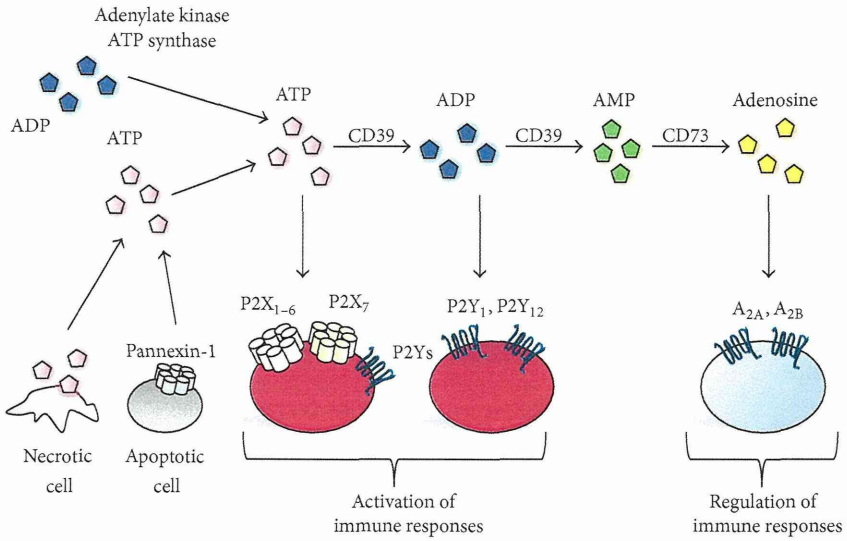

FIGURE 1: ATP is released from necrotic and apoptotic cells as extracellular ATP (eATP). Also, adenylate kinase and synthase mediate the generation of ATP in the extracellular compartment. Extracellular purines (e.g., ATP and ADP) stimulate their receptors and modulate various biological processes. Once eATP is released, the ATP is soon hydrolyzed to AMP and adenosine by the ectonucleotidases CD39 and CD73. Adenosine binds to adenosine receptors (e.g., A_1, A_{2A}, A_{2B}, and A_3).

responses [30]. Th2-type immune responses are also induced by dendritic cells expressing $P2X_7$. Indeed, depletion of eATP by apyrase treatment or $P2X_7$ deficiency reduces signs of inflammation in the upper respiratory tract [31]. It was recently found that the functional capacity of $P2X_7$ (i.e., its ability to promote pore formation) is associated with asthma risk or disease severity in humans [32]. Moreover, *in vivo* imaging analysis has revealed eATP release in the intestinal compartment and peritoneal cavity of mice with acute graft-versus-host disease (GVHD) [33]. Treatment with apyrase or with inhibitors of various purinergic receptors inhibits GVHD-associated intestinal inflammation. In this case, the eATP-P2X7 pathway activates dendritic cells and consequently induces Th1 immune responses (e.g., IFNγ production) and expansion of donor T cells, thus contributing to the onset of inflammation.

Several studies have revealed the pathologic roles of eATP and purinergic receptors (especially $P2X_7$) in the development of intestinal disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [1, 34] (Table 1). IBS is a common gastrointestinal disorder characterized by discomfort, chronic abdominal pain, and altered bowel habit. Sometimes it occurs after intestinal infection. One meta-analysis has demonstrated that the risk of IBS increases 600% after gastrointestinal infection [35]. Consistently, it has been reported that transient intestinal infection with *Trichinella spiralis* in mice causes postinflammatory visceral hypersensitivity, which is associated with IL-1β production mediated by eATP-$P2X_7$ pathways [34] (Table 1). Because mast cells are considered to play a critical role in the development of IBS and express high levels of $P2X_7$, it is possible that the eATP-$P2X_7$ pathway in mast cells is involved in the development of IBS [36].

The eATP-purinergic receptor pathway, especially the eATP-$P2X_7$ pathway, is also involved in the development of IBD. Overexpression of $P2X_7$ receptors has been observed in the intestinal mucosa of patients with IBD—especially Crohn's disease [44]. Experimentally, $P2X_7$-deficient mice do not develop experimental colitis, and inhibition of $P2X_7$ by A-740003, Brilliant Blue G, or KN-62 ameliorates experimental colitis by reducing the recruitment of neutrophils, T cells, and macrophages, as well as collagen deposition [44] (Table 1). The eATP-P2X7 pathway is therefore now considered to be a novel therapeutic target in the treatment of IBD [43, 44] (Table 1).

Several mechanisms of eATP-mediated inflammation in the development of IBD have been proposed. First, eATP from damaged intestinal epithelial cells, which are frequently observed in IBD patients, and inflammatory cells (e.g., neutrophils and macrophages) stimulates dendritic cells to produce IL-6, IL-12, and IL-23 and TGFβ, thus inducing the production of inflammatory Th1 and Th17 cells [42, 43] (Figure 2) (Table 1). Enteric neuronal cell death is frequently observed in intestinal inflammation and causes colonic motor dysfunction. The eATP–P2X7 pathway is involved in enteric neuronal cell death through the pannexin-inflammasome cascade, and thus colonic motor dysfunction during colitis is prevented by targeting these pathways [41] (Table 1). We previously established mast cell-specific antibody libraries and showed that $P2X_7$ is expressed at high levels in mast cells in the colonic tissues [40]. eATP stimulates mast cells to induce the production of inflammatory chemokines (e.g., CCL2, CCL4, CCL7, CXCL1, and CXCL2), cytokines (IL-1β, IL-6, and TNFα), and mediators (histamines and leukotriene). Thus, blockade of $P2X_7$ by a specific antibody (1F11 monoclonal antibody) inhibits mast cell activation in the colonic

Table 1: Recent reports indicating the critical roles of eATP in the adverse conditions of intestines (inflammatory bowel diseases and irritable bowel syndrome).

