This proliferative response was associated with Uhrf1 upregulation in Treg cells. In addition, ablation of Uhrf1 affects the development of functional Treg cells in the colonic lamina propria of young mice, but not the thymus and spleen, resulting in the spontaneous development of colitis. T cell-specific *Dnmt1*-deficient mice exhibited a similar phenotype (Obata et al., unpublished observation). These observations indicate that the DNA methylation machinery may be essential for the maintenance of Treg homeostasis after colonization by commensal microbiota. One of the principal targets of Uhrf1 is cyclin-dependent kinase inhibitor 1a (Cdkn1a), also known as $p21^{waf1/cip1}$ (Fig. 4). Cdkn1a is a negative regulator of G1 phase progression and harbors two CpG islands at proximal and distal regions of the 1000 bp preceding the transcription start site [67, 68]. Uhrf1 deficiency results in DNA hypomethylation of the *Cdkn1a* distal promoter region in embryonic stem and Treg cells [27, 69]. This results in a release of *Cdkn1a* transcriptional repression and leads to cell cycle arrest at the G1-S transition. Therefore, Uhrf1-dependent DNA methylation may ensure the highly proliferative nature of colonic Treg cells by epigenetically silencing *Cdkn1a* expression (Fig. 4). Given that Treg cells are under the influence of TGF-β signaling, which has the capacity to induce *Cdkn1a* expression [70], proliferating Treg cells might need to inhibit the recruitment of Smad3 (a downstream molecule for TGF-β signaling) to the *Cdkn1a* promoter via DNA methylation. In support of this notion, the GpG island proximal Smad3-binding element on the distal promoter of is highly methylated in wild-type Treg cells.

Commensals upregulate Uhrf1 expression most likely by the early production of IL-2 from conventional T cells in response to bacterial colonization [27]. A previous study on global gene expression analysis identified *Uhrf1* as an IL-2-responsive gene in T cells [71]. Furthermore, the promoter region of *Uhrf1* gene harbors two gamma-activated site (GAS)-like motifs (STAT5 tetramer motifs) in the promoter region that are directly bound by STAT5 in response to IL-2 [72] (ChIP data set number GSE26552) (Furusawa, unpublished observation).

