



# Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops

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**To explore the requirement for M cells and the Peyer's patch (PP) in induction of oral tolerance and address the potential in vivo role of intestinal epithelial cells as nonprofessional APCs, we have attempted to induce tolerance in mice with ligated small bowel loops without M cells and Peyer's patches. A 2-centimeter section of vascularized small bowel was spliced away from the gut without disruption of the mesenteric attachments. We introduced OVA directly into the lumen of the loop prior to footpad immunization. By excising segments of bowel that contain PPs in some mice and segments without patches in others, we could study the necessity of the M cell and the underlying patch versus epithelial cells in induction of mucosal tolerance. We show that OVA-specific T cell proliferation and serum antibody responses are reduced in mice that have previously been given OVA both in PP-containing loops and in loops without patches. Furthermore, both high- and low-dose tolerance could be induced in the absence of PPs. Low-dose tolerance is associated with bystander suppression and requires IL-10, which indicates active suppression and the induction of regulatory cells. These data suggest that there is a critical role for components of the mucosal immune system other than PPs in antigen sampling and induction of oral tolerance.**

## Introduction

The mucosal immune system utilizes a very complicated and tightly regulated system of suppression and controlled inflammation to distinguish between non-self that is harmless (dietary antigens [Ags] and normal enteric flora) and non-self that is harmful (pathogenic organisms) in the intestinal lumen. Although the exact mechanisms governing this regulation remain to be elucidated, evidence exists for the involvement of a variety of cell types, including CD8<sup>+</sup> suppressor T cells (1), CD4<sup>+</sup> regulatory T cells (2), B cells (3, 4), and DCs (5).

One phenomenon linked to this regulation is termed oral tolerance. Large quantities of non-self proteins are taken up daily from the intestinal lumen, yet they are presented to T cells in such a way as to promote tolerance. The systemic immune system is not ignorant of these foreign proteins, though, as in vitro stimulation with experimentally fed Ags can elicit Th2 cytokine responses (6, 7). Tolerance to soluble Ags that have been introduced at mucosal sites has been observed in many animal models as well as in human trials (8–10). It has been shown that low doses of fed Ag can suppress Th1 responses by swaying the response to a Th2 (IL-4, IL-5, IL-10), Th3 (TGF- $\beta$ ), or Treg 1 (Tr1) (IL-10) cytokine profile (11–13). High doses of fed Ag can induce tolerance by anergizing Ag-specific T cells or by clonal deletion (14).

Mucosal tolerance is thought to be contingent upon the very specific environment of the mucosa-associated lymphoid tissue. Regulation is most likely the result of the cytokine microenvironment as well as the immunoregulatory properties of the specialized cells that line the intestinal surface, the M cell and the absorptive epithelial cell.

The M cell resides only in the follicle-associated epithelium overlying a Peyer's patch (PP). M cells arise from undifferentiated enterocytes that overlie PPs (15), and differentiation is induced by B cells in the patch (16). M cells are very efficient at taking up particulate Ags such as virus particles, bacteria, and other macromolecules in the lumen and passing them through to the underlying patch, where they will be taken up, processed, and presented by the resident macrophages and DCs. Conversely, the M cell is inefficient at taking up soluble proteins. The absorptive intestinal epithelial cells (IECs), however, have been shown to take up soluble Ags and even process and present these Ags on their basolateral surface (17, 18). In humans, they express MHC class I, class II, and nonclassical class I molecules (19), as well as a unique costimulatory molecule, gp180, which acts to induce proliferation of CD8<sup>+</sup> T cells in culture (20). CD8<sup>+</sup> T cells activated in this manner could either play a role in local suppression (controlled physiologic inflammation) or systemic tolerance (21).

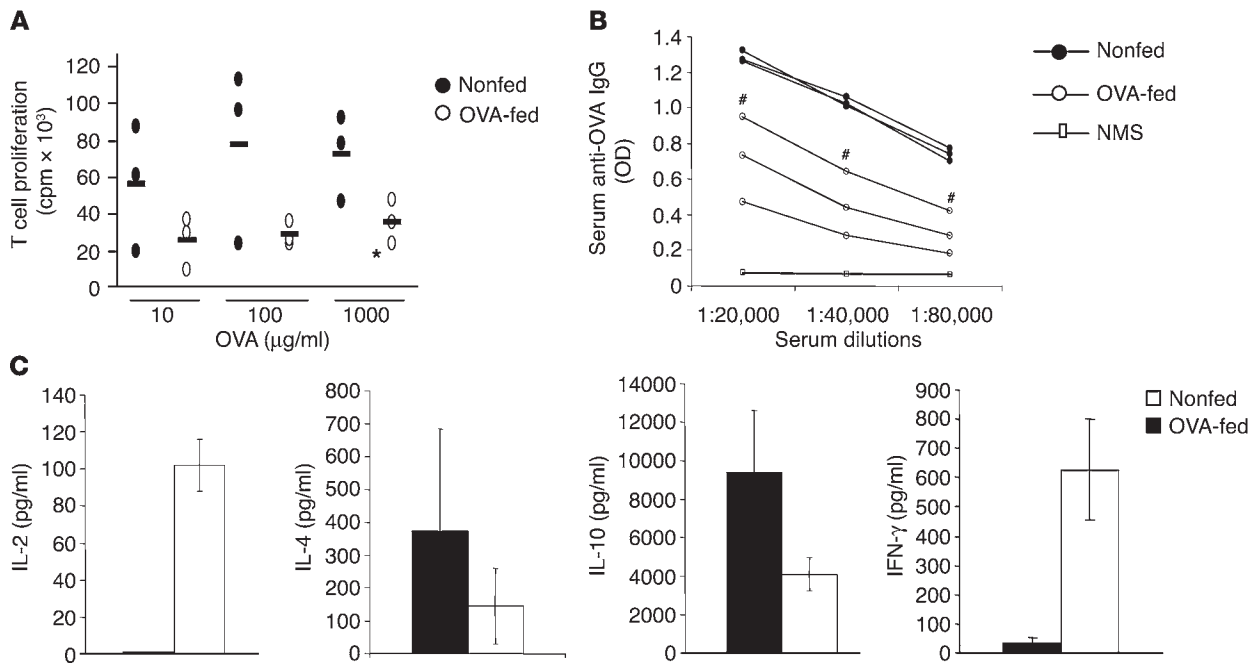
In attempting to understand the roles that the M cell and PP play in induction of oral tolerance, investigators have relied on gene-targeted knockouts, such as the lymphotoxin  $\alpha$  knockout (LT $\alpha$ <sup>-/-</sup>) and LT $\beta$ <sup>-/-</sup>, in which PPs are absent or aberrantly developed (22, 23), or else have treated mice in utero with LT $\beta$  receptor Ig fusion protein to generate mice lacking PPs (24). These mice fail to develop tolerance to orally administered Ags. However, in addition to undeveloped PP, these mice also have aberrantly developed splenic architecture that may result in altered immune responses. The LT $\alpha$ <sup>-/-</sup> mice also have no mesenteric lymph nodes, making interpretation of the results difficult. Others have suggested that PPs are not relevant to tolerance induction, although the findings in these studies were not absolute (25).

To understand the role of the M cell and the underlying PP, as well as the IEC in mucosal tolerance, we have surgically isolated a segment of small intestine and repositioned it in the abdomen so as to have access to the lumen. By choosing segments of small bowel that contain PPs and segments that do not, we were able

**Nonstandard abbreviations used:** CT, cholera toxin; IEC, intestinal epithelial cell; KLH, keyhole-limpet hemocyanin; LT $\alpha$ <sup>-/-</sup>, lymphotoxin  $\alpha$  knockout; OVA-pep, OVA peptide 323–339; PLN, popliteal lymph node; PP, Peyer's patch.

