Memory/effector T cells in TCR transgenic mice develop via recognition of enteric antigens by a second, endogenous TCR

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Abstract
The majority of clonotypic CD4+ T cells in the intestinal lamina propria of DO11.10 TCR transgenic mice have an activated/memory phenotype and produce effector cytokines despite the absence of prior exposure to ovalbumin (OVA), the transgene-specific antigen. A small number of splenic T cells have a similar phenotype. Clonotypic T cells from Peyer’s patch are intermediate in both phenotype and effector cytokine production. Flow cytometric analysis of cells isolated from thymectomized, OVA-naive DO11.10 mice treated with continuous administration of BrdU indicated that a significant fraction of clonotype-positive T cells in the lamina propria and Peyer’s patch were in the cell cycle, with significantly fewer cycling cells in the spleen. Most of the cycling cells from each anatomic site expressed low levels of CD45RB. Effector cytokine expression was enriched in the CD45RBlow populations. These memory/effector cell populations were eliminated in DO11.10/SCID and DO11.10/RAG-2−/− mice, suggesting that recognition of non-OVA antigens through a second, non-clonotypic TCR was driving differentiation of memory/effector cells in naive BALB/c DO11.10 mice. Clonotypic CD4+ T cells isolated from DO11.10, but not from DO11.10/SCID or DO11.10/RAG-2−/− mice, were stimulated to enter the cell cycle by antigen-presenting cells pulsed with an intestinal bacterial antigen extract. These data provide direct evidence that enteric bacterial antigens can activate transgenic T cells through a second, non-clonotypic TCR, and support the notion that the development and turnover of memory/effector cells in vivo is driven by the intestinal flora.

Introduction
The signals that drive T cell turnover in vivo and the mechanisms by which antigen-specific memory/effector T cells are maintained are poorly understood. It is generally accepted that recognition of specific antigen is required for the activation and differentiation of naive T cells into memory/effector cells in vivo, but the mechanism(s) by which the latter are maintained is less clear (1,2). The intrinsic capacity for prolonged survival in the absence of antigenic re-stimulation or cell cycling may represent one mechanism for memory cell maintenance. Alternatively, persistence of specific antigen, cross-reactivity with structurally related antigens or altered responsiveness to trophic factors that induce cell cycling in the absence of antigenic stimulation may also be contributory. The discovery that a significant fraction of post-thymic T cells express two TCR has raised the possibility that memory/effector differentiation driven in response to antigen recognition by one TCR may lead to memory maintenance through recognition of a distinct, perhaps self antigen by the second TCR (3–6).

Advances toward a better understanding of the relationship between naive and memory/effector cells have been facilitated using TCR transgenic mouse models. The availability of large populations of clonotypic, transgenic T cells previously unexposed to a defined, transgene-specific antigen has facilitated direct analyses of the development of naive precursors into memory/effector cells (7–12). However, although these models have proven invaluable for studies of T cell phenotype development, several studies have demonstrated the pres-
ence of small populations of clonotypic T cells with a memory phenotype in these mice (6,13,14). The origin of these cells has been poorly defined. It has been reported that a significant fraction of the circulating clonotypic T cells in DO11.10 TCR transgenic mice express a second, non-clonotypic TCR in addition to the ovalbumin (OVA)-specific αβ pair encoded by the DO11.10 transgenes (6,14). This is probably due to incomplete extinction of endogenous TCR α chain rearrangement during thymic development (15). For example, a small population of dual TCR spleen cells from OVA-naive DO11.10 mice had a memory phenotype and elimination of dual TCR T cells in DO11.10/RAG-2−/− mice resulted in loss of the memory phenotype (6).

In a previous study, we found that clonotype-positive CD4+ T cells isolated from the small intestinal lamina propria of DO11.10 TCR transgenic mice were markedly enriched for clonotypic cells with an activated/memory phenotype, despite the absence of prior exposure to the transgene-specific antigen, OVA (16). The cytokine phenotype of these cells was distinct. A predominance of IFN-γ and IL-10 expressed by distinct T cell subpopulations suggested two counter-regulatory memory/effector phenotypes (16). Others reported that dual TCR cells were enriched in the lamina propria of these mice, raising the possibility that mucosal T cells might be activated by recognition of enteric antigens by a second, non-clonotypic TCR (14).

