Suppression of Th1 and Th17, but not Th2, responses in a CD8⁺ T cell-mediated model of oral tolerance

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The role of CD8⁺ T cells in oral tolerance remains unclear. To address this, we developed a model to induce CD8⁺ Tregs by feeding the major histocompatibility complex class I immunodominant epitope of OVA, OVA_(257–264). OVA_(257–264) feeding induced tolerance similar to that observed in OVA protein-fed mice, capable of suppressing the production of Th1 and Th17 cytokines and inhibiting a Th1-driven delayed-type hypersensitivity response following immunization with whole OVA (wOVA) protein. OVA_(257–264) peptide-induced suppression could be transferred to naive mice with CD8⁺ cells, but not CD8-depleted cells, isolated from mesenteric lymph nodes of peptide-fed mice. Interestingly, while capable of inhibiting Th1 and Th17 responses, OVA_(257–264) feeding could not suppress any feature of a Th2 inflammatory response, though OVA protein feeding could, suggesting that these cells function through a different mechanism than their CD4⁺ counterparts generated in response to feeding with wOVA. Thus, CD8⁺ T cells are functionally capable of mediating tolerance to Th1 and Th17 responses.

INTRODUCTION

Tolerance to ingested antigens is an essential feature required for normal homeostasis and maintenance of barrier function in the gastrointestinal tract. Breakdown of tolerance is associated with the development of chronic inflammatory diseases, such as Crohn's disease and ulcerative colitis, as well as food allergies and celiac disease.¹ Although the specific mechanisms involved in the generation and maintenance of oral tolerance (OT) are poorly understood, numerous studies have suggested that it is mediated by a combination of clonal deletion, clonal anergy, and active suppression by regulatory T cells (reviewed in refs. 1–5). High doses of antigen are believed to induce clonal deletion and/or anergy of responding T cells,^{6–8} whereas multiple feedings with low doses of antigen stimulate active suppression of immune responses through the generation of regulatory T cells.^{1,3–5,9,10} Although high-dose OT is obviously important for normal gut homeostasis, low-dose OT is of particular therapeutic interest because it offers a simple, non-invasive method for generating specific regulatory cells for the treatment of autoimmune and chronic inflammatory diseases.

Early OT studies focused on CD8⁺ T suppressor cells as primary mediators of suppression (reviewed in ref. 3). Feeding

with low doses of myelin basic protein resulted in the generation of CD8⁺ regulatory T cells that suppressed the induction of experimental autoimmune encephalitis in both mice and rats through the production of transforming growth factor- β .^{3,9,11,12} Bland and Warren¹³ described a CD8⁺ suppressor T-cell population that obtained regulatory properties after co-culture with intestinal epithelial cells (IECs). CD8⁺ Tregs generated in low-dose OT models have been shown to inhibit CTL activity, suppress cytokine production, and alter antibody responses in the gut.^{3,11,14–16} Previous work from our lab identified a population of human regulatory CD8⁺ T cells, activated by co-culture with IECs, capable of suppressing through a contact-dependent mechanism.^{16,17} Defects in this cell population were observed in patients with inflammatory bowel disease, and correlated with a failure to induce OT in these patients, suggesting that they could have an important role in tolerance in the gut.¹⁸ We have also developed a murine model in which tolerance was induced by antigen uptake through IECs rather than through Peyer's patches or interdigitating dendritic cells,¹⁹ supporting a role for IECs in the induction of tolerance. As our own *in vitro* studies, as well as previous work by Bland and Warren,¹³ show that IECs can activate CD8⁺ T cells with regulatory activity, these findings

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can be interpreted to further support a role for CD8⁺ Tregs in OT. On the other hand, studies utilizing CD8⁺ T-cell-deficient mice have shown that they are not essential for the generation of low-dose OT, but that there is an absolute requirement for CD4⁺ T cells.^{9,20–24} As such, recent studies have focused on the generation and function of CD4⁺ regulatory T cells in tolerance, without taking into account the possible significant contributions of CD8⁺ T cells.

Though it has been established that CD8⁺ T cells are not required for the induction of OT in animal models, there is sufficient evidence to suggest that they can mediate tolerance effectively and that they could have an important role in tolerance in a normal setting. Studies using knockout and transgenic mice provide adequate information about the absolute requirement of cells in a particular immune response, but the developmental artifacts often associated with such mice preclude their use to address the relative contribution and subtle interactions of T-cell subsets in the development of tolerance with respect to an intact immune system. To this end, we have developed a novel model system in which CD8⁺ Tregs can be generated independently of CD4⁺ Tregs by antigen feeding in a normal, unaltered background. Using this model, we have now shown that CD8⁺ T cells are capable of mediating OT independently of their CD4⁺ counterparts.

RESULTS

SIINFEKL feeding induces OT comparable with feeding with whole OVA protein

To investigate the role of CD8⁺ T cells in tolerance in an immunologically intact mouse, we fed mice the major histocom-

