted to isolate and study lamina propria T lymphocytes. However, in the instances where such attempts have been made, only short-term *in vitro* assays of lamina propria T-cell function were utilized.[7–14] Those studies have provided limited information about phenotype, lymphokine production, activation and growth requirements of lamina propria T cells.

One of the major subpopulations of T cells found in the lamina propria consists of CD4$^+$ T cells. In general CD4$^+$ T cells are known to play important regulatory roles related to their interaction with B cells, other T cells, macrophages, dendritic cells, and NK cells. The CD4$^+$ T cells in the lamina propria are thought to be involved in several effector functions, including regulation of immunoglobulin secretion by B cells (especially IgA), induction of cytotoxic T-cell activity and modulation of epithelial cell function.[15–20] In order to better study these complex functions of CD4$^+$ lamina propria T cells, techniques to establish and maintain long-term *in vitro* CD4$^+$

Abbreviations: APC, avidin allophycocyanin; CMF-HBSS, Hank's balanced salt solution without calcium and magnesium; CT, cholera toxin; Con A, concanavalin A; FMF, flow microfluorimetry; HBSS, Hank's balanced salt solution; IEL, intra-epithelial lymphocytes; KLH, keyhole limpet haemocyanin; LP, lamina propria; PMA, phorbol myristate acetate; PHA, phytohaemagglutinin; PWM, pokeweed mitogen; TCR, T-cell receptor.

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lamina propria T-cell lines and clones are essential. We report here methods for successfully generating lamina propria CD4+ T-cell lines and further, we go on to analyse the surface phenotype and some of the functional characteristics of these T-cell lines.

MATERIALS AND METHODS

Animals

Male or female C57BL/6 mice were obtained from the animal breeding facilities (Department of Medical Microbiology and Immunology, University of Göteborg) and maintained under pathogen-free conditions until use. Mice were used between 7 and 14 weeks of age except for those used to obtain keyhole limpet haemocyanin (KLH) specific lamina propria (LP) T-cell lines which were 12–14 months of age at the time of death. The latter mice had been orally immunized by five oral priming immunizations with 2·5 mg of KLH (Sigma, St Louis, MO) and 10 μg of cholera toxin (CT) (List Biological Laboratories, Campbell, CA), used as an oral adjuvant, followed by oral boosting at the age of 13 months, 7 days before death.[21]

Preparation of freshly isolated cells

Spleen lymphocytes were isolated as previously described.[22] Briefly, the spleen tissue was gently forced through a fine nylon net into Hank's balanced salt solution (HBSS) (Gibco, Paisley, U.K.) to obtain a single cell suspension. Red blood cells were removed by hypotonic lysis as previously described.[22]

Lamina propria lymphocytes were prepared as previously described.[23] First, the small intestines were removed and flushed with HBSS to remove faecal contents. The Peyer's patches were carefully excised and discarded. The intestines were opened up lengthwise and cut into 5-mm pieces. The pieces were washed six times with HBSS without calcium and magnesium (CMF-HBSS) (Gibco), supplemented with 25 mM HEPES (Gibco) and incubated four times, 15 min each time, with prewarmed (37°) CMF-HBSS containing 5 mM ethylenediaminetetraacetic acid (EDTA) (Merck, Darmstadt, Germany), 10% heat inactivated horse serum (Gibco), gentamicin (50 μg/ml) (Gibco) and fungizone (0·5%) (w/v) (Gibco) in siliconized conical flasks (two intestines per flask) on a magnetic stirrer at room temperature. After each incubation the supernatant containing the sloughed epithelial cells and IELs was decanted and discarded. This treatment effectively removed epithelial cells and IELs.[24] To block any remaining EDTA activity the tissue pieces were incubated for 15 min at room temperature on the magnetic stirrer with RPMI 1640 (Flow Laboratories, Irvine, Ayrshire, U.K.) containing 25 mM HEPES and 10% heat inactivated horse serum.