In the present study, we have examined the in vivo turnover and functional phenotypes of clonotypic T cells in mucosal versus splenic tissues to determine if second, non-clonotypic TCRs are involved in the development of memory/effector cells. We find that in vivo turnover and effector cytokine expression of clonotypic T cells in DO11.10 mice is significantly increased in mucosal lymphoid tissues relative to spleen and correlates with the CD45RBlow phenotype in each site. Evidence for second TCRs with specificity for enteric antigens is presented. Thus, enteric antigens may activate transgenic T cells through a second, non-clonotypic TCR, supporting the notion that memory/effector cells develop in vivo in response to antigens derived from the intestinal flora.

**Methods**

**Mice**

DO11.10 TCR transgenic mice (17) were selected at age 4–6 weeks by flow cytometric analysis of peripheral blood leukocytes stained with the mAb, KJ1-26, that specifically recognizes the DO11.10 TCR (18). DO11.10/RAG-2 knockout mice and DO11.10/SCID mice were kindly provided by Drs Osami Kanagawa (Washington University, St Louis, MO) and Barry T. Rouse (University of Tennessee, Knoxville, TN) respectively. Female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice used in experiments were 6–11 weeks of age. In some experiments, DO11.10 mice were surgically thymectomized or sham-thymectomized and allowed to recover for 1 month prior to administration of BrdU (Sigma, St Louis, MO). BrdU was administered in the drinking water (0.8 mg/ml) as described by Tough et al. (19).

**Cell isolations**

Lamina propria lymphocytes were isolated as previously described (16). Briefly, the small intestines from five to eight mice were removed, and Peyer’s patches were carefully excised and kept on ice until used. For removal of epithelial cells and intraepithelial lymphocytes, the intestines were washed, cut into small pieces, and then the pieces were incubated with calcium- and magnesium-free HBSS supplemented with 5% bovine calf serum (HyClone, Logan, UT) and 5 mM EDTA (Sigma) on a magnetic stirrer at 37°C for 20 min. This was repeated 4–6 times. The tissues were then incubated with RPMI 1640 (Gibco/BRL, Gaithersburg, MD) containing 5% bovine calf serum, antibiotics, 25 mM HEPES and 90 U/ml collagenase (Sigma) for 30 min at 37°C with stirring. Isolated cells were separated on a 40/75% discontinuous Percoll gradient (Pharmacia, Piscataway, NJ) centrifuged at 600 g, 25°C for 20 min. The cell yield was typically ~2×10^8 lymphocytes per mouse with >90% cell viability. Peyer’s patch lymphocytes were isolated using the same conditions as for lamina propria lymphocytes. Splenic lymphocytes were obtained by gently teasing splenic fragments between sterile frosted slides and removing red blood cells by incubation in ACK lysis buffer (20). CD4+ T cells were purified using the MACS system (Miltenyi, Auburn, CA) according to the manufacturer’s protocol. Cells were incubated with biotinylated anti-CD8 (2.43), anti-B220 (14.8), anti-Mac-1 (M1/70) and anti-I-A^d^ (MKD6) for 30 min at 4°C, washed and incubated with streptavidin microbeads for 30 min at 4°C. The cells were again washed and passed through a magnetic column. The purity of CD4+ T cells was 92–97% by FACS analysis.

