

Interferon- λ Mediates Oral Tolerance and Inhibits Antigen-Specific, T-Helper 2 Cell-Mediated Inflammation in Mouse Intestine

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BACKGROUND & AIMS: Oral tolerance is an important component of gastrointestinal homeostasis, but mechanisms of its development are not fully understood. Loss of oral tolerance occurs during food allergen-related inflammation in the gastrointestinal tract. Interferon (IFN)- λ regulates immunity, but its role in oral tolerance is not clear. We investigated the role and the mechanism of IFN- λ in the development of oral tolerance and its effect on antigen-induced, T-helper (Th)-2 cell-mediated inflammation in the intestine. **METHODS:** Expression of IFN- λ and its receptor were analyzed by immunohistochemical, flow cytometric, or immunoblot analyses. Tolerogenic dendritic cells (DCs) and regulatory T cells were examined in vitro and in vivo. A mouse model of antigen-induced, Th2 cell-mediated intestinal inflammation used to examine the role of IFN- λ and T cells in oral tolerance in the intestine. **RESULTS:** CD3⁺ cells expressed the IFN- λ receptor, which was up-regulated following antigen-specific or nonspecific activation. Interaction between IFN- λ and its receptor induced apoptosis of T cells and their subsequent phagocytosis by DCs. This led to the generation of tolerogenic DCs and T regulatory cells in vitro and in vivo. Passive transfer of IFN- λ -primed CD3⁺ cells inhibited Th2 cell-mediated inflammation in the intestine. **CONCLUSIONS: IFN- λ is involved in development and maintenance of oral tolerance in the intestines of mice; it might be used to suppress antigen-specific Th2 cell-mediated inflammation in patients.**

Keywords: Food Allergy; Immune Response; Mucosal Immunity; Treg Cells.

Oral tolerance plays an important role in the maintenance of homeostasis in the body. Breakdown of oral tolerance may lead to hypersensitivity in the intestine and food allergy that affects 4% to 6% of the world population.¹ It is proposed that skewed T helper (Th)-2 cell polarization plays a critical role in the pathogenesis of food allergy. Recent advances further suggest a role of epithelial barrier dysfunction,² stress,³ and overreaction to microbial products⁴ in the development of food allergy. Despite these progresses, the molecular mechanisms of oral tolerance and food allergy associated with the breakdown of oral tolerance remain to be further understood. Enhanced knowledge in this regard will facilitate the

development of effective therapeutic strategies for allergic intestinal disorders.

Recent data indicate that the interferon (IFN) family plays an important role in the regulation of immune responses.⁵ In addition to their role in antipathogen⁶ and antitumor defense,⁷ recent studies also implicate IFN in the modulation of oral tolerance, based on its role in the maintenance of regulatory T-cell (Treg) function⁸ and suppressing interleukin (IL)-4 expression in Th1 cells.⁹ Furthermore, IFN- λ suppresses IL-13 expression in Th2 cells^{10,11} and modulates Treg function.^{12,13} IFN family includes type I and type III IFNs, and 14 subtypes of IFN have been identified as type I IFNs, including IFN- β and IFN- κ .¹⁴ Type III IFN, also called IFN- λ , is a newly described IFN that includes 3 subtypes, IFN- λ 1, IFN- λ 2, and IFN- λ 3,¹⁵ in humans and IFN- λ 2 and IFN- λ 3 in mice.¹⁶ All IFN- λ subtypes utilize a common receptor complex composed of the IL-28 receptor (R) α subunit and the IL-10R β subunit (hereafter referred to as IFN- λ R).^{15,17} IFN- λ R is found expressed on epithelial cells,¹⁸ cancer cells,¹⁹ and some immune cells, including monocytes, T cells, and natural killer cells.²⁰ Activation of IFN- λ R induces the activation of a series of genes, some of which encode antiviral proteins.⁵ However, much less is understood about the functional consequences in immune cells after IFN- λ R activation.²⁰

Dendritic cells (DC) and Tregs are important cellular components in the initiation and maintenance of oral tolerance.^{21,22} DCs express several Toll-like receptors (TLRs), such as TLR2, TLR3, and TLR4, that recognize and respond to the stimuli from pathogens. TLR2 recognizes gram-positive bacteria, TLR3 recognizes viral stimuli, and TLR4 recognizes gram-negative bacteria.²³ In general, the engagement of TLRs activates DCs and initiates immune responses to microbial invasion as well as induces the development of oral tolerance.^{24,25} Although activation of TLR3 can induce DCs

Abbreviations used in this paper: ALDH, aldehyde dehydrogenase; BmDC, bone marrow-derived dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; FACS, fluorescence-activated cell sorter; IFN, interferon; IL, interleukin; LPMC, lamina propria mononuclear cell; LPS, lipopolysaccharide; MACS, magnetic cell sorting; OVA, ovalbumin; R, receptor; RA, retinoic acid; TCR, T-cell receptor; TGF, transforming growth factor; Th, T-helper; TLR, Toll-like receptor; Treg, regulatory T cell.

to produce IFN- λ ,²⁶ its biological significance still remains completely understood.

Tregs encompass CD4⁺ CD25⁺ Foxp3⁺ Tregs, type 1 Tregs, and Th3 Tregs. Transforming growth factor (TGF)- β and IL-10 released from Tregs are the major immune suppressive mediators.¹⁴ TGF- β also has the capacity to induce Treg development from peripheral CD4⁺ CD25⁻ T cells.²⁷ Tolerogenic DCs can also be a source of TGF- β and retinoic acid (RA),^{21,22} both of which play an important role in the development of Tregs and oral tolerance.^{21,22,28} Development of tolerogenic DCs can be triggered by exposure to microbial products²⁹ or by DC phagocytosis of apoptotic CD3⁺ cells.³⁰ Recent evidence suggests that IFN- λ can induce Tregs via its effect on DCs in vitro,¹³ but the role and the mechanisms of this cytokine in oral tolerance and food antigen-induced intestinal inflammation are still largely unclear. In the current study, we set out to investigate whether IFN- λ modulates the interaction between DCs and CD3⁺ cells to facilitate the establishment of oral tolerance. Our results reveal that the interaction of IFN- λ /IFN- λ R facilitates the establishment of oral tolerance and inhibits Th2 inflammation in the intestine.

Materials and Methods

Mice

Balb/c mice, 6 to 8 weeks old, were purchased from Charles River Canada (St Constant, Quebec, Canada). Ovalbumin (OVA)/ T-cell receptor (TCR)/transgenic DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a pathogen-free environment. The procedures of animal experiments were approved by the Animal Care Committee at McMaster University.

Preparation of CD3⁺ Cells

Splenic cells were prepared. CD3⁺ cells were isolated with commercial reagent kits. Cells were cultured in RPMI 1640 medium in the presence of IL-2 (50 U/mL) and phytohemagglutinin (1 μ g/mL) for 5 days. Cells were then kept for further experiments.

CD3⁺ Cell Apoptosis Assay

CD3⁺ cells were isolated from lamina propria mononuclear cells (LPMCs) and cultured in RPMI 1640 complete media in anti-CD3 antibody (10 μ g/mL)-coated plates in the presence of anti-CD28 (1 μ g/mL) for indicated periods. The frequency of apoptotic cells was determined by Annexin V staining and then analyzed by flow cytometry.

