Table II. *Comparison of naive and memory CD4$^+$ T cells and cell yields from lymphoid tissues of young adult and aged mice*[a]

Source of Tissue	Age of Mice	CD45RB^{high}/CD44^{low} (% of CD4$^+$ T cells)	CD4$^+$ T Cells (% of Total lymphocytes)	Lymphocyte Counts/Mouse	Actual Numbers of CD4$^+$ Naive T Cells/Mouse
NALT	8–12 wk	76.5 ± 4.2	10.6 ± 5.6	~1.0×10^5	~8.1×10^3
	12–14 mo	29.5 ± 4.0	10.9 ± 0.7	$5.0–9.5 \times 10^5$	$16.1–30.5 \times 10^3$
Peyer's patches	8–12 wk	43.6 ± 8.8	17.2 ± 3.1	$15–20 \times 10^6$	$11.2–14.9 \times 10^5$
	12–14 mo	24.1 ± 6.6	12.1 ± 3.1	~10×10^6	~2.9×10^5
Spleen	8–12 wk	67.0 ± 2.0	26.1 ± 3.2	$60–75 \times 10^6$	$10.5–13.1 \times 10^6$
	12–14 mo	50.0 ± 0.1	19.4 ± 5.1	$20–26 \times 10^6$	$1.9–2.5 \times 10^6$

[a] Mononuclear cells from NALT, Peyer's patches, and spleen of nonimmunized mice were stained with FITC-conjugated anti-CD4, PE-labeled anti-CD45RB, and biotinylated anti-CD44 mAbs, followed by CyChrome-streptavidin, and were then subjected to flow cytometry analysis by FACSCalibur.

naive CD4$^+$, CD45RB$^+$ T cells in NALT are key players for the maintenance of the respiratory immune system in the induction of mucosal and systemic immune responses in 1-year-old mice.

Chimeric mCT-A E112K/LT-B induces OVA-specific Ab responses in 1-year-old mice

Our results to date indicate that nasal application of nCT induces mucosal and systemic Ab and T cell responses in 1-year-old mice. Our recent studies showed that newly constructed chimeric mCT-A E112K/LT-B elicits adjuvant activity without IgE Ab responses and potential toxicity in the CNS of young adult mice (30). Thus, it was important to examine whether similar adjuvant effects would be induced in 1-year-old mice when mCT-A E112K/LT-B was administered via the nasal route. In this regard, 1-year-old and young adult mice were nasally immunized with OVA plus mCT-A E112K/LT-B. When OVA-specific Ab responses in nasal washes, saliva, and fecal extracts were examined, significant S-IgA Ab responses occurred in mucosal secretions of both young adult and 1-year-old mice (Fig. 7). In addition, significant and comparable OVA-specific plasma IgA Ab responses were also seen in aged mice when compared with young adult mice (Fig. 7). Furthermore, both 1-year-old and young adult mice elicited similar levels of plasma IgG anti-OVA Ab responses.

Interestingly, both young adult and 1-year-old mice immunized nasally with OVA plus chimeric mCT-A E112K/LT-B showed a similar pattern of IgG subclass response. Thus, increased IgG1 and IgG2b, but essentially no IgG2a, anti-OVA Ab responses were detected (Fig. 7). Comparable levels of CD4$^+$ T cell proliferative responses were seen in CLNs of 1-year-old mice given nasal OVA plus chimeric mCT-A E112K/LT-B (stimulation index = ~10). Although stimulation indices in spleen of aged mice given nasal OVA plus chimeric mCT-A E112K/LT-B resulted in lower proliferative responses than occurred in young adult mice, the proliferative responses were nevertheless maintained (stimulation index = ~5–10). Taken together, these findings show that nasal administration of a nontoxic, safe mucosal adjuvant, mCT-A E112K/LT-B, effectively induced OVA-specific Ab and T cell responses in 1-year-old mice.

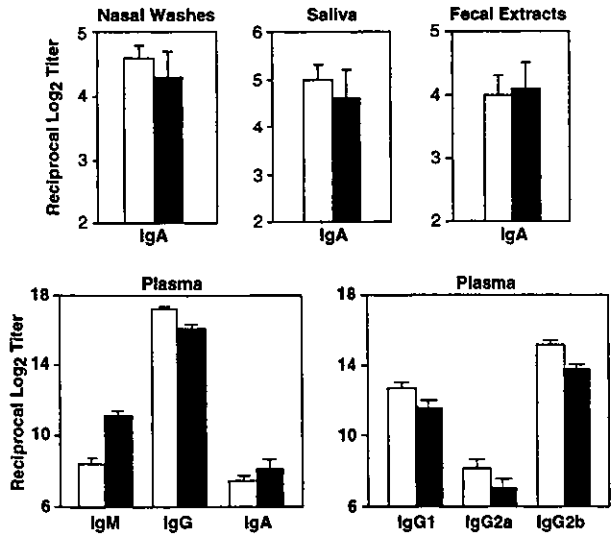

FIGURE 7. Comparison of OVA-specific IgA Ab responses in nasal washes, fecal extracts, saliva, and IgM, IgG, IgA, and IgG subclass Ab responses in plasma of 1-year-old (filled bars) and young adult (open bars) mice given nasal chimeric mutant toxin. Each mouse group was nasally immunized weekly for 3 consecutive wk with 100 μg of OVA plus 5 μg of chimeric mCT-A E112K/LT-B as mucosal adjuvants. Seven days later, Ab levels in nasal washes, fecal extracts, saliva, and plasma were determined by OVA-specific ELISA. The values shown are the mean ± SEM of 15 mice in each experimental group.

Induction of protective immunity

Because nasal immunization with OVA and chimeric enterotoxin as mucosal adjuvant induced both mucosal and systemic Ab responses in 1-year-old mice, it was important to determine whether

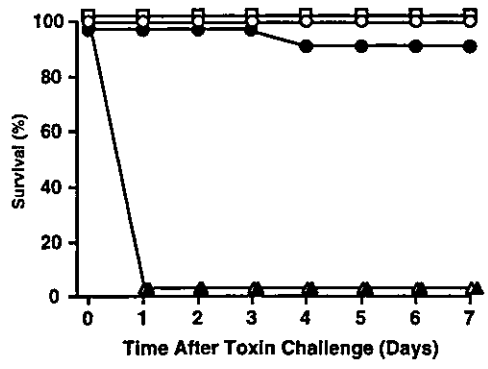

FIGURE 8. Nasal immunization with TT and chimera or nCT adjuvants elicited protective immunity in 1- and 2-year-old mice. Both groups of 1-year-old (● or ▲) and young adult (○ or △) mice were immunized nasally with 10 μg of TT and 5 μg of chimeric mCT-A E112K/LT-B (○ or ●) or 10 μg of TT alone (▲ or △) three times at weekly intervals. Two-year-old mice (□) were given nasal TT (10 μg) and nCT (0.5 μg) three times at weekly intervals. Mice were s.c. challenged with a lethal dose (130 LD_{50}) of tetanus toxin in 0.5 ml of PBS including 0.2% gelatin. Each group was comprised of five mice, and the data are representative of two separate experiments.

these Ag-specific Ab responses could protect mice from infection or intoxication. In this regard, both groups of 1-year-old and young adult mice were immunized nasally with TT and chimeric mCT-A E112K/LT-B or TT alone three times at weekly intervals. The mice were challenged s.c. with a lethal dose (130 LD_{50}) of tetanus toxin injected 1 wk after the last immunization. As expected, young adult mice given nasal TT plus chimeric enterotoxin as mucosal adjuvant were completely protected (Fig. 8). Importantly, 1-year-old mice given TT and mCT-A E112K/LT-B showed a degree of protection comparable to that seen in young adult mice, even though 1 of 15 mice died 4 days after challenge. Furthermore, 2-year-old mice were given nasal TT, and nCT showed complete protection from challenge (Fig. 8). In contrast, both 1-year-old and young adult mice given TT alone failed to protect from the paralysis and death that normally occurs within 2 days after tetanus toxin administration (Fig. 8). These findings clearly show that TT-specific, plasma IgG Abs in both 1- and 2-year-old mice induced by the chimeric mCT-A E112K/LT-B or nCT mucosal adjuvants were protective.

Discussion

This study has revealed that nasal administration of nCT or chimeric mCT-A E112K/LT-B, in contrast to oral immunization (15), effectively induced mucosal and systemic immune responses in 1-year-old mice. Thus, Ag-specific Ab responses were seen in plasma and mucosal secretions, and Ag-specific AFCs were detected in NPs, SMG, and the spleen, clearly showing that both mucosal and systemic immunity occurred in 1-year-old mice following nasal immunization. Furthermore, elevated levels of $CD4^+$ T cell proliferative and OVA-induced Th2-type cytokine responses were seen in CLN and spleen of 1-year-old mice given nasal OVA plus nCT or mCT-A E112K/LT-B chimera. In 2-year-old mice, Ag-specific plasma Ab responses were seen; however, mucosal S-IgA Ab responses were not induced when nCT was used as nasal adjuvant. When the frequencies of naive $CD4^+$ T cells in NALT, Peyer's patches, and spleen were compared in young adult and 1-year-old mice, reduced frequencies of $CD4^+$, $CD45RB^+$ T cells were seen in aged mice. Importantly, the actual cell counts of naive $CD4^+$ T cells in NALT of 1-year-old mice were higher than those seen in young adult mice. Finally, 1-year-old mice given a nasal TT vaccine and chimeric mCT-A E112K/LT-B as adjuvant were protected from systemic challenge with tetanus toxin.

It has been suggested that the mucosal immune system is compartmentalized and differs remarkably from the systemic one (34). In this regard, either nasal or oral immunization induces Ag-specific immune responses at mucosal surfaces as well as in systemic lymphoid tissues via the common mucosal immune system. In contrast, parenteral immunization does not effectively elicit mucosal immunity (34). It is evident that different types of mucosal immune responses occur when Ag is given either by the oral or nasal routes. Oral immunization effectively induces Ag-specific S-IgA Ab responses in the GI tract and saliva, but is less effective for S-IgA responses in the respiratory and reproductive tracts (31, 35, 36). In contrast, previous studies clearly showed that a nasal immunization regimen elicited significant immunity not only in the respiratory tract, but also in saliva, as well as in the GI and reproductive tracts (24, 25). These results suggest that distinct immune regulatory mechanisms in GALT vs NALT account for the induction of mucosal S-IgA and parenteral IgG Ab responses. Indeed, our current findings in 1-year-old mice given nasal vaccine revealed significant Ag-specific immune responses that provided effective protection, whereas our previous study had clearly shown that oral immunization with OVA plus nCT failed to elicit either OVA- or CT-B-specific Ab responses (28). Taken together, these two separate studies suggest that the regulation of the NALT-mediated mucosal immune system is distinct from the GALT-directed system. Furthermore, the nasal route may be the preferred method for administering vaccines to induce both Ag-specific mucosal and systemic immune responses in the elderly.