**Flow cytometry and BrdU staining**

Cells isolated from different anatomical sites were stained for 30 min at 4°C with the following mAb: anti-CD4 ( GK1.5)-phycoerythrin (PE) or –FITC, anti-CD45RB (23G2)-biotin or –FITC, CD44 (IM7)–FITC, anti-CD69 (H1.2F3)–FITC, anti-CD45RBlow CD4+ T cells were purified using the MACS system (Miltenyi, Auburn, CA) according to the manufacturer’s protocol. Cells were incubated with biotinylated anti-CD8 (2.43), anti-B220 (14.8), anti-Mac-1 (M1/70) and anti-I-A^d^ (MKD6) for 30 min at 4°C, washed and incubated with streptavidin microbeads for 30 min at 4°C. The cells were then incubated with streptavidin–Red670 (Gibco/BRL). To determine BrdU incorporation, cells were stained with the indicated surface markers as above, washed and fixed with 95% ethanol for 30 min (19). After incubation for 1 h in PBS supplemented with 3% paraformaldehyde and 0.1% Tween 20, the cellular DNA was denatured using 50 Kunitz units DNase I (Sigma) and cells were then stained with anti-BrdU–FITC (Becton Dickinson). For the flow cytometric sorting, magnetically purified CD4+ T cells were stained with KJ1-26–PE and CD45RB–FITC or CD4–FITC and sorted on a FACStar (Becton Dickinson).

**Cell culture and preparation of CBA**

In total, 5×10^5 sorted KJ1-26+ CD45RB^high^ or KJ1-26+ CD45RB^low^ CD4+ T cells isolated from DO11.10 mice or CD4+ T cells isolated from DO11.10/RAG-2 knockout or DO11.10/SCID mice were stimulated in 48-well plates (Costar, Cambridge, MA) with 1 μg/ml OVA peptide (OVA^233-339^) and 1.5×10^5/well irradiated (2500 rad) BALB/c splenic adherent cells (SAC) prepared as previously described (20). Culture
medium was RPMI 1640 containing 10% FCS (Hyclone), 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 2 mM L-glutamine (Gibco/BRL) and 50 mM 2-mercaptoethanol (Sigma). In some experiments, BALB/c SAC were incubated overnight at 37°C with 1 mg/ml cecal bacterial antigen (CBA) or 1 µg/ml OVA peptide. Preparation of CBA was as described (21). Briefly, normal BALB/c mice were sacrificed and their cecums removed. The cecum was opened, placed in PBS and, after the cecal contents were expelled by mixing in a vortex, the cecal tissue was removed. After addition of DNase (100 U/ml), 1 ml of the bacterial suspension was added to 1 ml of glass beads and the cells were disrupted for 3 min on ice using a minibead beater. The glass beads and unlysed cells were removed by centrifugation (5000 g for 10 min) and the lysates were filter sterilized. After overnight culture with CBA, SAC were washed and incubated with KJ1-26+ CD4+ T cells in 48-well plates (Costar) for 96 h. In preliminary experiments using splenic T cells isolated from DO11.10 mice, it was established that a pulse with BrdU (30 µg/ml) during the last 18 h of a 96 h proliferative response was required for maximal labeling of the dividing T cells (data not shown). The recovered cells were stained for BrdU incorporation and flow cytometric analyses were performed as above.

In situ hybridization and immunohistochemical analyses

The single-cell expression of cytokine mRNAs was detected by in situ hybridization, as previously described (20,22,23). Briefly, recovered cells were cytocentrifuged (Shandon, Pittsburg, PA) onto glass slides using RNase-free conditions. The slides were air-dried, fixed with 3% paraformaldehyde (1 h at 22°C) and hybridized overnight at 50°C with heat-denatured (80°C) RNA probes. The probes used were antisense, single-stranded RNA molecules labeled with digoxigenin-UTP by transcription from a T3 or T7 primer of a cDNA insert cloned into either pBSII (Stratagene, La Jolla, CA) or pGEM (Promega, Madison, WI) (26,28). After hybridization, slides were incubated with RNase A to remove non-hybridized probes, washed and incubated with Tris–NaCl, containing 2% normal horse serum to block non-specific binding. The slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) for 2 h at room temperature. Then 400 µl color solution (NBT/BCIP) was added to each slide and incubated overnight in the dark at 4°C. After incubation, the slides were placed in Tris–EDTA, pH 8.0, to stop the color reaction. Cells were quantitated using bright-field microscopy. Quantitation of transgene-positive cells in replicate slides was routinely determined by staining cells with KJ1-26+ mAb followed by detection with the ABC immunoperoxidase technique (20).