In order to determine the best enzymatic digestion method for optimal recovery and viability of LPL, without loss of surface markers, initial experiments were performed with different enzyme preparations in different concentrations, either alone or in various combinations. For example, the intestinal tissue pieces were enzymatically digested with either collagenase (0–600 U/ml) (Sigma, Cat. no. C2139), Dispase (0·5, 1 and 2 mg/ml) (Boehringer-Mannheim, Mannheim, Germany) or Thermolysin (0·5 mg/ml) (Boehringer-Mannheim)[20], dissolved in RPMI 1640 containing 25 mM HEPES, 20% heat inactivated horse serum, gentamicin (50 μg/ml) and 0·5% fungizone and incubated at 37° on a magnetic stirrer for 60 min. The suspension of cells was then decanted from the tissues, centrifuged and washed once in CMF-HBSS and stored on ice in RPMI 1640 with 10% heat inactivated foetal calf serum (FCS) (Flow Laboratories), 25 mM HEPES, 50 μg/ml gentamicin and fungizone (0·5%) (RPMI/10% FCS). The remaining tissue was returned for fresh enzyme-containing media and this process was repeated twice, 30 min each time, for a total time of 120 min. We found that collagenase alone, used at 300 U/ml, gave the best results.

After the third extraction, pooled cells were gently mixed and placed on ice for 10–15 min to let larger debris sediment. The cell suspension was then decanted and purified on a 40/100% discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient by pelleting the cells and resuspending in 100% isotonic Percoll (adjusted to pH 7·4), overlaying with 40% Percoll, followed by HBSS and centrifuging (Beckman TJ6 swing-out rotor, 2000 rpm for 20 min at 4°). After centrifugation, viable lymphocytes were recovered from the 100/40% interface and washed twice in CMF-HBSS. The number and viability of lymphocytes were determined by trypan blue exclusion.

For depletion of CD8+ lymphocytes, cells were incubated with anti-Lyt 2 (NEN, U.K.) (1:500 final dilution) for 45 min at 4° followed by Low-Tox rabbit complement (Cedarlane, Ontario, Canada) for 30 min at 37°, then washed twice in RPMI/10% FCS.

Cell culture

Cell culture and *in vitro* stimulations were performed in either Iscove's medium (Flow Laboratories) or RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol (Sigma), 1 mM L-glutamine (Gibco), gentamicin (50 μg/ml) and fungizone (0·5%) (complete media).

Flow microfluorimetry (FMF)

Lamina propria lymphocytes (10^6 cells/100 μl) were suspended in phosphate buffered saline (PBS) containing 0·1% BSA (Sigma) and labelled on ice for 30 min with combinations of the following antibodies: fluorescein conjugated anti-Thy 1.2 (clone 30-H12), fluorescein conjugated anti-Lyt 2 (clone 53-6·7), phycoerythrin conjugated anti-L3T4 (clone GK1·5) (all from Becton Dickinson, Mountain View, CA); fluorescein conjugated anti-CD3 (145-2C11)[25] or biotin conjugated anti-αβ TCR (H57-597)[26] (Pharmingen, San Diego, CA) followed by avidin allophycocyanin (APC) (Caltag, San Francisco, CA); or fluorescein conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL). All antibodies were used at 10 μg/ml final concentration while avidin APC was used at a 1:10 dilution. The cells were washed twice in PBS/0·1% BSA and analysed on a FACS Star or a FACS Star Plus (Becton Dickinson) using forward scatter and side scatter to gate out dead cells and debris. Ten thousand events were collected per sample.

Proliferation assays

Cells were cultured for either 48 or 72 hr in 96-well flat bottom plates (Nunc, Roskilde, Denmark) at 5×10^4 cells/well in 200 μl of complete media with various T-cell mitogens: anti-CD3 (145-2C11)[25] 2% supernatant; concanavalin A (Con A, Pharmacia) 2 μg/ml; pokeweed mitogen (PWM, Gibco) 1:200 final concentration; phytohaemagglutanin (PHA, Pharmacia) 1:100

final concentration; or with KLH 100 μg/ml. Where indicated 10% Con A supernatant (derived from 12×10^6/ml rat spleen cells stimulated with 2·5 μg/ml of Con A for 48 hr) or 10% rIL-4 derived from supernatant of the interleukin-4 (IL-4) transfected X-63 Ag 563 myeloma cell line[27] were added to the cultures. During the last 6 hr of culture 1 μCi of [^{3}H]thymidine (5 μCi/mmol, Amersham Lab., Amersham, Bucks, U.K.) was added to each well. The cells were then harvested with a cell harvester (Skatron, Lier, Norway) on to glass fibre filters and the incorporated radioactivity was measured in a scintillation counter (Beckman, LKB, Bromma, Sweden).