Additional evidence for differences between NALT and GALT has been provided by the finding that organogenesis of NALT occurs independently of the lymphotoxin β (LTβ) and LTβ receptor signaling pathways (37, 38). Thus, LT signaling pathways are essential for the genesis of Peyer's patches or GALT as well as for lymph nodes (LN) and spleen (39). For example, both LTα and LTβ gene knockout mice lack Peyer's patches and LNs (40–44). Furthermore, administration of LTβR-Ig during gestation disrupted the development of LNs and Peyer's patches (45, 46). It was also shown that IL-7 and the IL-7R pathway are essential for the development of GALT (47–50); however, an intact NALT system was present, but was reduced in size in IL-7Rα knockout mice (37, 38). Furthermore, organogenesis of NALT was shown to begin after birth, while GALT development began during gestation (38). These studies clearly point to significant differences in the development of the two major mucosal inductive tissues, e.g., GALT and NALT. Because of these differences, one would predict that age-associated alterations may independently occur in these mucosal inductive tissues. Our findings that nasal, but not oral immunization induced Ag-specific immune responses in 1-year-old mice strongly support the notion that the aging develops more slowly in NALT than GALT.

It has been shown that increased numbers of memory-type cells are associated with aging (51–54). Thus, aged mice showed decreased numbers of naive $CD4^+$ T cells that express CD45RB molecules. In this regard, overexpression of the Fas gene under the CD2 promoter resulted in reduced numbers of memory-type T cells in aged mice, and rejuvenated immune responses were seen in aged Fas-CD2 transgenic mice that resembled those of young adult mice (55). Furthermore, it was suggested that effector T cell production from naive T cells is also impaired in aged mice (56, 57). IL-2, but not other common γ-chain cytokine receptor-related ILs, e.g., IL-4, IL-7, or IL-15, effectively restored development of effector cells from naive precursors in aged mice (57). In this regard, it was important to investigate the frequencies of naive T cell populations in various mucosal tissues of aged mice. Our findings clearly show that the actual numbers of $CD4^+$, $CD45RB^+$ T cells in the NALT of aged mice were essentially the same as those of young adult mice. Thus, although the frequency of $CD4^+$, $CD45RB^+$ T cells was reduced, total numbers of lymphocytes in NALT were twice as high in 1-year-old mice as in young adult mice. In contrast, the total numbers of $CD4^+$, $CD45RB^+$ T cells were reduced, and $CD4^+$, $CD44^+$ T cell populations were increased in the spleen of aged mice. Furthermore, Peyer's patches of aged mice showed significant reductions in $CD4^+$, $CD45RB^+$ T cell frequencies in addition to total cell numbers when compared with young adult mice. These results suggest that the naive T cell population in NALT plays a pivotal role in the induction of both systemic and mucosal immune responses in 1-year-old mice.

Our results also suggest that naive T cell functions in NALT are maintained and these T cells are capable of developing into effector T cells. Further support for the presence of intact NALT T cell function in senescent mice, our results showed that high levels of Ag-specific $CD4^+$ T cell proliferative responses occur in CLN and spleen of 1-year-old mice given OVA and nCT. Furthermore, these $CD4^+$ T cells were capable of producing Th2-type cytokines when T cells were restimulated with OVA in vitro. Interestingly, when 2-year-old mice were given nasal OVA plus nCT, OVA-specific $CD4^+$ T cell immune responses were also maintained in the CLN

and splenic compartments. These results further confirm our prediction that naive $CD4^+$ T cells in the NALT of 1-year-old mice become effector $CD4^+$ T cells and migrate into both systemic and mucosal lymphoid compartments.

It is often suggested that experimental mice should be at least 2 years old to be suitable models for vaccine evaluation in elderly humans. In this regard, 2-year-old mice were immunized nasally with OVA and nCT as adjuvant. In terms of Ag-specific Ab responses, mice given nCT failed to undergo mucosal S-IgA Ab responses; however, and interestingly, mice that received nCT as mucosal adjuvant showed Ag-specific, systemic immune responses that were essentially identical with the responses seen in young adult mice. Similarly, $CD4^+$ T cell proliferative as well as Th1 and Th2 cytokine responses in the spleen of 2-year-old mice were comparable to those of young adult mice when nCT was used as nasal adjuvant. These results further confirm our previous findings that mucosal immunosenescence takes place before systemic immune dysregulation (15), even though nasal, but not oral immunization is still effective in 2-year-old mice. To overcome this mucosal immunodeficiency, it may be important to consider inducing innate and cellular immunity in mucosal compartments of 2-year-old mice to provide appropriate mucosal immunity. Although it has been suggested that CTL activity by $CD8^+$ T and NK cells in aged mice was altered (58, 59), nasal immunization using nCT as mucosal adjuvant may induce sufficient CTL responses in 2-year-old mice. Supporting this notion, our findings showed that intact Ag-specific T cell immune responses occur in 2-year-old mice given nasal OVA and nCT. Furthermore, nCT is known to elicit CTL activity when coadministered with viral protein Ag given by the nasal route (60). We are currently investigating Ag-specific CTL responses in NALT of 2-year-old mice given nCT as mucosal adjuvant.

Although mCT-A E112K/LT-B failed to elicit Ag-specific T and B cell and Ab responses in 2-year-old mice when given with OVA, this mucosal adjuvant successfully induced Ag-specific immune responses in 1-year-old mice. Importantly, these immune responses provided immune protection. Thus, 1-year-old mice given TT and mutant chimera toxin showed significant survival rates (>80%) when s.c. challenged with tetanus toxin. Based upon these results, mCT-A E112K/LT-B shows promise as a practical mucosal adjuvant to accompany vaccines for the elderly. Early vaccination in the elderly would be expected to be more effective, and vaccination in a middle-aged population may provide sufficient immunity later in life.

An additional reason for use of mCT-A E112K/LT-B chimera in a mucosal vaccine is its safety. Our separate studies showed that mCT-A E112K/LT-B chimera was essentially nontoxic when compared with nCT (29). In this regard, reduced levels of Ag-specific IgE Ab responses were noted in young adult mice given nasal TT and mCT-A E112K/LT-B chimera. Furthermore, this chimera toxin showed essentially no Ag redirection into CNS tissues in contrast to mice given nCT (29). Because safety is an important issue for nasal vaccines, the features of mCT-A E112K/LT-B as a nasal adjuvant offer significant advantages in the development of vaccines for the elderly.

Our current study has clearly shown the effectiveness of nasal immunization in senescent mice that is mediated through a naive $CD4^+$ T cell subset in NALT. Nasal immunization with protein Ag plus nCT or mCT-A E112K/LT-B as mucosal adjuvants was shown to induce protective Ag-specific Ab responses in 1-year-old mice. In contrast, our previous study showed that impaired Ag-specific Ab responses occur in 1-year-old mice given OVA plus nCT by the oral route (15). Taken together, these studies clearly show that the route of immunization is a critical factor for effective induction of protective mucosal immunity in the elderly. In addition, the age of initial mucosal immunization is another important factor, because our study showed that nCT failed to induce mucosal Ab responses in 2-year-old mice, but not in 1-year-old mice. Because the efficacy and safety of mucosal adjuvants are essential elements in mucosal vaccine development, it will be necessary to continue optimizing mucosal adjuvants that are suitable for use in the elderly.

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BASIC RESEARCH

Regulation of Mucosal Immune Responses by Recombinant Interleukin 10 Produced by Intestinal Epithelial Cells in Mice

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Background & Aims: Interleukin (IL)-10 is a cytokine with anti-inflammatory properties. The aim of this study was to explore the effect of a site-specific delivery of IL-10 on intestinal immune responses. *Methods:* Transgenic mice were created in which IL-10 is expressed by the intestinal epithelial cells. *Results:* Transgenic mice showed a marked increase in the number of intraepithelial lymphocytes in the small intestine. Mucosal lymphocytes of transgenic animals produced fewer T helper type 1 cytokines than wild-type lymphocytes. By contrast, the production of transforming growth factor β was increased. Moreover, the epithelial layer in transgenic mice was significantly enriched for $CD4^+CD25^+$ T cells. Furthermore, transgenic mice had increased numbers of immunoglobulin A–producing B cells in the small intestine. These effects were local because splenic lymphocytes were not affected. Studies in models of inflammatory bowel disease showed that transgenic IL-10 was able to attenuate the acute colitis induced by dextran sodium sulfate administration or by adoptive transfer of $CD4^+CD45RB^{high}$ splenocytes, with a modest effect on the chronic intestinal inflammation arising spontaneously in *IL-10*$^{-/-}$ mice. *Conclusions:* These observations provide evidence for an in vivo lymphoepithelial cross talk, by which cytokines locally produced by epithelial cells can regulate immune responses in the intestine without systemic modifications.

The gut-associated lymphoid system is well developed and consists mainly of intraepithelial (IEL) and lamina propria lymphocytes (LPL) in the mucosa, as well as scattered lymphoid organs (Peyer's patches). The only barrier between the antigen-rich luminal environment and the mucosal immune system is the single layer of columnar epithelial cells. Rather than forming a purely mechanical barrier, intestinal epithelial cells are capable of producing an array of cytokines, chemokines, and defensins.[1,2] By means of the lymphokines they produce, intestinal epithelial cells may be able to communicate with mucosal immune cells and thereby participate in the regulation of mucosal immune responses.[3] Although this hypothesis is compelling, the experimental evidence in support of it has been derived mainly from in vitro studies of intestinal epithelial cell lines. Therefore, as a model system to study epithelial–lymphocyte communication in vivo, we generated transgenic mice expressing interleukin (IL)-10 in the intestinal epithelium, by using the tissue-specific intestinal fatty acid binding protein (*Fabpi*) promoter.[4]

IL-10 is a homodimeric cytokine that has important anti-inflammatory and immunosuppressive properties. It is produced by a variety of cells, including activated T lymphocytes (primarily T helper type 2 cells), a subset of B lymphocytes, monocytes, and keratinocytes.[5–10] There is ample in vivo evidence for the important anti-inflammatory role of IL-10 in animal models of inflammatory bowel disease (IBD), the earliest being the observation that *IL-10*$^{-/-}$ mice spontaneously develop chronic colitis.[11,12] Systemic IL-10 treatment has been used in several animal models of colitis, such as the spontaneous colitis in *IL-10*$^{-/-}$ mice[12] and the induced colitis in the $CD4^+CD45RB^{high}$ T-cell transfer model,[13–15] with some amelioration of disease having been obtained from IL-10 treatment. Our studies showed that $CD4^+CD45RB^{high}$ T cells from transgenic mice with activation-induced IL-10

Abbreviations used in this paper: CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; Fabpi, intestinal fatty acid binding protein; IFN, interferon; IL, interleukin; PE, phycoerythrin; RT-PCR, reverse-transcription polymerase chain reaction; sIEL, small intestine intraepithelial lymphocyte; TCR, T-cell receptor; TGF, transforming growth factor.

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production were not able to induce colitis, and they even prevented colitis when cotransferred with pathogenic wild-type T cells.[16] Furthermore, IL-10 is necessary for the inhibition of colitis obtained by cotransfer of $CD4^+CD45RB^{low}$ cells from wild-type mice with the pathogenic $CD4^+CD45RB^{high}$ T-lymphocyte population.[13,14] It is not clear how, exactly, IL-10 exerts its action: it could prevent or reduce inflammation in the intestine by affecting a variety of cell types, including antigen-presenting cells, T cells, or epithelial cells. Moreover, IL-10 has been reported to influence intestinal epithelial cell barrier function in vitro.[17,18] Despite these promising results, the effectiveness of systemic administration of recombinant IL-10 as a treatment modality in animal models and in patients[19] is severely hampered by the short half-life of IL-10 and by possible systemic side effects.[20]

In the transgenic mouse model that we established, IL-10 is expressed constitutively by the intestinal epithelium. This model has allowed us to investigate the potential for epithelial–lymphocyte communication mediated by cytokines, by analyzing the effect of increased epithelial IL-10 production on mucosal lymphocytes. To delineate the means by which IL-10 might alter T-cell function, we also have studied the hypothesis that epithelial IL-10 production leads to the generation of increased numbers of T cells that produce transforming growth factor (TGF)-β1, IL-10, or both. Finally, we have analyzed the IL-10 transgene in the context of several IBD models, to determine the potential efficacy of the induction of local epithelial IL-10 production.