Development of a Mouse Model of Antigen-Specific Th2-Pattern Inflammation in the Intestine

Balb/c mice (10 mice per group) were subcutaneously injected with OVA (0.1 mg in 0.1 mL alum/mouse) on day 0, which was repeated on day 3. Mice were gavage fed with OVA (1 mg in 0.3 mL saline/mouse) on days 9, 11, and 13. Mice were killed on day 14. Blood samples were collected. The

serum levels of OVA-specific immunoglobulin E and IL-4 were measured by enzyme-linked immunosorbent assay.

Flow Cytometry

Cells were collected from culture and washed twice with fluorescence-activated cell sorter (FACS) buffer (2% fetal calf serum in phosphate-buffered saline), and 10⁶ cells were resuspended in 100 μ L FACS buffer. Cells were stained for 1 hour on ice with fluorescently conjugated monoclonal antibodies or isotype control immunoglobulin G and then washed twice and resuspended in FACS buffer for analysis. For intracellular staining, cell surface staining was performed first, followed by buffer fixation/permeabilization and intracellular staining. Cells were examined by a flow cytometer (FACSCanto; BD Bioscience, San Jose, CA). The aldehyde dehydrogenase (ALDH) activity in DCs was determined using an ALDEFLUOR staining kit (Stemcell Technologies, Vancouver, Canada) following the manufacturer's instructions.

Additional experimental procedures are presented in Supplementary Materials and Methods.

Results

Intestinal CD3⁺ Cells Express IFN- λ R

It is proposed that IFN- λ plays an important role in immune regulation.^{6,7} To elucidate if IFN- λ can directly act on T cells, we sampled small intestinal segments from naïve mice and examined the colocalization of IFN- λ R⁺ and CD3⁺ cells by double-staining immunohistochemistry and confocal microscopy. As shown in Figure 1A, a number of cells in the lamina propria were stained positive for both IFN- λ R and CD3. To further investigate, we isolated LPMCs from the small intestine of naïve mice and stained them with antibodies for IFN- λ R and CD3. By flow cytometry, about 6% to 8% LPMCs were shown to be both CD3⁺ and IFN- λ R⁺ (Figure 1C). These results suggest that some intestinal T cells express IFN- λ R and thus have the potential to respond to stimulation by IFN- λ .

TCR Activation Increases the Expression of IFN- λ R on Intestinal CD3⁺ Cells

To understand the regulation of IFN- λ R expression in intestinal T cells, we examined if TCR stimulation could increase the expression of IFN- λ R on intestinal T cells. To this end, OVA-TCR transgenic DO11.10 mice were treated with OVA by gavage. As shown by flow cytometry, the frequency of CD3⁺ IFN- λ R⁺ cells among isolated LPMCs was markedly increased in OVA-treated mice as compared with naïve mice (Figure 2A). We also isolated LPMCs from naïve transgenic DO11.10 mice and exposed the cells to OVA peptide in vitro and found a similar increase in the expression of IFN- λ R in CD3⁺ cells (Figure 2B). To see if nonspecific polyclonal activation of T cells could also increase IFN- λ R expression on T cells, we purified CD3⁺ cells from LPMCs of naïve wild-type Balb/c mice by magnetic cell sorting (MACS) and cultured in the presence of anti-CD3/CD28 antibodies. Consistent

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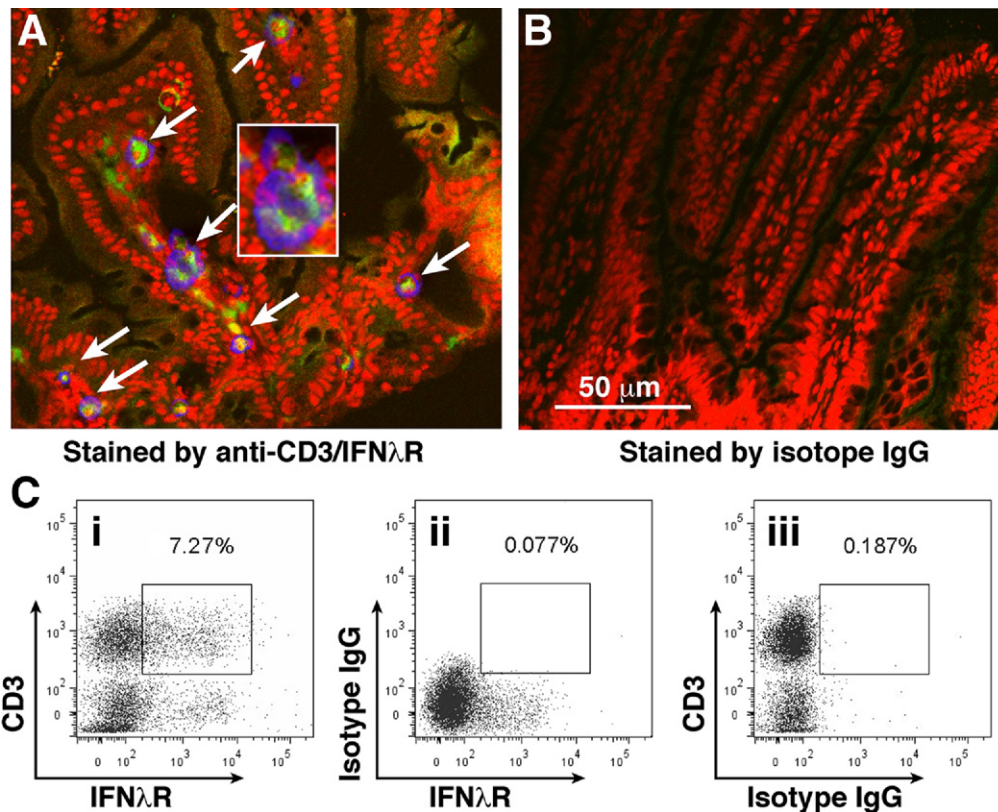


Figure 1. Intestinal T cells express IFN-λR. Naïve Balb/c mouse jejunal segments and LPMCs were prepared as described. Cryosections were examined by immunohistochemistry. (A) Representative confocal images show CD3⁺ (green) and IFN-λR⁺ (blue) staining in the lamina propria (arrows). The nuclei were stained with propidium iodide (red). (B) Negative isotype control staining. (C) LPMCs were examined by flow cytometry. (i) CD3⁺ IFN-λR⁺ cells. (ii) Negative isotype control staining for anti-CD3. (iii) Isotype control staining for anti-IFN-λR. Data represent 6 experiments. Original magnification: A and B, 200×; inset in A, 630×.

with the data in Figure 1C, a level of constitutive IFN-λR expression (about 7%) was detected on intestinal CD3⁺ cells without any stimulation (Figure 2C [a]), which was significantly increased on anti-CD3/CD28 stimulation (Figure 2C [b]). The inducibility of IFN-λR expression in D011.10 transgenic T cells was also independently verified by reverse-transcription polymerase chain reaction and Western blotting (Figure 2D). The results together suggest that the constitutive expression of IFN-λR on intestinal T cells can be further up-regulated by TCR activation via specific antigen or polyclonal stimulation.