patibility complex (MHC) class I immunodominant epitope of OVA, OVA_(257–264) (herein referred to by its amino-acid sequence, SIINFEKL), for 5 consecutive days (**Figure 1a**). Peptide purity was shown by high-performance liquid chromatography, and by the inability of SIINFEKL to induce proliferation of OT-II cells (specific for the OVA_(323–339) peptide) (data not shown). Mice were fed whole OVA (wOVA) or phosphate-buffered saline (PBS) as positive or negative controls for tolerance, respectively. To assess the establishment of OT, mice were immunized with wOVA in complete Freund's adjuvant (CFA) and draining lymph node (dLN) cells and splenocytes were subjected to *in vitro* restimulation with wOVA. dLN cells from PBS-fed, OVA-immunized mice produced substantial levels of inflammatory cytokines (interleukin (IL)-2, interferon (IFN)γ, and IL-17a) following *in vitro* restimulation with wOVA (**Figures 2 and 3**). Mice fed wOVA before immunization showed significantly reduced IFNγ, IL-17a ($P < 0.01$), and IL-2 ($P < 0.05$) production at all time points assayed (**Figures 2 and 3**). Cells isolated from mice fed SIINFEKL produced significantly less IFNγ and IL-17a at both 48 and 72 h ($P < 0.01$), as well as, diminished IL-2 at 48 h following *in vitro* restimulation with wOVA, compared with PBS-fed mice (**Figure 2**). A similar result was obtained when mice were both fed and immunized with SIINFEKL, although the overall cytokine response was substantially reduced in response to immunization with peptide rather than with whole protein (**Supplementary Figure S1** online); IL-17a levels were not determined. The inhibition of inflammatory cytokine production in dLN cell cultures from mice fed SIINFEKL closely modeled that observed in cultures

Figure 1 Oral tolerance model. (a) Mice were fed five consecutive doses of antigen, followed 3 days later (day 8) by immunization with wOVA or peptide emulsified in CFA. On day 20, serum and fecal pellets were collected for antibody analysis, and the mice were killed; dLNs were isolated and subjected to *in vitro* restimulation with wOVA. Delayed-type hypersensitivity model. (b) Mice were fed five consecutive doses of antigen, followed 3 days later (day 8) by immunization with wOVA or peptide emulsified in CFA. On day 20, the mice were injected ID with OVA in the right ear and PBS in the left ear. Ear thickness was measured both 24 and 48 h after injection. On day 22, the mice were killed; the ears were collected and fixed in formalin for histological analysis, and dLNs were isolated and subjected to *in vitro* restimulation with wOVA. Allergic lung inflammation model. (c) Mice were fed five consecutive doses of antigen, followed 3 days later (day 8) by sensitization with wOVA in alum. Beginning on day 22, the mice received five consecutive daily intranasal doses of OVA in saline. Twenty-four hours after the final dose, serum and fecal pellets were collected for antibody analysis, and the mice were killed. BAL washes were performed, and lung tissue was obtained for histological analysis. BAL, bronchoalveolar lavage; dLN, draining lymph node; ID, intradermal; PBS, phosphate-buffered saline; wOVA, whole OVA.

Figure 2 SIINFEKL feeding induces tolerance comparable to feeding with whole OVA. C57BL/6 mice were fed PBS ($n=31$), SIINFEKL ($n=33$), or wOVA ($n=31$) followed by immunization with wOVA in CFA. dLN cells were isolated and restimulated with 0, 10, or 100 $\mu\text{g ml}^{-1}$ wOVA for 48 or 72 h. IFN γ , IL-2, and IL-17a protein levels in culture supernatants were determined by ELISA at the indicated time points; data are reported in $\text{pg ml}^{-1} \pm \text{s.e.}$ * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$. The results are the combined data from seven independent experiments. dLN, draining lymph node; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; wOVA, whole OVA.

derived from mice fed wOVA protein (**Figure 2**). To confirm the ability of CD4 $^{+}$ T cells to function independently of CD8 $^{+}$ T cells in the development of tolerance in a normal immune system, mice were also fed OVA_(323–339), the MHC class II immunodominant epitope of OVA. Similar to tolerance induced in response to SIINFEKL feeding, dLN cells isolated from mice fed OVA_(323–339) produced lower levels of IFN γ , IL-17a, and IL-2 following *in vitro* restimulation with wOVA (**Figure 3**). IL-4, IL-10, IL-13, and transforming growth factor- β were undetectable by enzyme-linked immunosorbent assay at all time points assayed (data not shown). *In vitro* restimulation of splenocyte cultures resulted in a greatly attenuated response compared with that of dLN cells, making comparisons between feeding groups impossible (data not shown).

To show that the response to SIINFEKL feeding was mediated by CD8 $^{+}$ T cells, we first utilized CD8 $^{-/-}$ mice. It has been previously established that CD8 $^{+}$ T cells are not required for tolerance to protein antigens in animal models.^{9,20–24} Our results support these previous findings, as wOVA-fed CD8 $^{-/-}$ mice produced significantly reduced levels of IL-2 at 48 h, and IFN γ and IL-17a at 48 and 72 h, following *in vitro* restimulation with wOVA, compared with PBS-fed CD8 $^{-/-}$ mice (**Figure 4**). dLN cultures from SIINFEKL-fed CD8 $^{-/-}$ mice produced similar

levels of IL-2 at 48 h, and nearly identical levels of IFN γ and IL-17a at 48 and 72 h, compared with PBS-fed mice (**Figure 4**).

To confirm the development of a regulatory CD8 $^{+}$ T-cell population in response to SIINFEKL feeding, CD8 $^{+}$ cells were isolated from the mesenteric lymph nodes (MLNs) of SIINFEKL-fed mice on day 5 of peptide feeding and transferred intravenously into naive mice, rendering recipient mice tolerant to subsequent immunization with wOVA protein (**Figure 5**). dLN cells from naive recipients of SIINFEKL-fed CD8 $^{+}$ MLN cells produced significantly lower levels of IFN γ at 48 and 72 h and IL-17a at 72 h compared with recipients of CD8 $^{+}$ cells from OVA-fed mice (**Figure 5a**). IL-17a was not detected in 48 h cultures. A similar trend was observed when comparing SIINFEKL-fed and PBS-fed CD8 $^{+}$ -recipient mice, although this did not reach statistical significance. Reduced cytokine production was not observed when CD8-depleted MLN cells were transferred from SIINFEKL-fed mice; however, tolerance to wOVA could be transferred with CD8-depleted MLNs from OVA-fed mice compared with PBS- and SIINFEKL-fed cell transfer recipients (**Figure 5b**). Therefore, CD8 $^{+}$ T cells are required for SIINFEKL-mediated, but not OVA-mediated, tolerance.