Materials and Methods

Generation of Fabpi/IL-10 Transgenic Mice

A fragment of the rat *Fabpi* promoter containing nucleotides −1178 to +28 (a gift of Jeffrey I. Gordon, Washington University, St. Louis, MO)[4] was cloned upstream of the mouse *IL-10* genomic sequence. This *IL-10* sequence, a gift of Kevin Moore (DNAX Research Institute, Palo Alto, CA), contains positions 1568–6879 of a 7.2-kilobase Bgl II fragment, in which position 1568 of the *IL-10* gene corresponds to nucleotide 16 of the *IL-10* complementary DNA (cDNA).[21] Transgenic mice were made by standard methods at the University of California–Los Angeles transgenic mouse core facility. The transgenic mice were generated on a mixed C57BL/6 and C3H background and were then backcrossed onto the C57BL/6 background for 5 generations. *IL-10* transgenic mice were crossed with $IL\text{-}10^{-/-}$ mice on a mixed 129SV × C57BL/6 background to generate animals that express IL-10 exclusively in the intestine. *IL-10* transgenic mice were also crossed with $RAG2^{-/-}$ mice on a mixed 129SV × C57BL/6 background to generate transgenic recipients for adoptive transfer of $CD4^+CD45RB^{high}$ splenocytes. All mice were housed in microisolator cages under specific pathogen–free conditions. Animal care and use were performed in accordance with La Jolla Institute for Allergy and Immunology and National Institutes of Health guidelines.

Flow Cytometry

After blocking with anti-CD16/32 (2.4G2), cells were stained with 100 μL of properly diluted antibody. The antibodies were either directly labeled with fluorescein isothiocyanate, phycoerythrin (PE), allophycocyanin (APC), or CyChrome or they were biotinylated. For the latter, Streptavidin Tri-Color conjugate (Caltag, Palo Alto, CA) was used as a secondary reagent for detection. For detection of intracellular cytokines, cells were stimulated for 5–18 hours with plate-bound anti-CD3. All stimulation cultures contained 5 μg/mL of Brefeldin A (Calbiochem, San Diego, CA) to block protein transport into post-Golgi compartments. Cells were fixed and permeabilized after surface staining by using Cytofix/Cytoperm Plus (BD PharMingen, San Diego, CA) and stained with fluorescein isothiocyanate– or PE-labeled anti-cytokine antibodies according to the manufacturer's protocol. For analysis of intracellular cytotoxic T-lymphocyte–associated antigen (CTLA)-4, cells were fixed and permeabilized after surface staining by using Cytofix/Cytoperm Plus and stained with CTLA-4–specific antibody or isotype. All antibodies were purchased from BD PharMingen. Cells were acquired and analyzed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with Cell Quest software (Becton Dickinson).

Reverse-Transcription Polymerase Chain Reaction and Southern Blot

Spleen, heart, liver, and kidney were homogenized by using a TissueTearor. Suspensions enriched for epithelial cells from small and large intestine or from different regions of the small intestine were obtained by incubating the tissues in a 1 mmol/L dithiothreitol solution (Sigma Chemical Co., St. Louis, MO), shaking them at 200 rpm for 3 × 20 minutes, and passing them through a nylon mesh. Total cellular RNA was prepared by using TRIzol reagent (GIBCO BRL, St. Louis, MO). Five micrograms of total RNA was reverse transcribed by using *Not* I-$(dT)_{18}$ primer (Amersham Pharmacia Biotech, Piscataway, NJ) and AMV Reverse Transcriptase (Roche, Indianapolis, IN). The resulting cDNA was analyzed for the presence of IL-10 by PCR amplification of 2-μL cDNA aliquots in 50 μL of master mix consisting of nucleotides, buffer, 2.5 U of AmpliTaq Gold polymerase (Perkin Elmer, Shelton, CT), and primers. PCR amplification of the housekeeping gene β-actin was performed simultaneously. The sequence of amplification involved an initial preincubation at 95°C for 10 minutes to fully activate the AmpliTaq Gold, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes in a

Perkin-Elmer GeneAmp PCR System. Primer sequences used were the following: IL-10, sense 5′-TAT GCT GCC TGC TCT TAC TGA-3′, antisense 5′-GGT CTT CAG CTT CTC ACC CAG-3′; and β-actin, sense 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, antisense 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. The RT-PCR products were visualized by ethidium bromide staining of a 2% agarose gel and subsequently blotted and hybridized with an IL-10 cDNA probe labeled by using the Genius nonradioactive detection kit, which uses random-primed probes labeled with digoxigenin-deoxyuridine triphosphate (Boehringer Mannheim, Indianapolis, IN).

Immunohistochemistry

Animals were killed and perfused with periodate-lysine-paraformaldehyde as published.[22] Tissues were fixed in periodate-lysine-paraformaldehyde. Immunohistochemical detection of IL-10 was performed as described previously.[23] Briefly, sections were incubated with rat anti-mouse IL-10 monoclonal antibody (clone JES5-A25, dilution 1/300; Serotec, Raleigh, NC) overnight at 4°C. Slides were then incubated with biotinylated rabbit anti-rat immunoglobulins (Igs), followed by avidin-biotin–horseradish peroxidase complex and Vector VIP reagent, according to the Vectastain protocol (Vector Laboratories, Burlingame, CA). Slides were counterstained with 2% methyl green, dehydrated, and mounted. Appropriate positive and negative staining controls were included in each staining run.

For CD3 staining, sections were incubated for 30 minutes with a 1:100 dilution of rabbit anti-human CD3 (Dako Diagnostics, Carpinteria, CA), which recognizes the intracytoplasmic domain of the CD3ϵ chain and cross-reacts with murine CD3. The secondary antibody was biotin-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA), used at a 1:200 dilution. The sections were then incubated with avidin-biotin–horseradish peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride (Biogenex Laboratories, San Ramon, CA). Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Isolation of Lymphocytes

Splenocytes were isolated by conventional methods. Red blood cells were removed from spleen cell suspensions by using a standard Lympholyte gradient (Cedarlane, Ontario, Canada). Liver mononuclear cells were prepared as described previously.[24] Intestinal mucosal lymphocytes were isolated according to a previously published method.[25]

Enzyme-Linked Immunosorbent Assay

Lymphocytes were stimulated for 48 hours on anti-CD3–coated plates in complete Dulbecco's modified Eagle medium (GIBCO, Rockville, MD) containing 2.5% fetal calf serum. TGF-β1 levels in cell culture supernatants were detected after acid treatment by using a standard sandwich enzyme-linked immunosorbent assay (ELISA) with a coated capture antibody and a biotinylated detection antibody, according to the manufacturer's protocol (BD PharMingen). TGF-β levels in unstimulated control wells, after acidification, were less than 100 pg/mL.

Enzyme-Linked Immunospot Assay

Millititer HA Nitrocellulose plates (Millipore) were coated with anti-IgA, anti-IgG, or anti-IgM antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Plates were blocked with 10% fetal calf serum, and titrated concentrations of splenocytes or LPL were added for 4 hours at 37°C. After extensive washing, the wells were incubated with biotin-conjugated anti-IgA, anti-IgM, or anti-IgG (Southern Biotechnology Associates), followed by incubation with avidin-peroxidase (Vector Laboratories). Spots were developed by the addition of 400 μg/mL of 3-amino-9-ethylcarbazole substrate (Sigma) and enumerated by a computerized image analysis system (Lightools Research) by using the image analyzer program NIH Image 1.61 (National Institutes of Health).

Induction of Colitis

Colitis was induced by the administration of dextran sodium sulfate (DSS; 2.5% wt/vol; molecular weight, 40,000; ICN Biomedicals) in the drinking water for 5 days. DSS water consumption and weight were recorded daily. Animals were killed 8 days after termination of the DSS treatment, and H&E-stained tissue sections of the cecum and the proximal and distal colon were scored according to a previously published scoring system.[26] In another set of experiments, colitis was induced by transfer of 4×10^5 $CD4^+CD45RB^{high}$ splenocytes to *RAG2*$^{-/-}$ mice or *Fabpi/IL-10* transgenic *RAG2*$^{-/-}$ mice, as described previously.[25] Animals were killed 8 weeks after the transfer or earlier if they lost more than 20% of their starting weight. H&E-stained tissue sections from the proximal, middle, and distal colon were assessed in a blinded fashion according to the scoring system described previously.[25]

Statistical Analysis

Normally distributed continuous variable comparisons were performed with the Student *t* test, unless indicated differently.

Results

Intestine-Specific Expression of IL-10 in Transgenic Animals

The promoter fragment used to direct IL-10 expression has been extensively characterized previously by analyzing reporter gene expression.[4] According to these studies, transgene-derived protein should be found exclusively in mature enterocytes but not crypt epithelial cells, with the highest levels of expression in the distal small intestine and lower levels in the remainder of the small intestine and the proximal colon. RT-PCR analysis

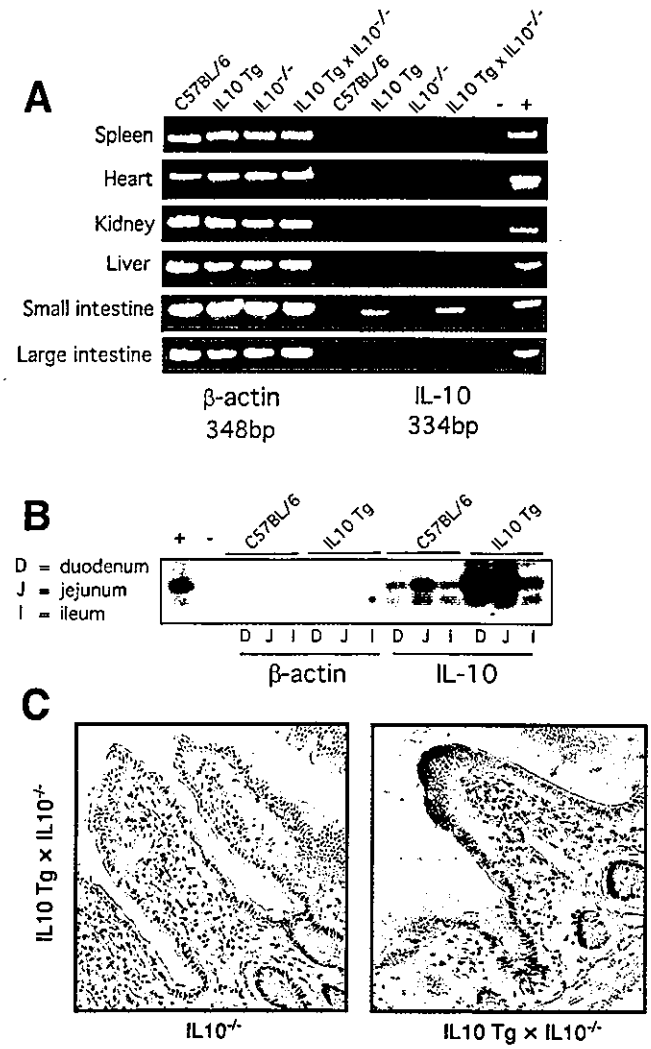

Figure 1. Pattern of *IL-10* messenger RNA expression in *Fabpi/IL-10* transgenic mice. (*A*) RT-PCR detection of *IL-10* and *β-actin* messenger RNA in the indicated mice. Total RNA was prepared and reverse transcribed from the tissues indicated. A plasmid containing purified *IL-10 cDNA* served as a positive control, and a reaction mix without cDNA served as a negative control (indicated by + and −, respectively). Representative data for 1 of 3 independent experiments are shown. Tg, transgenic. (*B*) Southern blot analysis of IL-10 expression in different parts of the small intestine. Total RNA was prepared and reverse transcribed from the different regions of the small intestine indicated. RT-PCR for *β-actin* and *IL-10* was performed, and PCR products were electrophoresed on an agarose gel. The gel was stained with ethidium bromide to detect β-actin message to confirm equal loading (data not shown) and was then hybridized with an *IL-10*-specific cDNA probe. (*C*) In situ detection of IL-10 protein by immunohistochemistry. Jejunal sections of the indicated mouse strains were stained with an anti–IL-10 antibody followed by biotinylated rabbit anti-rat Ig and horseradish peroxidase complex as described in Materials and Methods. The staining was developed with VIP reagent, and tissues were counterstained with methyl green (original magnification: 200×).