Double-label immunohistochemistry was performed on a BioTek Solutions TechMate automated staining system (Ventana, Tucson, AZ), as previously described (24,25). Intestinal and splenic tissues were embedded in OCT embedding medium (Miles, Naperville, IL) and snap-frozen in liquid nitrogen. Then 5 µm cryostat sections were cut and fixed in cold 95% ethanol. DO11.10 clonotype-positive cells were detected with sequential KJ1-26–FITC, biotinylated anti-FITC (Boehringer Mannheim) and streptavidin-conjugated horseradish peroxidase, using dianinobenzidine as chromagen. B cells were stained with anti-IgD (JAI2.5), biotinylated anti-rat IgG and streptavidin-conjugated alkaline phosphatase, using nitroblue tetrazolium dimethylformamide as chromagen.

Results

Increased turnover of clonotypic T cells in the small intestine

Clonotypic CD4+ T cells isolated from the small intestinal lamina propria of OVA-naïve DO11.10 mice demonstrate nearly uniform expression of ‘activated/memory’ surface phenotype markers (CD45RBhi/CD44hi/L-selectinlo/CD69hi) and produce a mix of effector cytokines when activated by OVA in vitro (14,16). This is in contrast to clonotypic cells isolated from peripheral lymph nodes and spleen, where only a small minority expresses a memory phenotype and effector cytokines (6,14,16,20,25). To study the turnover of clonotype-positive CD4+ T cells (KJ1-26+ CD4+) in different lymphoid tissues, BrdU was administered to DO11.10 mice in their drinking water and incorporation of label by cycling T cells was analyzed as previously described (19). Cells were isolated from the lamina propria, Peyer’s patches and spleen of individual BrdU-fed mice, and analyzed by flow cytometry to determine the fraction of cells that were in cell cycle during the period of BrdU administration. Initial experiments confirmed that a subpopulation of BrdUlow cells was present in spleen from sham-thymectomized, but not thymectomized, mice, and likely represent recent thymic emigrants (19 and data not shown). All subsequent experiments were performed with thymectomized DO11.10 mice to eliminate this background. BrdU was administered for 9 days in the drinking water, a time sufficient to label almost all bone marrow cells and double-positive thymocytes (19), and mice were sacrificed for analysis.

Lymphocytes isolated from lamina propria, Peyer’s patches and spleen were stained with anti-CD4 and KJ1-26+ mAb, and total CD4+ T cells or KJ1-26+ CD4+ T cells were gated for determination of BrdU incorporation by flow cytometry. The fraction of total CD4+ T cells that expressed the DO11.10 TCR (KJ1-26+) was reduced in the small intestinal isolates compared to spleen (41 and 44 versus 65%; Fig. 1, left panel). The KJ1-26+ CD4+ cells isolated from both the lamina propria and Peyer’s patches showed reduced levels of DO11.10 TCR expression (14). Despite the absence of prior exposure to the transgene-specific antigen OVA, a significant fraction of clonotypic T cells was in cell cycle during the 9 day period of BrdU administration, irrespective of the lymphoid tissue examined. The fraction of clonotype-negative CD4+ T cells in cell cycle over the same time period paralleled the clonotype-positive population in each tissue site. The fraction of Peyer’s patches and lamina propria cells that incorporated BrdU was 3- to 5-fold greater than splenic cells, both within the clonotype-positive and -negative CD4+ T cell populations (Fig. 1, right panel). Between 20 and 30% of the clonotypic CD4+ T cells in the lamina propria and Peyer’s patch was labeled with BrdU, compared to 6–8% of SP clonotype-positive CD4+ T cells. Thus, both clonotype-positive and -negative CD4+ T cells from small intestinal tissues demonstrated more rapid cell turnover rates than their splenic counterparts.
Increased turnover of clonotypic T cells occurs in the CD45RBlow subset, regardless of tissue site