Establishment of polyclonal and KLH-specific CD4$^+$ lamina propria T-cell lines

For the generation of polyclonal or KLH-specific CD4$^+$ T-cell lines, lamina propria lymphocytes were first depleted of CD8$^+$ T cells. These CD4$^+$ T cells were then stimulated with anti-CD3 (2%) or KLH (100 μg/ml) and either Con A sup (10%) or rIL-4 (10%). Every 3–4 days 50% of the media was discarded and fresh media along with either Con A sup or rIL-4 was added. Every 7–10 days the cells were restimulated with anti-CD3 or KLH, Con A sup or rIL-4 and spleen feeder cells (10:1, feeder cells:T cells) obtained from fresh spleen cells treated with mitomycin C (Sigma) or irradiated with 3000R (^{137}Cs source).

As an additional step, because our cell lines contained variable numbers of Thy-1$^+$, CD4$^-$, CD8$^-$ cells in addition to the CD4$^+$ T cells, we further purified CD4$^+$ T cells by staining with FITC anti-Thy-1 and PE anti-CD4, then sorting for Thy-1$^+$, CD4$^+$ cells on a FACS Star Plus (Becton Dickinson). This procedure provided essentially pure CD4$^+$ lamina propria T-cell lines.

Lymphokine assays

Freshly isolated lamina propria lymphocytes (5×10^5 cells/ml) or CD4$^+$ lamina propria T-cell lines (1×10^6 cells/ml) were stimulated with 5% anti-CD3 supernatant and 10 ng/ml PMA (Boehringer) for 48 hr. The cell-free supernatants were then harvested and frozen at −20° until assayed for lymphokines.

Interleukin-2 (IL-2) activity was assayed using a CTLL subline which is responsive to IL-2 but not IL-4.[28] CTLL cells were washed twice with HBSS then 2×10^4 cells per well in 200 μl of complete media were added to 96-well flat bottom plates along with serial dilutions of T-cell supernatants. A standard curve was established using rIL-2 (Genzyme, Boston, MA). Cells were cultured for 24 hr, [^{3}H]thymidine was added during the final 6 hr and the cells were then harvested as described above for proliferation assays.

IL-4 activity was assayed using an anti-IgM costimulation assay as previously described.[29] Briefly, resting mouse spleen B cells were obtained by treatment of whole spleen cells with anti-Thy 1.2 (NEN) and Low-Tox rabbit complement to remove T cells, followed by isolation of small B cells on a discontinuous Percoll gradient. The cells were then washed with HBSS and cocultured at 1×10^5 cells per well in 96-well flat bottom plates with 5 μg/ml of anti-mouse IgM (Jackson Laboratories, West Grove, PA) and serial dilutions of T-cell supernatants or rIL-4 (as a standard) for 72 hr. [^{3}H]thymidine was added during the final 16 hr of culture and the cells were harvested and [^{3}H]thymidine uptake was determined as above.

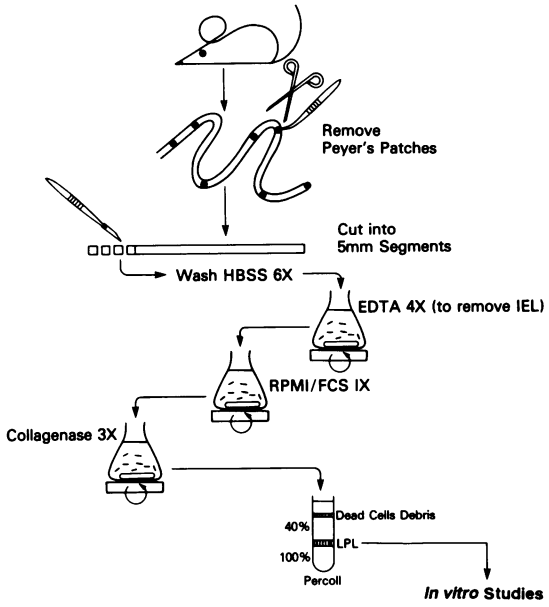

Figure 1. Preparation of lamina propria lymphocytes. The method for isolation of highly purified, viable lamina propria lymphocytes is schematically represented in this figure.

Interleukin-5 (IL-5) activity was assayed using a BCL1 proliferation assay or IL-5 ELISA as described ([22,30] respectively).