These data clearly show that tolerance can be induced in wild-type mice by feeding OVA-derived peptides at a level

Figure 3 Feeding with OVA₍₃₂₃₋₃₃₉₎ induces tolerance comparable to feeding with wOVA. C57BL/6 mice were fed PBS ($n=12$), OVA₍₃₂₃₋₃₃₉₎ ($n=11$), or wOVA ($n=12$) followed by immunization with wOVA in CFA. dLN cells were isolated and restimulated with 0, 10, or 100 $\mu\text{g ml}^{-1}$ wOVA for 48 or 72 h. IFN- γ , IL-2, and IL-17a protein levels in culture supernatants were determined by ELISA at the indicated time points; data are reported in $\text{pg ml}^{-1} \pm \text{s.e.}$ * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$. The results are the combined data from three independent experiments. dLN, draining lymph node; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; wOVA, whole OVA.

comparable with that observed in mice fed the intact protein. They suggest that tolerance occurs through the induction of peptide-specific regulatory cells because feeding with either SIINFEKL or OVA₍₃₂₃₋₃₃₉₎ suppresses the response to other OVA epitopes presented following systemic immunization with wOVA. As (1) SIINFEKL binds only to MHC class I and is presented only to CD8⁺ T cells, (2) tolerance is not observed in CD8^{-/-} mice, and (3) tolerance can be transferred to naive mice with CD8⁺ cells, but not CD8-depleted cells, isolated from SIINFEKL-fed mice, these data strongly suggest that SIINFEKL feeding induces CD8⁺ T cells with regulatory activity that are effective at mediating OT, independently of the induction of CD4⁺ Tregs.

Systemic antibody levels remained unaffected by the induction of OT using SIINFEKL-fed wild-type mice. Reproducible differences were not observed when comparing total, IgG1, or IgG2c OVA-specific antibody levels in serum from PBS- or antigen-fed mice (**Figure 6a**). The ability of OT to effectively modulate B-cell responses has been controversial. Some studies have shown reduced antibody production in response to feeding, whereas others have reported a limited effect on serum antibody levels; thus, these findings are not surprising and are consistent with previously published observations (reviewed in ref. ⁵). On the other hand, local IgA production was enhanced in antigen-fed

mice compared with mice fed PBS (**Figure 6b**). Significantly higher levels of total IgA were observed in fecal extracts from wOVA-fed mice compared with those from PBS-fed mice. Increases were also observed in SIINFEKL- and OVA₍₃₂₃₋₃₃₉₎-fed mice, but did not reach statistical significance. Though the relationship between the induction of OT and mucosal IgA production remains unclear,²⁵ these data support previously published reports showing increased mucosal IgA production in conjunction with suppression of systemic responses following antigen feeding.^{5,26,27} Further study is required to clarify the importance of SIgA with respect to tolerance induction.

SIINFEKL-induced regulatory cells can suppress Th1-mediated inflammation *in vivo*

To determine the ability of SIINFEKL-induced regulatory cells to suppress inflammation *in vivo*, we employed a mouse model of delayed-type hypersensitivity (DTH) (**Figure 1b**). Twelve days after immunization, antigen-fed mice had wOVA injected into the right ear, whereas PBS was injected into the left ear as a control. The induction of a DTH response was shown by an increase in ear thickness in the right ear (OVA) as compared with that in the left ear (PBS). Twenty-four hours after injection of wOVA, both SIINFEKL- and OVA-fed mice had significantly reduced ear swelling compared with PBS-fed mice ($P < 0.05$ and

Figure 4 SIINFEKL-induced tolerance requires the presence of CD8⁺ T cells. CD8^{-/-} mice were fed PBS, SIINFEKL, or wOVA followed by immunization and boost with wOVA. dLN cells were isolated and restimulated with 0, 10, or 100 μg ml⁻¹ wOVA for 48 or 72 h. IFN γ , IL-2, and IL-17a protein levels in culture supernatants were determined by ELISA at the indicated time points; data are reported in pg ml⁻¹ $\pm$ s.e. * P < 0.05, ** P < 0.01, † P < 0.001. The results are representative of five experiments (n = 5 mice/group). dLN, draining lymph node; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; wOVA, whole OVA.

P < 0.001, respectively), though wOVA-fed mice had a more pronounced reduction (Figure 7a). A similar trend was observed at 48 h, although the difference did not reach statistical significance when comparing SIINFEKL-fed and PBS-fed mice (Figure 7a). When ear swelling was monitored over the course of 4 days, at no time did swelling in SIINFEKL- or wOVA-fed mice reach the level observed in PBS-fed mice (data not shown). Reduced ear swelling was observed following the induction of a DTH response in transfer recipients of CD8⁺ MLN cells from SIINFEKL-fed mice, compared with recipients of CD8⁺ MLN cells from PBS-fed or OVA-fed mice (Figure 7b). Ear swelling was not reduced when CD8⁺-depleted MLN cells were transferred from SIINFEKL-fed mice, but was observed when cells were transferred from OVA-fed mice, compared with PBS-fed recipients (Figure 7c). Thus, SIINFEKL-mediated suppression of inflammation is mediated by CD8⁺ cells. In most experiments, mice were killed at 48 h and the ears and popliteal LNs (draining the site of primary immunization) were isolated. The presence of inflammatory cells in the ears was confirmed histologically by hematoxylin and eosin staining, with a clear reduction in cell infiltrates observed in OVA-fed mice and a modest reduction observed in SIINFEKL-fed mice compared with PBS-fed mice (data not shown). Tolerance was also confirmed by the reduced production of IFN γ and IL-17a following *in vitro* restimulation of popliteal LN cells as described in Figure 2 (data not shown).