was performed on samples from various tissues of *Fabpi/IL-10* transgenic mice, on either the wild-type or the *IL-10*$^{-/-}$ background. Controls included C57BL/6 and *IL-10*$^{-/-}$ mice. On ethidium bromide–stained gels, *IL-10* messenger RNA was detected only in epithelial cell suspensions from the small intestine of *Fabpi/IL-10* transgenic mice (Figure 1*A*). Low levels of *IL-10* messenger RNA could be detected in the small intestine of wild-type mice in some PCR reactions or when the gels were blotted and hybridized with an *IL-10*–specific probe (Figure 1*B*). The data from Southern blot hybridization indicate higher levels of *IL-10* expression in the intestine of transgenic mice compared with wild-type

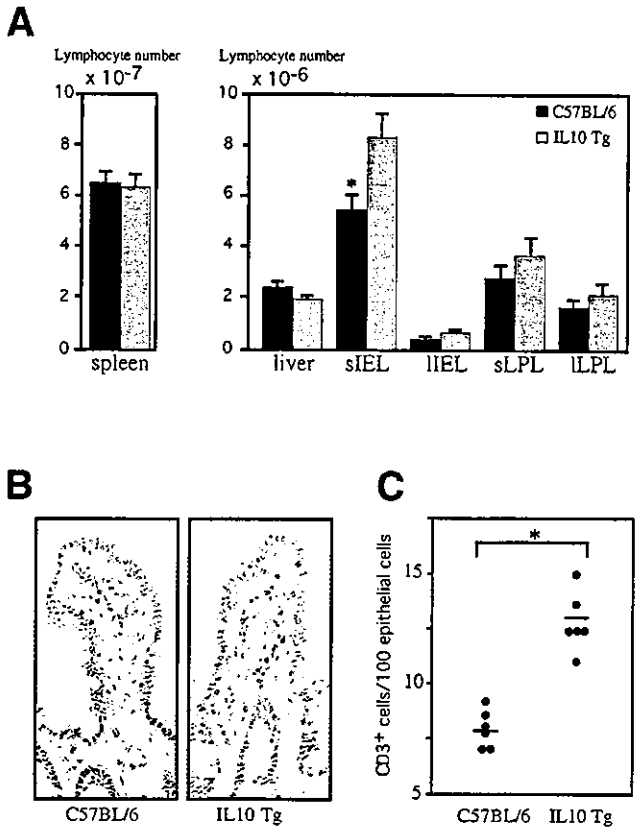

Figure 2. Increased mucosal T-cell numbers in *IL-10* transgenic mice. (*A*) Mononuclear cells were isolated from the organs indicated. Results are represented as the mean number of viable cells in the lymphocyte gate as assessed by analysis of forward and side angle light scatter ± SEM. Each number represents the mean of 16–24 animals per group, except for the liver, in which 4 mice in each group were tested. *$P < 0.05$, as determined by the Student two-tailed *t* test; no other differences were statistically significant. sLPL, small intestine lamina propria lymphocyte; lIEL, large intestine intraepithelial lymphocyte; lLPL, large intestine lamina propria lymphocyte. (*B*) In situ detection of CD3$^+$ cells in the small intestine of C57BL/6 and transgenic mice by immunohistochemistry. Paraffin-embedded sections were prepared and stained as described (original magnification: 200×). (*C*) In situ quantification of CD3$^+$ IEL in the small intestine. Sections from small bowel were stained for CD3 expression as shown in panel *B*. The number of positive-staining cells per 100 epithelial cells was determined in 5 different objective fields. Each number represents the mean score for an individual section. For each group, sections from 2 different sites of the small intestine of 3 different mice were analyzed. *$P < 0.005$ as determined by the Student 2-tailed *t* test.

mice. Comparing different regions of the intestine in *Fabpi/IL-10* transgenic mice, there was decreased expression in the ileum relative to the duodenum and jejunum (Figure 1*B*). On increasing the number of amplification cycles to 45, message for *IL-10* could also be detected in the cecum of transgenic animals, but it could not be consistently detected in the colon (data not shown). Western blot analysis also showed expression of *IL-10* protein in the intestine of *IL-10* transgenic mice, but not in *IL-10*$^{-/-}$ mice (data not shown). Immunohistochemical staining of the jejunum of transgenic mice on the *IL-10*$^{-/-}$ background confirmed transgene-derived IL-10 expression in the intestine and permitted identification of the positive cells. As shown in Figure 1*C*, mature enterocytes along the villi express IL-10, but the crypts do not. In agreement with our previous article,[23] in the intestine of wild-type mice, only scattered cells, with the morphology of lymphocytes, express IL-10 in the epithelium (data not shown). The expression of transgene-derived IL-10 had no effect on gross gut and Peyer's patch morphology, and IL-10 could not be detected in the serum of transgenic mice by ELISA (data not shown).

Fabpi/IL-10 Transgenic Mice Have Increased Numbers of Lymphocytes in the Small Intestine

To determine whether constitutive expression of IL-10 by mature epithelial cells in the small intestine leads to alterations in lymphocyte homeostasis, analyses of the number and phenotype of different lymphocyte populations were performed. Cell suspensions were isolated from the small and large intestine, spleen, and liver, and the percentage of lymphocytes was measured by flow cytometry. The total number of lymphocytes was obtained from the total count of viable cells, as determined by trypan blue exclusion. *Fabpi/IL-10* transgenic mice had approximately 1.5 times more small-intestine IEL (sIEL) than control mice (Figure 2*A*), whereas there were no significant changes in the numbers of lymphocytes in other sites. We confirmed these findings by in situ immunohistochemical detection of CD3$^+$ lymphocytes in paraffin sections, which also showed an approximately 1.5-fold increase in the number of CD3$^+$ cells in the presence of the *IL-10* transgene (Figure 2*B* and *C*).

Analysis of the phenotype of sIEL by flow cytometry showed that the proportions of T-cell receptor (TCR)αβ (38.9% ± 2.2% of total IEL in wild-type mice; 44.1% ± 1.4% in *IL-10* transgenic mice) and TCRγδ lymphocytes (37.0% ± 4.6% of total IEL in wild-type; 30.9% ± 2.3% in *IL-10* transgenic mice) were maintained. However, in the TCRαβ sIEL of *IL-10* transgenic animals, there was a significant increase in CD4$^+$ T cells (22.8% ± 1.5% of TCRαβ lymphocytes in wild-type mice, compared with 31.5% ± 1.7% of TCRαβ lymphocytes in *IL-10* transgenic mice; $P < 0.005$) and a concomitant, reciprocal decrease in the percentage of TCRαβ CD8 cells. Mucosal lymphocytes from the small intestine of *Fabpi/IL-10* transgenic mice generally showed the same cell-surface phenotype as do IEL and LPL from control, wild-type mice. In both types of mice, IEL and LPL typically have an activated phenotype characterized by the expression of CD69, high levels of CD44, and low levels of CD45RB (data not shown). It is interesting to note that there is an increased population of CD4$^+$CD25$^+$ T cells in the small intestine of transgenic mice—a phenotype characteristic of regulatory T cells. As shown in Figure 3*A*, the average percentage of CD25$^+$ cells among the CD4$^+$ IEL population is increased from 5.2% in wild-type mice to 8.1% in IL-10 transgenic mice. Because the total number of CD4$^+$ sIEL in these mice is already increased, the difference in the

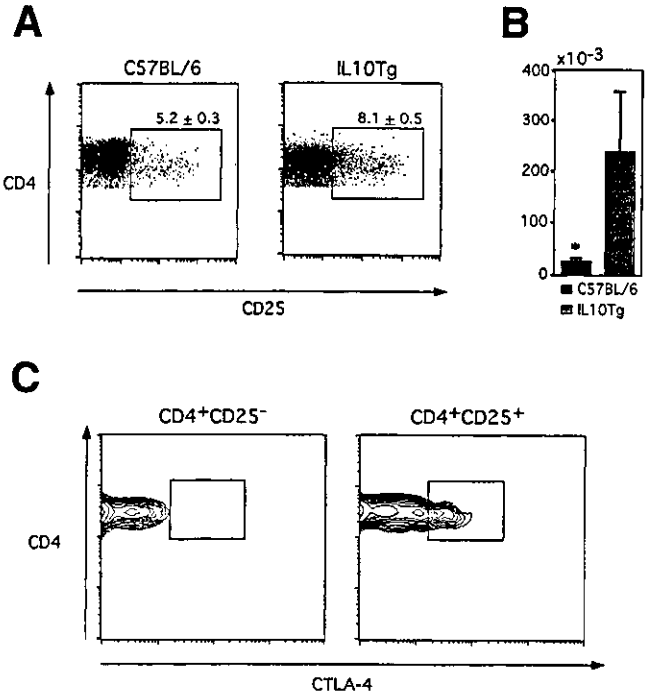

Figure 3. (*A*) Flow cytometric analysis of CD25 expression on TCR-β$^+$CD4$^+$ IELs isolated from the small intestine. A representative example is shown; numbers represent mean ± SEM of 5 mice in each group. $P < 0.05$. (*B*) Absolute numbers of CD4$^+$CD25$^+$ sIEL, obtained by multiplying the percentage of CD4$^+$CD25$^+$ cells by the total number of viable cells. Numbers represent the mean ± SEM of 5 mice in each group. $P < 0.05$. (*C*) Staining for intracellular CTLA-4 on CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ sIEL isolated from a *Fabpi/IL-10* transgenic mouse. For both wild-type and transgenic mice, only CD25$^+$ T cells were positive for intracellular CTLA-4. Five wild-type and transgenic mice were analyzed in each group, with comparable results.