Gamma interferon activity was assayed using a gamma interferon ELISA assay as described.[22]

RESULTS

Isolation of lamina propria lymphocytes

Previous studies from this laboratory described a methodology for isolating lamina propria antibody producing B cells.[23] In order to establish an optimal procedure for isolation of lamina propria T cells, extensive studies were performed to evaluate kinetics of release and viability of lymphocytes using different enzyme preparations (E. Hörnqvist and N.Y. Lycke, manuscript in preparation). The preparation which was found to be optimal is explained in detail in the Materials and Methods section and outlined in Fig. 1. Collagenase alone was found to give equal, if not greater, cell recovery compared to Dispase or Thermolysin, either alone or in combination. In addition, unlike the latter two enzymes, collagenase did not remove or damage cell surface antigens (e.g. Thy-1, CD3, CD4, CD8 or sIg) and therefore allowed surface phenotypic characterization, as well as *in vitro* mitogenic and antigenic activation of freshly isolated LPL. Approximately 2×10^6 viable lymphocytes were recovered per intestine.

Surface phenotype of freshly isolated lamina propria lymphocytes

LPL were evaluated by FMF for expression of B- and T-cell surface markers. The results of five separate experiments are summarized in Table 1. A substantial fraction of LPL, $57 \pm 11\%$, were Thy-1$^+$. An average of $29 \pm 8\%$ of the LPL were CD4$^+$ while $18 \pm 7\%$ were CD8$^+$. Of note, a small but consistent number of LPL (approximately 10%) which were Thy-1$^+$

Table 1. Surface phenotype of freshly isolated lamina propria lymphocytes[a]

	%
Thy-1+	57±11
CD4+	29±8
CD8+	18±7
mIg+	21±5

[a] LPL single cell suspensions, prepared as described in Methods, were stained with fluorochrome-labelled antibodies and evaluated by single or dual colour analysis on a FACS Star microfluorimeter.

Table 2. Proliferation of freshly isolated lamina propria T cells

Experiment 1: Proliferation of whole or CD8-depleted LPL[a]

	Whole LPL	CD8-depleted LPL
Media	167±41	245±73
Anti-CD3	17,124±2066	12,850±745
Anti-CD3 & Con A sup	29,415±2564	15,711±1730
Anti-CD3 & rIL-4	11,285±1222	7901±563

Experiment 2: LPL proliferation to different T-cell mitogens[b]

	CD8-depleted LPL	Spleen
Media	162±40	980±130
Anti-CD3	4660±654	153,644±24,316
Con A	3362±700	40,928±10,522
PHA	1476±125	35,051±7769
PWM	957±70	9077±2·216

[a] LPL cells were stimulated with Anti-CD3 (2%) in the presence or absence of 10% Con A spleen cell supernatant (Con A sup) or 10% rIL-4 supernatant (rIL-4) and cultured for 72 hr, pulsed for the final 6 hr with [^{3}H]thymidine. Results are mean (cpm) ±SD of triplicate wells, $2{\cdot}5 \times 10^4$ cells/well.

[b] LPL or spleen lymphocytes were treated similarly with EDTA and collagenase. Mitogens used were: Anti-CD3 (2%), Con A (2 μg/ml), PHA (1:100) and PWM (1:200). Cells were cultured, in the presence of Con A sup, as in experiment 1.

apparently did not express CD4 or CD8 since in each of five experiments the number of CD4+ cells and CD8+ cells was always less than the number of Thy-1+ cells. Finally, 21±5% of the lamina propria cells were surface Ig+.

Optimal conditions for *in vitro* lamina propria T-cell proliferation

A series of experiments were performed to determine optimal *in vitro* conditions for lamina propria T-cell growth. As shown in Table 2 (experiment 1), both whole LPL and CD8-depleted LPL

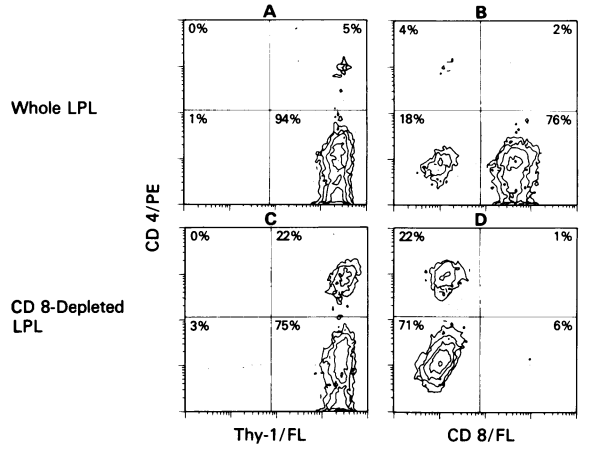

Figure 2. Surface phenotype of whole lamina propria lymphocytes or CD8-depleted lamina propria lymphocytes 7 days after *in vitro* stimulation. Whole or CD8-depleted lamina propria lymphocytes were stimulated with anti-CD3 and Con A sup for 7 days. The surface expression of Thy-1, CD4 and CD8 by these cells was then determined by FMF. Panel A: Thy-1 and CD4 expression, whole LPL. Panel B: CD8 and CD4 expression, whole LPL. Panel C: Thy-1 and CD4 expression, CD8-depleted LPL. Panel D: CD8 and CD4 expression, CD8-depleted LPL.