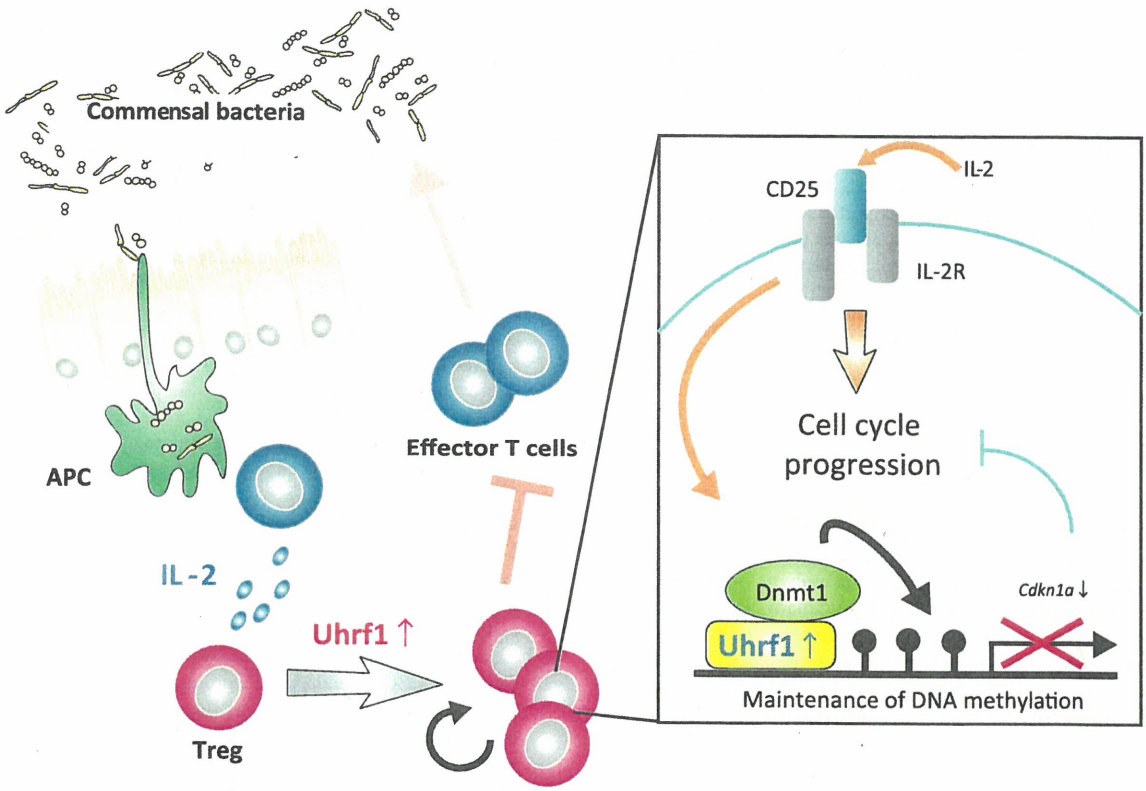

Fig. 4 A model of the Uhrf1-dependent mechanism of colonic Treg expansion in response to colonization by gut microbiota. (1) Early in life, colonizing bacteria are initially recognized by antigen-presenting cells (APC), such as DCs; (2) antigen-loaded APC evoke an IL-2 response by stimulating T effector cells through antigen presentation; (3) IL-2 provides a proliferative cue and simultaneously upregulates Uhrf1 expression in Tregs; (4) Uhrf1 represses the expression of cell cycle-dependent kinase inhibitor, Cdkn1a, via DNA methylation to safeguard the active proliferation of Tregs; and (5) actively proliferating Tregs become functionally mature and, in turn, prevent excessive immune responses to the colonizing microbiota. This model illustrates the establishment of gut immune homeostasis based on the reciprocal interaction between Tregs and T effector cells. Note that commensal bacteria-derived butyrate facilitates peripheral generation, but not proliferation, of Tregs [34]

Conclusion

Colonization by commensal microbiota influences the cell fate decisions of intestinal $CD4^+$ T cells through multiple mechanisms. Commensals are of prime importance as antigens, whereas their components also stimulate TLR signaling. Furthermore, commensal microbiota-derived SCFAs serve as ligands for Gpr41, 43, and 109a on myeloid cells and potential epithelial cells. In addition, butyrate and propionate are sufficient to modify the epigenetic status of several genes in $CD4^+$ T cells. Currently, several lines of new "omics" technologies utilizing next generation sequencing, NMR, and mass spectrometry are increasing in popularity. In particular, a metagenomic investigation enabled us to understand the complexity of microbial community. Moreover, the integrated analysis of omics-style data should provide significant insights into the mechanisms by which microbes and their metabolites influence host metabolism and immunity. Furthermore, we consider commensal microbiota as one of the most important environmental factors modulating the epigenetic status of host cells. These epigenetic modifications can be further defined by genomewide epigenetic analysis, namely, chromatin immunoprecipitation sequencing (ChIP-seq) and methylated DNA precipitation sequencing (MeDP-seq). Importantly, these *trans*-omic studies have the potential to uncover the elaborate interactions that exist between intestinal microbiota and host immune cells.

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The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells

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Intestinal regulatory T cells (T_{reg} cells) are necessary for the suppression of excessive immune responses to commensal bacteria. However, the molecular machinery that controls the homeostasis of intestinal T_{reg} cells has remained largely unknown. Here we report that colonization of germ-free mice with gut microbiota upregulated expression of the DNA-methylation adaptor Uhrf1 in T_{reg} cells. Mice with T cell–specific deficiency in Uhrf1 (*Uhrf1*$^{fl/fl}$*Cd4*-Cre mice) showed defective proliferation and functional maturation of colonic T_{reg} cells. Uhrf1 deficiency resulted in derepression of the gene (*Cdkn1a*) that encodes the cyclin-dependent kinase inhibitor p21 due to hypomethylation of its promoter region, which resulted in cell-cycle arrest of T_{reg} cells. As a consequence, *Uhrf1*$^{fl/fl}$*Cd4*-Cre mice spontaneously developed severe colitis. Thus, Uhrf1-dependent epigenetic silencing of *Cdkn1a* was required for the maintenance of gut immunological homeostasis. This mechanism enforces symbiotic host-microbe interactions without an inflammatory response.