Interaction of IFN-λ/IFN-λR Induces CD3⁺ Cell Apoptosis

Given significant IFN-λR expression on CD3⁺ cells in the gut, we were interested in understanding the functionality of IFN-λ and IFN-λR interaction. Based on the previous reports that IFN-λ/IFN-λR interaction can induce cancer cell death⁷ and that DC uptake of apoptotic T cells facilitates oral tolerance,³⁰ we wondered if IFN-λ/IFN-λR interaction could induce T-cell apoptosis. To this end, CD3⁺ cells purified from isolated LPMCs were treated with IFN-λ in culture for 16 hours. By using propidium iodide and Annexin V immunostaining, we found that on exposure to recombinant IFN-λ, but not to the control albumin stimulation, close to 20% of CD3⁺

cells became apoptotic (Figure 3A and B). To investigate this further, we cultured LPMCs in the presence of polyIC, which was previously shown to activate DCs to produce IFN-λ.³¹ As shown in Figure 3C and D, addition of polyIC to LPMC culture increased the frequency of apoptotic CD3⁺ cells (Figure 3C [4]) that was abolished by pretreatment with anti-IFN-λ antibody (Figure 3C [5]). To verify if DCs and macrophages were indeed the source of IFN-λ in polyIC-stimulated LPMC cultures, we depleted CD11c⁺ and F4/80⁺ cells from LPMC preparations by positive selection with MACS and then cultured the collected cells in the presence of polyIC. Indeed, the removal of DCs and macrophages from the LPMC preparations resulted in the loss of CD3⁺ cell apoptosis induced by polyIC (Figure 3C [6]). The previously described results together suggest that either exogenously introduced IFN-λ or antigen-presenting cell-derived IFN-λ can interact with IFN-λR expressed on intestinal T cells, leading to T-cell apoptosis.

DC Phagocytosis of Apoptotic CD3⁺ Cells Induced by IFN-λ/IFN-λR Interaction

The published data have suggested that the phagocytosis of apoptotic T cells facilitates the development of tolerogenic DCs.³⁰ Having shown that DC-derived IFN-λ induced intestinal T-cell apoptosis (Figure 3), we determined the relationship between DC activation, T-cell ap-

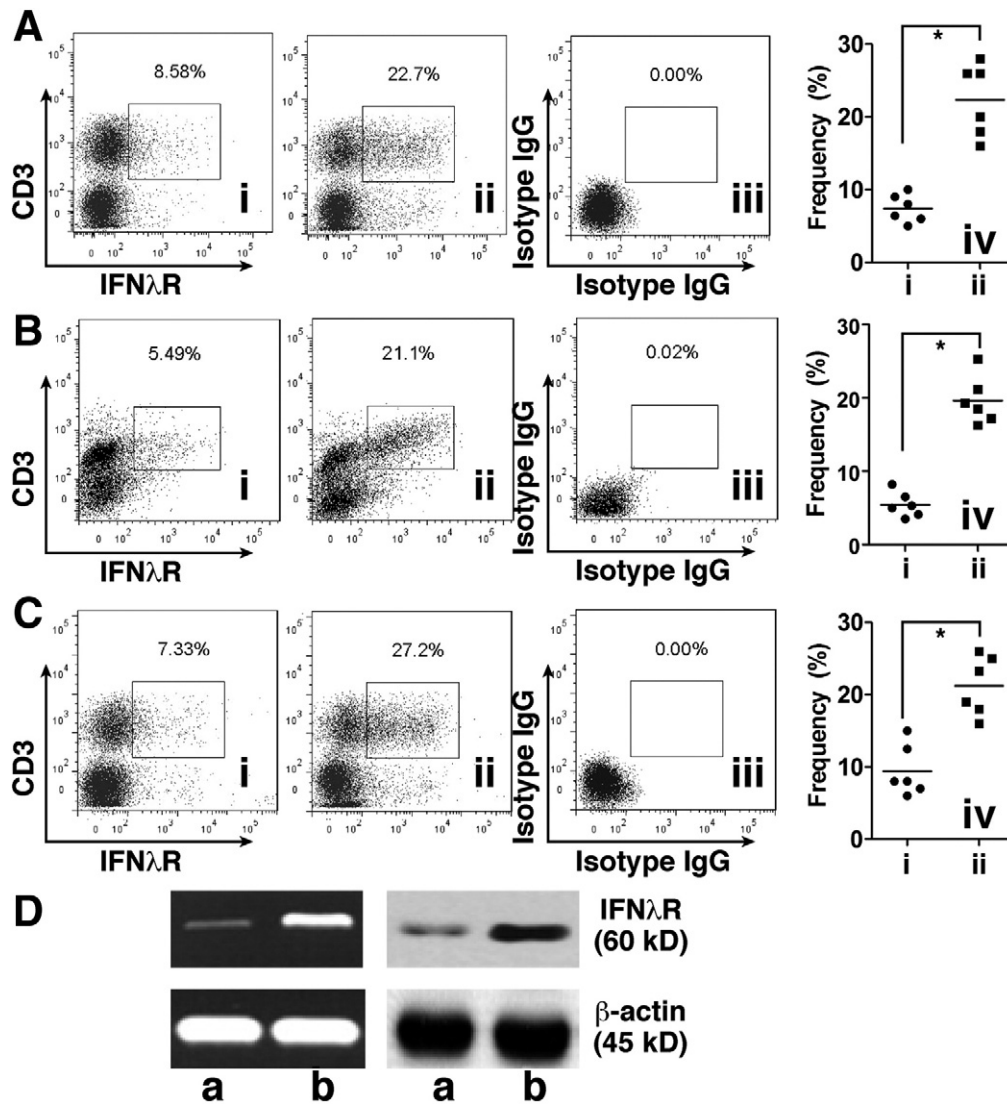


Figure 2. TCR activation increases IFN-λR expression in intestinal CD3⁺ cells. Expression of IFN-λR in LPMCs was examined by flow cytometry. (A) LPMCs were collected from DO11.10 mice fed with OVA, with no further antigen stimulation in vitro. (B) LPMCs were collected from naïve DO11.10 mice and stimulated in vitro with OVA peptide 323–339. (C) LPMCs were collected from naïve Balb/c mice and cultured for 3 days and then exposed to anti-CD3 (10 μg/mL)/CD28 (1 μg/mL) antibodies or saline for 18 hours. Column i, unstimulated LPMCs from the corresponding naïve mice; column ii, in vivo or in vitro TCR-activated cells; column iii, isotype immunoglobulin G control staining; column iv, individual data points in columns i and ii. (D) After TCR activation, CD3⁺ cells were isolated by MACS and analyzed for the IFN-λR expression by reverse-transcription polymerase chain reaction and Western blotting. The gels show results of reverse-transcription polymerase chain reaction (left panels; 215 base pairs) and Western blotting (right panels) of IFN-λR expression in naïve (lane a) or activated CD3⁺ cells (lane b). Data are representative of 3 experiments. **P* < .01.

optosis, and DC phagocytosis of apoptotic T cells. To this end, we first cocultured immature bone marrow-derived dendritic cells (BmDCs) with anti-CD3/CD28-activated CD3⁺ cells for 24 hours. On confocal microscopy, as expected (due to the lack of IFN-λ), we found few CD3⁺ cells being phagocytosed by BmDCs (Figure 4A). We then examined the phagocytosis of anti-CD3/CD28-activated CD3⁺ cells by either polyIC- or lipopolysaccharide (LPS)-activated DCs. Of interest, while abundant polyIC-treated DCs were found to have phagocytosed apoptotic CD3⁺ cells (Figure 4B), few LPS-treated DCs phagocytosed CD3⁺ cells (Figure 4C). It is known that both polyIC and LPS can activate DCs but they engage TLR3 and TLR4,

respectively. Because polyIC is known to stimulate DCs to produce IFN-λ, as shown in this study (Supplementary Figure 1) and by others,³¹ we speculated that polyIC-stimulated DCs released IFN-λ and led to the apoptosis of CD3⁺ cells and their subsequent phagocytosis by DCs, whereas LPS-stimulated DCs failed to release IFN-λ and were thus unable to induce CD3⁺ cell apoptosis and DC phagocytosis. To test this, we stimulated BmDCs with polyIC or LPS and subsequently measured IFN-λ expression by flow cytometry. Indeed, we found the high frequency of IFN-λ-expressing DCs only by polyIC stimulation, but not by LPS stimulation (Supplementary Figure 1). We next performed the same experiment as for Figure