proliferated in response to anti-CD3 and this proliferation was enhanced by Con A sup but suppressed slightly by IL-4. The ability of CD4+ lamina propria T cells to respond to polyclonal activation was tested by stimulating CD8-depleted LPL with various T-cell mitogens. As shown in Table 2 (experiment 2), anti-CD3 gave the greatest amount of proliferation, followed by Con A, PHA and PWM in that order. Spleen lymphocytes, which had been treated in an identical fashion as the LPL (including enzyme digestion), demonstrated much greater proliferation in response to these T-cell mitogens, although the hierarchy of proliferation was similar.

Generation of polyclonal and antigen-specific CD4+ lamina propria T-cell lines

Having defined *in vitro* conditions which promoted lamina propria T-cell proliferation, we undertook a series of experiments to derive and characterize lamina propria T-cell lines. Initial studies, utilizing anti-CD3, irradiated syngeneic spleen cells and either Con A sup or rIL-4, demonstrated that polyclonal lamina propria T-cell lines could be generated. When whole LPL were stimulated with anti-CD3 the majority of responding T cells were CD8+ 1–2 weeks later (Fig. 2). These T-cell lines proliferated well upon restimulation with anti-CD3 for 3–4 weeks but then ceased proliferating and died off. On the other hand, when CD8-depleted LPL were used, significant numbers of CD4+ T cells were obtained (Fig. 2) and these cells survived beyond 4 weeks. In both cases substantial numbers of Thy-1+, CD4−, CD8− cells were also present. While the majority of these latter cells were CD3− when evaluated after 12 weeks of culture (data not shown), with longer time periods the majority of double negative cells present in these cultures expressed the αβ TcR, as shown in Fig. 3. In order to obtain CD4+ T-cell lines free of the double negative cells, FMF cell sorting was performed. Cells were stained with FITC anti-Thy-1

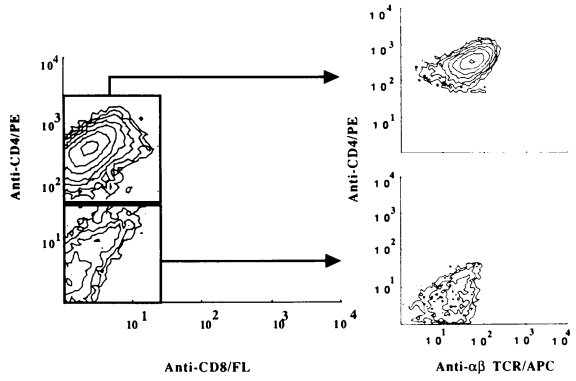

Figure 3. Expression of CD4, CD8 and the αβ TCR on a polyclonal LPL T-cell line. A polyclonal LPL T-cell line derived from CD8-depleted LPL and maintained *in vitro* for 4 weeks, as described in Methods, was analysed by FMF on a FACS Star Plus. The majority of the cells were $CD4^+CD8^-$ (77%), and of these >95% were $\alpha\beta$ TCR^+. A significant minority of the cells were $CD4^-CD8^-$ (23%), and of these 73% were $\alpha\beta$ TCR^+.

and phycoerythrin anti-CD4, then sorted for $Thy\text{-}1^+$, $CD4^+$ cells. The surface phenotype of one such polyclonal $CD4^+$ T-cell line, MLP 3A, is shown in Fig. 4. This T-cell line was uniformly $Thy\text{-}1^+$, $\alpha\beta$ TcR^+, $CD4^+$, and $CD8^-$.