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**Figure 1**

Induction of low-dose oral tolerance in normal mice. **(A)** Antigen-specific T cell proliferation in nonfed and OVA-fed mice. The tolerance protocol was performed as described in Methods. \* $P < 0.035$ . **(B)** Serum anti-OVA antibody levels in nonfed and OVA-fed mice. Serum from mice that were (open circles) or were not (filled circles) fed OVA prior to OVA immunization was diluted ( $1.25 \times 10^{-5}$  to  $5 \times 10^{-5}$ ) and analyzed for the presence of anti-OVA IgG by ELISA. Serum from a nonimmunized animal (normal mouse serum [NMS]; open squares) was used as a negative control. The titers of OVA-specific IgG were significantly lower in mice fed OVA compared with nonfed mice. # $P < 0.007$ . **(C)** Cytokine profile of cells from OVA-fed or nonfed mice. Cells from the PLN of OVA-fed (black bars) and nonfed (white bars) OVA-immunized mice were cultured with 100 µg/ml OVA for 24–96 hours, and cytokine secretion was measured by ELISA. The results indicate that the OVA-fed mice had increased secretion of IL-4 (72 hours) and IL-10 (72 hours) and a decreased secretion of IFN-γ (24 hours) and IL-2 (24 hours) compared with nonfed mice.

study the necessity for M cells and that of the PP in mucosal tolerance in an immunologically intact animal. We administered multiple doses of either whole OVA, OVA fragments, or OVA peptides via the loops followed by immunization in the footpad. Our results show that mice can be tolerized by Ag administration through both M cell and PP-containing and M cell and PP-deficient loops. We show that administration of peptide into either loop leads to the induction of bystander suppression, which provides evidence for the generation of regulatory cells. In addition, we show that IL-10 is a critical cytokine for low-dose tolerance in both PP-independent and -dependent tolerance induction. Moreover, oral tolerance was not achieved using whole OVA as the tolerogen in the loops, which supports the concept that gastric digestion is needed in induction of oral tolerance.

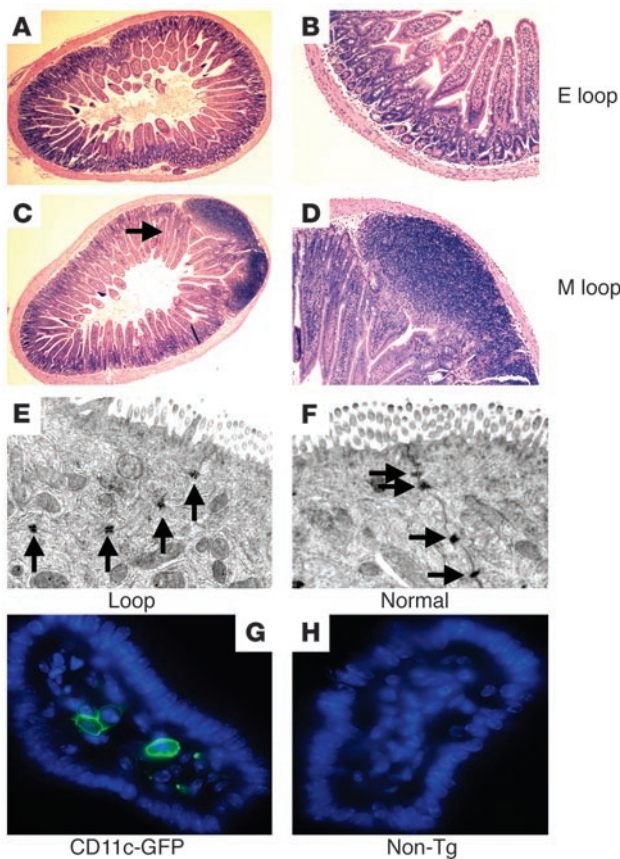
## Results

**Oral tolerance in normal mice.** First, using the protocol developed by Liu et al. (26), we tested the low-dose tolerance of mice with unligated loops. One group of mice was fed 1 mg OVA by gastric intubation every day for 5 days, then immunized in both footpads 3 days after the last feeding. Two weeks later, mice were bled and sacrificed; the lymphocytes from the popliteal lymph nodes (PLNs) were restimulated in vitro with 10–1,000 µg/ml OVA, and the response was compared with that of mice that were immunized without OVA feeding. The results (Figure 1A) show that T cell proliferative responses were reduced 52–64% in mice that were fed OVA compared with nonfed mice. Serum was analyzed

for the presence of anti-OVA IgG, and as shown in Figure 1B, the levels of OVA-specific IgG were reduced by 45–61% in mice fed OVA compared with nonfed mice. Cytokine production by T cells from OVA-fed and nonfed mice upon in vitro stimulation was also measured. As can be seen in Figure 1C, OVA-fed mice displayed a decreased secretion of IFN-γ and IL-2 and enhanced secretion of IL-4 and IL-10 compared with nonfed mice. The above results are consistent with those of previously published reports (26) and confirm the effectiveness of the tolerance protocol in our study.

**Isolated loops remain intact, without evidence of inflammation.** Before initiating studies involving Ag administration in the loops, we needed to ensure that the surgical procedure itself would not induce local inflammation or disruption of mucosal architecture, as this could prevent tolerance induction by altering Ag trafficking patterns. Furthermore, we needed to confirm the presence or absence of PPs. Therefore, 10 days after surgery, we excised loops for histologic analysis. As shown in Figure 2, H&E staining of a cross-section of a representative loop without PPs (E loop; Figure 2, A and B) and a representative M loop (containing PPs and M cells; Figure 2, C and D) revealed normal architecture and no evidence of active inflammation.

It was also important to assess the possibility that the surgical procedure had damaged the mucosal barrier, allowing for paracellular transport of the OVA. Therefore, we analyzed the loops for the presence of tight junctions by electron microscopy. Figure 2E shows a representative section of the loop 10 days after surgery. The arrows indicate the presence of desmosomes. The cell-cell con-



**Figure 2**

Microscopic evaluation of intestinal loops. E and M loops were excised 10 days after surgery and fixed in 10% formalin for H&E staining or immediately fixed in glutaraldehyde for electron microscopy. Cross-sections were cut and stained with hematoxylin and counterstained with eosin. (A and B) Representative sections from the E loop (no PP). (C and D) Representative sections from the M loop. The PP is indicated by an arrow in C. Original magnification,  $\times 10$  (A and C) and  $\times 40$  (B and D). (E and F) Electron micrographs of a loop 10 days after surgery (E) compared with normal bowel (F) at  $\times 40,000$  magnification. Desmosomes (arrows) indicate the presence of tight junctions. As shown, cross-sections of the loops revealed normal architecture as well as an intact epithelium and no evidence of active inflammation. (G and H) Deconvolution micrograph demonstrating the presence of DCs in the lamina propria of distal jejunal segments in CD11c-GFP mice (G) and in controls (H). Note that the dendrites failed to reach the epithelium in these segments (in contrast to the distal ileum). A 3D reconstruction (supplemental data) further demonstrates the failure of DCs to invade the epithelium in this part of the small intestine.

meability has been associated with a loss of tolerance, and this would have prevented us from drawing valid conclusions from this study. Mice were sacrificed within 2 weeks after surgery, and the permeability of the isolated loops was compared with that seen in intact bowel segments from either the operated mice (bowel in continuity) or nonoperated control mice. As shown in Table 1, the resistance across the barrier within the loops was comparable to that seen across the intact intestine. These findings as well as the failure to generate an immune response to intact OVA in the loops (see below) provided further evidence that the epithelium of the loops exhibited normal barrier function during the interval of the experiment.

tacts between epithelial cells in the loop appear normal when compared with normal small bowel from mice that had not undergone surgery (Figure 2F). Importantly, no subepithelial DCs were noted on any of the sections.