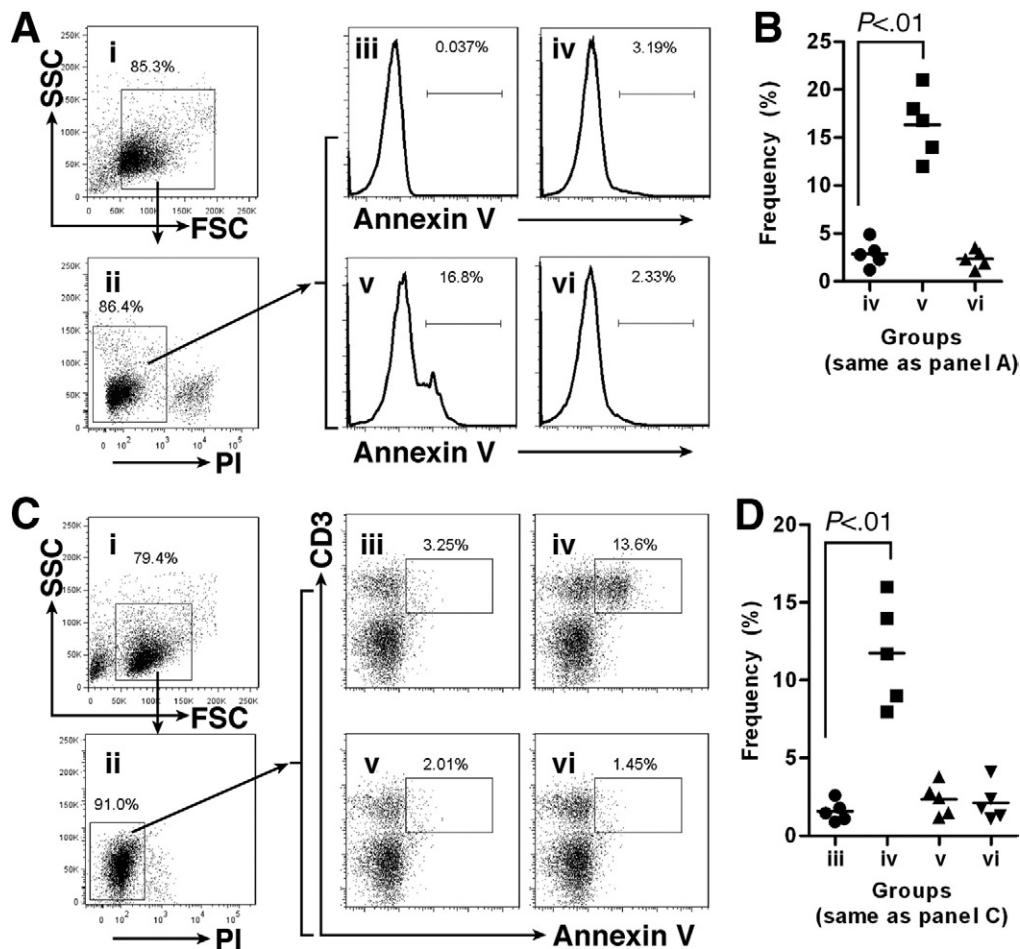


Figure 3. Role of IFN- λ in the induction of T-cell apoptosis. (A and B) CD3⁺ cells were purified from LPMCs of naive mice and treated with IFN- λ (100 ng/mL) in culture for 16 hours. Cells were collected and stained with propidium iodide (PI) to stain the dead cells and Annexin V (to stain apoptotic cells). Live cells were separated from the dead cells (i); live cells (PI⁻ cells; ii) were further analyzed for the frequency of Annexin V-positive cells (apoptotic cells). (ii) Live cells (PI⁻) were further analyzed for the frequency of Annexin V-positive cells (apoptotic cells). (iii–vi) The frequency of apoptotic CD3⁺ cells (the gated CD3⁺ Annexin V-positive cells) in naive cells (iii; no treatment), cells treated with polyIC alone (iv) or pretreated with anti-IFN- λ antibody, and then treated with polyIC (v) or predepleted DCs and macrophages; the remaining cells were then treated with polyIC (vi). (D) Individual data points in C [iii–vi].

4B but with addition of anti-IFN- λ antibody. Indeed, the phagocytosis of CD3⁺ cells by polyIC-treated DCs was abrogated by anti-IFN- λ treatment (Figure 4D). On the other hand, the similarly reduced phagocytosis was observed in the coculture using CD3⁺ cells with knocked-down IFN- λ R gene expression and polyIC-stimulated DCs (Figure 4E). The previously described data support a DC and T-cell loop regulated by IFN- λ and IFN- λ R interaction; IFN- λ production of DCs is under tight control. Properly activated DC-derived IFN- λ interacts with IFN- λ R on intestinal T cells, leading to T-cell apoptosis. Apoptotic T cells are subsequently phagocytosed by DCs.

Phagocytosis of Apoptotic CD3⁺ Cells Induces the Development of Tolerogenic DCs and Regulatory T Cells in the Intestine

Because DC phagocytosis of apoptotic T cells was previously shown to facilitate the development of tolero-

genic DCs,³⁰ we examined whether polyIC-treated DCs with phagocytosed apoptotic T cells might have assumed the property of tolerogenic DCs. Thus, we first measured the level of TGF- β 1 by these DCs and found that the levels of TGF- β were increased only in the supernatants of cocultures with DCs treated with polyIC but not those treated with LPS (Supplementary Figure 2). There was also a lack of TGF- β production when IFN- λ R knock-down T cells were used (Supplementary Figure 2).

To investigate if apoptotic T cells could be phagocytosed by DCs and subsequently render these DCs to possess the tolerogenic property in vivo, we prepared anti-CD3/CD28 antibody-activated and IFN- λ -treated intestinal CD3⁺ cells (about 50% cells were apoptotic as checked by flow cytometry with Annexin V staining). Cells were then labeled with carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred to mice by tail vein injection.