To determine the relationship between cell turnover and cell surface phenotype of clonotype-positive T cells in the intestinal and splenic tissues, CD4⁺ T cells were isolated from thymectomized mice fed BrdU for 9 days, and stained for expression of CD45RB, DO11.10 TCR and BrdU (Fig. 2). In agreement with previous studies by our group (16) and others (14), the majority of clonotype-positive T cells from lamina propria had a memory phenotype and expressed low levels of CD45RB (Fig. 2, left panel). In contrast, few
clonotypic T cells in spleen were CD45RB\textsuperscript{low}, while Peyer’s patch clonotypic T cells demonstrated an intermediate phenotype (Fig. 2, left panel) (16). The incorporation of BrdU by clonotypic T cells correlated with CD45RB expression levels and was remarkably similar for each population in the different tissue sites (Fig. 2, right panel). There was significantly greater cell turnover within the CD45RB\textsuperscript{low} population from each tissue. Approximately 40\% of CD45RB\textsuperscript{low} clonotype-positive cells were labeled with BrdU, compared with \textasciitilde5\% of CD45RB\textsuperscript{high} cells in each site.

**Cytokine expression patterns by CD45RB\textsuperscript{high} and CD45RB\textsuperscript{low} subsets**

KJ1-26\textsuperscript{+} CD4\textsuperscript{+} T cells isolated from lamina propria, Peyer’s patches and spleen of OVA-naive DO11.10 mice were sorted on the basis of CD45RB expression to study the cytokine phenotypes of memory and naive cells from each site. Unsorted and sorted clonotype-positive CD4\textsuperscript{+} T cells were stimulated with OVA peptide (OVA\textsubscript{323-339}) presented by BALB/c SAC and analyzed for single-cell cytokine mRNA expression by \textit{in situ} hybridization (16, 20, 22). As previously reported,
DO11.10 mice eliminated the memory phenotype population observed in normal DO11.10 mice in this and other studies (6,14). Shown in Fig. 4 are flow cytometric analyses of clonotypic T cells isolated from DO11.10/RAG-2-/+ produced by backcrossing the DO11.10 transgenes into RAG-2 deficient BALB/c mice. Clonotypic CD4+ T cells isolated from both the lamina propria (upper panel) and spleen (lower panel) of DO11.10/RAG-2-/+ expressed a naive, non-activated surface phenotype (CD45RBhigh CD44low CD69low). BrdU incorporation by KJ1-26+ T cells populating the small intestinal lamina propria was markedly reduced, but detectable, in thymectomized DO11.10/RAG-2-/+ mice, when compared to DO11.10 mice (5- to 7-fold decrease; cf. Fig. 1). Cell turnover in the spleens of thymectomized DO11.10/RAG-2-/+ mice was essentially eliminated. Identical results were found in analyses of DO11.10/SCID mice (data not shown). Because DO11.10/ RAG-2-/+ and DO11.10/SCID mice fail to develop Peyer’s patches, this tissue could not be examined.

To determine the cytokine phenotype of clonotypic T cells from recombinase-deficient DO11.10 mice, lamina propria and spleen CD4+ T cells isolated from DO11.10/RAG-2-/+ or DO11.10/SCID mice were stimulated with OVA peptide presented by BALB/c antigen-presenting cells and analyzed by in situ hybridization (Fig. 5). Unsorted CD4+ T cells isolated from the lamina propria of DO11.10 mice that have a memory/effector cytokine phenotype with a greater frequency of IFN-γ and IL-10-positive cells and significantly fewer IL-2-positive cells (Fig. 3). In contrast, CD4+ T cells isolated from the lamina propria of DO11.10/RAG-2-/+ expressed primarily IL-2, consistent with their naive surface phenotype. Notably, however, there were small, but consistent, populations of DO11.10/RAG-2-/+ CD4+ T cells in the lamina propria that expressed IFN-γ or IL-10. Only rare splenic CD4+ cells from DO11.10/RAG-2-/+ mice expressed effector cytokines (IFN-γ < 0.3%; IL-10 < 0.1%), in contrast to the small, but significant numbers of IFN-γ- and IL-10-positive cells found in unsorted DO11.10 splenic CD4+ T cells (Fig. 3).