Enteric diseases	Receptors	Functions	Reference
Inflammatory bowel disease	P2R/A2BR	Enhance co-transmigration of neutrophils and platelets across intestinal epithelial cells in IBD patients. Platelets release large amount of ATP in the lumen metabolite to adenosine via CD73 and ecto-NTPDases expressed in epithelial cells. Adenosine-A2BR pathway induces electrogenic Cl- secretion with water movement to lumen.	[37]
	P2XR	T cell receptor stimulation induces ATP synthesis and release from activated T cells through pannexin-1 hemichannels. Released ATP activates T cells and produce IL-2 and proliferation in autocrine manner. Blockage of P2X receptors (oxidative ATP) impairs the development of colitis in mice.	[38]
	P2R	ATP released from commensal bacteria acts on CD70+ CD11c+ cells reside in the intestinal lamuna propria and induces Th17 cells in mice; degradation of ATP (by apyrase treatments) ameliorates colitis in mice.	[9]
	P2Y2	Increase of P2Y2 expression in epithelial cells is observed during colitis. P2Y2 stimulation induces release of prostaglandin E2 release from the cells and promotion of intestinal microtubule stabilization and mucosal reepithelization. Those pathways take part in the wound healing during colonic inflammation. Treatment with P2Y2 agonist improves recovery from colitis in mice.	[39]
	P2X7	ATP induces activation of mast cells and enhances inflammatory responses, upregulation of P2X7 in mast cells of Crohn's disease patients, anti-P2X7 antibody treatment inhibits colitis in mice.	[40]
	P2X7	Induction of enteric neuronal cell death and alteration of intestinal motility.	[41]
	P2R	ATP induces IL-6 and CXCL1 productions from epithelial cells; ATP influences the response of epithelial cells to various TLR ligands and induces inflammatory T cells by affecting DC maturations.	[42]
	P2X7	Prophylactic systemic P2X7 blockade (A740003 and brilliant blue G) reduces inflammatory cytokines in rats.	[43]
	P2X7	Upregulation of P2X7 in epithelium, macrophage, and dendritic cells of Crohn's disease patients, P2X7-deficient mice did not develop colitis.	[44]
Irritable bowel syndrome	P2X7	Induction of IL-1β and the development of postinflammatory visceral hypersensitivity in the *Trichinella spiralis*-infected mouse	[34]

tissues and consequently prevents the development of intestinal inflammation [40] (Table 1). In this pathway, $P2X_7$ expression on mast cells is important for the development of colitis, because mast cell-deficient mice reconstituted with $P2X_7$-deficient mast cells show amelioration of inflammatory signs. Of clinical relevance, we have found that the number of $P2X_7^+$ mast cells is increased at sites of inflammation in Crohn's disease patients [40]. eATP is produced by injured epithelial cells and inflammatory cells, including neutrophils, via gap junction molecules such as connexin 43 [45]. It was reported that P2Y2 and P2X7 receptors are important for the migration of neutrophils and macrophages. In the inflammatory condition, neutrophils transmigrated between epithelial cells to the luminal part of the intestine. In this condition, platelets translocate along with neutrophils and released eATP at the mucosal surface (Figure 2) (Table 1). Additionally, mast cells express ectoadenylate kinase and ATP synthase to mediate the extracellular conversion of ADP to ATP, which in turn promotes mast cell activation in an autocrine and paracrine manner (Figure 2). We have recently found that, in contrast to the abundance of $P2X_7$ expression on mast cells in the colon, there are limited levels of $P2X_7$ expression on skin mast cells, which is regulated by skin fibroblasts [46]. Skin fibroblasts uniquely express Cyp26b1 to

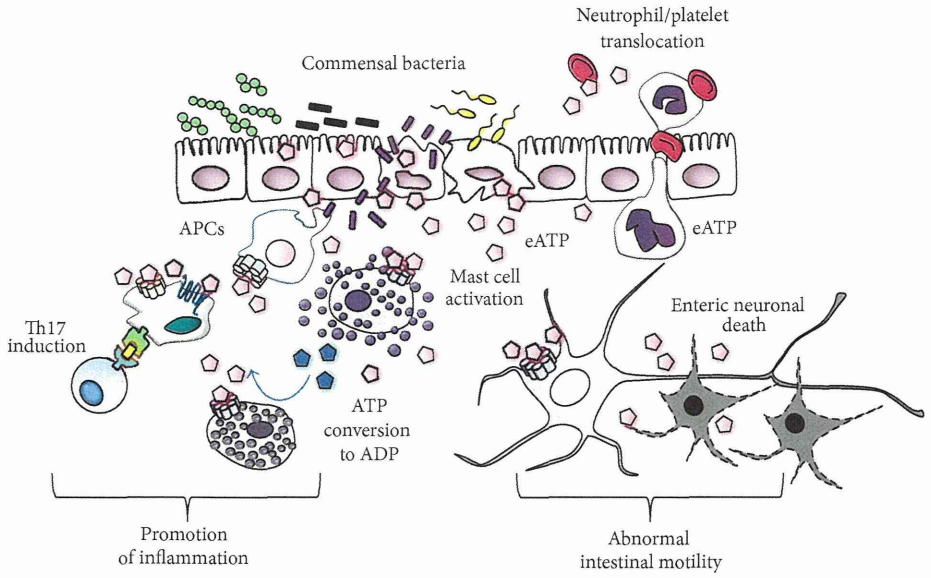

FIGURE 2: In the intestinal compartment, extracellular ATP (eATP) is released from damaged epithelial cells and commensal bacteria. Macrophages, platelets, mast cells, and neutrophils are potential source of eATP upon their activation. Neutrophils facilitate translocation of platelets across intestinal epithelium. eATP also induces Th17 cell generation, activation of mast cells, and neuronal cell death, promoting intestinal inflammation. APCs: antigen-presenting cells. eATP stimulates mast cells to induce the production of inflammatory chemokines (e.g., CCL2, CCL4, CCL7, CXCL1, and CXCL2), cytokines (IL-1β, IL-6, and TNFα), and mediators (histamines and leukotrienes).

degrade retinoic acid within tissues or microenvironments; Cyp26b1 is responsible for inhibiting $P2X_7$ expression [46]. Thus, unique tissue environments determine $P2X_7$ expression on mast cells, which is a critical factor in the development of local inflammation.

4. Resolution of eATP-Mediated Inflammation for Maintenance of Mucosal Homeostasis

Once eATP is released, it is soon hydrolyzed to ADP, AMP, and adenosine by the ectonucleotidases CD39 and CD73; this is essential for resolving inflammatory responses (Figure 1). Indeed, CD39-deficient mice, as well as humans who have CD39 polymorphism and thus low levels of CD39 expression, have increased susceptibility to IBD [47]. Similarly, CD73 deficiency or administration of CD73 inhibitor (e.g., α,β-methylene ADP) enhances susceptibility to intestinal inflammation in mice [48–50].