Previous studies have suggested that intestinal DCs express tight junction proteins and can intercalate their dendrites between absorptive epithelial cells (27). These findings suggest that subepithelial/intraepithelial DCs might sample Ag from the lumen and carry these Ags to local draining lymph nodes. More recent studies suggest that these DCs are present predominantly in the distal small bowel (ileum). The loops generated in our model are from the jejunoileal junction. In order to determine whether intraepithelial DCs were present in the loops, we used 2 different approaches. We used CD11c-GFP transgenic mice, originally generated by the Littman laboratory (28). DCs from these mice fluoresce spontaneously. Using deconvolution microscopy, we were able to demonstrate DCs within the lamina propria of the jejunum. Fluorescent dendrites did not extend into the epithelium in any section (Figure 2G and supplemental data; supplemental material available online with this article; doi:10.1172/JCI19102DS1). Direct staining with a FITC-conjugated anti-CD11c mAb also failed to demonstrate the presence of intraepithelial DCs (data not shown). In summary, we analyzed loops using fluorescence microscopy and electron microscopy and could not document intraepithelial dendrites using either of these approaches.

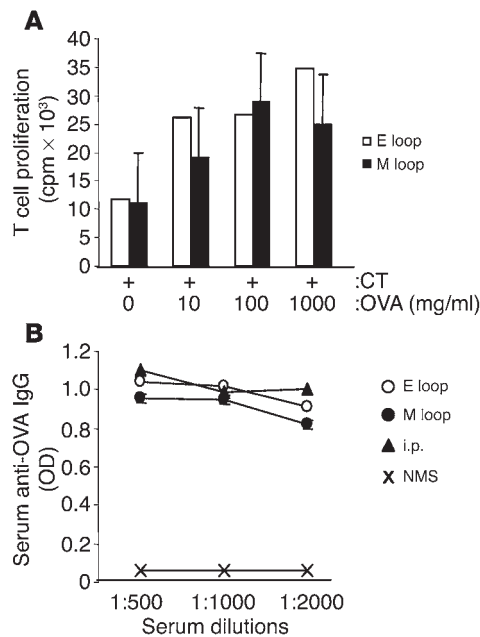
*Permeability in loops remains unaltered.* To eliminate the possibility that the surgical procedure could have influenced the permeability of the loops to Ag, we performed electrical resistance studies, analyzing the tissues in Ussing chambers. Epithelial per-

**Table 1**

Permeability of bowel segments is unaltered after surgery

Mice <sup>A</sup>	Segment <sup>B</sup>	$\Omega \times \text{cm}^2$ <sup>C</sup>
Control 1	Intact	12.4
Control 1	Intact	12.4
Control 2	Intact	12.4
Control 2	Intact	12.4
Mouse 4	Loop	12.4
Mouse 4	Loop	12.4
Mouse 4	Intact	12.4
Mouse 4	Intact	12.4
Mouse 5	Loop	12.4
Mouse 5	Loop	10.1
Mouse 5	Intact	12.1
Mouse 5	Intact	11.8
Mouse 6	Loop	11.8
Mouse 6	Loop	12.4
Mouse 6	Intact	9.3
Mouse 6	Intact	12.4

<sup>A</sup>Ten days after surgery, 3 loop mice (mouse 4–6) were sacrificed and the loops and intact bowel were excised and subject to measurement of intestinal permeability using an Ussing chamber. <sup>B</sup>Segments from the loop or from intact small intestine from the same experimental mice were used, as well as bowel from 2 non-surgically altered mice (controls 1 and 2). <sup>C</sup>Following tissue equilibration (20–30 minutes), passive ion transport, or transmural resistance ( $\Omega \times \text{cm}^2$ ), was measured as an indicator of barrier function. Readings from 2 sections of each tissue were taken. Controls were intact BALB/c mice sacrificed at 10 weeks of age. Mouse 4–6 were mice with surgically developed loops.



Loops are functional and respond as well as intact small bowel to cholera toxin and OVA. We next assessed whether the loops, 10 days after surgery, were immunologically intact. Cholera toxin (CT) is a known mucosal adjuvant that stimulates both local and systemic immune responses when given orally, alone or in association with soluble Ag. CT and OVA were coadministered into the loop on days 1, 21, and 28. Two weeks later, the mice were bled, sacrificed, and tested for serum anti-OVA IgG levels and anti-OVA splenic T cell proliferation. As shown in Figure 3, mice immunized with CT/OVA in the loops responded by OVA-specific T cell proliferation (Figure 3A) and production of anti-OVA antibodies (Figure 3B). The responses were similar in E and M loops, which suggests that repositioned bowel loops both with and without PP responded normally to Ag in the presence of the mucosal adjuvant CT.

Tolerance could be achieved with OVA fragments but not whole OVA in both E and M loops. We next attempted to induce mucosal tolerance by administering OVA (without CT) directly into the ligated loops. As shown in Figure 4, A and B, neither tolerance nor immunization was seen in either the M loops or the E loops when whole OVA was used as the tolerogen. T cell proliferative responses were variable but not significantly decreased in the “loop-fed” mice (Figure 4A), and the serum anti-OVA IgG levels were unchanged (Figure 4B). These data suggest that the epithelial barrier is intact in the surgically repositioned intestinal loops, as described above, and that alteration of the native Ag may be required for tolerance induction. Since early reports have suggested that gastric digestion might be critical for achieving mucosal tolerance (29–31), we repeated the above experiment using pepsin-treated fragments of OVA as the tolerogen. Interestingly, administration of these fragments into the loop prior to systemic immunization did cause a significant reduction in the OVA-specific T cell proliferation (Figure 4C) as well as a reduction in the levels of serum anti-OVA antibodies (Figure 4D). This could be seen both in E and M loop mice, but it appears that pepsin-digested Ag introduced into M loops induced greater tol-

**Figure 3**

Response to immunization with OVA and CT in E and M loops. Ten micrograms CT and 5 mg OVA were coadministered in the loop on days 1, 21, and 28. Mice were bled and sacrificed 2 weeks later. (A) OVA-specific T cell proliferation. Splenocytes from mice immunized with OVA and CT in either E loops (white bars) or M loops (black bars) were cultured with or without 10–1,000  $\mu\text{g/ml}$  OVA for 72 hours, and this was followed by addition of [<sup>3</sup>H]thymidine for 16 hours. Cells were harvested, and incorporated thymidine was read on a MicroBeta counter. The results show that splenocytes from mice immunized with CT plus OVA in an E loop or an M loop proliferated similarly in response to OVA. (B) Serum anti-OVA levels. Serum was diluted to the indicated titers and analyzed for the presence of OVA-specific IgG by ELISA. Normal mouse serum (indicated by an X) and serum from mice immunized i.p. with OVA in complete Freund’s adjuvant (triangles) were used as negative and positive controls, respectively. OVA-specific antibody titers from E loop (open circles) or M loop (filled circles) mice were statistically similar and not different from those from normal mice at the indicated dilutions.