Immunohistochemical analyses of the small intestine and spleen from DO11.10/SCID and normal DO11.10/BALB/c mice were performed to characterize the localization of the clonotypic T cell populations in these sites (Fig. 6). Although the architecture of lymphoid tissues is distorted in SCID mice by the absence of normal B and T cell populations, the distribution of clonotypic T cells in both lamina propria and spleen of DO11.10/SCID animals paralleled that for normal DO11.10 mice. In both sites, the density of clonotypic T cells was decreased, although their localization was similar. Thus the great majority of KJ1-26+ T cells in spleen were found in a periarterial lymphoid sheath and in the small intestine they were found in the lamina propria.

Reactivity to enteric antigens is eliminated in DO11.10/RAG-2 knockout and DO11.10/SCID mice

To directly examine the possibility that DO11.10 KJ1-26+ T cells might recognize enteric lumenal antigens through an endogenous, non-clonotypic TCR, an assay for reactivity to enteric antigens was developed, and the responses of clonotypic T cells isolated from DO11.10 and DO11.10/SCID mice were compared (Fig. 7). SAC isolated from BALB/c mice were cultured overnight with an extract of CBA and assayed...
for their capacity to stimulate a proliferative response. Flow cytometric determination of BrdU incorporation by KJ1-26+ CD4+ gated cells limited analysis to the clonotypic T cell population. SAC loaded with OVA peptide were used as a control for the proliferative capacities of isolated DO11.10 or DO11.10/SCID clonotypic T cells.

Cell turnover in response to SAC pulsed with OVA peptide was similar for splenic KJ1-26+ CD4+ T cells isolated from either DO11.10 or DO11.10/SCID mice (Fig. 7, left panel). Greater than 85% of the clonotype-positive CD4+ T cells in both populations incorporated BrdU and the intensity of BrdU staining was similar. In contrast, only the DO11.10 cell population demonstrated BrdU incorporation in response to CBA-pulsed SAC (Fig. 7, right panel). As many as 8% of the DO11.10 KJ1-26+ CD4+ T cells progressed through cell cycle during the 18 h pulse with BrdU, compared with no incorporation above background for KJ1-26+ CD4+ T cells from DO11.10/SCID mice. Similar results were obtained using DO11.10/RAG-2+ mice and proliferative responses assayed by [3H]thymidine incorporation were comparable (data not shown). Responses to CBA prepared from SCID mice were similar to that shown for BALB/c antigen preparations, suggesting that the stimulating antigenic specificities were not strain-specific. These data demonstrate that clonotypic T cells isolated from DO11.10, but not DO11.10/SCID or DO11.10/RAG-2+ mice, recognize intestinal antigens derived from the normal enteric flora.

Discussion

The present study was initiated to examine the origin of memory/effector T cells in antigen-naive DO11.10 mice. Past studies suggested the possibility that dual TCR cells were the source for activation and differentiation of memory/effector cells in the DO11.10 mouse, probably driven by recognition of environmental antigens by the second, non-clonotypic TCR. The demonstration here of differential reactivity of single and dual TCR DO11.10 populations to enteric antigens, but not OVA peptide, provides direct evidence for the function of a second, non-clonotypic TCR population in the response to enteric antigens. These data support the premise that memory/effector cells in DO11.10 mice develop from dual TCR
precursors, and establish that both the clonotypic and non-clonotypic TCRs expressed by the same T cell population can react independently to separate antigens. Although the specific enteric antigens that are recognized by non-clonotypic TCRs are unknown, characterization of the cecal antigen preparation indicates that the reactivity is directed against peptides derived from the bacterial flora, not mitogens or superantigens (21). Thus, reactivity of dual TCR precursors to peptide antigens expressed by the endogenous bacterial flora appears to be the major source of the memory/effector cell populations that develop in TCR transgenic mice.