Adenosine, which is derived from the dephosphorylation of eATP via CD39 and CD73 or diffuses directly from the intracellular compartment via equilibrative nucleoside transporters, binds to adenosine receptors such as A_{2A} and A_3 receptors, which are involved in both the promotion and the resolution of inflammatory responses [51–53]. A_{2A} and A_3 receptor expression on T cells and myeloid cells is a prerequisite for the inhibition of intestinal inflammation [54]. In fact, A_{2A} and A_3 adenosine receptor-selective agonists (e.g., ATP-146e and IB-MECA, resp.) ameliorate intestinal inflammation by impairing the recruitment of inflammatory cells and the production of inflammatory cytokines [55, 56]. In addition, cyclosporine, salicylates, methotrexate, and sulfasalazine, which are used to treat IBD in humans, all decrease eATP levels and increase adenosine production, partly via the stimulation of CD73-dependent adenosine production [57]. Similarly, upregulation of CD39 expression induced on dendritic cells by IL-27 hampers Th1 and Th17 cell production and consequently prevents eATP-mediated inflammation [58]. All of this evidence indicates that inhibition of eATP signaling, together with the promotion of adenosine-mediated regulatory pathways by targeting receptors or ectoenzymes, would be a beneficial strategy for the treatment of intestinal inflammation.

5. Closing Remarks

The importance of purinergic signaling was recognized almost 70 years ago. Accumulating evidence has since revealed the underlying molecular and cellular mechanisms of purinergic signal-mediated maintenance and disruption of mucosal homeostasis. Currently, the clinical relevance of some of the drugs used to treat intestinal inflammation is explained by their regulation of eATP-adenosine balance. Additionally, drugs that target purinergic receptors have now undergone clinical trials [11]. Notably, ATP-adenosine balance, as well as receptor expression levels and the cells expressing these receptors, differs among tissues and environmental conditions. Further investigations using new technologies such as *in vivo* monitoring of eATP release [59, 60] will clarify the complex mechanisms of purinergic signal-mediated immune regulation. This in turn will provide further advances in the design of drugs for preventing and

treating inflammatory diseases and maintaining immunologic health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Some of the work featured in this review article was supported by grants from the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries, and the Food Industry; the Program for Promotion of Basic and Applied Research for Innovations in Bio-Oriented Industry; Japan Science and Technology Agency's Core Research for Evolutional Science and Technology (CREST); the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Ministry of Health and Welfare of Japan; the Yakult Bio-Science Foundation; Kowa Life Science Foundation; a Kishimoto Foundation Research Grant; and The Naito Foundation.

References

[1] M. Idzko, D. Ferrari, and H. K. Eltzschig, "Nucleotide signalling during inflammation," *Nature*, vol. 509, no. 7500, pp. 310–317, 2014.

[2] G. Burnstock, "Purinergic signalling and disorders of the central nervous system," *Nature Reviews Drug Discovery*, vol. 7, no. 7, pp. 575–590, 2008.

[3] G. Burnstock, "The journey to establish purinergic signalling in the gut," *Neurogastroenterology and Motility*, vol. 20, no. 1, pp. 8–19, 2008.

[4] J. A. Roberts, M. K. Lukewich, K. A. Sharkey, J. B. Furness, G. M. Mawe, and A. E. Lomax, "The roles of purinergic signaling during gastrointestinal inflammation," *Current Opinion in Pharmacology*, vol. 12, no. 6, pp. 659–666, 2012.

[5] W. G. Junger, "Immune cell regulation by autocrine purinergic signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 201–212, 2011.

[6] A. Piccini, S. Carta, S. Tassi, D. Lasiglié, G. Fossati, and A. Rubartelli, "ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1β and IL-18 secretion in an autocrine way," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 23, pp. 8067–8072, 2008.

[7] S. E. Adamson and N. Leitinger, "The role of pannexin1 in the induction and resolution of inflammation," *FEBS Letters*, vol. 588, no. 8, pp. 1416–1422, 2014.

[8] T. Iwase, H. Shinji, A. Tajima et al., "Isolation and identification of ATP-secreting bacteria from mice and humans," *Journal of Clinical Microbiology*, vol. 48, no. 5, pp. 1949–1951, 2010.

[9] K. Atarashi, J. Nishimura, T. Shima et al., "ATP drives lamina propria T_H17 cell differentiation," *Nature*, vol. 455, no. 7214, pp. 808–812, 2008.

[10] T. Kusu, H. Kayama, M. Kinoshita et al., "Ecto-nucleoside triphosphate diphosphohydrolase 7 controls Th17 cell responses through regulation of luminal ATP in the small intestine," *Journal of Immunology*, vol. 190, no. 2, pp. 774–783, 2013.

[11] H. K. Eltzschig, M. V. Sitkovsky, and S. C. Robson, "Purinergic signaling during inflammation," *The New England Journal of Medicine*, vol. 367, no. 24, pp. 2322–2333, 2012.

[12] L.-H. Jiang, M. Kim, V. Spelta, X. Bo, A. Surprenant, and R. A. North, "Subunit arrangement in P2X receptors," *The Journal of Neuroscience*, vol. 23, no. 26, pp. 8903–8910, 2003.

[13] G. E. Torres, T. M. Egan, and M. M. Voigt, "Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners," *The Journal of Biological Chemistry*, vol. 274, no. 10, pp. 6653–6659, 1999.

[14] H. M. Costa-Junior, F. S. Vieira, and R. Coutinho-Silva, "C terminus of the P2X7 receptor: treasure hunting," *Purinergic Signalling*, vol. 7, no. 1, pp. 7–19, 2011.

[15] H. L. Wilson, S. A. Wilson, A. Surprenant, and R. North, "Epithelial membrane proteins induce membrane blebbing and interact with the P2X7 receptor C terminus," *The Journal of Biological Chemistry*, vol. 277, no. 37, pp. 34017–34023, 2002.

[16] F. Scheuplein, N. Schwarz, S. Adriouch et al., "NAD+ and ATP released from injured cells induce P2X 7-dependent shedding of CD62L and externalization of phosphatidylserine by Murine T cells," *Journal of Immunology*, vol. 182, no. 5, pp. 2898–2908, 2009.

[17] C. Lin, S. Ren, L. Zhang, H. Jin, J. Sun, and Y. Zuo, "Extracellular ATP induces CD44 shedding from macrophage-like P388D1 cells via the P2X7 receptor," *Hematological Oncology*, vol. 30, no. 2, pp. 70–75, 2012.

[18] Y. Chen, Y. Yao, Y. Sumi et al., "Purinergic signaling: a fundamental mechanism in neutrophil activation," *Science Signaling*, vol. 3, no. 125, p. ra45, 2010.