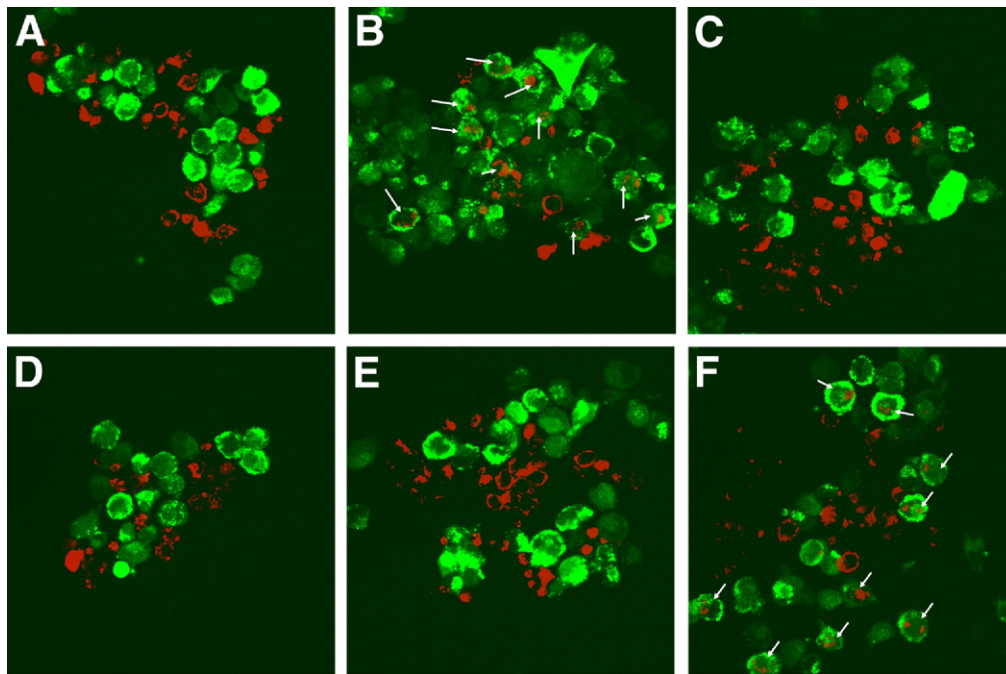


Figure 4. DC-derived IFN- λ plays a critical role in DC phagocytosis of apoptotic T cells. BmDCs were treated with saline (A), polyIC (B, E, and F; 50 μ g/mL), LPS (C; 1 μ g/mL), or polyIC/anti-IFN- λ antibody (D; 1 μ g/mL) in culture. Apoptotic CD3 $^{+}$ cells were prepared by exposing naïve spleen CD3 $^{+}$ cells (A–D), IFN- λ R-deficient CD3 $^{+}$ cells (E; by small interfering RNA IFN- λ R), or control small interfering RNA-treated CD3 $^{+}$ cells (F; also refer to [Supplementary Figure 2](#)) to anti-CD3/CD28 antibodies and staining with Annexin V-PE. DCs and CD3 $^{+}$ cells were cocultured at a ratio of 1:1 for 24 hours and then stained by anti-CD11c antibody; cells were smeared on slides and observed by confocal microscopy. Representative confocal images show DCs (stained in green) and apoptotic CD3 $^{+}$ cells (stained in red). Some CD3 $^{+}$ cells were phagocytosed by DCs (pointed by arrows). Data are representative of 3 experiments.

By confocal microscopy, the CFSE-positive cells (IFN- λ -treated CD3 $^{+}$ cells) were observed in the lamina propria of the small intestine, some of which were found to be phagocytosed by DCs (Figure 5A). Furthermore, we isolated the DCs from the small intestine of these mice and examined the level of their expression of both ALDH, an enzyme that can catalyze retinal acid into RA, and TGF- β . Tolerogenic DC-derived ALDH and TGF- β were shown to play a critical role in the generation of inducible regulatory T cells.^{21,22} By Western blotting, we found a much higher level of ALDH and TGF- β expression in the DCs isolated from apoptotic T-cell recipient animals as compared with those isolated from the control hosts (Figure 5B). The similar finding was obtained by flow cytometry (Figure 5C).

To investigate whether increased tolerogenic DCs in the intestinal mucosa, as a result of phagocytosing IFN- λ -triggered apoptotic T cells, could lead to increased immunosuppressive Tregs, we isolated the CD4 $^{+}$ T cells from the small intestine of the mice treated as described previously and analyzed their immune phenotype by flow cytometry. We found significantly more Foxp3 $^{+}$ CD4 $^{+}$ Treg cells in the mice receiving IFN- λ -triggered apoptotic CD3 $^{+}$ cells than in naïve mice (Figure 5D [d]). We further examined the role of DC-derived ALDH and TGF- β in the generation of Foxp3 $^{+}$ Tregs in the intestine. Thus, for the mice set up as in Figure 5D [d], we treated them with an RA receptor inhibitor and/or anti-TGF- β antibody. Compared with the data in Figure 5D [d], blockade of the RA

pathway (Figure 5D [e]) or TGF- β (Figure 5D [f]) significantly inhibited the frequency of Foxp3 $^{+}$ Tregs, and blockade of both RA and TGF- β further reduced the level of Tregs in the intestine (Figure 5D [g]) (Figure 5E). These results together suggest that phagocytosis of apoptotic intestinal T cells induced by IFN- λ /IFN- λ R interaction leads to the generation of tolerogenic DCs capable of ALDH and TGF- β production and the subsequent development of Tregs in the intestine.

Administration of IFN- λ -Primed CD3 $^{+}$ Cells Inhibits Antigen-Specific Th2 Inflammation in the Intestine

Having shown that phagocytosis of apoptotic intestinal T cells leads to the generation of tolerogenic DCs and Tregs in the intestine, we examined whether this could functionally inhibit intestinal inflammation of Th2 nature as pertaining to food allergy. To this end, the mice were first sensitized subcutaneously with OVA and then challenged via gavage with OVA. A group of mice were injected intravenously, beginning 2 days before the first OVA challenge, with saline control- or IFN- λ -treated activated T cells every other day for 3 times. We found that OVA-sensitized mice that received saline-primed T cells had typical Th2 inflammation in the intestine, whereas those that received IFN- λ -primed T cells had markedly reduced Th2 inflammation in the intestine. Specifically, transfer of IFN- λ -primed T cells to OVA-sensitized mice showed markedly suppressed OVA-specific Th2 immune