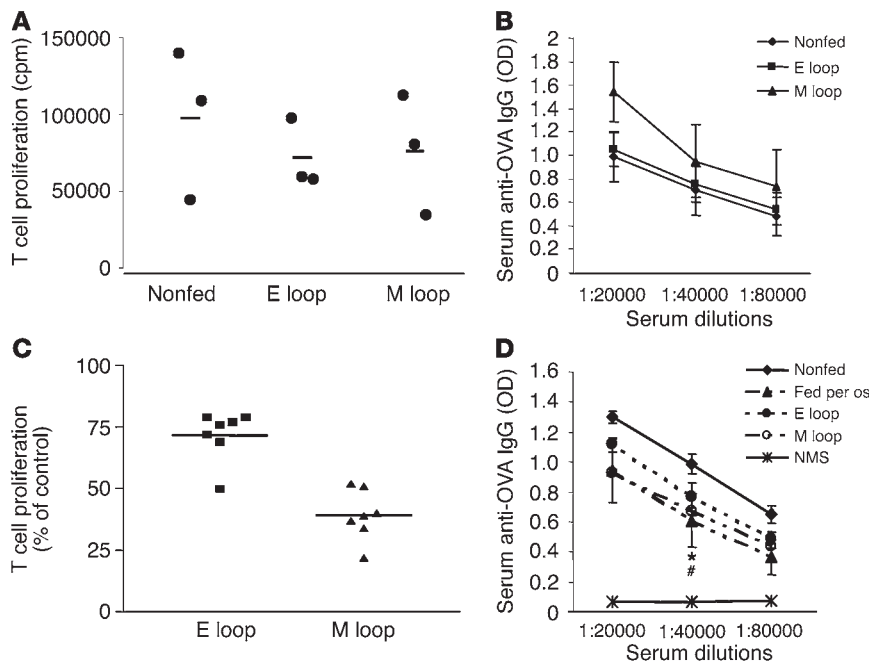
erance (40% of control) compared with that achieved with fragments introduced into loops without PPs (72% of control).

High- and low-dose oral tolerance are induced in small bowel loops. In order to study the induction of high- and low-dose tolerance in the small bowel loops, we switched to a more controllable system using OVA peptide 323–339 (OVA-pep). We explored the induction of high-dose oral tolerance by feeding 0.5 mg OVA-pep into the lumen of either M loops or E loops. This dose was chosen following a series of dose-ranging studies (data not shown). OVA-specific in vitro proliferation of LN cells from both E and M loop mice that were fed OVA-pep into the loop was decreased more than 50% ( $P < 0.01$ ) compared with that of LN cells from nonfed mice (Figure 5A). Furthermore, we observed a reduction of the IFN- $\gamma$  and IL-2 secretion by LN cells from both E and M loop mice compared with nonfed mice, whereas the levels of IL-4 and IL-10 secretion were unchanged (Figure 5, B–E).

In order to investigate the induction of low-dose tolerance in the presence and absence of PP, we reduced the dose 50-fold and administered 0.01 mg OVA-pep into the loops. Upon restimulation with OVA-pep in vitro, the proliferation of LN cells both from OVA-fed E loop and M loop mice was reduced by more than 50% compared with loop that did not receive OVA-pep ( $P < 0.01$ ) (Figure 6A). The levels of IFN- $\gamma$  and IL-2 were significantly suppressed, whereas the IL-4 levels remained unchanged, and the IL-10 levels were increased in mice fed OVA-pep (Figure 6, B–E).

These results indicate that both high- and low-dose tolerance to OVA peptide can be induced equally well in the presence and absence of PP.

Bystander suppression in low-dose tolerance. Low-dose tolerance is thought to be dependent upon the generation of regulatory T cells. In order to investigate whether the generation of Th3/Tr1 cells is dependent on the presence of PP, we examined the induction of bystander suppression in loop mice. This was performed by administering OVA-pep (0.01 mg) into the loops as before, then immunizing with either a mixture of OVA-pep and another immunogen, keyhole-limpet hemocyanin (KLH), or immunizing with KLH alone. We then restimulated cells from the draining LN in vitro with KLH to assess whether there was bystander suppression. The results showed a marked decrease in KLH responsiveness in T cells in the animals that were immunized with both OVA and KLH (Figure 7A), whereas no tolerance to



**Figure 4** The effect of administration of whole OVA or OVA fragments in E or M loops. **(A)** OVA-specific proliferation of PLN cells was measured as in Figure 2. As shown, T cell proliferation was not significantly suppressed in the mice with intact OVA administered through the E or M loop compared with OVA-nonfed animals. **(B)** Serum anti-OVA IgG levels. Serum from loop-fed and control mice was analyzed for OVA-specific IgG by ELISA. As shown, OVA-specific IgG levels were not reduced in mice that had received OVA either in M loops (triangles) or in E loops (squares) compared with nonfed animals (diamonds). These results indicate that neither mucosal tolerance nor immunization could be achieved by administration of whole OVA directly into either E or M loops. **(C and D)** OVA was digested by pepsin, and purified OVA fragments (3–30 kDa) were administered via either E loops or M loops prior to immunization and in vitro stimulation as described in Methods. **(C)** OVA-specific proliferative responses were reduced in T cells from the PLN of mice administered OVA through the E loop by 28% ( $P = 0.0016$  compared with nonfed controls). Mice fed through the M loop had T cell proliferative responses reduced by 60% ( $P < 0.001$  compared with nonfed controls). **(D)** Reduced serum anti-OVA antibody levels in E and M loop mice. Serum from mice that were fed OVA fragments per os (triangles) or in E loops (filled circles) or M loops (open circles) was diluted and analyzed for anti-OVA IgG and compared with serum from nonfed mice (diamonds). Serum anti-OVA antibody levels were reduced in E loops, and the reduction was enhanced in M loops. \* $P < 0.03$ , E loop compared with nonfed controls; # $P < 0.001$ , M loop compared with nonfed controls.

KLH was induced in mice fed OVA-pep and immunized with KLH alone (Figure 7B). The results were similar in M and E loop mice, which suggests that bystander suppression does occur in both PP-independent and PP-dependent systems.

*IL-10 but not TGF- $\beta$  is critical for low-dose tolerance through M and E loops.* Since regulatory cells were generated by feeding through both PP- and non-PP-containing loops, we wanted to investigate the necessity for TGF- $\beta$  and IL-10 for induction of tolerance in the loop mice. We first asked whether TGF- $\beta$  secreting Th3 cells were critical to M loop or E loop tolerance. We treated mice with neutralizing anti-TGF- $\beta$  mAbs prior to and during Ag administration (1 mg every other day for 10 days starting 1 day prior to Ag administration). This protocol has previously been shown to block TGF- $\beta$  activity (26). As seen in Figure 8, when compared with treatment with an isotype-matched control mAb, this treatment failed to inhibit tolerance induction in either loop mice or intact mice. Thus TGF- $\beta$  may not be critical for tolerance induction.

We then performed tolerance studies in IL-10<sup>-/-</sup> mice and wild-type littermates (C57BL/6 mice). In contrast to the results of the studies using anti-TGF- $\beta$  mAbs, there was a failure to generate low-dose tolerance in the IL-10<sup>-/-</sup> mice, whether mice were fed OVA-pep orally (data not shown) or via the loops (Figure 9, A and B), which demonstrates that low-dose tolerance generated both in the presence and absence of PPs is dependent on IL-10. Neither T cell proliferation nor IFN- $\gamma$  secretion was decreased upon OVA administration (Figure 9, A and B). These data are consistent with those of previous studies that reported that low-dose tolerance could not be achieved in IL-10<sup>-/-</sup> mice (32) and support a role for this cytokine in induction of oral tolerance. In contrast, high-dose tolerance (0.5 mg/d for 5 days) was independent of IL-10, regardless of whether it was induced in the presence or absence of PP. T cell proliferation and IFN- $\gamma$  secretion were significantly decreased upon OVA administration in both E and M loops (Figure 9, C and D).

In summary, IL-10, but not TGF- $\beta$ , appears to be a critical component of oral tolerance in a PP-independent model as well as a PP-dependent model.