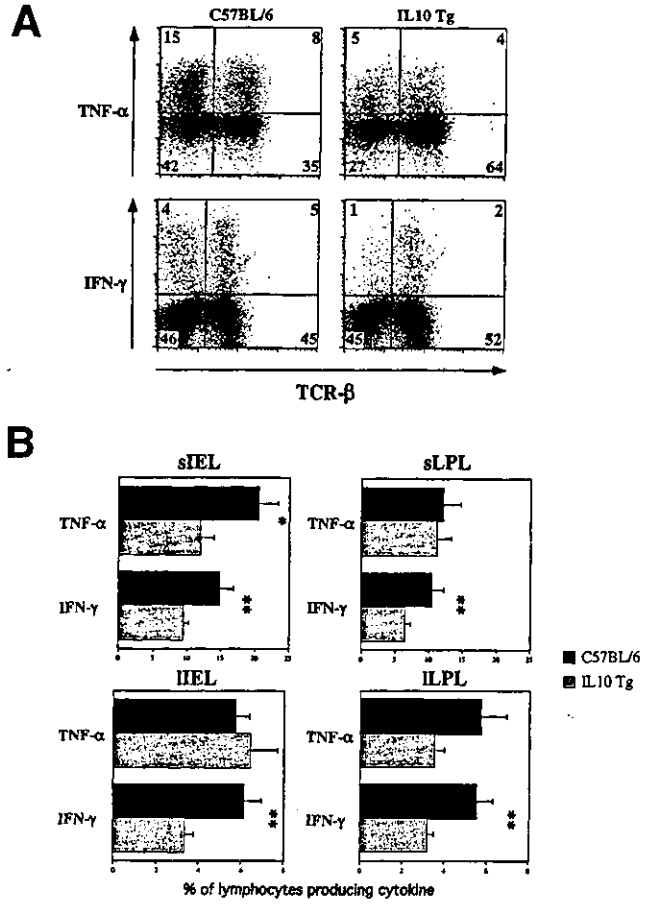

Figure 4. Reduced production of proinflammatory cytokines by mucosal lymphocytes from *IL-10* transgenic mice. (*A*) Intracellular staining for IFN-γ and TNF-α on sIEL isolated from transgenic and nontransgenic mice. TCR-β vs. intracellular cytokine staining was conducted as described. One representative example is shown. (*B*) Mean cytokine production by sIEL, sLPL, iIEL, and iLPL. The mean percentage (±SEM) of lymphocytes producing IFN-γ and TNF-α, as determined by intracellular cytokine staining, is depicted. The number of mice tested was 14 for the sLPL and 16 for the sIEL. $*P < 0.005$; $**P < 0.05$ (Student paired two-tailed *t* test).

absolute number of $CD4^+CD25^+$ T cells is even more pronounced (Figure 3*B*). It is also interesting that the $CD4^+CD25^+$ lymphocytes among the sIEL constitutively express CTLA-4 (Figure 3*C*), which has been reported to be essential for the in vivo suppressive activity of these regulatory cells,[27,28] whereas the $CD4^+CD25^-$ cells do not.

Mucosal Lymphocytes From *Fabpi/IL-10* Transgenic Mice Produce Lower Levels of T Helper Type 1 Cytokines But More TGF-β1

To determine whether the transgene-derived IL-10 induced functional changes in lymphocytes, we analyzed the cytokine profile of activated cells by intracellular cytokine staining and ELISA. Assays were performed after stimulation of cells with plate-bound anti-CD3 antibody. The results from intracellular cytokine stainings are shown in Figure 4*A*. There was a consistent decrease in the percentage of cells producing the T helper type 1 cytokines interferon (IFN)-γ (in both the small and large intestine) and TNF-α (small intestine only) when mucosal lymphocytes from transgenic mice were compared with wild-type lymphocytes. As shown in Figure 4*A*, these decreases could be observed among both the TCR-β^+ and TCR-β^- T cells after CD3 stimulation. The latter population presumably is composed mostly of TCRγδ T cells. Overall, these changes were more pronounced in IEL compared with LPL (Figure 4*B*); no decrease was observed in the spleen or mesenteric lymph nodes of IL-10 transgenic mice (data not shown).

By contrast, the production of the anti-inflammatory cytokine TGF-β1, as measured by ELISA (intracellular staining for this cytokine is not technically possible), was increased both in sIEL and small-intestine LPL of transgenic mice (Figure 5). This effect was local, because there

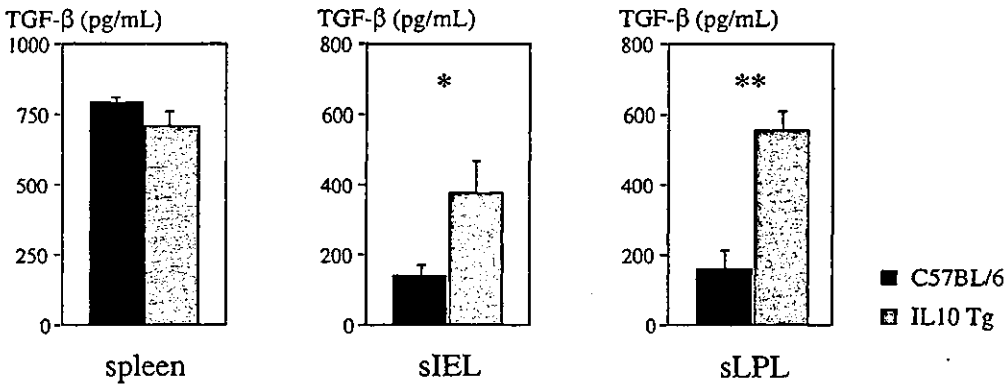

Figure 5. Increased TGF-β1 production by mucosal lymphocytes from transgenic mice upon CD3 cross-linking. Splenocytes, sIEL, and sLPL were stimulated for 48 hours, after which total TGF-β1 levels in the supernatants were measured by ELISA. Data shown are representative of 1 of 6 independent experiments. Cytokine levels are expressed as mean ± SEM of culture triplicates. $*P < 0.05$; $**P < 0.005$.

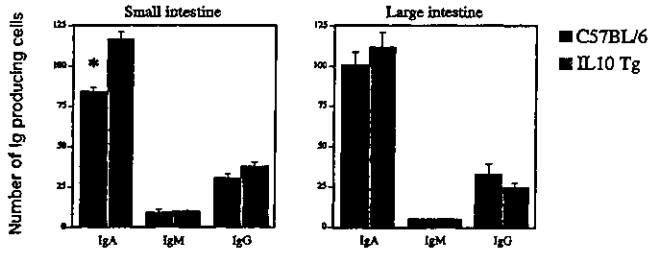

Figure 6. Increased numbers of IgA-secreting cells in the lamina propria of *IL-10* transgenic mice. Ig production was determined by enzyme-linked immunospot in LPL from the small and large intestine. Data shown are the number of Ig spots per 1250 LPL (IgA) or 10^4 LPL (IgG and IgM). Numbers shown represent the mean number of spots ± SEM. From 3 to 9 individual transgenic and nontransgenic mice were analyzed in triplicate for each Ig. $*P < 0.005$.

was no change in the TGF-β1 levels produced by in vitro stimulated T cells from the spleen. Relatively few cells could be detected that produce IL-4 or IL-10, measured by both ELISA and intracellular cytokine staining, in stimulated T cells from either wild-type or transgenic mice (data not shown).

Fabpi/IL-10 Transgenic Mice Have Increased Mucosal IgA Responses

IL-10 has been described as a differentiation factor for B cells,[29] and TGF-β promotes the switching to IgA production.[30,31] We therefore investigated whether epithelial IL-10 has functional effects on B cells in the lamina propria. We initially quantified the total number of B cells among the LPL and found the number to be in the reference range (data not shown). To evaluate B-cell function, Ig isotype production by LPL of the large and small intestine, as well as the spleen, was analyzed by enzyme-linked immunospot. There were significantly more IgA-producing B cells in small intestinal LPL from *IL-10* transgenic mice (Figure 6). The number of *IgG* and IgMs producing LPL in the small intestine, however, was not affected by the local intestinal production of IL-10 in these transgenic mice (Figure 6). Additionally, there were no significant differences in Ig production between splenocytes from *IL-10* transgenic and wild-type mice (data not shown).

Effect of Transgene-Derived IL-10 on Intestinal Inflammation

To evaluate the effect of epithelial-derived IL-10 on the spontaneous chronic colitis that arises in *IL-10*$^{-/-}$ mice,[11] colons of 5- to 6-month-old transgenic mice that express IL-10 only in the intestinal epithelium (Fabpi/*IL-10* transgenic *IL-10*$^{-/-}$ mice) were histopathologically scored and compared with the colons of age-matched *IL-10*$^{-/-}$ mice. As shown in Table 1, histopathological scores for the proximal and distal colon were not significantly different. However, 5 of the 6 *IL-10*$^{-/-}$ mice analyzed had rectal prolapse, whereas only 2 of the 6 *Fabpi/IL-10* transgenic IL-10$^{-/-}$ mice did. Rectal prolapse often accompanies the colitis seen in *IL-10*$^{-/-}$ mice and provides a way to screen large numbers of individual animals over many weeks. Therefore, all *IL-10*$^{-/-}$ mice and *Fabpi/IL-10* transgenic *IL-10*$^{-/-}$ mice born in our colony were observed prospectively for the development of rectal prolapse. The chance for *IL-10*$^{-/-}$ mice to be prolapse free at 5 months of age was 50%; this chance was increased to 75% for *Fabpi/IL-10* transgenic *IL-10*$^{-/-}$ mice. Moreover, *Fabpi/IL-10* transgenic *IL-10*$^{-/-}$ mice also showed a delay in the onset of rectal prolapse when compared with the *IL-10*$^{-/-}$ mice (Figure 7*A*). Therefore, although the Fabpi/IL-10 transgene does not completely prevent the induction of colitis, the prolapse data suggest that the frequency and severity of disease may be reduced.

We also assessed the in vivo effects of IL-10 in 2 induced models of colitis, in which the disease tends to be more acute. The adoptive transfer of wild-type

Table 1. Histopathologic Score of IL-10$^{-/-}$ and IL-10 Transgenic IL-10$^{-/-}$ Mice

Variable	Total score (*0–14*)	Inflammatory cell infiltrate (*0–3*)	Mucin depletion (*0–2*)	Epithelial cell-reactive atypia (*0–3*)	Number of IEL in epithelial crypts (*0–3*)	Number of inflammatory foci (*0–3*)
IL-10$^{-/-}$ (n = 6)						
Proximal colon	5.8 ± 1.3	1.3 ± 0.3	1.1 ± 0.2	1.3 ± 0.4	0.7 ± 0.2	1.5 ± 0.3
Distal colon	9.2 ± 2.0	2.1 ± 0.5	1.5 ± 0.3	2.0 ± 0.5	1.3 ± 0.5	2.3 ± 0.4
Mean	7.5 ± 1.2	1.7 ± 0.3	1.3 ± 0.2	1.7 ± 0.3	1.0 ± 0.3	1.9 ± 0.3
Transgenic IL-10$^{-/-}$ (n = 6)						
Proximal colon	7.5 ± 1.4	2.1 ± 0.4	1.1 ± 0.2	1.8 ± 0.4	0.7 ± 0.3	2.1 ± 0.3
Distal colon	9.3 ± 1.0	2.2 ± 0.3	1.5 ± 0.2	2.5 ± 0.2	1.1 ± 0.1	1.9 ± 0.3
Mean	8.4 ± 0.9	2.1 ± 0.2	1.3 ± 0.2	2.1 ± 0.2	0.9 ± 0.2	2.0 ± 0.2

NOTE. Results are expressed as means ± SEM.