SIINFEKL-induced regulatory cells do not suppress Th2-driven inflammation

Having shown that SIINFEKL feeding could suppress Th1 and Th17 cytokine production *in vitro* and inhibit Th1-driven inflammation *in vivo*, we next assessed its ability to suppress Th2-mediated inflammation using a mouse model of allergic lung inflammation. Allergic lung inflammation was induced in antigen-fed mice by sensitizing with 10 μg wOVA in alum on day 8 followed 14 days later by five consecutive daily intranasal instillations of wOVA in saline (Figure 1c). Control mice received wOVA intranasally in the absence of sensitization. Contrary to our Th1-driven DTH results, SIINFEKL-fed mice (7 out of 10) had a robust inflammatory response (inflammatory score = 2.65 $\pm$ 0.95) comparable to PBS-fed mice (inflammatory score = 3.38 $\pm$ 0.55), consisting of eosinophil-rich infiltrates in the peribronchiolar and perivascular regions of the lung, hypertrophy of the bronchiolar epithelium, and plugging of some airways (Figure 8a and Supplementary Figure S2a online). By contrast, OVA-fed mice had much fewer and smaller infiltrates, involving less of the total lung tissue, and no plugging of the airways (inflammatory score = 1.85 $\pm$ 0.66, P < 0.05 vs. PBS- and SIINFEKL-fed mice) (Figure 8a and Supplementary Figure S2a online). Correspondingly, there were significantly fewer inflammatory cells in bronchoalveolar lavage (BAL) washes derived from OVA-fed mice, which were themselves comprised

Figure 5 Tolerance can be transferred to naive mice with CD8⁺ cells from SIINFEKL-fed mice, but not with CD8-depleted cells. CD8⁺ (5a) and CD8-depleted cells (5b) were isolated 3h after the final feed from C57BL/6 mice fed PBS, SIINFEKL, or wOVA for 5 consecutive days. Naive mice received 2×10^5 CD8⁺ cells (PBS $n=8$, SIIN $n=10$, and OVA $n=10$) or 1×10^6 CD8-depleted cells (PBS $n=7$, SIIN $n=5$, and OVA $n=10$) IV by retro-orbital injection and were immunized 24h later in the hindleg footpad with wOVA in CFA. dLN cells were isolated and restimulated with 0, 10, or $100 \mu\text{g ml}^{-1}$ wOVA for 48 or 72h. IFN γ and IL-17a protein levels in culture supernatants were determined by ELISA at the indicated time points; data are reported in $\text{pg ml}^{-1} \pm \text{s.e.}$ ** $P < 0.01$, * $P < 0.001$. The results are the combined data from two independent experiments. dLN, draining lymph node; ELISA, enzyme-linked immunosorbent assay; IV, intravenous; PBS, phosphate-buffered saline.

of a significantly smaller percentage of eosinophils, compared with PBS- and SIINFEKL-fed mice ($P < 0.01$) (Figure 8b). Mucus production was assessed by periodic acid-Schiff staining. Positive staining was generally limited to larger airways, and substantial differences in staining were not observed between feeding groups (Supplementary Figure S2b online); however, staining was much more prominent in sensitized mice compared with that in non-allergic controls (Supplementary Figure S2b online). A trend toward diminished IL-4 levels was observed in BAL fluids from OVA-fed mice as compared with PBS- and SIINFEKL-fed mice, whereas IL-10 levels were increased (Figure 8c). IL-13 was not detected in any group of mice (data not shown), and, interestingly, IL-5 was elevated only in BAL fluids derived from SIINFEKL-fed mice (Figure 8c). Finally, the level of OVA-specific total and IgG1 antibodies, and total IgE in serum as well as OVA-specific IgG1 antibody in BAL fluids was significantly reduced in OVA-fed mice compared with PBS- and SIINFEKL-fed mice (Figure 8d).

Though effective at suppressing Th1 and Th17 cytokine production and inhibiting Th1-induced inflammation *in vivo*, SIINFEKL feeding was incapable of modulating Th2-driven

inflammation. On the other hand, a marked decrease in inflammatory cells was observed in the lungs of OVA-fed mice, though the response was not completely ablated. As wOVA feeding likely induces the generation of CD4⁺ Tregs, this could suggest that SIINFEKL-induced CD8⁺ cells function through a different regulatory mechanism.

SIINFEKL-specific cells are not deleted following feeding with SIINFEKL or wOVA

Though the specific mechanisms involved in the induction and maintenance of OT are poorly understood, it is generally accepted that high doses of antigen induce tolerance through anergy and clonal deletion, whereas low doses induce active suppression through the development of regulatory cells. Nevertheless, it is unlikely that these mechanisms are exclusive of one another, and it remains unclear as to what constitutes a high-dose vs. a low-dose for any given antigen. One milligram of wOVA is a low dose for OT induction specifically because of its ability to induce OVA-specific regulatory cells, rather than deletion/anergy. Our current studies were conducted using molar equivalents of SIINFEKL to induce tolerance; a dose-response using a wide

Figure 6 Fecal IgA is elevated in antigen-fed mice, whereas serum antibody levels remain unchanged. Blood and fecal pellets were collected from C57BL/6 mice fed PBS, SIINFEKL, OVA₍₃₂₃₋₃₃₉₎, or wOVA followed by immunization and boost with wOVA at the time of killing. (a) Serum total, IgG1 and IgG2a OVA-specific antibody titers were determined by ELISA. Data are reported as 50% reciprocal maximum binding titer. The results are representative of three experiments ($n=5$ mice/group). (b) Total IgA in fecal extracts was determined by ELISA. An IgA myeloma protein was incorporated on each plate as a standard; data are reported as ng of fecal IgA/mg of fecal pellet weight. * $P<0.05$. The results were compiled from five independent experiments ($n=3-5$ mice/group/experiment), each symbol represents a single mouse. ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; wOVA, whole OVA.