### Discussion

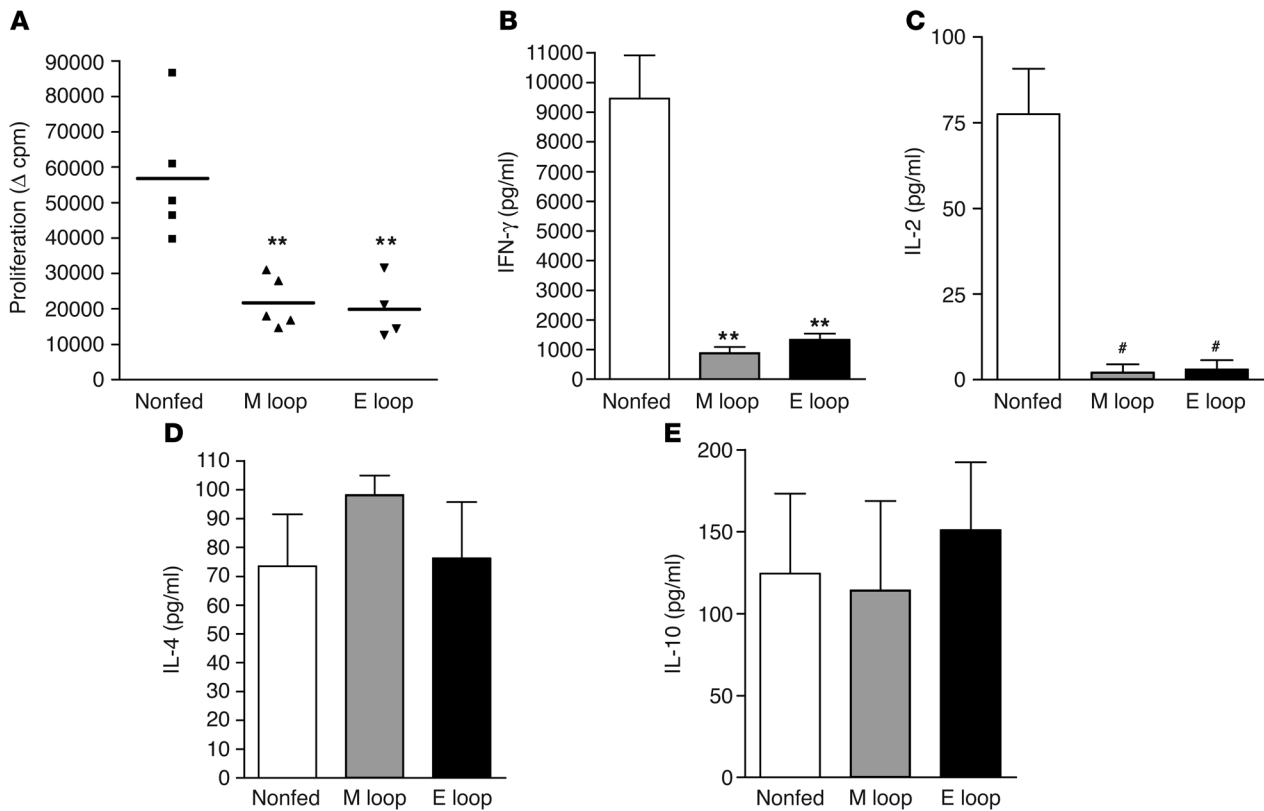
M cells are quite efficient at taking up luminal bacteria and specific viruses and transporting them into the PP for presentation by the resident APCs to T cells. Because of this capacity, the M cell has been proposed as the obvious candidate for transport of proteins in mucosal tolerance induction.

Previous studies from our laboratory have focused on the potential role of the IEC as an APC (17, 33). Another study suggests that normal IEC-T cell interactions result in the activation of regulatory CD8<sup>+</sup> T cells

controlled by the class Ib molecule CD1d and a novel CD8 ligand, gp180 (34). These studies were performed in vitro, in human systems, and there have been no in vivo studies to suggest a role for the IEC in mucosal Ag presentation, regulation of physiological inflammation in the gut, or oral tolerance.

In the present study, we took a different approach in order to study the role of PPs and M cells versus epithelial cells in the genesis of oral tolerance. By administering Ag directly into isolated noncontiguous segments of the small intestine, in which a PP was either present or absent, we could study the necessity of PPs for mucosal tolerance in an otherwise normal mouse.

Using this loop model to study oral tolerance induction, we demonstrated that OVA-specific antibody production as well as T cell proliferative responses are reduced in mice that received the tolerogen (pepsin-digested OVA fragments) in loops devoid of M cells and PP, although greater suppression was seen after administration of Ag into loops with M cells and PP. However,

**Figure 5**

High-dose tolerance to OVA-pep can be achieved in both E and M loops. Loop mice were either not fed or fed 0.5 mg OVA-pep into E or M loops and then immunized with the same antigen in the footpads. Twelve days later, PLN cells were harvested and restimulated with OVA-pep in vitro. Proliferation (A) and cytokine production (B–E) were measured after 72 hours of incubation. Each data point represents an individual mouse, while each column represents the mean value of 4 mice, and error bars indicate SEM. \*\* $P < 0.01$ , # $P < 0.001$  vs. nonfed.

E and M loop mice that received OVA-pep tolerized equally well and to a greater extent (up to 80% inhibition) than those receiving pepsin-digested fragments. This suggests that tolerance to mucosally administered Ag can occur without the presence of M cells to absorb the soluble protein or PPs. Most investigators have noted that M cells do not develop in the absence of PPs or lymphoid follicles, although the existence of M cells in the villi (in the absence of PPs) has been suggested (35).

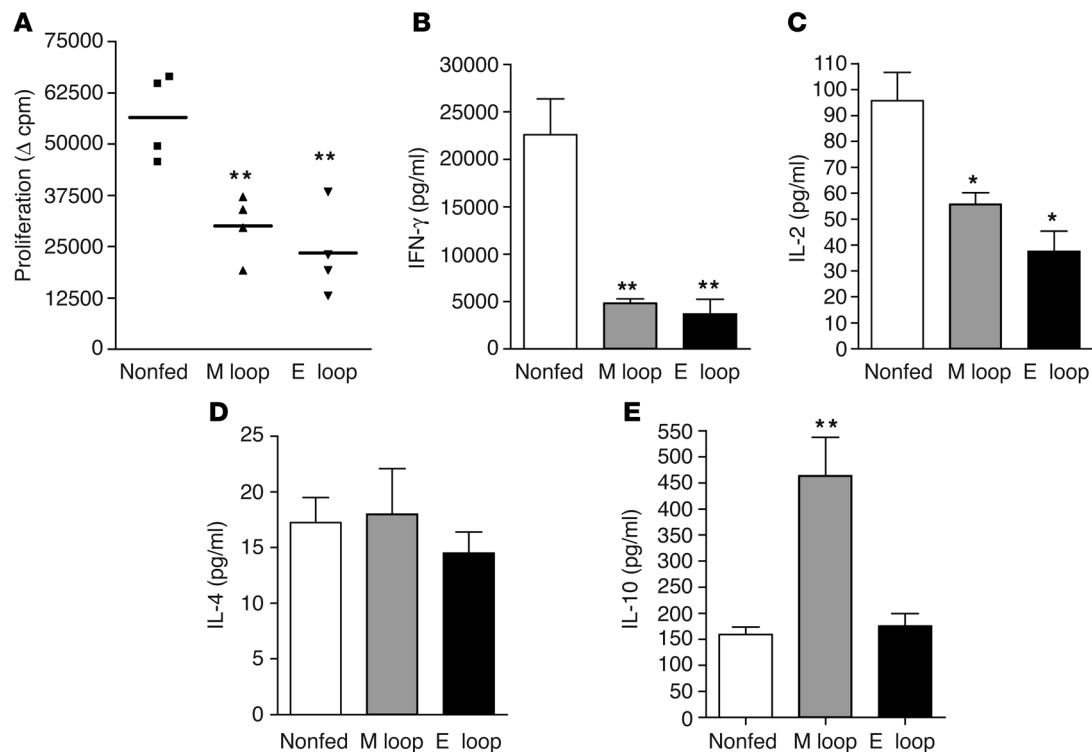
Several lines of evidence support the fact that the mucosal barrier was intact in both E and M loops. First, by electron microscopy, we demonstrated that the tight junctions were intact. Second, the administration of intact OVA into the loops failed to elicit any form of immune response. Moreover, H&E staining showed normal mucosa free of inflammation. Last, barrier integrity of the loops, as assayed by Ussing chamber studies, was comparable to that seen in intact bowel. Thus, taken together, these data support, but do not unequivocally prove, a direct role for the IEC in the sampling of Ag involved in the regulation of mucosal immune responses.