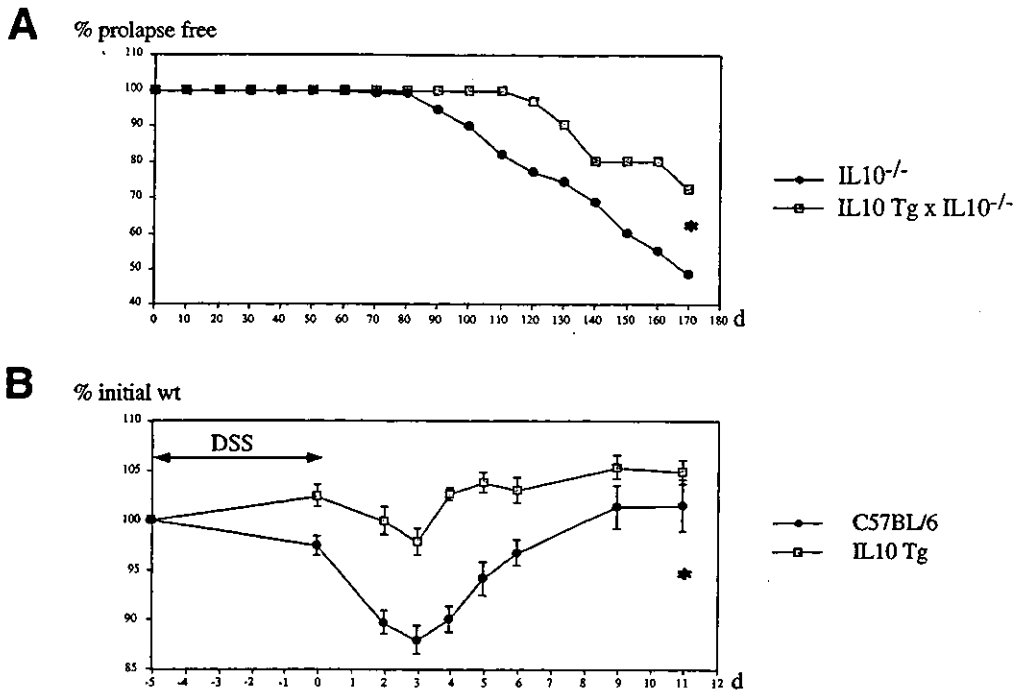

Figure 7. (*A*) Effect of intestinal epithelium-derived IL-10 on mouse models of colitis. Reduced rectal prolapse in *IL-10*$^{-/-}$ mice in the presence of the *Fabpi/IL-10* transgene. Sixty-two *IL-10* transgenic × *IL-10*$^{-/-}$ mice and 132 *IL-10*$^{-/-}$ mice were observed for the incidence of rectal prolapse from birth until the moment they were removed from the colony (mean follow-up, 105 days; range, 26–246 days). Data shown represent the chances of prolapse-free survival in terms of percentage obtained by Kaplan–Meier analysis. *$P < 0.05$ as determined by the log-rank test (Mantel-Cox-Savage). (*B*) Influence of the *IL-10* transgene on colitis induced by administration of DSS. Wild-type (n = 19) and transgenic (n = 18) mice were treated for 5 days with 2.5% DSS. Body weight was measured daily. Data shown represent the mean weight ± SEM of mice in each group. $P = 0.001$, as determined by the Student paired two-tailed *t* test.

$CD4^+CD45RB^{high}$ splenocytes to immunodeficient recipient mice induces intestinal inflammation (see reviews[32,33]). To study the effect of local intestinal expression of IL-10 in this adoptive transfer model, sorted $CD4^+CD45RB^{high}$ cells from immunocompetent donors were transferred into either *IL-10* transgenic or nontransgenic *RAG2*$^{-/-}$ recipient mice. The epithelial-produced IL-10 significantly reduced the severity of colitis, assessed by histological scoring, in all regions of the intestine in the *IL-10* transgenic *RAG2*$^{-/-}$ mice compared with *RAG2*$^{-/-}$ mice (Table 2). This may reflect the broad recirculation of a few pathogenic T-cell clones in these recipients. The overall difference in the histological scores, 10.7 for the controls compared with 7.0 for the

Table 2. Histopathologic Score of RAG2$^{-/-}$ and IL-10 Transgenic RAG2$^{-/-}$ Recipients After Transfer of $CD4CD45RB^{high}$ T Cells

Variable	Total score (*0–14*)	Inflammatory cell infiltrate (*0–3*)	Mucin depletion (*0–2*)	Epithelial cell-reactive atypia (*0–3*)	Number of IEL in epithelial crypts (*0–3*)	Number of inflammatory foci (*0–3*)
RAG2$^{-/-}$ (n = 6)						
Cecum	11.3 ± 1.0	2.7 ± 0.2	1.8 ± 0.2	2.7 ± 0.2	1.5 ± 0.3	2.7 ± 0.2
Proximal colon	9.6 ± 1.7	2.2 ± 0.5	1.7 ± 0.2	2.1 ± 0.5	1.3 ± 0.4	2.3 ± 0.5
Distal colon	11.2 ± 0.8	2.7 ± 0.2	2.0 ± 0.0	2.5 ± 0.3	1.3 ± 0.2	2.7 ± 0.2
Mean	10.7 ± 0.7	2.5 ± 0.2	1.8 ± 0.1	2.4 ± 0.2	1.4 ± 0.2	2.6 ± 0.2
Transgenic RAG2$^{-/-}$ (n = 6)						
Cecum	10.5 ± 0.6	2.2 ± 0.2	1.5 ± 0.2	2.1 ± 0.1[a]	1.0 ± 0.0	2.3 ± 0.3
Proximal colon	4.0 ± 1.8	0.8 ± 0.4	0.6 ± 0.3[a]	0.9 ± 0.4	0.5 ± 0.2	0.8 ± 0.4[a]
Distal colon	8.8 ± 2.1	2.2 ± 0.5	1.5 ± 0.3	1.9 ± 0.5	0.7 ± 0.2[a]	2.1 ± 0.5
Mean	7.0 ± 1.0[a]	1.7 ± 0.3[a]	1.2 ± 0.2[b]	1.6 ± 0.2[a]	0.7 ± 0.1[b]	1.8 ± 0.3[a]

NOTE. Results are expressed as means ± SEM.
[a]$P < 0.05$ vs. RAG2$^{-/-}$; [b]$P < 0.005$ vs. RAG2$^{-/-}$.

Table 3. Histopathologic Score of DSS-Treated Wild-Type and IL-10 Transgenic Mice

Variable	Total score	Inflammation severity (0–3)	Inflammation extent (0–3)	Crypt damage (0–4)	Percentage involvement (0–4)
Wild-type (n = 8)					
Cecum	11.2 ± 0.6	2.6 ± 0.2	2.4 ± 0.2	3.4 ± 0.3	2.8 ± 0.2
Proximal colon	7.7 ± 1.0	1.8 ± 0.2	1.9 ± 0.3	2.4 ± 1.6	1.7 ± 0.3
Distal colon	5.0 ± 1.5	1.3 ± 0.2	1.2 ± 0.2	1.6 ± 1.5	0.9 ± 0.1
Mean	8.0 ± 0.8	1.9 ± 0.2	1.8 ± 0.2	2.5 ± 0.3	1.8 ± 0.2
Transgenic (n = 6)					
Cecum	6.3 ± 1.1[a]	1.3 ± 0.2[b]	1.6 ± 0.3[a]	1.7 ± 0.6[a]	1.6 ± 0.2[b]
Proximal colon	6.2 ± 1.2	1.3 ± 0.2	1.8 ± 0.3	1.5 ± 0.6	1.5 ± 0.2
Distal colon	2.3 ± 0.8	0.6 ± 0.2	1.2 ± 0.4	0.0 ± 0.0[a]	0.6 ± 0.2
Mean	4.9 ± 0.7[a]	1.1 ± 0.1[b]	1.5 ± 0.2	1.1 ± 0.3[b]	1.2 ± 0.2

NOTE. Results are expressed as means ± SEM.
[a]$P < 0.05$ vs. wild-type; [b]$P < 0.005$ vs. wild-type.

IL-10 transgenic mice, was statistically significant. Acute colitis can also be induced by short-term administration of DSS in the drinking water.[34] Unlike the T-cell transfer model, which is induced by a defective regulation of T-cell responses, the DSS model reflects an inflammatory response resulting from epithelial damage. *Fabpi/IL-10* transgenic and wild-type mice were treated with DSS for 5 days, and their weight evolution during DSS administration and recovery was observed. The weight loss caused by DSS treatment was significantly reduced in the *Fabpi/IL-10* transgenic mice as compared with wild-type mice, and their recovery after termination of DSS treatment was markedly enhanced (Figure 7*B*). Consistent with this finding, the *Fabpi/IL-10* transgenic mice also showed significantly less histological damage to the intestine (Table 3). The average score for the cecum and proximal and distal colon was 8.0 ± 0.8 for wild-type mice (n = 8) and 4.9 ± 0.7 for *IL-10* transgenic mice (n = 6), the maximum possible score being 14 ($P < 0.05$). Differences were most pronounced in the cecum, with an average score of 11.2 ± 0.6 in wild-type mice compared with 6.3 ± 1.1 in IL-10 transgenic mice ($P < 0.005$).

Discussion

It is remarkable that large numbers of lymphocytes are located in the intestine, coexisting peaceably with the external environment that is only a single cell layer away. To achieve this, the immunologic tone of the intestinal tract has to be one of suppressed or highly regulated responses. The intestinal epithelial cell has been proposed as a key player in this process. It can take up, process, and present antigens to the mucosal immune system.[1,35,36] Furthermore, epithelial cells can produce a variety of cytokines and chemokines as a result of activation of the nuclear factor-κB pathway by invasive microorganisms or other stimuli.[2,37–41] These findings have given rise to the concept of cross talk or mutual regulation between epithelial cells and lymphocytes.[3,42] Although this concept is widely accepted in mucosal immunology, most of the evidence for it has been generated from observations on transformed epithelial cell lines in vitro. We used an in vivo approach to investigate whether epithelium-derived cytokines have an effect on the function of mature, mucosal lymphocytes. In the transgenic mice we have analyzed, epithelial cells of the small intestine constitutively produce IL-10. This led to a series of changes in mucosal lymphocyte populations.