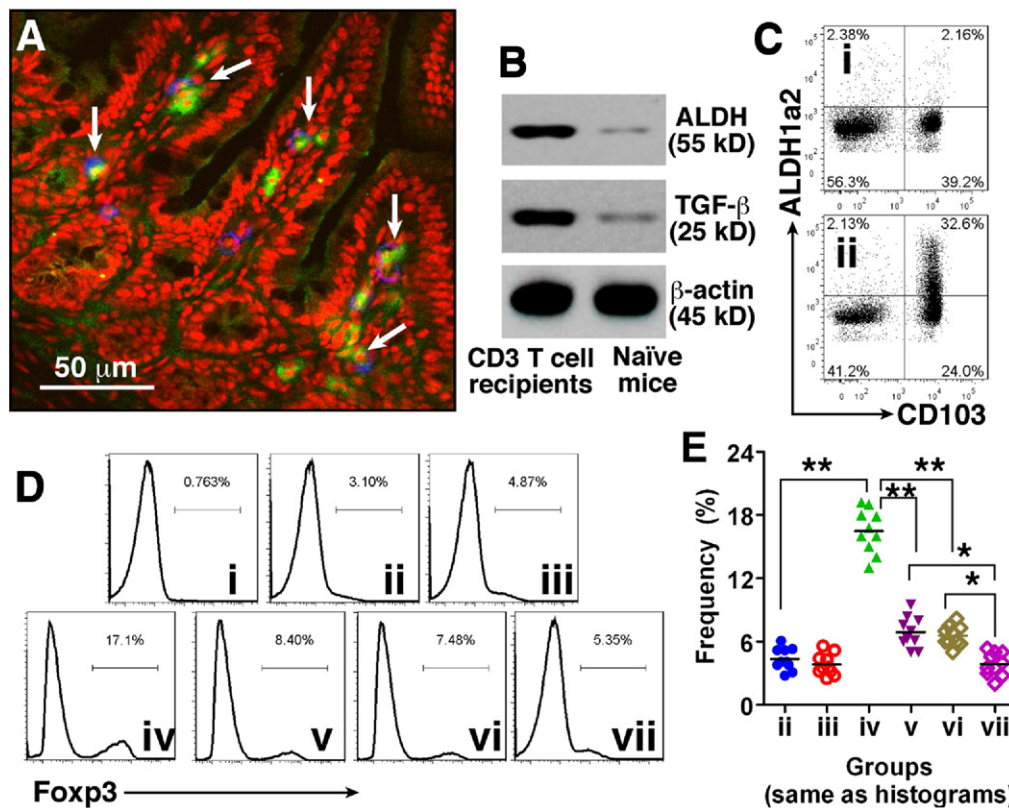


Figure 5. IFN- λ -primed T cells facilitate the development of tolerogenic DCs and Tregs in the intestine. Anti-CD3/CD28 antibody-activated CD3⁺ cells isolated from the spleen of naïve Balb/c mice were exposed to IFN- λ in culture for 24 hours. The IFN- λ -primed CD3⁺ cells were labeled with or without CFSE and transferred to naïve Balb/c mice (10^6 cells/mouse; 10 mice per group) via tail vein injection every other day for 3 times. Mice were killed on day 7. (A) Cryosections of the jejunum were stained with anti-CD11c antibody and propidium iodide (to stain the nuclei). The transferred IFN- λ -primed CD3⁺ cells (in green) were observed in the jejunal sections. DCs were stained in blue. Some CD3⁺ cells were localized inside DCs (the green cells surrounded by blue color; pointed to by arrows). (B and C) CD11c + MHCII + DCs were isolated by MACS from LPMCs (isolated from the excised small intestine). (B) DC protein extracts were analyzed by Western blotting for ALDH and TGF- β contents. (C) Isolated DCs were analyzed by flow cytometry for the frequency of ALDH⁺ or/and CD103⁺ DCs from naïve mice (i) or mice that received IFN- λ -primed CD3⁺ cells (ii). (D and E) CD4⁺ T cells were isolated by MACS from excised small intestine, stained with fluorescence-labeled anti-Foxp3 antibody, and analyzed by flow cytometry. The histograms show the frequency of Foxp3⁺ CD4⁺ T cells in the intestine. i, isotype control staining; ii, CD4⁺ T cells from naïve mice; iii, CD4⁺ T cells from mice receiving CD3⁺ cells activated by anti-CD3/CD28 antibodies; iv–vii, CD4⁺ T cells from mice receiving CD3⁺ cells activated by anti-CD3/CD28 antibodies and primed with IFN- λ . Some groups of mice were also treated with RA receptor β inhibitor LE540 at 50 μ g/mouse intraperitoneally (v) or anti-TGF- β at 100 μ g/mouse intraperitoneally (vi) or both LE540 and anti-TGF- β (vii) 2 hours before the CD3⁺ cell transfer. (E) The individual data points of Foxp3⁺ T cells. The group annotations on the x-axis are the same as for D. * $P < .05$; ** $P < .01$.

and inflammatory responses, including (1) decreased levels of serum OVA-specific immunoglobulin E (Figure 6A), (2) decreased IL-4 protein levels in intestinal extracts (Figure 6B), (3) decreased OVA-specific CD4 T-cell proliferation measured in an ex vivo DC/T-cell stimulation assay (Figure 6C and D), and (4) reduced inflammatory cell infiltration in the intestinal mucosa (Figure 6E). These results together indicate that passive in vivo transfer of IFN- λ -primed T cells is able to not only elicit the development of tolerogenic DCs and Tregs but also to facilitate the establishment of oral tolerance and inhibit the development of Th2 immune responses and gut inflammation associated with food antigen exposure in sensitized hosts.

Discussion

Oral tolerance can be induced by one large dose of antigen or repeat exposures to small doses of antigen. However, the molecular mechanisms that help maintain

oral tolerance still remain incompletely understood. Enhanced knowledge in this regard will also help understanding of the mechanisms of breach of oral tolerance and food allergy. The present study has provided novel information on the role and mechanism of IFN- λ in the induction of oral tolerance. We show that intestinal T cells express IFN- λ AR that can be up-regulated on activation. TLR3 activation increases the expression of its ligand IFN- λ in DCs. The interaction of IFN- λ /IFN- λ AR induces T-cell apoptosis and the apoptotic T cells can then be phagocytosed by DCs, which leads these DCs to become tolerogenic DCs. Tolerogenic DCs subsequently facilitate the induction of Tregs. Furthermore, we have provided evidence that administration of IFN- λ -treated T cells is able to inhibit antigen-specific Th2 inflammation in the intestine. The present data are in line with a previous study that IFN- λ -treated DCs specifically induce proliferation of a CD4⁺ CD25⁺ Foxp3⁺ T-cell subset with con-