Previous studies have demonstrated that cholera toxin (CT) acts as a strong oral adjuvant and can give rise to long-lived memory B cells in the lamina propria.[6,21,31] Reasoning that a similar phenomenon might occur with lamina propria T cells, we orally immunized mice with KLH and CT. When lamina propria cells, depleted of $CD8^+$ T cells, were cultured with KLH, irradiated syngeneic spleen cells and Con A supernatant, antigen (KLH)-specific $CD4^+$ T-cell lines were obtained. Figure 4 demonstrates the surface phenotype of one such line, MLP 2B. This T-cell line, similar to the polyclonal LPL $CD4^+$ T-cell line, was $Thy\text{-}1^+$, $\alpha\beta$ TcR^+, $CD4^+$ and $CD8^-$. In contrast to the polyclonal LPL T-cell lines, very few $Thy\text{-}1^+$, $CD4^-$, $CD8^-$ cells were present in this LP T-cell line. This antigen-specific $CD4^+$ T-cell line was KLH specific in that it proliferated in response to KLH (in the presence of irradiated syngeneic spleen cells) but not in the absence of KLH (Table 3). Likewise the polyclonal $CD4^+$ T-cell line proliferated in response to anti-CD3 (in the presence of irradiated syngeneic spleen cells) but not in the absence of anti-CD3 or if KLH, instead of anti-CD3, was used. The polyclonal and antigen-specific $CD4^+$ lamina propria T-cell lines have been maintained *in vitro* for greater than 16 weeks by weekly restimulation with anti-CD3 or KLH, respectively, irradiated syngeneic spleen cells and Con A sup.

Lymphokine secretion by freshly isolated lamina propria T cells and $CD4^+$ T-cell lines

In order to evaluate the profile of lymphokines secreted by lamina propria T cells, freshly isolated lamina propria T cells or *in vitro* $CD4^+$ T-cell lines were cultured with anti-CD3 and PMA for 48 hr. The supernatants were then harvested and assayed for lymphokines. As shown in Table 4, freshly isolated LPL T cells produced significant amounts of IL-2, IL-4 and gamma interferon. They also produced detectable amounts of IL-5. The polyclonal $CD4^+$ T-cell lines produced substantially less IL-2, undetectable amounts of gamma interferon but large amounts of IL-4 and IL-5. The KLH-specific $CD4^+$ T-cell line produced large amounts of IL-2, IL-4 and gamma interferon but very little IL-5.

DISCUSSION

This is to our knowledge the first study to describe a methodology for generating and characterizing *in vitro* $CD4^+$ lamina propria T-cell lines. By modifying a protocol originally designed for isolation of antibody secreting cells from the lamina propria,[23] we have obtained highly purified, viable lamina propria (LP) lymphocytes. This has allowed us to characterize the surface phenotype and lymphokine secretion of freshly isolated LP T cells, but more importantly, this has permitted the development of a method for generating *in vitro* long-term polyclonal and antigen-specific $CD4^+$ LP T-cell lines.

To ensure that lamina propria lymphocytes were free of contaminating lymphocytes from other mucosal sites, great care was taken to exclude cells from the Peyer's patches (PP) and the intra-epithelial compartment of the mucosa. This was done by careful excision of the PP using a glass rod and sharp scissors as described[23] and by thorough treatment of the pieces of small intestine with EDTA-containing medium for several rounds until no further release of epithelial cells and intraepithelial lymphocytes could be detected.[23,24] The lamina propria lymphocytes were separated from epithelial cells and debris by Percoll gradient centrifugation as described.[23] Given the fact that $Thy1^+$, $CD4^+$ or $CD8^+$ T cells and sIg^+ B cells, which represented the majority of our LPL, are present in relatively small numbers within the IEL population ([33] and G. R. Harriman and N. Y. Lycke, unpublished observations), it is unlikely that our lamina propria lymphocytes were contaminated by substantial numbers of IEL.

In initial experiments we found that polyclonal stimulation of whole LPL with anti-CD3 gave rise to the preferential proliferation and expansion of $CD8^+$ lamina propria T cells. These cultures proliferated well during the first restimulation with anti-CD3 but on subsequent restimulation the cells became progressively unresponsive and eventually died off after 3–4 weeks. However, by first depleting $CD8^+$ T cells, we strongly enhanced expansion of $CD4^+$ T cells after stimulation with anti-CD3 and importantly these cultures escaped anergy and spontaneous death. To obtain pure $CD4^+$ lamina propria T-cell lines from these original T-cell lines we utilized cell sorting. Moreover, using a similar protocol with LPL from animals previously immunized with KLH and cholera toxin as an oral adjuvant, we also generated antigen-specific (KLH) lamina propria $CD4^+$ T-cell lines. The polyclonal and KLH-specific $CD4^+$ lamina propria T-cell lines thus derived have been maintained *in vitro* for longer than 16 weeks by periodic restimulation with anti-CD3 or KLH, respectively.