[19] D. Ferrari, P. Chiozzi, S. Falzoni, S. Hanau, and F. Di Virgilio, "Purinergic modulation of interleukin-1β release from microglial cells stimulated with bacterial endotoxin," *Journal of Experimental Medicine*, vol. 185, no. 3, pp. 579–582, 1997.

[20] G. Corrêa, C. Marques da Silva, A. C. de Abreu Moreira-Souza, R. C. Vommaro, and R. Coutinho-Silva, "Activation of the $P2X_7$ receptor triggers the elimination of *Toxoplasma gondii* tachyzoites from infected macrophages," *Microbes and Infection*, vol. 12, no. 6, pp. 497–504, 2010.

[21] T. Darville, L. Welter-Stahl, C. Cruz, A. A. Sater, C. W. Andrews Jr., and D. M. Ojcius, "Effect of the purinergic receptor $P2X_7$ on *Chlamydia* infection in cervical epithelial cells and vaginally infected mice," *The Journal of Immunology*, vol. 179, no. 6, pp. 3707–3714, 2007.

[22] M. Vono, M. Taccone, P. Caccin et al., "The adjuvant MF59 induces ATP release from muscle that potentiates response to vaccination," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 52, pp. 21095–21100, 2013.

[23] J. K. Crane, R. A. Olson, H. M. Jones, and M. E. Duffey, "Release of ATP during host cell killing by enteropathogenic *E. coli* and its role as a secretory mediator," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 283, no. 1, pp. G74–G86, 2002.

[24] J. K. Crane, T. M. Naeher, S. S. Choudhari, and E. M. Giroux, "Two pathways for ATP release from host cells in enteropathogenic *Escherichia coli* infection," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 289, no. 3, pp. G407–G417, 2005.

[25] A. Puhar, H. Tronchère, B. Payrastre, G. Tran van Nhieu, and P. J. Sansonetti, "A Shigella effector dampens inflammation by regulating epithelial release of danger signal ATP through

production of the lipid mediator PtdIns5P," *Immunity*, vol. 39, no. 6, pp. 1121–1131, 2013.

[26] A. Firon, M. Dinis, B. Raynal, C. Poyart, P. Trieu-Cuot, and P. A. Kaminski, "Extracellular nucleotide catabolism by the Group B *Streptococcus* ectonucleotidase NudP increases bacterial survival in blood," *The Journal of Biological Chemistry*, vol. 289, no. 9, pp. 5479–5489, 2014.

[27] R. Coutinho-Silva and D. M. Ojcius, "Role of extracellular nucleotides in the immune response against intracellular bacteria and protozoan parasites," *Microbes and Infection*, vol. 14, no. 14, pp. 1271–1277, 2012.

[28] S. Brumfield, J. J. Matasi, D. Tulshian et al., "Synthesis and SAR development of novel P2X7 receptor antagonists for the treatment of pain: part 2," *Bioorganic and Medicinal Chemistry Letters*, vol. 21, no. 24, pp. 7287–7290, 2011.

[29] L. Antonioli, R. Colucci, C. Pellegrini et al., "The role of purinergic pathways in the pathophysiology of gut diseases: pharmacological modulation and potential therapeutic applications," *Pharmacology & Therapeutics*, vol. 139, no. 2, pp. 157–188, 2013.

[30] T. Müller, B. Robaye, R. P. Vieira et al., "The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation," *Allergy*, vol. 65, no. 12, pp. 1545–1553, 2010.

[31] T. Müller, R. P. Vieira, M. Grimm et al., "A potential role for $P2X_7R$ in allergic airway inflammation in mice and humans," *American Journal of Respiratory Cell and Molecular Biology*, vol. 44, no. 4, pp. 456–464, 2011.

[32] D. M. Manthei, D. J. Jackson, M. D. Evans et al., "Protection from asthma in a high-risk birth cohort by attenuated P2X 7 function," *Journal of Allergy and Clinical Immunology*, vol. 130, no. 2, pp. 496–502, 2012.

[33] K. Wilhelm, J. Ganesan, T. Müller et al., "Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R," *Nature Medicine*, vol. 16, no. 12, pp. 1434–1439, 2010.

[34] C. Keating, P. Pelegrin, C. M. Martínez, and D. Grundy, "$P2X_7$ receptor-dependent intestinal afferent hypersensitivity in a mouse model of postinfectious irritable bowel syndrome," *The Journal of Immunology*, vol. 187, no. 3, pp. 1467–1474, 2011.

[35] M. Thabane, D. T. Kottachchi, and J. K. Marshall, "Systematic review and meta-analysis: the incidence and prognosis of post-infectious irritable bowel syndrome," *Alimentary Pharmacology & Therapeutics*, vol. 26, no. 4, pp. 535–544, 2007.

[36] L. Ohman and M. Simren, "Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions," *Nature Reviews Gastroenterology & Hepatology*, vol. 7, no. 3, pp. 163–173, 2010.

[37] T. Weissmüller, E. L. Campbell, P. Rosenberger et al., "PMNs facilitate translocation of platelets across human and mouse epithelium and together alter fluid homeostasis via epithelial cell-expressed ecto-NTPDases," *Journal of Clinical Investigation*, vol. 118, no. 11, pp. 3682–3692, 2008.

[38] U. Schenk, A. M. Westendorf, E. Radaelli et al., "Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels," *Science Signaling*, vol. 1, no. 39, article ra6, 2008.

[39] E. Degagné, J. Degrandmaison, D. M. Grbic, V. Vinette, G. Arguin, and F.-P. Gendron, "P2Y2 receptor promotes intestinal microtubule stabilization and mucosal re-epithelization in experimental colitis," *Journal of Cellular Physiology*, vol. 228, no. 1, pp. 99–109, 2013.

[40] Y. Kurashima, T. Amiya, T. Nochi et al., "Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors," *Nature Communications*, vol. 3, article 1034, 2012.

[41] B. D. Gulbransen, M. Bashashati, S. A. Hirota et al., "Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis," *Nature Medicine*, vol. 18, no. 4, pp. 600–604, 2012.

[42] Y. Yao, M. K. Levings, and T. S. Steiner, "ATP conditions intestinal epithelial cells to an inflammatory state that promotes components of DC maturation," *European Journal of Immunology*, vol. 42, no. 12, pp. 3310–3321, 2012.

[43] C. C. Marques, M. T. Castelo-Branco, R. G. Pacheco et al., "Prophylactic systemic P2X7 receptor blockade prevents experimental colitis," *Biochimica et Biophysica Acta: Molecular Basis of Disease*, vol. 1842, no. 1, pp. 65–78, 2014.