range of SIINFEKL concentrations (0.0217–217.0 μg) showed that both 2.17 and 21.7 μg induced a tolerogenic response comparable to feeding 1 mg of OVA (data not shown). To clarify

Figure 7 SIINFEKL feeding suppresses the induction of delayed-type hypersensitivity *in vivo*. (a) C57BL/6 mice were fed PBS ($n=15$), SIINFEKL ($n=15$), or wOVA ($n=15$) followed by immunization with wOVA in CFA. On day 15, 20 μg of wOVA was injected into the right ear of each mouse, whereas 20 μl of PBS was injected into the left ear. Ear thickness was measured at 24 and 48 h after ear injection. (b) Ear thickness was measured at 24 and 48 h after injection of OVA in recipient mice immunized with wOVA in CFA 24 h after IV injection of 2×10^5 CD8⁺ cells from PBS-fed ($n=8$), SIINFEKL-fed ($n=10$), or wOVA-fed ($n=10$) mice. (c) Ear thickness was measured at 24 and 48 h after injection of OVA in recipient mice immunized with wOVA in CFA 24 h after IV injection of 1×10^6 CD8-depleted cells from PBS-fed ($n=7$), SIINFEKL-fed ($n=5$), or wOVA-fed ($n=10$) mice. Data are reported as a change in ear thickness (thickness of the right ear (OVA) – thickness of the left ear (PBS)). Data are reported in mm $\pm$ s.e. * $P<0.05$, ** $P<0.01$, † $P<0.001$. The results are the combined data from three independent experiments. IV, intravenous; PBS, phosphate-buffered saline; wOVA, whole OVA.

the general mechanism of tolerance induced by SIINFEKL feeding, we determined the presence of SIINFEKL-specific cells in dLNs and spleens of mice after feeding and immunization, with flow cytometry using fluorescently labeled SIINFEKL-loaded H2-K^b pentamers. Splenocytes from OT-I Rag-1^{-/-} mice, which contain CD8⁺ T cells of singular specificity for SIINFEKL, were used as a positive control for staining, whereas cells from CD8^{-/-} mice were used as a negative control. Evaluation of dLN cells

(Table 1, Supplementary Figure S3 online) and splenocytes (data not shown) from PBS-, SIINFEKL-, or OVA-fed, OVA-immunized and -boosted mice revealed no significant differences in the numbers of total CD4⁺ or CD8⁺ T cells, or SIINFEKL-

specific CD8⁺ T cells in the different feeding groups. Limited numbers of antigen-specific cells were detected in both dLNs and spleens; however, this range was well within the expected value of antigen-specific cells for a normal, non-transgenic,

Table 1 SIINFEKL-positive cells following antigen feeding

Draining lymph node	Feeding group ^a	Total number of pentamer+ cells ^{a,b,c}	Percent CD8 ⁺ T cells ^{a,b,d}	Ratio of pentamer+ cells to total CD3 ⁺ cells ^{a,b,e}
Exp 1 ^f	PBS fed	46.4±14.3	12.3±2.5	725.1±216.4
	SIINFEKL fed	45.6±10.9	14.2±1.1	823.3±191.4
	OVA fed	37.4±7.4	16.3±2.76	1018.5±206.8
Exp 2 ^g	PBS fed	100.8±31.1	10.16±1.58	278.2±125.7
	SIINFEKL fed	91.6±25.3	9.64±2.20	274.2±55.1
	OVA fed	148.5±39.8	12.85±0.65	210.1±33.9

^aMean of five mice per group.^b100,000 cells were collected in the lymphocyte gate.^cNumber of CD8, pentamer double-positive cells in the lymphocyte gate after subtraction of CD19⁺ and CD49b⁺ cells±s.d.^dPercent CD3, CD8 double-positive cells in the lymphocyte gate.^eThe number of CD3⁺ cells in the lymphocyte gate divided by the total number of pentamer-positive cells±s.d.^fP=0.40, as determined by analysis of variance.^gP=0.12, as determined by analysis of variance.

antigen-immunized mouse. Positive staining was not observed in CD8^{-/-} mice, nor was it readily detectable in spleens from unimmunized C57BL/6 mice (data not shown). Similar numbers of SIINFEKL-specific T cells in SIINFEKL-, PBS-, and OVA-fed mice indicate that SIINFEKL feeding does not result in T-cell depletion at the concentrations used in this study, and further support the premise that CD8⁺ T cells are actively regulating the tolerogenic response to OVA in our system. As a whole, these data provide clear evidence for the ability of CD8⁺ T cells to regulate tolerance independently of CD4⁺ T-cell help in a normal immune setting.

DISCUSSION

The majority of studies investigating the regulation of OT have relied on the use of manipulated immune systems to study T-cell function. These systems include the use of knockout mice, where the absence of an entire lineage of cells during development often results in additional unintended impairments in immune function or the development of compensatory mechanisms that are not present in a normal setting. Other studies have employed antibodies to deplete entire cell lineages, or more commonly, transgenic mice with clonal populations of T cells that have singular specificity for the antigen (peptide) of interest. Although these systems are valuable for the gross assessment of immune function of specific cell types, artifacts inherent to them do not allow for an intricate understanding of the immune response as it relates to a normal immune system.