When re-stimulated in vitro, mucosal lymphocytes from the small intestine of the *IL-10* transgenic mice produced lower amounts of the proinflammatory cytokines TNF-α and IFN-γ, concordant with the cytokine synthesis–inhibiting properties of IL-10.[5,43,44] T cells from the large intestine, but not from mesenteric lymph nodes or spleen, also displayed a decreased percentage of IFN-γ–secreting cells. Under the same conditions, IL-10 could not be detected by ELISA or by intracellular cytokine staining. Recently, it has been shown that IL-10 can drive the generation of a $CD4^+$ T-cell subset, designated Tr1 cells, which secretes predominantly IL-10 and therefore suppresses antigen-specific immune responses.[45] The fact that IL-10 could not be detected in our experiments suggests that the constitutive production of IL-10 by epithelial cells did not lead to the in vivo generation of Tr1 cells among IEL and LPL. It remains possible, however, that higher levels of IL-10 could induce these cells in vivo or that mucosal T cells are not highly efficient at secreting IL-10 compared with peripheral T lymphocytes. However, we observed an increase in the subset of $CD4^+CD25^+$ T cells in the small intestine

of the *IL-10* transgenic mice. T cells with this surface phenotype have been described to have immunoregulatory properties, both in autoimmune diseases and intestinal inflammation.[27,46–49] In some studies, the immunosuppressive function of these regulatory cells in vivo is dependent on the secretion of TGF-β1.[27] Recently, Nakamura et al.[50] elegantly showed that $CD4^+CD25^+$ T cells, when optimally stimulated in vitro, are capable of producing high amounts of both IL-10 and TGF-β1, whereas $CD4^+CD25^-$ T cells are not. Moreover, they found that $CD4^+CD25^+$ T cells not only secrete TGF-β1, but also express high and persistent levels of it on the cell surface. Consistent with a possible increase in these regulatory T cells in the transgenic mice, TGF-β1 production by mucosal lymphocytes from these *IL-10* transgenic animals was increased after in vitro stimulation. These data also indicate that TGF-β1 may be capable of acting downstream of IL-10 in the pathway, preventing chronic inflammation in the intestine, and corroborate earlier results obtained in vitro showing that the production of TGF-β1 in culture was increased in the presence of exogenous IL-10.[51] The importance of IL-10 for the production of TGF-β1 is further underlined by the finding that lymphocytes from *IL-10*$^{-/-}$ mice produce less TGF-β1 than those of control mice (data not shown; see also Seder et al.[51]). Another article suggested that the administration of a *TGF-β1*–encoding plasmid mediated the suppression of colitis by stimulating IL-10 production.[52] Therefore, a positive feedback loop may more accurately describe the relationship between these 2 anti-inflammatory cytokines.

Unexpectedly, transgenic animals also had increased numbers of lymphocytes in the intestinal epithelial layer. This is reminiscent of the findings in transgenic mice expressing IL-10 in the islets of Langerhans, where a pronounced leukocyte extravasation into the pancreatic tissue was observed, although this did not cause either inflammation of the islets of Langerhans themselves or diabetes.[53] In vivo BrdU labeling of intestinal lymphocytes showed comparable proliferation in both *Fabpi/IL-10* transgenic and nontransgenic animals, and combined annexin V and propidium iodide staining did not show significant differences in apoptosis (data not shown). This suggests an effect of epithelial IL-10 on the recruitment of T cells to the mucosa, perhaps by influencing chemokine or chemokine receptor expression or the expression pattern of adhesion molecules. However, the exact mechanism responsible for the increase in lymphocytes in the small intestine remains unexplained.

The influence of transgene-encoded IL-10 was concentrated on, but not limited to, the epithelial layer, because the lamina propria clearly was also affected. Specifically, LPL from the small intestine of the transgenic mice produced less IFN-γ and more TGF-β1 after stimulation. Both IEL and LPL from the large intestine also produced less IFN-γ. Additionally, the number of IgA-producing B cells in LPL was significantly increased in the IL-10 transgenic animals. This is consistent with previous reports that IL-10 is a maturation factor for B cells[29] and also with the finding that TGF-β1 promotes switching to IgA production.[30,31]

From a theoretical point of view, the anti-inflammatory cytokine IL-10 should offer a promising treatment modality for a variety of immunologic and inflammatory disorders, including IBD. Although our own and previously published data[23,54] suggest that there is relatively little constitutive production of IL-10 protein in vivo by any cell type in the intestine, including epithelial cells, IL-10 production could be upregulated in response to inflammation. The spontaneous colitis observed in *IL-10*$^{-/-}$ mice clearly shows an important physiological role for IL-10 in the prevention of disease at this site. So far, however, clinical trials with systemic administration of IL-10 have fallen short of expectations, because they are hampered by the short half-life of the cytokine and by the risk of systemic side effects.[19,20] Therefore, there has been increasing interest in administration routes to deliver IL-10 locally and continuously.[55–57]

Because most models of intestinal inflammation primarily involve the large intestine, it might be expected that transgene-derived IL-10 expressed predominantly by the epithelial cells of the small intestine would be less optimal. Despite this, we were able to observe some attenuation of disease in each of the 3 models, consistent with the finding that T cells in the large intestine of the IL-10 transgenic mice secrete less IFN-γ.

There are several plausible scenarios that could explain the positive effect of transgenic IL-10 produced mainly in the distal small intestine in the colitis models. First, it could reflect the fact that inflammation in these models originates close to the distal small intestine in the cecum, as has been described for the *IL-10*$^{-/-}$ mice.[12] Low levels of *IL-10* messenger RNA could be detected in the cecum by RT-PCR. Second, it is possible that because of peristalsis, IL-10–containing enterocytes shed in the small intestine, or IL-10 itself, end up in the cecum and the large intestine. Both explanations are based on the premise that low levels of IL-10 are sufficient to partially prevent inflammation. It is interesting to note that by

using a *Lactococcus*-based system for delivery of IL-10 in the intestine lumen, it was found that very low levels of IL-10 were sufficient to prevent inflammation in both the DSS and *IL-10*$^{-/-}$ models.[56] Third, the small intestine is the major site for the uptake of nutrients, as well as antigens. Enhancement of the barrier function by transgenic IL-10 could lead to systemic changes in antigen presentation and mucosal immune responses. Finally, although studies of parabiotic mice indicate that IELs do not circulate systemically,[58] there could be local recirculation of T cells between the small and large intestine. If this were the case, T cells exposed to IL-10 in the small intestine might also be found in the large intestine. Although there is no direct evidence for such a circulation pattern, the same T-cell clones are found in the small and large intestine in recipients of $CD4^+CD45RB^{high}$ T cells,[59] and T cells from one part of the intestine can colonize the length of the intestine after transfer to immune-deficient recipients (H.C. and M.K., unpublished data).

A number of potential delivery methods could be used to achieve expression of IL-10 in the intestine to obtain the local beneficial regulatory effects of this cytokine. These include the use of IL-10–encoding DNA in gene therapy, probiotic bacteria, or genetically engineered bacteria. While this manuscript was in preparation, it was shown that mice treated with IL-10–transfected *Lactococcus* strains could constitutively produce IL-10 in the intestine, resulting in reduced inflammation; however, the effects of bacteria-derived IL-10 on mucosal lymphocytes were not characterized.[56] Collectively, our results suggest that the local expression of anti-inflammatory cytokines within the intestine is effective at modulating local immune responses while circumventing systemic side effects. Therefore, targeted cytokine production could be of significant benefit in the treatment of IBD.

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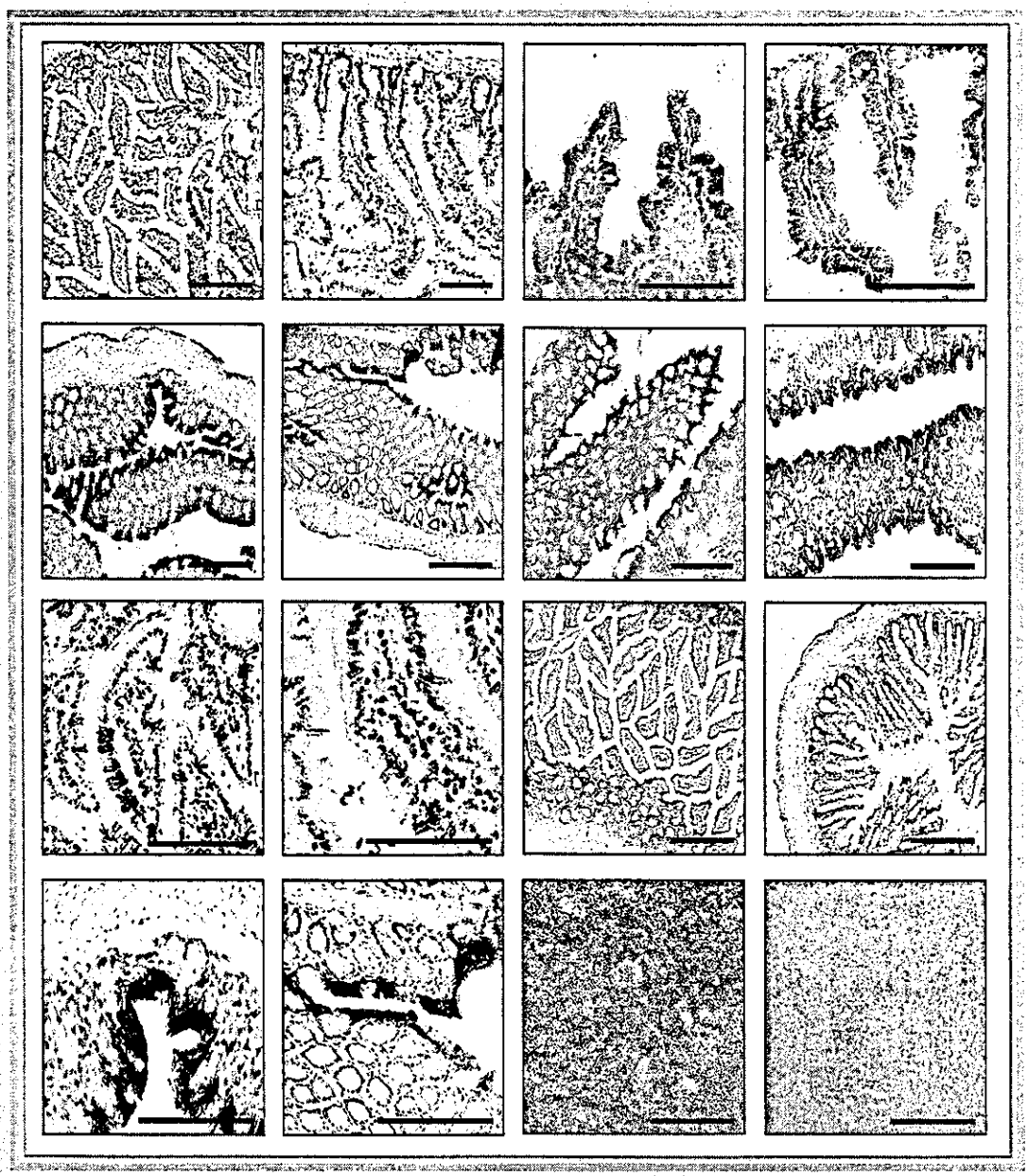

Clonal Expansion of Double-Positive Intraepithelial Lymphocytes by MHC Class I-Related Chain A Expressed in Mouse Small Intestinal Epithelium[1]

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Expression of a distant homologue MHC class I molecule, MHC class I-related chain A (MICA), has been found to be stress inducible and limited to the intestinal epithelium. This nonclassical MHC molecule is associated with various carcinomas in humans. To understand the biological consequences of MICA expression in the gut, we generated transgenic (Tg) mice ($T3^b$-MICA Tg) under the control of the $T3^b$ promoter. The $T3^b$-MICA Tg mice expressed MICA selectively in the intestine and had an increased number of TCR$\alpha\beta$ CD4CD8$\alpha\alpha$, double-positive (DP) intraepithelial lymphocytes (IELs) in the small bowel. These MICA-expanded DP IELs exhibited a bias to Vβ8.2 and overlapped motifs of the complementarity-determining region 3 region among various Tg mice. Hence, the overexpression of MICA resulted in a clonal expansion of DP IELs. Studies in model of inflammatory bowel disease showed that transgenic MICA was able to attenuate the acute colitis induced by dextran sodium sulfate administration. Therefore, this unique in vivo model will enable investigation of possible influences of stress-inducible MICA on the gut immune surveillance. ***The Journal of Immunology,*** **2003, 171: 4131–4139.**

Intestinal epithelia contain a developmentally and functionally specialized T cell pool, the so-called intraepithelial lymphocyte (IEL)[3] (1, 2). Because of their specific and unique location in the mucosal epithelium, IELs are often regarded as a first line of mucosal barrier against enteric flora (3). With respect to IEL ontogeny, it can be divided into $\alpha\beta$ T cells bearing CD4 or CD8$\alpha\beta$ coreceptors (thymus dependent) and $\gamma\delta$ or $\alpha\beta$ T cells bearing CD8$\alpha\alpha$ coreceptors (thymus independent) (4–8). However, a recent report suggests that the thymus is critical for generation of TCR$\alpha\beta$ CD8$\alpha\alpha$ IELs also (9). Little is known about locally expressed key molecules that may be involved in the selection and maturation of extrathymic IEL; reportedly though, some integrins or chemokine receptors facilitate the migration of TCR$\gamma\delta$ IELs or their precursors (10).