Lymphoid aggregates are occasionally seen in the small intestine mucosa. Although their function is unknown, it is possible that they are sites of luminal Ag presentation to T cells. Recent studies by Lorenz and colleagues have suggested that there is a compensatory increase in lymphoid nodules in  $LT\beta R$ -Ig fusion protein-treated mice (36). These nodules are predominantly located in the terminal ileum and not in the more proximal segments of the

small intestine (R. Lorenz, personal communication). The loops in the mice described in this study were derived from the distal jejunum/proximal ileum and therefore would be less likely to contain such aggregates/nodules. However, to exclude the possibility that lymphoid aggregates were in our PP-deficient loops, we performed multiple (serial) sectioning of both E and M loops. In no section did we find evidence for lymphocyte clusters.

We also found no evidence for subepithelial DCs in any of these loops. Rescigno et al. have reported that DCs can reside below the basement membrane and extend processes between the epithelium (expressing tight junction proteins) to “sample” luminal Ags (27). Subepithelial DCs typically reside in the distal small bowel, and the loops in the present study were generated from the distal jejunum/proximal ileum. However, we still looked for the presence of such DCs using 2 different approaches. In neither case could we identify dendrites from DCs extending into the epithelium. These findings provide further support for a more primary role of IECs or M cells in Ag sampling. Indeed, previous studies by our laboratory as well as others (37, 38) have demonstrated that IECs could take up soluble Ag either in intact mice or in surgical loops via an endolysosomal pathway.

Other laboratories have also shown that some form of tolerance can be induced at mucosal sites free of M cells and PPs. Nasal tolerance (39), as well as vaginally induced tolerance (40), have been demonstrated in a murine system. In our model, the enhancement of



**Figure 6**

Low-dose tolerance to OVA-pep can be achieved in both E loops and M loops. Mice with small bowel loops were either not fed or fed 0.01 mg OVA-pep into E or M loops and then immunized with the same antigen in the footpads. Twelve days later, PLN cells were harvested and restimulated *in vitro*. Proliferation of PLN cells upon *in vitro* restimulation with OVA-pep (**A**) and cytokine production after 72 hours of incubation (**B–E**). Each data point represents an individual mouse, while each column represents the mean value of 4 mice, and error bars indicate SEM. \* $P < 0.05$ , \*\* $P < 0.01$  vs. nonfed.

tolerance to pepsin-digested OVA fragments in the M loops suggests that there might be multiple mechanisms, both M cell/PP-dependent and -independent, involved in oral tolerance.

Interestingly, we were not able to induce tolerance by administering whole OVA into the loops. This is in agreement with previously published data (30, 31) that report that direct administration of whole OVA into the jejunum or ileum cannot elicit tolerance, while OVA fragments or peptide administered similarly can tolerize.

In the present study, the cytokines analyzed that were significantly different in both E and M loop mice compared with nonfed mice were IFN- $\gamma$  and IL-2. The levels of IL-10 were only increased in mice fed low-dose OVA peptide via the E loop. The decreased levels of IFN- $\gamma$  and the moderately increased levels of IL-10 suggest that regulatory cells might be generated during tolerance induction. In support of this was the finding that low-dose tolerance could not be achieved in either E or M loops in IL-10<sup>-/-</sup> mice, while tolerance was achieved in IL-10<sup>-/-</sup> mice with high-dose OVA-pep. In contrast, neutralization of TGF- $\beta$  had no effect in this system. Thus, IL-10 appears to be a more critical cytokine in our model of induction of oral tolerance. Attempts to define the nature of the cells generated by administration of Ag into the loops were thwarted by the low yield of cells isolated from the loops. Further studies using *in situ* hybridization and immunohistochemistry should shed some light on the cytokines induced following Ag administration into the loops.

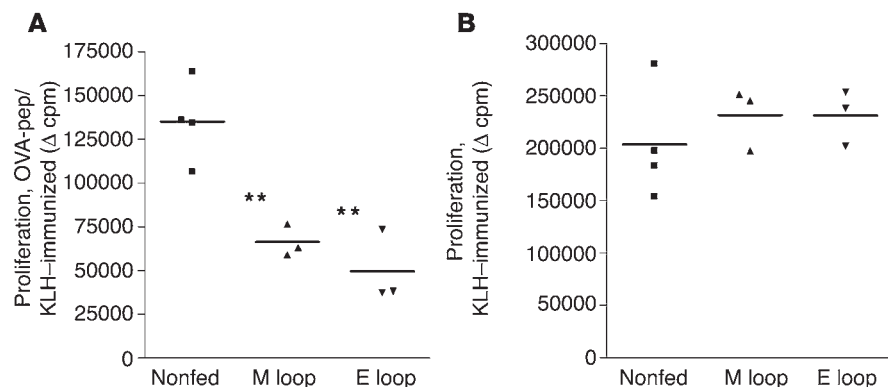
The fact that the absorptive area in the loop is much smaller than that in the intact small bowel (by approximately 10%) could influence the amount of Ag that is absorbed from the lumen. On

the other hand, as the gastric digestion processes are bypassed, it is very likely that much more immunologically intact Ag is in contact with the mucosa after loop administration than after gastric feeding. Importantly, we were able to demonstrate that OVA-pep administered into either E or M loops induced tolerance as well. This allowed for specific dose-ranging studies and the demonstration of both low- and high-dose tolerance in the loops. Although E and M loops are of the same size and both act as internal controls for absorption, it will be important for us to estimate the amount of protein in the circulation immediately following administration and compare it with that following gastric feeding.

Thus far we have not identified differences in tolerance induction generated via either E or M loops (other than an increase in IL-10 in E loop-tolerized mice). If in fact there are distinct mechanisms involved (e.g., direct presentation of Ag by IEC to lamina propria lymphocytes in E loops), we should be able to identify these using different transgenic and knockout mice (e.g., CD28<sup>-/-</sup>, CD2<sup>-/-</sup>, CD8<sup>-/-</sup>, CD4<sup>-/-</sup>, OVA TCR-transgenic mice, etc.). Such studies are currently in progress.

**Methods**

*Surgical procedure.* The studies were approved by the Mount Sinai Medical Center Institutional Animal Care and Use Committee. Eight week-old BALB/c mice (Jackson Laboratory) were used in this study. The mice were taken off solid food 18 hours before surgery. Under anesthesia, a 2-cm section of small intestine from approximately midway between the ileum and jejunum either with a PP (M loop) or without (E loop) was clamped and



**Figure 7** Bystander tolerance. Mice with small bowel loops were either fed or not fed 0.01 mg OVA-pep into loops with or without PP. The mice were then immunized in the footpads either with a mixture of OVA-pep and KLH (A) or with KLH alone (B). PLN cells were isolated 12 days later and restimulated in vitro with KLH. (A) KLH-specific proliferation of LN cells from mice immunized with OVA-pep and KLH together. (B) KLH-specific proliferation of LN cells from OVA-pep fed mice immunized with KLH alone. Proliferation was measured after 72 hours of incubation. Each data point represents an individual mouse. \*\**P* < 0.01 vs. nonfed.

excised from the intestine without disruption of the mesenteric attachments or vascularization. The remaining intestine was religated and released from clamps. The bypassed loop was closed at one end with surgical sutures; the other end was sutured to the abdominal wall, and a small opening (ostomy) was brought to the skin surface, which allowed access to the lumen of the loop. Oral feeding with mouse chow was resumed 2 days after surgery. Histological analysis of the loops confirmed the presence or absence of PPs. No inflammation or disruption of mucosal architecture was noted. The survival rate was 50–60% over the 3-week study and 90% after the first 3 days following surgery. Mice that failed to survive died of intestinal obstruction. The isolated loops appeared viable and well vascularized. The surviving mice were not malnourished nor did they show signs of inflammation or infection.