To circumvent the need for a manipulated immune system, we developed an OT model designed to induce the develop-

ment of a CD8⁺ Treg population, independently of CD4⁺ Tregs, in a normal immune system through the feeding of the MHC class I immunodominant epitope of OVA, SIINFEKL. Feeding with SIINFEKL peptide suppressed the production of pro-inflammatory cytokines (IFN γ , IL-17a, and IL-2) *in vitro* and inhibited Th1-driven DTH *in vivo* (Figures 2 and 6). These data suggest that this occurs through the induction of a CD8⁺ regulatory cell population. The OVA protein is comprised of several highly immunogenic epitopes in addition to SIINFEKL, most notably OVA₍₃₂₃₋₃₃₉₎, against which the majority of the CD4⁺ T-cell response to OVA is generated. If SIINFEKL feeding induced anergy or deletion of peptide-specific cells, the T-cell response to other OVA epitopes would continue unabated, resulting in a similar inflammation/cytokine production compared with PBS-fed mice. Clearly, neither anergy nor deletion was the primary mechanism of tolerance in this case, as the response to immunization with wOVA was significantly diminished, and staining with SIINFEKL-loaded H2-K^b pentamers clearly showed that SIINFEKL-specific cells were not deleted following peptide feeding (Table 1). Furthermore, SIINFEKL-induced tolerance required the presence of CD8⁺ T cells as peptide-induced tolerance could not be achieved in CD8^{-/-} mice. Finally, and most importantly, tolerance to SIINFEKL could be transferred to naive mice with CD8⁺ cells isolated from MLNs of SIINFEKL-fed mice, but not with CD8-depleted cells (Figures 5 and 7). In total, these data strongly suggest that peptide feeding establishes a CD8⁺ Treg population that actively suppresses the response to other immunogenic OVA epitopes (bystander suppression).

Figure 8 SIINFEKL feeding does not inhibit Th2-driven inflammation. C57BL/6 mice were fed PBS ($n=9$), SIINFEKL ($n=10$), or wOVA ($n=10$) followed by IP sensitization with wOVA in alum. Beginning on day 15, mice received 100 μ g wOVA in 50 μ l saline intranasally for 5 consecutive days. Non-allergic controls received OVA intranasally in the absence of sensitization ($n=9$). (a) Representative hematoxylin and eosin-stained lung sections from mice fed PBS, SIINFEKL, wOVA, or non-allergic controls ($\times 100$ magnification). Disease score was determined as described in Methods. (b) Differential staining of inflammatory cells isolated from the BAL fluids of antigen-fed mice and non-allergic controls. Data are reported as the percent of the total cell population. (c) IL-4, IL-5, and IL-10 levels in BAL washes were determined by ELISA. Data are reported as pg ml⁻¹±s.e. (d) OVA-specific Ab and total IgE levels were measured by ELISA in sera and BAL fluids. The data are reported as mean absorbance±s.e. * $P<0.05$, ** $P<0.01$, † $P<0.001$. The results are the combined data from two independent experiments. BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; wOVA, whole OVA.

CD8⁺ cells from SIINFEKL-fed mice were capable of suppressing inflammation *in vivo* in a Th1-driven DTH response; however, the level of suppression observed in SIINFEKL-fed mice was reduced compared with that observed in OVA-fed mice. This was anticipated as feeding with wOVA likely induced the generation of a mixture of regulatory cells of different specificities in conjunction with anergy and/or deletion of other OVA epitope-specific T-cell populations. SIINFEKL feeding would generate a regulatory cell population of singular specificity, likely with a single mechanism of action, which, comparatively, would have a more localized effect. Interestingly, our transfer of tolerance data suggest that feeding with wOVA does not induce the development of CD8⁺ regulatory cells, as transfer of CD8⁺ MLN cells from OVA-fed mice did not transfer tolerance, whereas transfer of CD8⁺-depleted cells did (most likely due to the presence of CD4⁺ Tregs). This was not unexpected; despite the fact that mice were administered molar equivalents of SIINFEKL and wOVA, the bioavailability of peptide derived through the digestion of whole protein vs. the bioavailability of freely administered peptide is likely to be different. The induction of low-dose OT is tightly controlled by antigen dose; thus, although a molar equivalent was used (compared with SIINFEKL peptide), the amount of SIINFEKL (or other CD8 peptides) derived from the wOVA protein for presentation could be outside of the range necessary to induce tolerance in CD8⁺ cells.

Of significant interest was the finding that SIINFEKL feeding did not suppress any aspect of a Th2-driven model of inflammation, whereas OVA feeding did substantially reduce (though it did not completely prevent the response) the recruitment of inflammatory cells to the lungs. One explanation is that the CD8⁺ Treg cell population generated in response to SIINFEKL feeding was not recruited to the site of inflammation and, therefore, was not capable of mediating suppression in this instance. However, it has been shown that previously activated CD8⁺ T cells can be switched to produce IL-5 in the presence of a Th2 microenvironment.²⁸ Therefore, it is possible that the SIINFEKL-specific cells are being recruited to the site of inflammation in our model, but are being switched to an IL-5-producing profile in response to the Th2 microenvironment. As a result, they would be unable to suppress the Th2 response. This hypothesis is supported by the increased level of IL-5 observed in BAL fluids from SIINFEKL-fed mice, as compared with all other groups. Nevertheless, if these cells were switched to an IL-5-producing phenotype, we would have expected an increased level of eosinophilia in the lungs of SIINFEKL-fed mice compared with that in other groups, which was not observed. We are currently designing experiments to investigate the specific method of suppression used by SIINFEKL-induced CD8⁺ Tregs, as well as, attempting to create a phenotypic profile of these cells (including chemokine receptors and homing molecules) to address their ability to be recruited to different sites of inflammation. It is important to note, however, that we do not believe that the suppression of IFN γ or IL-17a in our dLN cell cultures is mediated through the production of Th2 cytokines. We have never detected the production of Th2 cytokines (IL-4 and IL-13) at any time point in any of our dLN cell cultures (data not shown).