Several previous studies have looked at the phenotype of LPL.[7,9,32-40] Interpretation of their results and comparisons among the studies are made difficult by the fact that these studies have looked at different species, have evaluated different regions of the intestinal tract, have used different techniques for evaluating surface antigen expression and finally have looked at normal tissues in some cases and diseased tissue in other instances. These variables have given rise to widely divergent estimates of the size of various T- and B-lymphocyte popula-

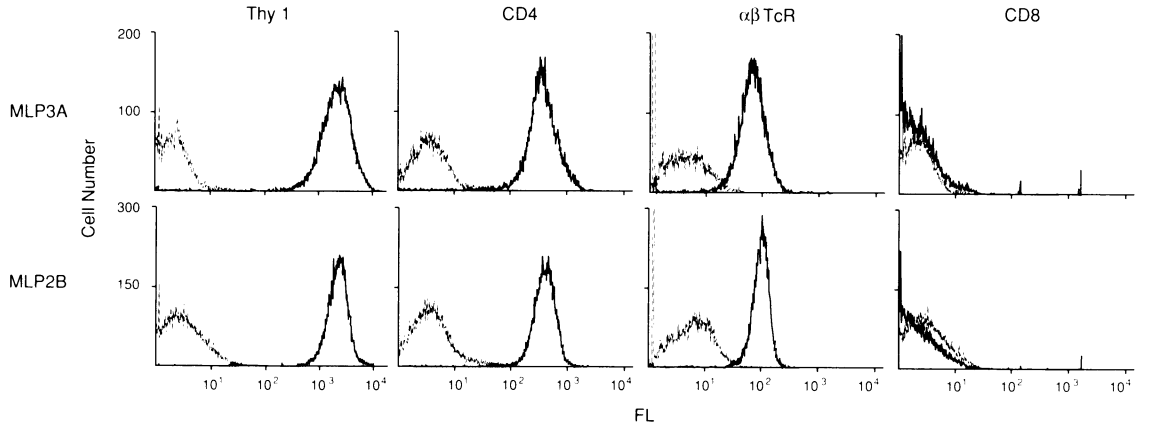

Figure 4. Surface phenotype of polyclonal and antigen-specific (KLH) CD4+ lamina propria T-cell lines. The surface expression of Thy-1, CD4, $\alpha\beta$ TCR and CD8 was analysed after 4 months of *in vitro* cultures by FMF on a FACS Star Plus. The upper panels show analysis of a polyclonal CD4+ T-cell line (MLP3A). The lower panels show analysis of a KLH-specific CD4+ T-cell line; (MLP2B). (- - - -) unstained cells; (——) FITC anti-Thy-1, PE anti-CD4, biotin-anti-$\alpha\beta$ TCR +avidin APC or FITC anti-CD8.

Table 3. Proliferation of lamina propria T-cell lines[a]

	MLP 3A[b]	MLP 2B[c]
Media only	978±634	372±290
KLH only	ND	634±482
Anti-CD3 only	491±224	ND
Feeder cells only	783±371	968±359
Anti-CD3 & feeder cells	12,341±540	5293±620
KLH & feeder cells	536±312	5355±914

[a] Lamina propria T-cell lines (5×10^4 cells/well) were stimulated with anti-CD3 (2%) or KLH (100 μg/ml) in the presence or absence of $2{\cdot}5 \times 10^5$ spleen feeder cells for 48 hr, pulsed for the final 6 hr with [^{3}H]thymidine. Results are mean (cpm) ± SD of triplicate wells.

[b] MLP 3A is a polyclonal CD4+ lamina propria T-cell line.

[c] MLP 2B is a KLH-specific CD4+ lamina propria T-cell line.

tions in the lamina propria. Estimates of the percentage of LPL which are T cells (using different T-cell antigens such as Thy-1, CD3 or CD2 as markers) have ranged from 30 to 80%, CD4+ T cells from 8 to 50%, CD8+ T cells from 9 to 54% and sIg+ B cells from 7 to 40%. Our findings fall within these ranges and are consistent with the very limited previous studies of murine LPL.[7,32,33] We also found populations within the LP that were Thy-1+, CD4− and CD8− of which some expressed CD3 while a majority were CD3−. Both these populations of LP cells could be expanded *in vitro* and studies are now under way to characterize them further.