Similar studies were performed in IL-10<sup>-/-</sup> mice (Jackson Laboratories) and wild-type littermates (C57BL/6). These mice were operated on at 8 weeks of age. Since IL-10<sup>-/-</sup> mice housed at the Mount Sinai Animal Facility have been shown to develop colitis by 14 weeks of age, the studies were completed by 11 weeks of age. These mice showed no macroscopic signs of colitis (i.e., weight loss, diarrhea, ruffled fur) at the time of sacrifice, and histological examination of the colon and small intestine did not reveal any inflammation.

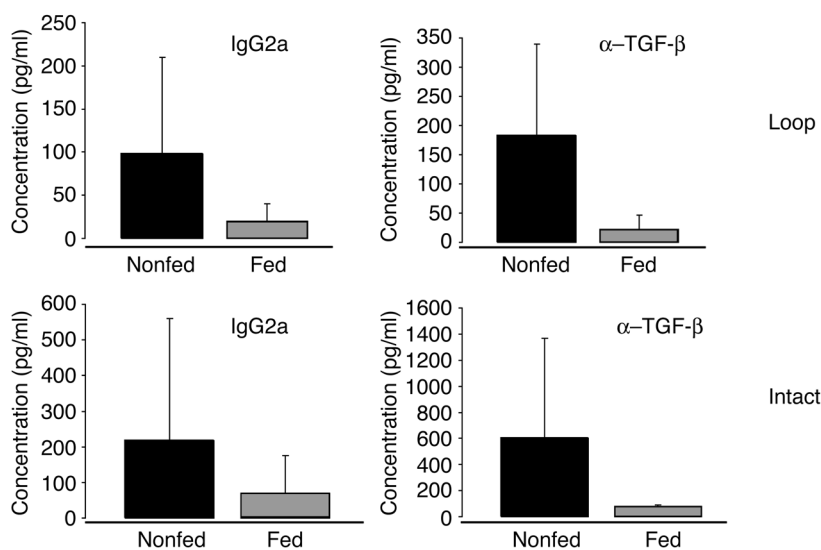
**Mucosal tolerance protocol.** All mice were rested for 10 days after surgery to allow for recovery. Tolerance induction was attempted with repeated administrations of OVA (Sigma-Aldrich). The Ag administration schedule was 1 mg whole OVA or 0.25 mg of 3–30 kDa fragments of pepsin-treated OVA either by gastric intubation or by administration of Ag using a flexible tube directly into the lumen of the loop for 5 consecutive days (days 1–5). On day 8, mice were immunized with 2 footpad injections of 100 μg OVA emulsified in complete Freund’s adjuvant (Sigma-Aldrich). On day 18, blood was collected from the retro-orbital plexus or tail vein, and the mice were sacrificed. Splens and

the PLNs were aseptically removed and teased into single-cell suspensions. Each mouse was analyzed separately.

In some experiments, OVA-pep was used as the Ag. OVA-pep was purchased from the peptide synthesis core at the Mount Sinai Medical Center. The purity was greater than 90%. Oral tolerance was induced as described above using 0.01–0.5 mg of peptide in 100 μl of PBS.

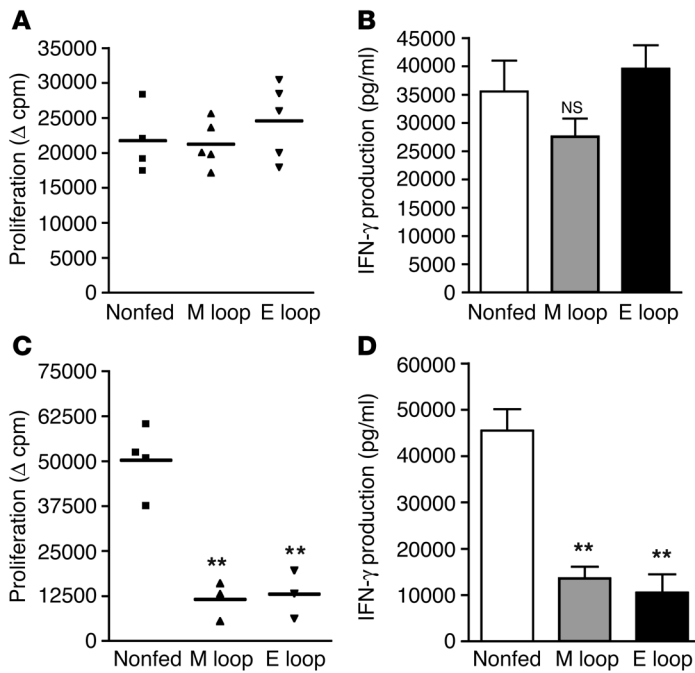
In a separate series of experiments, mice with E or M loops or without loops were treated with neutralizing anti-TGF-β mAbs (1 mg i.p. every other day for 10 days just prior to and during Ag administration; a kind gift of Ivan Fuss, NIH, Bethesda, Maryland, USA). This regimen had previously been shown to block TGF-β in vivo (41).

**Pepsin digestion of OVA.** OVA was resuspended in deionized water and adjusted to pH 2.0 with HCl. OVA was incubated with insoluble pepsin-agarose beads (Sigma-Aldrich) overnight at 37°C, and an aliquot was run on SDS-PAGE to determine efficiency of digestion. A smear centered at 8 kDa was seen after digestion. This is in agreement with previous studies that resolved pepsin-treated OVA fragments at 8 kDa and less than 2 kDa. The 8-kDa fragment, previously found to be tolerogenic if administered orally and immunogenic if injected with adjuvant (42), was purified by consecutive centrifugations through Centricon columns (Millipore) with membrane cutoffs of 3 kDa and 30 kDa. The purified fragment was dialyzed against PBS and sterile filtered, and the concentration was determined by optical absorbance (Spectronic 601; Milton Roy) at 280 nm.



**Figure 8** Low-dose tolerance is independent of TGF-β. Intact or loop mice were treated with either an anti-TGF-β mAb or an isotype-matched control for 1 day prior and every other day during OVA-pep administration (total of 5 mg over 10 days). This dosing regimen had previously been shown to neutralize TGF-β activity in vivo (24). Mice were immunized on day 11 as described above, and T cell proliferation (data not shown) and IFN-γ secretion by draining LN T cells were assessed 5 days later (as described for Figures 4–6). Neutralization of TGF-β failed to inhibit tolerance induction in either intact or loop (results for E loop are shown here) mice.





**Figure 9**  
IL-10 is necessary for the induction of low-dose tolerance both in the presence and absence of PP. IL-10<sup>-/-</sup> mice with small bowel loops were either not fed or fed a low dose (0.01 mg) (A and B) or high dose (0.5 mg) (C and D) of OVA-pep into E or M loops and then immunized in the footpads with OVA-pep in Freund's complete adjuvant. Twelve days after immunization, PLN cells were restimulated by OVA-pep in vitro, and proliferation and IFN-γ production were measured after 72 hours of incubation. (A and C) Proliferation of LN cells from IL-10<sup>-/-</sup> mice. (B and D) IFN-γ production from IL-10<sup>-/-</sup> mice. Each data point represents an individual mouse, while each column represents the mean of 4 mice. Error bars indicate SEM. As previously reported, low-dose tolerance cannot be achieved in intact IL10<sup>-/-</sup> mice fed orally (data not shown). \*\*P < 0.01.