[44] A. R. Neves, M. T. L. Castelo-Branco, V. R. Figliuolo et al., "Overexpression of ATP-activated P2X7 receptors in the intestinal mucosa is implicated in the pathogenesis of Crohn's disease," *Inflammatory Bowel Diseases*, vol. 20, no. 3, pp. 444–457, 2014.

[45] H. K. Eltzschig, T. Eckle, A. Mager et al., "ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function," *Circulation Research*, vol. 99, no. 10, pp. 1100–1108, 2006.

[46] Y. Kurashima, T. Amiya, K. Fujisawa et al., "The Enzyme Cyp26b1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis," *Immunity*, vol. 40, no. 4, pp. 530–541, 2014.

[47] D. J. Friedman, B. M. Künzli, Y. I. A-Rahim et al., "CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 39, pp. 16788–16793, 2009.

[48] M. S. Bynoe, A. T. Waickman, D. A. Mahamed, C. Mueller, J. H. Mills, and A. Czopik, "CD73 is critical for the resolution of murine colonic inflammation," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 260983, 13 pages, 2012.

[49] N. A. Louis, A. M. Robinson, C. F. MacManus, J. Karhausen, M. Scully, and S. P. Colgan, "Control of IFN-αA by CD73: implications for mucosal inflammation," *The Journal of Immunology*, vol. 180, no. 6, pp. 4246–4255, 2008.

[50] K. Synnestvedt, G. T. Furuta, K. M. Comerford et al., "Ecto-5′-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia," *Journal of Clinical Investigation*, vol. 110, no. 7, pp. 993–1002, 2002.

[51] S. P. Colgan and H. K. Eltzschig, "Adenosine and hypoxia-inducible factor signaling in intestinal injury and recovery," *Annual Review of Physiology*, vol. 74, pp. 153–175, 2012.

[52] L. Antonioli, P. Pacher, E. S. Vizi, and G. Haskó, "CD39 and CD73 in immunity and inflammation," *Trends in Molecular Medicine*, vol. 19, no. 6, pp. 355–367, 2013.

[53] L. Antonioli, R. Colucci, C. la Motta et al., "Adenosine deaminase in the modulation of immune system and its potential as a novel target for treatment of inflammatory disorders," *Current Drug Targets*, vol. 13, no. 6, pp. 842–862, 2012.

[54] J. H. Ye and V. M. Rajendran, "Adenosine: an immune modulator of inflammatory bowel diseases," *World Journal of Gastroenterology*, vol. 15, no. 36, pp. 4491–4498, 2009.

[55] M. Odashima, G. Bamias, J. Rivera-Nieves et al., "Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease," *Gastroenterology*, vol. 129, no. 1, pp. 26–33, 2005.

[56] C. C. Kurtz, "Extracellular adenosine regulates colitis through effects on lymphoid and nonlymphoid cells," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 307, no. 3, pp. G338–G346, 2014.

[57] F. Ochoa-Cortes, A. Liñán-Rico, K. A. Jacobson, and F. L. Christofi, "Potential for developing purinergic drugs for gastrointestinal diseases," *Inflammatory Bowel Disease*, vol. 20, no. 7, pp. 1259–1287, 2014.

[58] I. D. Mascanfroni, A. Yeste, S. M. Vieira et al., "IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39," *Nature Immunology*, vol. 14, no. 10, pp. 1054–1063, 2013.

[59] T. Takahashi, Y. Kimura, K. Niwa et al., "*In vivo* imaging demonstrates ATP release from murine keratinocytes and its involvement in cutaneous inflammation after tape stripping," *Journal of Investigative Dermatology*, vol. 133, no. 10, pp. 2407–2415, 2013.

[60] P. Pellegatti, L. Raffaghello, G. Bianchi, F. Piccardi, V. Pistoia, and F. Di Virgilio, "Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase," *PLoS ONE*, vol. 3, no. 7, Article ID e2599, 2008.

RESEARCH ARTICLE

MUCOSAL IMMUNOLOGY

Innate lymphoid cells regulate intestinal epithelial cell glycosylation

Yoshiyuki Goto,[1,2,3] Takashi Obata,[1,3] Jun Kunisawa,[1,4,5] Shintaro Sato,[1,2] Ivaylo I. Ivanov,[6] Aayam Lamichhane,[1] Natsumi Takeyama,[1,7] Mariko Kamioka,[1] Mitsuo Sakamoto,[3] Takahiro Matsuki,[8] Hiromi Setoyama,[8] Akemi Imaoka,[8] Satoshi Uematsu,[9,10] Shizuo Akira,[11] Steven E. Domino,[12] Paulina Kulig,[13] Burkhard Becher,[13] Jean-Christophe Renauld,[14] Chihiro Sasakawa,[7,15,16] Yoshinori Umesaki,[8] Yoshimi Benno,[17] Hiroshi Kiyono[1,2,5]

Fucosylation of intestinal epithelial cells, catalyzed by fucosyltransferase 2 (Fut2), is a major glycosylation mechanism of host–microbiota symbiosis. Commensal bacteria induce epithelial fucosylation, and epithelial fucose is used as a dietary carbohydrate by many of these bacteria. However, the molecular and cellular mechanisms that regulate the induction of epithelial fucosylation are unknown. Here, we show that type 3 innate lymphoid cells (ILC3) induced intestinal epithelial *Fut2* expression and fucosylation in mice. This induction required the cytokines interleukin-22 and lymphotoxin in a commensal bacteria–dependent and –independent manner, respectively. Disruption of intestinal fucosylation led to increased susceptibility to infection by *Salmonella typhimurium*. Our data reveal a role for ILC3 in shaping the gut microenvironment through the regulation of epithelial glycosylation.

In the gastrointestinal tract, bilateral regulation between the gut microbiota and the host creates a mutually beneficial environment. The intestinal epithelium is a physical barrier that separates the environments inside and outside the mucosal surface. Intestinal epithelial cells (ECs) are the first line of defense against foreign antigens, including those from commensal and pathogenic bacteria. ECs play key roles in initiating and maintaining an immunologically appropriate and balanced environment in reaction to constant foreign stimulation (*1*). Resident commensal bacteria support the development of this functional mucosal immune system, and in turn, mucosal immune cells control the homeostasis of the gut microbiota and protect against pathogenic bacterial infection through intestinal ECs. In particular, type 3 innate lymphoid cells (ILC3) produce interleukin-22 (IL-22), which not only regulates the homeostasis of the commensal microbiota but also protects against *Citrobacter rodentium* infection, presumably by inducing EC-derived antimicrobial molecules such as RegIIIγ (*2*–*5*).