It is also an interesting finding that OVA_(323–339) feeding required a 10-fold lower molar concentration of peptide to induce OT compared with feeding with SIINFEKL. When mice were fed a molar equivalent of OVA_(323–339) (40.1 μ g rather than 4.01 μ g), tolerance could only be generated to subsequent immunization with OVA_(323–339) peptide, but not to wOVA (data not shown). This suggests the induction of high dose tolerance, i.e., the functional inactivation or deletion of OVA_(323–339)-responsive cells, rather than the generation of OVA_(323–339)-specific Tregs. High-dose OT was not observed with up to a 10-fold greater molar concentration of SIINFEKL peptide (217.0 μ g). These data may suggest that CD4⁺ T cells are more sensitive to the induction of tolerance than their CD8⁺ T-cell counterparts. Alternatively, and perhaps more likely, different sensitivities in the induction of CD4⁺ vs. CD8⁺ T-cell-mediated tolerance could be attributed to differences in the antigen-presenting cell population involved in the activation of each cell type following antigen feeding. Previous work from our laboratory has shown that CD8⁺ Tregs can be activated by co-culture with IECs. It is possible that IECs are having a primary role in the activation of CD8⁺ Tregs in our model, whereas CD4⁺ T-cell activation is reliant on conventional antigen-presenting cells, such as dendritic cells. In addition, it is possible that different subtypes of dendritic cells might also be involved, as recent studies have provided intriguing evidence into a role for plasmacytoid dendritic cells in the induction of CD8⁺ Tregs.^{29,30} We are currently designing experiments to assess the trafficking and uptake of our peptide antigens following feeding.

Historically, the goal of OT has been the inhibition of Th1-mediated immune responses, as these were believed to be the primary mediators of most autoimmune and chronic inflammatory diseases. More recently, however, a new inflammatory T-cell “lineage,” Th17, has emerged as a key protagonist in a number of inflammatory diseases, including multiple sclerosis, Crohn’s Disease, and rheumatoid arthritis.^{31,32} In fact, in experimental autoimmune encephalitis, an animal model of multiple sclerosis, members of the IL-17 family, IL-17a and IL-17f, have been recognized as the primary cytokines responsible for disease progression.³¹ The ability of myelin basic protein feeding to suppress the development of experimental autoimmune encephalitis in mice and rats^{3,11} suggests that OT is capable of suppressing the production of IL-17. However, to our knowledge, this has never been directly shown in a model of OT. Here, for the first time, we have documented that feeding soluble antigen (peptide or protein) suppresses the production of the pro-inflammatory cytokine IL-17a. IL-17a production is equally suppressed by both CD4⁺ and CD8⁺ T cells, as feeding with either OVA_(323–339) or SIINFEKL, respectively, resulted in significantly lower levels of IL-17a following *in vitro* restimulation. Thus, OT is capable of suppressing both Th1 and Th17 immune responses.

In summary, the data presented here suggest that we can induce the development of CD8⁺ Tregs by feeding the MHC class I immunodominant epitope of OVA, SIINFEKL. We have shown that CD8⁺ T cells are fully capable of mediating tolerance independently of their CD4⁺ counterparts in a normal immune

system. Although low-dose OT has been extensively studied, the specific mechanisms involved in its induction remain unclear. A complete understanding of these mechanisms is required for OT to be used effectively as therapy for chronic inflammatory/autoimmune diseases. By first establishing a functional understanding of CD8⁺ Tregs in OT, we can then identify how the function of these cells intersects with that of CD4⁺ Tregs, in turn, giving us a more complete picture of the function of regulatory T cells in mucosal tolerance.

METHODS

Animals. C57BL/6 mice, originally obtained through the NCI-Frederick Animal Production Program (NCI, Frederick, MD), and CD8^{-/-} mice, originally obtained from Jackson Laboratories (Bar Harbor, ME), were bred and housed in the SPF barrier facility at The Mount Sinai School of Medicine. The institutional animal care and use committee approved all procedures concerning the use of these mice.

OT model. OT was induced by feeding 1 mg of OVA (Sigma, St Louis, MO), 21.7 µg of OVA_(257–264) peptide (SIINFEKL), or 4.01 µg of OVA_(323–339) (Peptides International, Louisville, KY or Anaspec, Fremont, CA) in 250 µl of PBS for 5 consecutive days by intragastric intubation with a 20-gauge feeding needle (Figure 1a). Control mice received PBS alone. SIINFEKL is the MHC class I immunodominant epitope of the OVA protein presented in H2-K^b,³³ stimulating CD8⁺ T cells. Conversely, OVA_(323–339) is the MHC class II immunodominant epitope of OVA presented in both I-A^{d34} and I-A^b, stimulating CD4⁺ T cells. A dose-response was performed to obtain the tolerogenic doses for each peptide by feeding mice 0.01–10× the molar equivalent of 1 mg of wOVA, where 21.7 and 40.1 µg represent the molar equivalent dose of SIINFEKL and OVA_(323–339), respectively. Purity of the peptides was confirmed by high-performance liquid chromatography and mass spec analysis conducted by the manufacturer (Peptides International) (Anaspec). Mice were immunized 3 days after the final feeding (day 8) in both hindleg footpads with 100 µg of OVA or SIINFEKL emulsified in CFA (50 µg in 50 µl per footpad) (Pierce, Rockford, IL). Twelve days after primary immunization, the mice were bled and killed, and dLNs (popliteal and inguinal) and spleens were collected and teased into single-cell suspensions. Red blood cells were lysed with 0.8% NH₄Cl, and 2×10⁵ cells/well were cultured in 96-well U-bottom plates with 5 µg ml⁻¹ Concanavalin A (positive control) (Sigma), media alone (negative control), 10 µg ml⁻¹ OVA, or 100 µg ml⁻¹ OVA at 37°C. The response of individual mice was assayed in triplicate. Supernatants were collected at 24, 48, and 72 h and stored at -20°C.