**Serum anti-OVA antibody measurements.** Serum anti-OVA antibody levels were measured by ELISA. Serum was separated from blood collected from the tail vein or from the retro-orbital plexus at the time of sacrifice. Diluted serum samples were incubated for 1 hour on ELISA plates (Nalge Nunc) previously coated overnight at 4°C with 5 μg/ml OVA in 0.01 M carbonate buffer, pH 9.5. Plates were washed and incubated for 1 hour with 100 μl/well HRP-conjugated goat anti-mouse IgG (Roche Diagnostics Corp.) diluted to 0.2 μg/ml. A/B substrate (BD Biosciences – Pharmingen) was added, and colorimetric analysis was performed on an ELISA reader (Bio-Tek Instruments Inc.) at a wavelength of 650 nm. As controls, sera from nonimmunized mice (normal mouse sera) and OVA-immunized (by i.p. injection) mice were used at concentrations identical to those of the samples.

**In vitro analysis of T cell anti-OVA response.** PLNs were teased into single-cell suspensions and washed twice with PBS. Cells were then cultured at 2 × 10<sup>6</sup> cells/ml in RPMI 1640 supplemented with 10% FCS (Atlantic Biologicals), 2-mercapto-ethanol (5 × 10<sup>-5</sup> M), and 1% penicillin–1% streptomycin–glutamine (2 mM) (Invitrogen Corp.) and analyzed for Ag-specific cytokine release and T cell proliferation.

**Cytokine measurements.** Cells (2 × 10<sup>6</sup>/ml) were cultured in the presence or absence of OVA (10–1,000 μg/ml) for 96 hours, and supernatants were collected at 24-hour intervals. Culture supernatant was analyzed for the presence of IL-10, IL-4, IL-2, and IFN-γ by OptEIA ELISA kits (BD Biosciences – Pharmingen)

following the manufacturer's protocol. A standard curve was generated using recombinant cytokines, and concentrations of samples were determined by a polynomial curve fit analysis.

**T cell proliferation.** Cells from the PLN were cultured in the presence or absence of OVA (10–1000 μg/ml) for 72 hours, followed by a 16-hour pulse with 1 μCi [<sup>3</sup>H]thymidine (ICN Pharmaceuticals). Incorporated radioactivity was measured on a flatbed MicroBeta counter (Wallac). As a positive control for T cell proliferative capacity, cells were stimulated with Concanavalin A (Sigma-Aldrich) at 1 μg/ml.

**Bystander suppression.** In order to assess induction of bystander suppression in loop mice, we administered 0.01 mg OVA-pep into the loops as described above and immunized mice in the footpads and the flank with 50 μl of either KLH (Sigma-Aldrich) alone or a mixture of KLH and OVA-pep, both at a protein concentration of 4 mg/ml and emulsified 1:1 in complete Freund's adjuvant. KLH and OVA-pep-specific T cell proliferation was measured by culturing cells from the PLN in the presence or absence of KLH or OVA (10–100 μg/ml), respectively, for 72 hours, followed by a 16-hour pulse with 1 μCi [<sup>3</sup>H]thymidine (ICN). Incorporated radioactivity was measured as described above.

**Immunohistochemistry.** Upon sacrifice, bowel loops were removed and immediately fixed in 10% formalin or snap-frozen for immunohistochemical analysis. For analysis of loop architecture and inflammatory infiltrates, sections were stained and counter-stained with H&E.

**Electron microscopy.** Small (1 mm<sup>3</sup>) blocks of tissue were fixed in 3% formaldehyde plus 2% glutaraldehyde in PBS, pH 7.4, for 2 hours at room temperature. After fixation, the tissue was washed 3 times in PBS and postfixed in 1% OsO<sub>4</sub> in PBS, pH 7.4, for 60 minutes. The tissue was then washed 3 times in PBS, en bloc stained in 1% aqueous uranyl acetate for 2.5 hours, and subsequently dehydrated through a graded ethanol series (50%, 70%, 95%, 100%, and 100%; 10 minutes for each step). After dehydration, the samples were placed in a 2:5 mixture of ethanol/propylene oxide followed by a change through propylene oxide. The tissue was infiltrated overnight (on a rotator) with EMbed 812 (Electron Microscopy Sciences). The tissue was embedded in EMbed 812 and polymerized for 24 hours at 68°C. Eighty-nanometer sections were cut (using a Reichert Ultracut E ultramicrotome), collected on 200 mesh copper grids, and stained with uranyl acetate (15% saturated in 30% ethanol) for 10 minutes followed by Reynolds lead citrate for 1.5 minutes. Sections were viewed and photographed with a Hitachi H7000 transmission electron microscope operated at 75 kV.

**Deconvolution microscopy.** CD11c-GFP transgenic mice (28) and were the kind gift of Akiko Iwasaka (Yale University, New Haven, Connecticut, USA). Jejunal segments were isolated, fixed in formalin, sectioned following embedding in paraffin (6-μm sections), and stained with DAPI. In some experiments, isolated loops were snap-frozen in Tissuetek OCT (Bayer Corp.), sectioned (4-μm sections), and stained with anti-CD11c FITC. The images were captured using an Olympus BX61WI work station with a motorized XY stage allowing lateral movement between the X and Y positions and a Z focusing drive to allow the focal plane to be rapidly changed. The microscope was equipped with a Sutter Lambda DG-4 high-speed wavelength changer (Sutter Instrument Co.) and 175-W xenon light source with excitation filters (360 nm, 480 nm, and 590 nm) matched to a triple-band filter (DAPI, FITC, and Cy5) and individual filter sets for DAPI, FITC, Cy3, TexasRed, and Cy5 in the body of the microscope. The image was collected using a ×60 objective (NA1.4) and Coolsnap camera (Roper Inc.) (1,392 × 1,040 pixels) and analyzed by deconvolution microscopy using Slidebook 4.0 (Intelligent Imaging Innovations Inc.).



*Assessment of intestinal permeability (barrier function) in loops.* Techniques assessing gastrointestinal function through measurement of ion flux have been previously described (43). The intestine was mounted in modified Ussing chambers (Physiologic Instruments Inc.) with 2.0 mm (0.031/cm<sup>2</sup>) of the mucosal and serosal surfaces exposed to 4 ml oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) modified Krebs buffer maintained at 37°C with a circulating water bath (Fischer Scientific International). The buffer contained 140 mM Na, 119.8 mM Cl, 25 mM HCO<sub>3</sub>, 12 mM Mg, 1.2 mM Ca, 4.8 mM K, 2.4 mM HPO<sub>4</sub>, and 0.4 mM H<sub>2</sub>PO<sub>4</sub>, pH 7.4. Mannitol (10 mM) was added to the mucosal side, and glucose (10 mM) was added to the serosal side of the tissue for osmotic balance. Following tissue equilibration (20–30 minutes), passive ion transport, or transmural resistance ( $\Omega \times \text{cm}^2$ ), was measured as an indicator of barrier function.

*Statistical analyses.* In all experiments, each mouse was analyzed separately. In the figures showing results for oral tolerance/T cell proliferation analyses, data points represent values for individual mice; for cytokine and serum dilution analyses, values from mice within the same group were averaged, and error bars represent the SEM. *P* values represent the probability associated with the 2-tailed Student's *t* test. *P* < 0.05 was considered statistically significant.

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