Fucosylated carbohydrate moieties expressed on intestinal ECs are involved in the creation of an environmental niche for commensal bacteria in mice and humans (*6*–*10*). Fucosylated glycans are generated by the addition of an L-fucose residue via an α1-2 linkage to the terminal β-D-galactose residues of glycan in a process catalyzed by fucosyltransferase. Two fucosyltransferases, Fut1 and Fut2, mediate intestinal epithelial fucosylation, and each enzyme acts on a distinct subset of epithelial cells. Fut1 regulates fucosylation of Peyer's patch (PP) M cells, whereas Fut2 is a key enzyme regulating intestinal columnar epithelial fucosylation and the production of secretory fucosylated ABO(H) histo-blood group antigens (*11*). Defective Fut2 has been shown to result in susceptibility to *Candida albicans* infection in mice (*12*). In addition, inactivating polymorphisms of *FUT2* are associated with metabolic abnormalities and infectious and inflammatory diseases in humans (*13*–*19*).

The importance of epithelial fucose has been explored through studies of host–microbe interactions. Signals from commensal bacteria are required for epithelial fucosylation (*6*). Specific commensals, in particular *Bacteroides*, have been shown to induce epithelial fucosylation and are able to catabolize fucose for energy or incorporate it into bacterial cellular components—capsular polysaccharides—that give microbes a survival advantage in competitive environments (*8*, *9*). Indeed, a lack of Fut2 alters the diversity and composition of the fecal microbiota in humans and mice (*20*, *21*). Therefore, epithelial fucose functions as a mediator between the host and commensal microbiota. Although a previous report proposed a model in which *Bacteroides*–EC interaction mediates epithelial fucosylation (*7*), the precise mechanisms by which Fut2 regulates fucosylation remain largely unknown.

Microbiota induces epithelial fucosylation

Epithelial fucosylation, a major glycosylation process, occurs in the small intestine (*10*, *11*). To assess the inductive mechanism of intestinal epithelial fucosylation, we first investigated the localization of fucosylated ECs (F-ECs) along the length of the small intestine, divided equally into four parts from the duodenum (part 1) to the terminal ileum (part 4), in naïve mice (Fig. 1A). The frequency of F-ECs, detected with the α(1,2)-fucose-recognizing lectin *Ulex europaeus* agglutinin-1 (UEA-1), was low in the duodenum and jejunum (part 1 and a portion of part 2; <15% F-ECs) and gradually increased toward the ileum (part 4; 40 to 90% F-ECs) (Fig. 1, A to C). Consistent with epithelial fucosylation, epithelial Fut2 expression was also higher in the ileum (Fig. 1D). Because greater numbers of microorganisms are present in the distal ileum than in the duodenum (*22*), it may be possible that high numbers of ileal F-ECs are induced and maintained through microbial stimulation. To test this hypothesis, we examined the fucosylation status of ileal ECs (part 4) in mice treated with a mixture of antibiotics (AB), as well as in germ-free (GF) mice. The number of F-ECs was dramatically reduced in AB-treated and GF mice (Fig. 2A and fig. S1A). Furthermore, expression of epithelial *Fut2* was also reduced in AB-treated mice (Fig. 2B). Epithelial fucosylation was restored after cessation of AB treatment and in conventionalized GF mice (Fig. 2A and fig. S1A). In addition, fucosylation of goblet cells, but not Paneth cells, was lost in AB-treated and GF mice (Fig. 2C), indicating that commensal bacteria induce fucosylation of columnar epithelial cells and goblet cells, but not Paneth cells.

It has been shown that epithelial fucosylation can be induced by the mouse and human commensal *Bacteroides thetaiotaomicron* (*6*). However, on the basis of bacterial 16*S* ribosomal RNA (rRNA) gene clone library data obtained from

[1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan. [2]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan. [3]Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba 305-0074, Japan. [4]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation, Osaka 567-0085, Japan. [5]Division of Mucosal Immunology, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan. [6]Department of Microbiology and Immunology, Columbia University Medical Center, New York, NY 10032, USA. [7]Nippon Institute for Biological Science, Tokyo 198-0024, Japan. [8]Yakult Central Institute, Tokyo 186-8650, Japan. [9]Division of Innate Immune Regulation, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan. [10]Department of Mucosal Immunology, School of Medicine, Chiba University, 1-8-1 Inohana, Chuou-ku, Chiba, 260-8670, Japan. [11]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan. [12]Department of Obstetrics and Gynecology, Cellular and Molecular Biology Program, University of Michigan Medical Center, Ann Arbor, MI 48109-5617, USA. [13]Institute of Experimental Immunology, University of Zürich, Winterthurerstrasse 190, Zürich CH-8057, Switzerland. [14]Ludwig Institute for Cancer Research and Université Catholique de Louvain, Brussels B-1200, Belgium. [15]Division of Bacterial Infection, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan. [16]Medical Mycology Research Center, Chiba University, Chiba 260-8673, Japan. [17]Benno Laboratory, Innovation Center, RIKEN, Wako, Saitama 351-0198, Japan.

isolated ileal mucus samples from naïve mice (Fig. 2D), we did not detect *B. thetaiotaomicron* in our colony, suggesting that other commensals can induce epithelial fucosylation. To identify which indigenous bacteria are responsible for the induction of F-ECs, we analyzed mucus-associated bacterial populations residing in the mouse duodenum (part 1) and ileum (part 4). In contrast to the predominance of *Lactobacillus* in the duodenum, segmented filamentous bacteria (SFB) predominated in the ileum (Fig. 2D); this is consistent with previous studies (*23*, *24*). SFB are Gram-positive bacteria that preferentially colonize the epithelial surface of the terminal ileum, where they induce T helper 17 (T_H17) cells (*25*, *26*). Similar to their effect on T_H17 cell–inducing microbiota (*27*), vancomycin, ampicillin, and to some extent metronidazole—but not neomycin—extinguished epithelial fucosylation (fig. S1, B and C). Furthermore, consistent with the emergence of SFB, epithelial fucosylation is initiated after weaning (*6*, *28*). To investigate whether SFB have the potential to induce F-ECs, we examined mono-associated gnotobiotic mice and found that F-ECs were induced in SFB but not in *Lactobacillus murinus* mono-associated mice (Fig. 2E). Together, these results suggest that epithelial fucosylation in the terminal ileum is induced by commensal bacteria, including SFB, under physiological conditions.