A nonclassical MHC class I chain A (MICA) molecule is stress inducible and mainly expressed on intestinal epithelium and various epithelial tumors (11–13). Because of the discovery that intraepithelial Vδ1 T cells recognize MICA (14), interaction of the NKG2D receptor on these T cells with MICA has been suggested to be important for activation of NK and T cell responses against MICA-bearing tumors (15). In addition, bacterial infection has enhanced the expression of MICA on target cell surface and up-regulated Vγ2δ2 T cell activation by nonpeptide Ags (16).

The increase in MICA expression induced by various stresses, including heat shock, oxidative stress, and virus or bacteria, together with the expression of MICA locally in gut epithelium prompted us to consider the possibility that interaction of MICA with IEL is important for the development and effector functions of IEL. In particular, because the gut is in a permanent state of mild inflammation or immunological stress due to exposure to commensal microflora and food Ags, lymphocytes struggling against damaged cells on the frontline may be necessary for maintaining host homeostasis (17, 18). However, the function of MICA expressed in the human intestinal tracts and consequences of the increase in MICA observed in vivo are undefined.

To evaluate these issues, in this work, we generated a transgenic model ectopically expressing human MICA, under the control of the $T3^b$ promoter in the mouse intestine. The transgenic mice expressed human MICA specifically on their intestinal epithelium and possessed numerous CD4CD8$\alpha\alpha$ (double-positive (DP)) IELs in their small intestine. We thus inquired into the characteristics of this expanded DP subset, examining the clonotype and DNA sequence of complementarity-determining region 3 (CDR3) to determine whether MICA exposure biased these cells toward a unique Vβ repertoire. Moreover, we introduced an experimental inflammatory bowel disease model into the transgenic (Tg) mice

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[3] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; APC, allophycocyanin; CDR3, complementarity-determining region 3; DP, double positive; DSS, dextran sodium sulfate; IEC, intestinal epithelial cell; MICA, MHC class I-related chain A; Tg, transgenic; WT, wild type.

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and showed a substantial attenuation of the development of intestinal disorder.

Materials and Methods

DNA construct and the generation of T3^b-MICA Tg mice

The 2.8-kb promoter region of the T3^b gene was shown to direct transgene expression exclusively in the epithelial cells of the small and large intestines (19). The upstream end of the T3^b promoter region in the T3^b transgene vector was originally an *Sph*I site, but was changed to a *Kpn*I site using a linker, because of the presence of an *Sph*I site in the MICA cDNA. The human MICA cDNA, including 1.2 kb of the whole MICA coding sequence, was cloned by PCR, and its sequence was confirmed (11). The T3^b-MICA transgene was constructed by inserting this MICA cDNA into the unique *Eco*RI site of the T3^b transgene vector, which contains the T3^b promoter and the rabbit β-globin gene sequences from the second exon to the third exon, including the polyadenylation signal. The transgene vector was digested with *Kpn*I and *Xho*I, and the resulting 5.5-kb fragment of the T3^b-MICA transgene was isolated and used for microinjection into the pronuclei of one-cell embryos of BDF$_1$ mice to produce T3^b-MICA transgenic mice, as described previously (20). ICR (recipient) and C57BL/6 (backcrossing partner) mice purchased from Japan SLC (Shizuoka, Japan) were used throughout this study. The mice were maintained under specific pathogen-free conditions in the animal facility of the Research Institute for Microbial Diseases, Osaka University. For screening of founder mice, tail DNA was isolated by use of the SDS-proteinase K method. Founders were genotyped by PCR using specific primers for the transgene, MICA cDNA. The oligonucleotide 5′-GCTGGTTATTGTGCTGTCTC-3′ was used as a forward primer, and 5′-GGATCTCACAGACCCTAATC-3′ was used as a backward primer.

RT-PCR

Total RNA was extracted from various tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. DNase digestion of extracted RNA was performed before cDNA synthesis. A total of 1 μg of total RNA was reverse transcribed into cDNA using Omniscript reverse transcriptase (Qiagen, Valencia, CA). PCR amplification (GeneAmp PCR system 9700) (PerkinElmer, Foster City, CA) was performed initially at 95°C for 5 min and then in sequential cycles at 95°C for 30 s, 61°C for 30 s, and 72°C for 40 s, followed by an extension for 10 min at 72°C. The oligonucleotide primers used for the determination of MICA expression were 5′-CTCGAGGAGCCCCACAGTCTTCGTTATA AC-3′ as a forward primer and 5′-CTCGAGCTAGTGATTCCCCCTGT GTTCCATGTAG-3′ as a backward primer. The equal amount of PCR products was used to electrophoresis on 1% agarose gel.

Immunoblotting

Whole cell lysates (20 μg of proteins) were separated on 1-mm-thick 4–20% Tris-glycine gels and then transferred to nitrocellulose. Equal protein loading in each of the lanes was confirmed by staining the same gel with GelCode Blue Stain Reagent (Pierce, Rockford, IL). The filters were blocked with 5% (w/v) nonfat dry milk powder in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Tween 20). Anti-MICA mAb 2C10 (mouse IgG3) (13) was diluted 1/2000 in TBST containing 3 mg/ml BSA and incubated with the filter at 2 h. As a loading control, anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO) was also incubated with a separate filter. After washing with TBST, the filters were incubated for 1 h with HRP-conjugated goat anti-mouse IgG3 (1/3000 in TBST) (Southern Biotechnology Associates, Birmingham, AL). Filters were washed extensively with TBST, and immunoreactive bands were visualized by chemiluminescence reagent (NEN Life Science, Boston, MA).

Immunohistochemical assay

Blocks of intestine, spleen, and thymus were removed, fixed in 4% paraformaldehyde, embedded, and snap frozen in OCT compound (Tissue-Tek, Torrance, CA). Sections (5 μm) were cut in a cryostat and air dried. The sections were quenched with H_2O_2, treated with 10% FBS, and then incubated with a titrated dilution of anti-MICA mAb 6D4 (mouse IgG2a) (21). For the detection of bound Abs, Vectorstain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate kit (Funakoshi, Tokyo, Japan) were used. Slides were then counterstained with hematoxylin. Control sections without the primary Ab or with an isotype control were run in parallel.

Isolation of splenocytes and IELs and flow cytometry analysis

Lymphocytes were isolated from spleens and IELs, as described previously (22). In case of IELs, after Peyer's patches and fatty tissues were removed, a standard mechanical dissociation method was performed and followed by a Percoll discontinuous density gradient separation. After blocking with anti-CD16/CD32 FcR mAb (2.4G2), the cells were stained using following labeled mAb conjugates: FITC-conjugated anti-CD4 mAb (L3T4; RM4-5), Vβ4 (KT4), Vβ5.1, 5.2 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8.1, 8.2 (MR5-2), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (MR14-2), TCR$\gamma\delta$ (GL3), PE-conjugated anti-TCRβ mAb (H57-597), TCR$\gamma\delta$ (GL3), CD69 (H1.2F3), CD44 (Ly-24; IM7), CD62L (MEL-14), and allophycocyanin (APC)-conjugated anti-CD8α (Ly-2; 53-6.7) mAb. All mAbs were purchased from BD PharMingen (San Diego, CA). The stained cells were then washed and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Immunoscope analysis for Vβ repertoire

As previously described, cDNA synthesized from total RNA of the sorted cells was used for Vβ-Cβ amplification (23). The oligonucleotide primers used for these reactions were: forward (Vβ8.2), 5′-CATTATTCATATG GTGCTGGC-3′; reverse (Cβ145), 5′-CACTGATGTTCTGTGTGACA-3′. The amplified products were then used to run off reactions with an oligonucleotide primer labeled with a fluorescent tag (Cβ5-6-carboxyfluorescein; 5′-6-carboxyfluorescein-CTTGGGTGGAGTCACATTTCTC-3′). The runoff products were subjected to capillary electrophoresis in an automated DNA sequencer (PE Applied Biosystems, Foster City, CA), and CDR3 size distribution and signal intensities were analyzed with GeneScan software (PE Applied Biosystems).

Cloning and sequencing of selected Vβ-Jβ rearrangements

Each Vβ-Jβ-amplified product was shotgun cloned with the pGEM-T Easy TA cloning kit (Promega, Madison, WI) (23). Resulting colonies were randomly selected for plasmid DNA isolation by using ABI Prism Miniprep kits (PE Applied Biosystems). Sequencing reactions were performed with an IRDye AFLP Kit (LI-COR, Lincoln, NE) and analyzed on an LI-COR4000 sequencer (LI-COR).

Animal experiment for induction and analysis of dextran sodium sulfate (DSS)-induced colitis

Colitis was induced by the administration of DSS (2.5% w/v; m.w., 40,000; ICN Biomedicals, Irvine, CA) in the drinking water for 5 days (24). DSS water consumption and weight were recorded daily. For the assessment of the severity of colitis, animals were sacrificed on days 5, 9, and 17 after beginning of the DSS treatment, and the colons of the Tg mice and C57BL/6 mice were examined histologically. Tissue samples obtained from the proximal and distal colon were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned at a thickness of 6 μm. The tissue sections were stained with H&E. Severity of colitis was evaluated by the standard scoring system, as described previously (25). Each region of the colon (ascending proximal and descending distal colon) was graded semiquantitatively from 0 (no change) to 3 (most severe change) per examination item. The grading represents a degree of monocyte and/or neutrophil infiltration, goblet cell and/or mucous loss, epithelial erosion, and ulceration. The scoring was performed by a blinded manner.

Statistical analysis

Significant differences between mean values were determined by Student's t test. $p < 0.05$ was considered to be statistically significant.

Results

Selective expression of human MICA in the intestine of T3^b-MICA Tg mice

Human MICA cDNA, expressed under the control of the T3^b promoter, was specifically expressed in intestinal epithelial cells (Fig. 1*A*). From 10 founder mice, three representative lines (Tg-07-5, Tg-07-6, and Tg-09-4) were maintained by mating to C57BL/6 mice. We mainly used F_2 and F_3 MICA Tg mice in this study. Thus, in addition to the background effect of backcross partner, C57BL/6 strain, the effect of the DBA2 strain that was used to make a donor for BDF$_1$ embryos also might have been involved in the phenotype of the Tg mice. To determine the tissue specificity of the transgene expression, total RNA was isolated from various