Fecal pellets were collected, weighed, and stored in 1 ml of PBS containing 0.02% NaN₃. Fecal extracts were generated by vortexing for 5 min and centrifuging to remove solid waste. Serum was isolated by allowing blood to clot overnight at 4°C, followed by centrifugation, and stored at -20°C for antibody analysis.

Transfer of tolerance. MLNs were collected and pooled from antigen-fed mice 3 h after the final feed and teased into single-cell suspensions. CD8⁺ T cells were purified using a CD8⁺ cell magnetic bead isolation kit (Stem Cell Technologies, Vancouver, CA) according to the manufacturer's instructions. Cells were confirmed to be >95% pure by flow cytometry. In all, 2×10⁵ CD8⁺ cells were transferred intravenously through retro-orbital injection. Separate groups of mice received 1×10⁶ CD8⁺-depleted cells. Twenty-four hours after transfer, the mice were immunized and tolerance was assessed as described above.

DTH model. Antigen-fed mice were injected intradermal with 20 µg of OVA in 20 µl of PBS in the right ear and 20 µl of PBS in the left ear 12 days after feeding (Figure 1b). Ear thickness was measured under anesthesia at both 24 and 48 h, by carefully placing a digital caliper

(World Precision Instruments, Sarasota, FL) along the midline of the ear, over the site of injection. The amount of swelling was ascertained by subtracting the thickness of the left ear (vehicle) from the thickness of the right ear (antigen) (right ear thickness – left ear thickness). At 48 h, the mice were killed, the ears were removed and fixed in 10% formalin for histological analysis, and dLNs were isolated and restimulated *in vitro* as described above.

Allergic lung inflammation model. Allergic lung inflammation was induced as previously described³⁵ (Figure 1c). Briefly, antigen-fed mice, intraperitoneally sensitized with 10 µg OVA in alum (Reheis, Berkely Heights, NJ) on day 8, were anesthetized and challenged intranasally for 5 consecutive days (days 22–25) with 100 µg OVA in 50 µl normal saline. Twenty-four hours after the final intranasal dose, the mice were bled and killed. BAL was performed by washing the lungs twice with 500 µl of 5 mM EDTA in saline (Sigma). The BAL fluids were centrifuged and the numbers of viable cells were determined by trypan blue exclusion. BAL fluids were stored at -20°C, whereas the cells were mounted on slides for analysis. Lung tissue was fixed in formalin (Fisher Scientific, Pittsburgh, PA) for histological analysis. Formalin-fixed tissues were processed, sectioned, and hematoxylin and eosin stained by the Biorepository Cooperative and Histology Service Shared Resource Facility of the Mount Sinai School of Medicine. The presence of inflammatory cells was assessed using standard light microscopy at the MSSM-Microscopy Shared Research Facility and inflammation manifested as infiltrates in the perivascular and peribronchiolar regions of the lung. Stained slides were coded and blindly examined, and the relative degree of inflammation was graded in a semiquantitative manner by assigning a score of 0–4 based upon the number, location, and size of the inflammatory infiltrates (0, no visible lesion; 1, multifocal inflammation; 2, locally extensive inflammation; 3, diffuse inflammation; and 4, severe diffuse inflammation).

Enzyme-linked immunosorbent assay. Cytokine and antibody levels were determined by enzyme-linked immunosorbent assay. For OVA-specific antibody levels, 96-well plates were coated with 10 µg ml⁻¹ OVA overnight at 4°C. For fecal IgA and total IgE levels, plates were coated with 5 µg ml⁻¹ goat anti-mouse IgA or goat anti-mouse IgE overnight (Southern Biotech, Birmingham, AL) at 4°C. Plates were washed three times and blocked with 10% fetal bovine serum in PBS for 1 h at room temperature. Following another wash step, samples were serially diluted across the plate and incubated for 2 h at room temperature. After another wash step, the plates were incubated with optimized dilutions of horseradish peroxidase-linked goat anti-mouse total Ig or goat anti-mouse IgG1, or alkaline phosphatase-conjugated goat anti-mouse IgG2a, goat anti-mouse IgA, or goat anti-mouse IgE (Southern Biotech) for 1 h at room temperature. The plates were washed again and developed with TMB microwell peroxidase substrate (KPL, Gaithersburg, MD) or *p*-nitrophenyl phosphate (Sigma). Absorbance was measured using a µQuant Microplate Reader and KC4 software (Bio-tek Instruments, Winooski, VT). Absolute levels of fecal IgA were determined by inclusion of a mouse IgA myeloma protein (S107) (Southern Biotech) as a standard on each plate.

IL-2, IL-4, IL-10, IL-13, IFNγ (BD Biosciences, San Jose, CA), IL-17a (eBiosciences, San Diego, CA), and transforming growth factor-β levels (R&D Systems, Minneapolis, MN) were determined using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Flow cytometry. In all, 1×10⁶ cells were incubated with unlabeled SIINFEKL-loaded H2K^b pentamers (Proimmune, Oxford, UK) for 10 min at room temperature. The cells were washed once with PBS containing 0.1% BSA and 0.1% NaN₃, and incubated with one test of allophycocyanin-labeled pentamer conjugate, 0.25 µg Pacific Blue-labeled anti-mouse CD3, 0.25 µg fluorescein isothiocyanate-labeled anti-mouse CD8 (eBiosciences), 0.25 µg phycoerythrin-labeled anti-mouse CD19,

0.25 µg phycoerythrin-labeled anti-mouse CD49b, and 0.25 µg PerCP-labeled anti-mouse CD4 (BD Biosciences) for 20 min at 4°C. The cells were washed twice, resuspended in 1.5% paraformaldehyde containing 1% FBS, and analyzed using an LSR II flow cytometer (BD Biosystems) and FlowJo analysis software (Treestar, Ashland, OR). 100,000 events were collected in the lymphoid gate for analysis.

Statistics. One-way analysis of variance with a Bonferroni *t*-test post-test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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