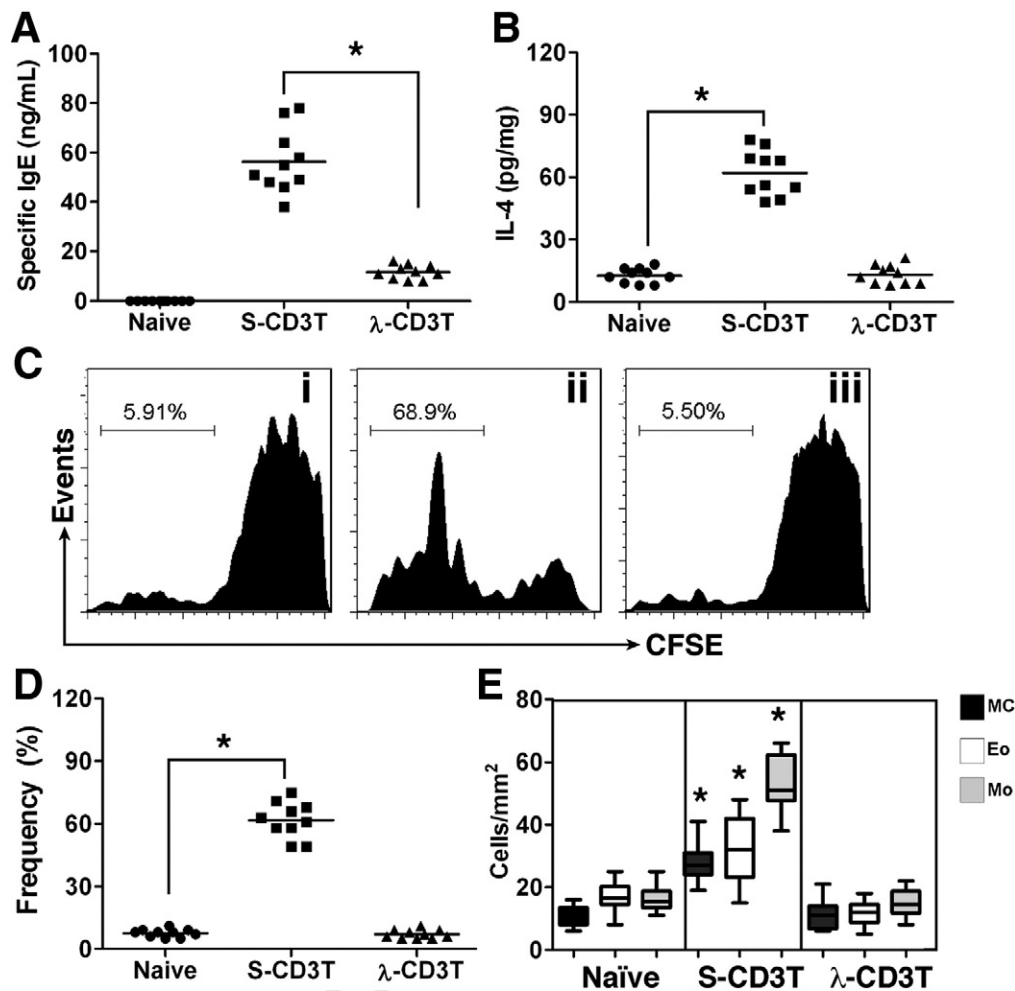


Figure 6. IFN- λ -primed T cells inhibit Th2 inflammation in the intestine. CD3⁺ cells were prepared as described in the legend to Figure 4. Balb/c mice (10 mice/group) were treated with saline (Naive group) or OVA/alum as described in Materials and Methods. In addition, S-CD3T group mice were injected with saline-treated anti-CD3/CD28-activated CD3⁺ cells (10^6 cells/mouse); λ -CD3T group mice were injected with IFN- λ -primed anti-CD3/CD28-activated CD3⁺ cells (10^6 cells/mouse). Collected samples were examined for Th2 pattern inflammation in the intestine. (A) Serum level of OVA-specific IgE measured by enzyme-linked immunosorbent assay. Naive, naive mice; S-CD3T, mice receiving saline-treated CD3⁺ cells; λ -CD3T, mice receiving IFN- λ -primed CD3⁺ cells. * $P < .01$. (B) IL-4 levels in protein extracts from mouse small intestine determined by enzyme-linked immunosorbent assay. Each dot in panels A and B represents a single data point from one mouse. * $P < .01$. (C and D) CD4⁺ T cells were isolated from the small intestine, labeled with CFSE, and cocultured with isolated splenic DCs (T cell/DC = 10:1) in the presence of specific antigen OVA (10 μ g/mL) for 4 days. The CFSE-dilution assay was measured for T-cell proliferation by flow cytometry. i, naive mice; ii, mice receiving saline-treated CD3⁺ cells; iii, mice receiving IFN- λ -primed CD3⁺ cells. (D) Individual data points of the frequency of proliferated CD4⁺ cells. (E) Jejunal segments were processed for inflammatory cell counting. Floating boxes represent the counts of mast cells (MC), eosinophils (Eo), and mononuclear cells (Mo) in the intestine. Data are presented as mean $\pm$ SD. * $P < .01$, compared with naive group.

tact-dependent suppressive effect on T-cell proliferation.¹³ On the other hand, our results are somewhat different from a recent report that using IFN- λ as an adjuvant in a DNA vaccine suppressed CD4⁺ CD25⁺ Foxp3⁺ T cells in the spleen.¹² This suggests that the immune regulatory effect of IFN- λ may differ when concurrently used with other immune-activating agents.

DCs express several TLRs, such as TLR2, TLR3, and TLR4, that actively recognize microbial stimuli to initiate certain immune responses. Previous reports indicate that viral infection can induce DCs to produce IFN- λ .³¹ Our study supports this by showing that activation of TLR3 by polyIC exposure up-regulates the production of IFN- λ by DCs. However, we further show that different from TLR3 activation, activation of TLR4 by a bacterial product LPS

does not increase IFN- λ expression in DCs. Our results thus support the notion that the production of IFN- λ by DCs is more likely induced by viral, but not bacterial, infection. These findings, however, warrant further investigation. DCs are distributed throughout the subepithelial region, where they have the opportunity to contact other immune cells. Like DCs, there are abundant T cells in the subepithelial mucosal region. Such a special anatomic feature provides an optimal opportunity for DCs and T cells to interact with each other. In this case, DC-produced IFN- λ may easily reach T cells. In turn, IFN- λ -modified, apoptotic T cells interact with mucosal DCs and render them immune tolerogenic properties, as shown in our study. One of the novel findings in this study is that intestinal CD3⁺ T cells express IFN- λ R. This

special feature enables intestinal T cells to respond to DC-derived IFN- λ . Thus, conceivably any mechanisms that can turn on the production of IFN- λ in intestinal DCs may go on to induce tolerogenic DCs and Tregs, contributing to the maintenance of intestinal homeostasis and oral tolerance. Indeed, one way for the host to maintain the immune homeostasis is that some immune cells undergo apoptosis. In line with current knowledge about the activation-induced T-cell death,³² our study provides the mechanistic insight that such activated-induced T-cell death could be due to increased expression of IFN- λ R on T cells and their subsequently increased responsiveness to IFN- λ . Our findings also provide an explanation as to why human blood immune cells, when nonactivated, do not respond to stimulation by even high doses of IFN- λ .^{20,33}

Phagocytes are the major scavenger to clean up apoptotic cells from the body. The present study not only supports previous studies by showing DCs and macrophages phagocytosing apoptotic T cells,³⁰ but has also provided novel information that such phagocytosis is modulated by the interaction of IFN- λ /IFN- λ R. The biological significance of this phenomenon may be that during viral infection, T cells are activated and initiate necessary protective immune responses. On the other hand, as a counter mechanism, their expression of IFN- λ R is increased and their interaction with IFN- λ produced by virus-activated DCs will result in subsequent apoptosis of T cells that will then be taken up by DCs. The generation of tolerogenic DCs and Tregs will keep the immune activation in check and help restore immune homeostasis.

Oral tolerance plays a critical role in the maintenance of homeostasis in the body. Tregs are one of the major components in the mechanisms of oral tolerance. The development of Tregs is important in establishment and maintenance of oral tolerance. It is reported that RA and TGF- β work synergistically in the development of Tregs.^{21,22,27,34} DCs can be a major source of RA and TGF- β for the development of Tregs³⁵ that can be a result from various biologic events such as phagocytosing apoptotic T cells, as reported by others³⁰ and revealed by the present study. As mounting evidence suggests that the breakdown of oral tolerance plays a critical role in the pathogenesis of a number of intestinal immune disorders³⁶ and there is a lack of satisfactory remedies for such disorders, our results suggest that in addition to its potential application for treating viral infectious disease,³⁷ IFN- λ may also be used to treat Th2 type of immune disorders in the gut. Furthermore, any strategies designed to enhance IFN- λ production by intestinal DCs may also help control these conditions.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.04.006.

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Acknowledgments

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Conflicts of interest

The authors disclose no conflicts.

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AQ:1

AQ:2

AQ:3

Supplementary Materials and Methods

Reagents

Antibodies of MHC II (30.2), CD80 (H-208), CD86 (D-6), Foxp3 (2A11G9), IL-28, IL-28R (L-17), CD3, CD28, TGF- β , immunoglobulin (Ig) E (6HD5), CD11c, and IFN- λ R small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PolyIC and LPS were purchased from Sigma Aldrich (Oakville, Ontario, Canada). IL-4 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Burlington, Ontario, Canada; sensitivity of 2 pg/mL). OVA-specific IgE ELISA kit was purchased from AbD Serotec (Raleigh, NC; sensitivity of 8 ng/mL). TGF- β 1 ELISA kit was purchased from AbAffinity (Rantoul, IL; sensitivity of 2 pg/mL). DC and T-cell isolated reagents were purchased from Miltenyi Biotec (Auburn, CA). Cell culture reagents and precast NuPAGE gel system were purchased from Invitrogen (Mississauga, Ontario, Canada). Recombinant IL-28 protein was purchased from eBioscience (San Diego, CA). Antibodies used for flow cytometry and permeabilization reagents were purchased from BD Bioscience (San Jose, CA). Annexin V (FITC and PE) was purchased from Abcam (Boston, MA). RA receptor β inhibitor LE540 was purchased from Wako Chemicals USA (Richmond, VA). α -TGF- β monoclonal antibody (1D11.16.8) and isotype control monoclonal antibody MOPC were purchased from ATCC (Manassas, VA). ALDEFLUOR staining kit was purchased from Stemcell Technologies (Vancouver, Canada). Endotoxin levels in all reagents were detected using the limulus assay (Limulus amoebocyte lysate QCL 1000; BioWhittaker, Walkersville, MD). LPS-contaminated reagents were treated with polymyxin B-agarose to eliminate the LPS. The reagents used in this study contained <0.2 U endotoxin/10 μ g reagents.