These studies have evaluated the capacity of murine lamina propria T cells to respond to various T-cell mitogens. Consistent with what has been reported previously,[7,9,13] we found that lamina propria T cells proliferated less well compared to T cells from spleen in response to mitogens and that this difference was not a consequence of treatment with collagenase. However, whether LP T cells are inherently less responsive to mitogenic stimulation, or alternatively require additional signals/costimuli remains an unresolved question. Evidence in support of the latter possibility has recently been provided by studies in which human LP T cells were less responsive to anti-CD3 than

Table 4. Lymphokine production by freshly isolated lamina propria lymphocytes and CD4+ lamina propria T cell lines (in U/ml)

	IL-2[a]	IL-4[b]	IL-5[c]	IFN-γ[d]
Freshly isolated LPL[e]	30±6	39±13	5±1	50±5
Polyclonal CD4+ LP T-cell lines[e]	9±7	104±127	45±21	<3
KLH-specific CD4+ LP T-cell line[e]	119±4	138±18	3±2	>300

[a] IL-2 activity was assayed with a CTLL line responsive to IL-2 but not IL-4.[28]

[b] IL-4 activity was assayed in an anti-IgM costimulation assay.[29]

[c] IL-5 activity was assayed with either a BCL_1 assay or an IL-5 ELISA.[22,30]

[d] Gamma interferon was assayed with a gamma interferon ELISA.[22]

[e] Fresh whole lamina propria lymphocytes (LPL), polyclonal or KLH-specific CD4+ lamina propria (LP) T-cell lines were stimulated with anti-CD3 (5%) and PMA (10 ng/ml) for 48 hr then supernatants were harvested and assayed for lymphokines.

peripheral blood T cells, but were as responsive, if not more so, to mitogenic combinations of anti-CD2 antibodies.[13]

Evaluation of lymphokine secretion demonstrated that freshly isolated LPL and the KLH-specific CD4+ LPL T-cell line both produced large amounts of IL-2, IL-4 and gamma interferon, but produced less IL-5. In contrast, the polyclonal CD4+ LPL T-cell lines all produced significant amounts of IL-4 and IL-5 but only small amounts of IL-2 and no detectable amounts of gamma interferon. These differences in lymphokine secretion between polyclonal versus antigen-specific CD4+ LPL T-cell lines might reflect either an *in vitro* selection bias for cells of a particular T-helper phenotype, or for secretion of particular cytokines, depending on whether polyclonal T-cell expansion with anti-CD3 or antigen-specific stimulation with KLH is used. Thus, anti-CD3 may induce release of cytokines from CD4+ T cells, for example interleukin-10 (IL-10), which would select for a T_H2 phenotype by inhibiting secretion of IL-2 or gamma interferon.[41] Likewise, antigen might cause substantial amounts of gamma interferon secretion which could inhibit the outgrowth of T_H2 cells.[42] Alternatively, these results may represent an *in vivo* selection bias for antigen-specific memory T cells of the T_H1 phenotype after oral immunization with antigen and cholera toxin. However, it should be noted that these LP T-cell lines are not clones. Therefore, they are likely to consist of mixtures of T-helper cell subsets including T_H0, T_H1 and T_H2 cells.

Previous studies from our laboratory have demonstrated the ability to generate lifelong memory B cells within the lamina propria through use of cholera toxin as an oral immunogen and adjuvant.[31] In the studies reported here, our ability to obtain antigen-specific lamina propria CD4+ T cells from mice which had been immunized with antigen and cholera toxin 12 months previously suggests that long-term memory T cells are also generated by use of cholera toxin as an oral adjuvant. This opens up the possibility for use of cholera toxin as an oral adjuvant in the induction of cell-mediated, as well as humoral, mucosal immunity. Evidence in support of this notion is now accumulating (N. Y. Lycke, unpublished observations).

The authors are aware of the potential bias involved in the study of *in vitro* propagated LP T-cell lines which might not always correctly reflect the normal phenotype and function of these cells in the gut mucosa. However, freshly isolated LP T cells and in particular antigen-specific T cells are difficult to obtain in large numbers and will only allow limited analysis. We therefore believe that the ability now to generate *in vitro* long-term polyclonal and antigen-specific CD4+ LP T-cell lines should greatly facilitate the study of their function and their regulatory role in the gut mucosa.

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