As demonstrated for DO11.10 mice in this and previous
studies (14,16), a number of reports have identified the lamina propria of the small intestine as a site typically populated by memory/effector cells with features of on-going activation (26–28). It has been shown that there is preferential homing of certain memory/effector cells to this site (reviewed in 29). The dense population of the lamina propria by clonotype-positive cells with a predominantly naive surface and cytokine phenotype was therefore a surprising feature of the DO11.10/LAM or DO11.10/SCID TCR transgenic mice. Although recoveries of clonotypic CD4 T cells from the small intestines of these animals were typically 25–40% less than from normal DO11.10 mice, immunohistochemical analyses confirmed that there was diffuse population of this tissue compartment by KJ1-26 mice, immunohistochemical analyses confirmed that there were typically 25–40% less than from normal DO11.10 1 T cells from the small intestines of these animals were typically 25–40% less than from normal DO11.10 mice, immunohistochemical analyses confirmed that there was diffuse population of this tissue compartment by KJ1-26 mice. Several possibilities are consistent with this observation, none of which are mutually exclusive. First, it is unlikely that expression by the lamina propria endothelium of addressins selective for immigration of memory/effecter cells is dependent on local production of factors produced by activated memory/effecter T cells, B cells or both (29). In the absence of activated B or T cell populations, the lamina propria endothelium may express an ensemble of targeting molecules that favors trafficking of naive cells into this site. Alternatively, the normal predominance of memory/effecter cells in the lamina propria might reflect a kinetic competition for cell entry between naive and memory/effecter populations that favors the latter. In the absence of a competing memory/effecter population, naive cells might accumulate to higher numbers, despite their relatively poorer homing efficiency. A similar mechanism has been proposed to explain the population of B cell follicles by anergic B cells only in the absence of a competing memory/effecter population (30,31). Finally, although controversial, it has been suggested that a major fraction of the αβ CD4 T cells that populate the lamina propria may differentiate locally in the intestinal epithelium, instead of trafficking in from the general circulation (32). The recent discovery of small intestinal ‘cryptopatches’ that support local mucosal T cell development might also explain population of the lamina propria in recombinase-deficient mice (33,34). The relative contribution of each of these factors to the localization of naive, single TCR clonotypic cells in the lamina propria of DO11.10/RAG-2−/− and DO11.10/SCID mice is unclear. However, data in this report, and similar data from others (14), suggest that there is no intrinsic inability of naive αβ CD4 T cells to localize to this site.

In accordance with the increased population of intestinal immune tissues by memory/effecter cells, the in vivo turnover rates of clonotypic T cells from thymectomized DO11.10 mice were significantly increased in these sites relative to spleen. However, in each tissue site, the relative frequency of BrdU-labeled cells correlated extremely well with the CD45RB expression pattern, suggesting that the turnover of naive and memory/effecter cells is similar in each microenvironment. Notably, the turnover rates of clonotypic (CD4 T cells) and non-clonotypic (CD4 T cells) populations were comparable in each tissue, consistent with the parallel surface phenotypes of these populations identified previously (16). In agreement with studies using non-transgenic mice (19), approximately half of the clonotype-positive and clonotype-negative memory/effecter (CD4RBlow) cells from each tissue failed to incorporate BrdU over the 9 day period of labeling, and there was incomplete labeling of these cells for even longer periods of BrdU administration (up to 30 days; data not shown). Thus, a significant number of the memory/effecter cells can remain in interphase for a prolonged period (19). This was particularly surprising for the lamina propria population, since these cells demonstrated nearly uniform expression of CD69, a sensitive early marker for T cell activation (16). This apparent discrepancy may reflect heterogeneity in the progression of activated cells through cell cycle, and suggest that there are differential activation thresholds for cell surface marker modulation and cell cycle progression (35).