ILC3 are required for epithelial fucosylation

We next investigated the cellular and molecular mechanisms of F-EC induction. Commensal bacteria, including SFB, induce the proliferation of intraepithelial lymphocytes and immunoglobulin A (IgA)–producing cells and the development of T_H17 cells; they also modulate the function of ILCs (*3*, *4*, *25*–*27*, *29*). To assess whether epithelial fucosylation is induced directly by commensal bacteria or is mediated by mucosal immune cells, we first analyzed the epithelial fucose status of T cell–, B cell–, and Rag-deficient mice. The number of F-ECs was not decreased in T cell– or B cell–deficient mice (fig. S2), indicating that T cells and B cells are dispensable for the induction of epithelial fucosylation. Although SFB induce T_H17 cells (*25*, *26*), T_H17 cells are not required for epithelial fucosylation because IL-6, a critical cytokine for T_H17 cell differentiation in the intestine (*30*), was also not necessary for the induction of F-ECs (fig. S3, A to C). We next analyzed RAR-related orphan receptor–γt (RORγt)–deficient mice, which lack the ILC3 subset, in addition to T_H17 cells (*30*, *31*). RORγt-deficient mice exhibited a marked decrease in the number of F-ECs, accompanied by a decrease in *Fut2* expression in ileal ECs (Fig. 3, A to D). These findings suggest that ILC3 are critical inducers of F-ECs. This was further supported by our observation of few F-ECs in the ileum of Id2-deficient mice, which do not develop any of the ILC subsets (Fig. 3, E to G) (*31*, *32*). Although both RORγt- and Id2-deficient mice lack PPs (*33*, *34*), PPs are not necessary for epithelial fucosylation because PP-null mice, generated by treatment with monoclonal antibody (mAb) to IL-7R during fetal growth, had normal levels of F-ECs (fig. S4). ILC3 in the small intestine are aberrantly expanded in Rag-deficient mice (*35*), and elevated numbers of F-ECs were observed in these mice (Fig. 3, H and I), supporting the notion that F-ECs are induced by ILC3. Because ILC3 express higher levels of CD90, they

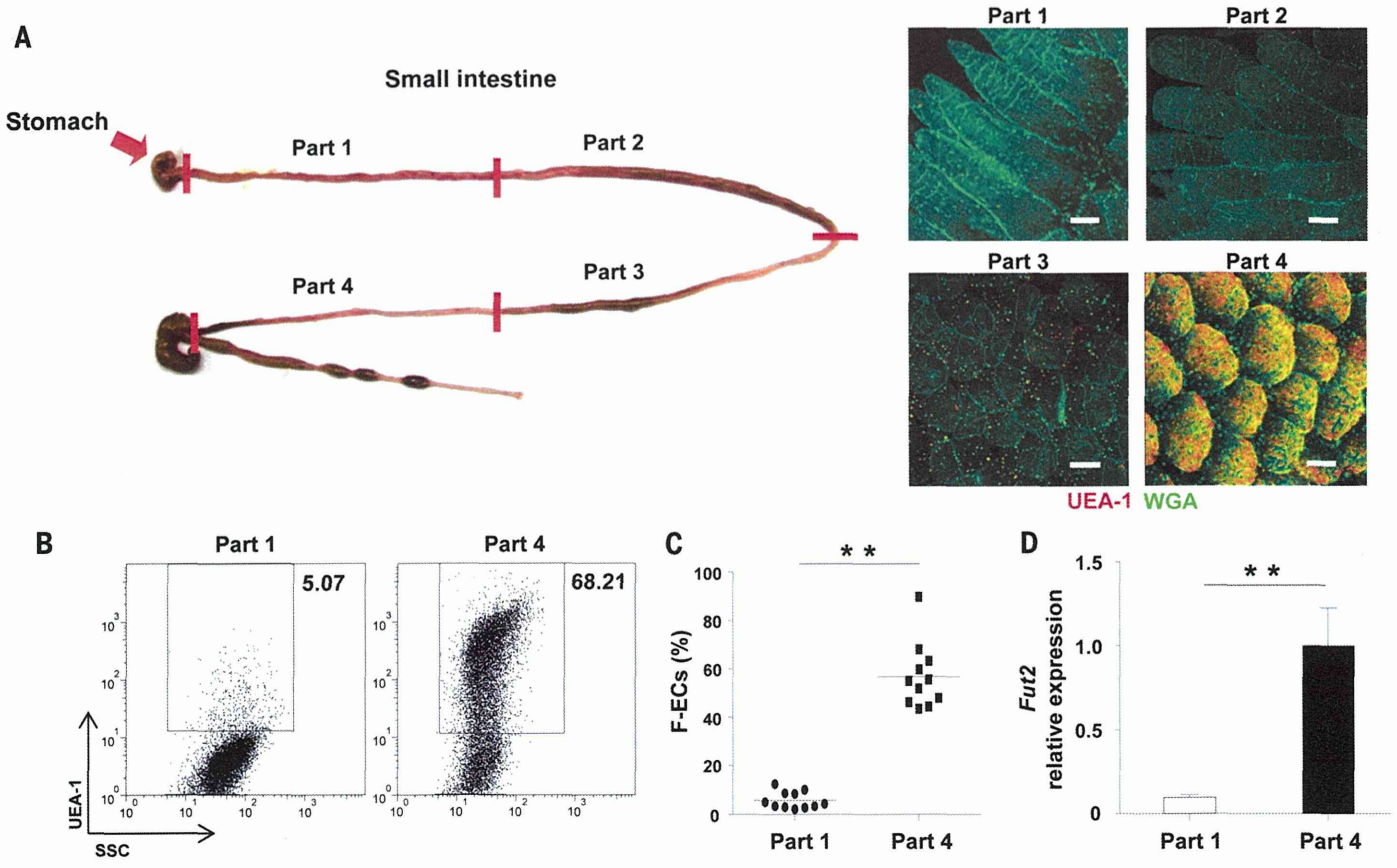

Fig. 1. F-ECs are dominant in the ileum. (**A**) Mouse small intestines were divided equally into 4 parts (parts 1, 2, 3, and 4), from the proximal (duodenum) to the distal (ileum) ends (left), and whole-mount tissues were stained with UEA-1 (red) and WGA (green) to detect F-ECs (UEA-1^+ WGA^+ cells) (right). Scale bars, 100 μm. Data are representative of three independent experiments. (**B** and **C**) Flow cytometric analysis of intestinal ECs isolated from part 1 and part 4 of the small intestines of C57BL/6 (B6) mice. Representative dot-plots are shown in (B). Percentages and mean numbers (horizontal bars) of fucosylated epithelial cells (n = 11 mice per group) are shown (C). SSC, side scatter. Data of two independent experiments are combined. (**D**) Expression of *Fut2* in ECs isolated from part 1 and part 4 of the small intestine isolated from five to six mice per group. Error bars indicate SD. $^{**}P < 0.01$ by using Student's t test. Data are representative of two independent experiments.