Generation of Bone Marrow-Derived DC

The bone marrow was obtained from Balb/c mouse femurs and was depleted of red blood cells with hypotonic-buffered Tris/ammonium chloride (0.83%, pH 7.21). The cells were washed twice in RPMI 1640 medium. Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 20 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor, and 10 ng/mL IL-4. On day 2, nonadherent cells were removed; the remaining cells were continued to culture by changing culture media with granulocyte-macrophage colony-stimulating factor and IL-4 every 2 to 3 days.

Histopathology

After the animals were killed, the excised jejunal segments were fixed with 10% buffered neutral formalin or Carnoy solution and processed for paraffin embedding. Sections were stained with H&E or 0.5% toluidine

blue. Mononuclear cells and eosinophils were counted in H&E-stained sections. Mast cells were counted on sections stained with toluidine blue. Twenty fields (200 $\times$) were counted for each mouse.

Western Blotting

Cells were lysed in lysis buffer (50 mmol/L Tris Cl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40), including protease and phosphatase inhibitors, for 15 minutes on ice and insoluble debris was removed by centrifugation at 10,000g for 10 minutes. Proteins were separated by electrophoresis on 12% sodium dodecyl sulfate/polyacrylamide gels and then blotted onto nitrocellulose membrane and probed with anti-TGF- β at 1:5000 dilution. Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG (Fab). Signals were visualized using the ECL Western Blotting Detection Kit (Baie d'Urfe, Quebec, Canada).

Isolation of LPMCs

Freshly excised small intestinal segments were cut into small pieces and treated with 1 mmol/L EDTA. After stirring for 1 hour at 37°C, the supernatant was removed and the remaining tissue was treated with type IV collagenase (1 mg/mL) for 2 hours with stirring at 37°C. The resulting cell suspension was filtered through a 100- μ m cell strainer first and then through a 40- μ m cell strainer. After centrifugation, gradient density centrifugation with Percoll was performed on the cells. Cells were then washed twice and resuspended in RPMI 1640 medium. Viability of the cells was checked with trypan blue exclusion method, and only those with viability exceeding 90% were used in experiments.

Immunohistochemistry

Cryosections (or cells smeared on glass slides) were fixed with cold acetone for 20 minutes, blocked with 1% bovine serum albumin for 30 minutes at room temperature, and incubated with the primary antibodies in 1% bovine serum albumin at 4°C overnight. After washing with phosphate-buffered saline (5 minutes $\times$ 3 times), sections were incubated with fluorescence-labeled second antibodies in 1% bovine serum albumin at room temperature for 1 hour. After washing with phosphate-buffered saline, sections were mounted with anti-fade aqueous mounting media and coverslips and observed under a confocal microscope (LSM 510; Carl Zeiss Micro Imaging GmbH, Jena, Germany). Negative control staining was performed by using isotype IgG to replace the primary antibodies. For the double staining, the hosts of the primary antibodies were different stains; the second antibodies were labeled by different fluorescence.

ELISA

Levels of OVA-specific IgE and IL-4 in serum samples were measured by ELISA with commercial reagent kits following the manufacturer's instructions.

AQ: 11

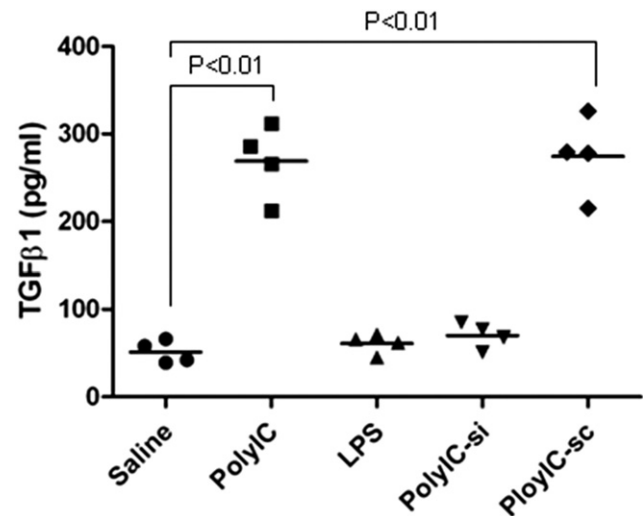
AQ: 12

Reverse-Transcription Polymerase Chain Reaction

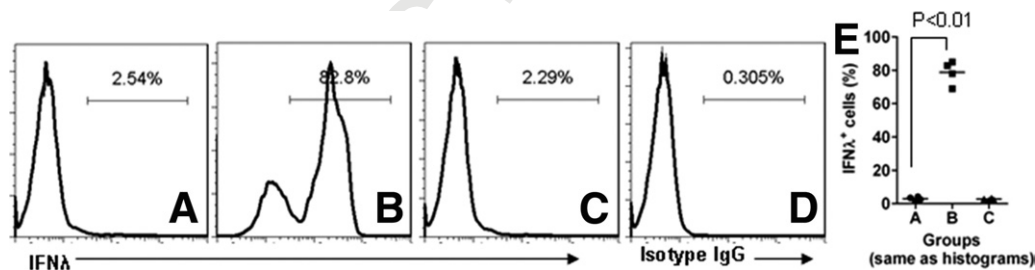
Cells were collected from culture and washed 3 times with phosphate-buffered saline. The total RNA was extracted from the cells using an RNeasy Mini Kit (QIAGEN, Toronto, Ontario, Canada). One microgram of RNA was reverse transcribed using an iScript cDNA Synthesis Kit (QIAGEN). The resulted complementary DNA was then subjected to polymerase chain reaction using a HotStarTaq Master Mix Kit (QIAGEN). The primers of IFN- λ R used in this study were as follows: forward, ggtgcaaccagaagttcat; reverse, atacagggcagatttcgac (NCBI: NM_174851; start code, 3715; end code, 3929; size, 215 base pairs). The annealing temperature was 60°C. Amplifying cycles were 35 cycles.

Statistics

Data are expressed as means $\pm$ SD. The values were analyzed using the 2-tailed unpaired Student *t* test when data consisted of 2 groups or by analysis of variance when 3 or more groups were compared. The difference is considered statistically significant when $P < .05$.



Supplementary Figure 2. Levels of TGF- β in culture supernatant. The experimental procedures were described in the legend to Figure 4. Supernatant was collected at the end of culture and subjected to analysis of TGF- β levels by ELISA following the manufacturer's instructions. The scatter dot plots indicate the levels of TGF- β in supernatant. Each dot represents an individual data point. The annotations of the x-axis indicate the treatment for DCs. si, CD3⁺ cells were treated with small interfering RNA of IFN- λ R; sc, CD3⁺ cells were treated with control small interfering RNA.



Supplementary Figure 1. Activation of TLR3 increases IFN- λ expression in DCs. BmDCs were cultured in the presence of (A) LPS or (B) polyIC for 24 hours. The histograms show frequency of IFN- λ -positive DCs. Panel C is naïve control (naïve BmDCs). Panel D is isotype IgG control. The scatter dot plots in panel E show individual data points of panels A-C.