While the highest in vivo turnover rates were found in the memory/effecter population, a small, but consistent fraction of the ‘naive’ (CD45RBhigh) clonotypic population in DO11.10 mice incorporated BrdU. Similar findings by Tough and Sprent were taken as evidence for cell cycling without conversion to a memory phenotype or reversion of a subpopulation of memory/effecter cells to a naive phenotype (19). Adoptive transfer studies by Bunce et al. (36) showed that reversion of CD45RClow memory cells in rats can occur in vivo and are consistent with the latter interpretation, but do not exclude the possibility of cell cycling in naive post-thymic cells. A study by Swain showed that TCR transgenic effecter cells derived in vitro underwent significant expansion after adoptive transfer into thymectomized, irradiated hosts, independently of exposure to the transgene-specific antigen (37). However, the recovered cells expressed a memory phenotype not dissimilar from that found in normal TCR transgenic mice, raising the possibility that clonal expansion in vivo might have resulted from recognition of enteric antigens by dual TCR cells. The absence of detectable cell cycling by single TCR clonotypic cells in the spleen of thymectomized mice.
DO11.10/RAG−/− or DO11.10/SCID mice (Fig. 4 and data not shown) argues that naive CD4+ T cells in this tissue do not undergo cell cycling in the absence of specific antigen. This suggests that the labeling of CD45RBhigh cells in the spleens of DO11.10 mice is most likely due to reversion of dual receptor ‘memory’ cells to a ‘naïve’ phenotype. Interestingly, however, there was reproducibly detectable, albeit significantly decreased, cell cycling by single TCR clonotypic cells in the lamina propria of thymectomized DO11.10/RAG−/− or DO11.10/SCID mice, suggesting that turnover of naive cells independently of specific antigen may be dependent on the microenvironment. Alternatively, it is possible that extrathymic T cell development in the lamina propria is responsible for turnover in this site (32–34). Further studies will be necessary to resolve this issue. In any case, our findings indicate that the major component of cell division by clonotype-positive CD4+ T cells in vivo is due to recognition of enteric bacterial antigens by a second, non-clonotypic TCR and does not require transgene-specific antigen recognition.

The present analysis indicates that the IFN-γ/IL-10 effector phenotype identified previously in the lamina propria of DO11.10 mice is not unique to that site (16), but instead, like the in vivo cell turnover rate, correlates with CD45RBlow expression in each of the tissues. The loss of this effector population in RAG−/− or SCID DO11.10 mice suggests that, at least in the DO11.10 transgenic model, development of this cytokine phenotype is driven by recognition of non-OVA peptides by a second, non-clonotypic TCR. Although the source of the effector populations in each tissue site cannot be identified on the basis of these data, the similarity of the cytokine phenotype in both intestinal and splenic CD45RBlow populations suggests that memory/effector cells populating both sites may develop in response to enteric antigens. This might be due to antigen recognition in the intestinal lymphoid tissues of cells that then circulate to spleen, to reactivity of cells in the spleen to enteric antigens that have circulated there or both. The ability of CD45RBlow cells isolated from the spleen or peripheral lymph nodes of normal mice to inhibit the colitic inflammation in a CD45RB transfer model (38,39) argues that regulatory cells reactive to enteric antigens do circulate in the periphery (see below). Thus, at least on the basis of cell frequencies, the dominant cytokines produced by memory/effector cells in both intestinal and splenic tissues are IFN-γ and IL-10, as opposed to dominant IL-2 production by naive cells. Further, the primary source of these cells is reactivity to enteric antigens (16). Production of the T₄₂ effector cytokines, IL-4 and IL-5, is limited to a small minority of the memory/effector cells.

Although it remains conjectural what portion of dual receptors are functional in normal and TCR transgenic animals, data from this study provide direct evidence that there exist dual TCR cells with specificity for enteric antigens. Recognition of enteric antigens by these cells promotes the development and increased turnover of an IL-10/IFN-γ-producing memory/effector population that is enriched in the intestinal lamina propria, but may also populate peripheral immune tissues. The development of memory/effector cells with this cytokine phenotype may play a critical role in the maintenance of peripheral tolerance and the enteric flora may provide an important reservoir of antigens capable of educating peripheral T cells in favor of a tolerogenic, rather than immunogenic, functional repertoire. The propensity for development in the DO11.10 model of dual TCR memory/effector cells that recognize enteric antigens, yet retain specificity for OVA, makes this a particularly powerful system for further studies of the role of the enteric flora in the development and maintenance of memory/effector populations in the intestinal and non-intestinal immune tissues.

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Abbreviations

CBA: cecal bacterial antigen
OVA: ovalbumin
PE: phycoerythrin
SAC: splenic adherent cell

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