can be depleted with a mAb to CD90 (*36*, *37*). To identify whether ILC3 induce F-ECs, we treated wild-type and Rag-deficient mice with a mAb to CD90. *Fut2* expression and the number of F-ECs were markedly decreased after depletion of ILCs in both wild-type and Rag-deficient mice (Fig. 3, J to M, and fig. S5, A and B). Substantial numbers of SFB were still observed in RORγt-, Id2-, and CD90$^+$ ILC–depleted mice (fig. S6, A and B). Therefore, the defective epithelial fucosylation in these models was not attributable to the absence of F-EC–inducing commensals. Collectively, these results indicate that CD90$^+$ ILC3 are required for the induction and maintenance of F-ECs.

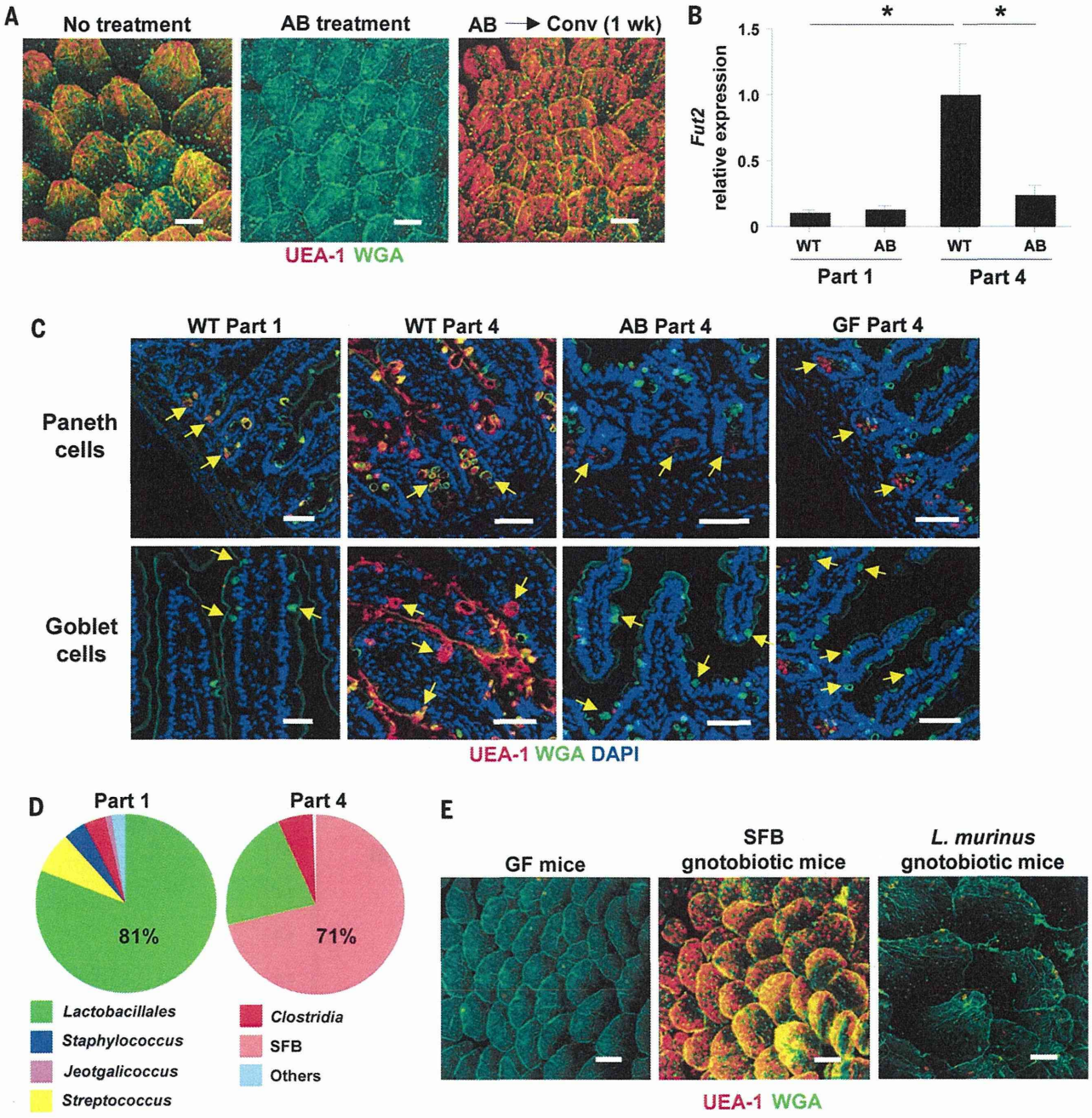

Fig. 2. Commensal bacteria induce epithelial fucosylation under homeostatic conditions. (**A**) Whole-mount ileal tissues of AB-treated mice and conventionalized AB-treated mice were stained with UEA-1 (red) and WGA (green) (n = 3 mice per group). Scale bars, 100 μm. Data are representative of two independent experiments. (**B**) *Fut2* expression in ECs isolated from part 1 (duodenum) and part 4 (ileum) of the small intestines of wild-type (WT) and AB-treated mice (n = 3 mice per group). Error bars indicate SD. *P < 0.05 by using Student's t test. Data are representative of two independent experiments. (**C**) Tissues from part 1 and part 4 of the small intestines of WT, AB-treated, and GF mice were stained with UEA-1 (red), WGA (green), and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrows show Paneth cells (top) and goblet cells (bottom). Scale bars, 50 μm. Data are representative of two independent experiments. (**D**) Bacterial populations isolated from the mucus fraction of part 1 and part 4 of mouse small intestine were analyzed by means of 16S rRNA gene clone library. Representative graphs were constructed from samples (part 1, n = 480 clones; Part 4, n = 477 clones) isolated from five different mice (95 or 96 samples were obtained from each mouse). (**E**) Ileal tissues of GF, SFB, or *L. murinus* mono-associated mice (n = 3 mice per group) were stained with UEA-1 (red) and WGA (green). Scale bars, 100 μm. Data are representative of two independent experiments.