The mammalian fetus is maintained under sterile conditions in the uterus. However, immediately after birth, it is exposed to a multitude of environmental microbes, some of which colonize the skin and mucosal surfaces. In particular, the lumen of the human distal intestine harbors trillions of microorganisms. Notably, despite such a tremendous microbial burden in close proximity to the intestinal epithelial cells, the colonizing microbiota seldom causes inflammatory diseases. This is mainly due to the establishment of an immunoregulatory system characterized by the accumulation of mucosal Foxp3$^+$ regulatory T cells (T_{reg} cells)[1–4], which serve a pivotal role in the containment of potentially pathogenic inflammatory responses[1,5,6]. T_{reg} cells arise both in the thymus and in the periphery as a consequence of exposure to microbial antigens (for example, antigens from clusters IV and XIVa of the bacterial class Clostridia, altered Schaedler flora and *Bacteroides fragilis*)[1–4,7–10]. Although much has been learned about the development, migration[11] and function of intestinal T_{reg} cells, the molecular mechanisms by which these cells establish symbiotic host-microbe relationships without inflammation still remains to be elucidated.

Epigenetic regulation serves important roles in controlling gene expression in a heritable manner[12]. Compelling evidence has revealed active contribution of epigenetic regulation to cell-fate 'decisions' as well as to the stabilization of cell lineages during the development of various cells of the immune system, including T_{reg} cells[13–15]. Butyrate derived from Clostridia bacteria upregulates acetylation of histone H3 at the promoter and conserved-noncoding-sequence regions of the locus encoding the transcription factor Foxp3 and eventually facilitates *Foxp3* expression in naive T cells[7]. That finding supports the idea that the epigenetic status of T_{reg} cells and potentially other T cell subsets may be influenced by environmental factors, such as the cytokine milieu and microbial factors. The spatiotemporal control of the epigenetic status of T_{reg} cells should be clarified for full understanding of local development of these cells and their homeostasis in the intestine.

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Uhrf1 ('ubiquitin-like, with pleckstrin-homology and RING-finger domains 1'; also known as Np95 in mice and ICBP90 in humans) is an epigenetic regulator that forms gene-repression complexes through its interaction with the DNA methyltransferase Dnmt1 and the histone deacetylase HDAC1 (refs. 16–19). Uhrf1 'preferentially' binds hemimethylated DNA via the SET- and RING finger–associated domain and contributes substantially to the accurate maintenance of DNA methylation by recruiting Dnmt1 to the hemimethylation sites. Therefore, ablation of Uhrf1 results in the hypomethylation of retrotransposons and 'imprinted' genes in embryonic stem cells[17].

Here we sought to elucidate the molecular entity responsible for the population expansion of T_{reg} cells on the basis of host-microbe interactions and found upregulation of Uhrf1 expression in colonic T_{reg} cells in response to bacterial colonization. The upregulation of Uhrf1 expression was essential for vigorous proliferation of colonic T_{reg} cells in response to bacterial colonization through its epigenetic silencing of the gene that encodes the cyclin-dependent kinase inhibitor p21 (*Cdkn1a*). Accordingly, mice with T cell–specific deletion of Uhrf1 spontaneously developed colitis due to defects in the proliferation and suppressive function of T_{reg} cells. We therefore reason that Uhrf1-dependent regulation of the proliferation of T_{reg} cells via this epigenetic mechanism is essential for containment of the inflammatory response to gut microbiota.

RESULT

Gut bacteria induce proliferation of colonic T_{reg} cells

To gain mechanistic insight into the maintenance of gut immunological homeostasis during the establishment of symbiotic host-microbe interactions, we orally inoculated germ-free (GF) mice of the IQI strain with commensal microbiota and monitored changes in interleukin 2 (IL-2)-expressing $CD4^+$ T cells and $Foxp3^+$ T_{reg} cell populations in the colonic lamina propria (cLP) of these formerly germ-free ('ex–germ-free' (exGF)) mice. The frequency of $IL\text{-}2^+CD4^+$ T cells peaked within 3 d of bacterial colonization and then gradually decreased to the basal frequency by day 7 (**Fig. 1a**). The kinetics of the T_{reg} cell population expansion paralleled that of the $IL\text{-}2^+CD4^+$ T cells up until day 3, but then the T_{reg} cell populations continued to expand (**Fig. 1a**) and became the dominant $CD4^+$ T cell population in the colon. The rapid population expansion of T_{reg} cells after bacterial colonization raised the possibility that the commensals may induce not just the differentiation[7,9] and migration[8] but also the local proliferation of T_{reg} cells in the cLP. Indeed, there was considerable population expansion of $Ki67^+$ proliferative T_{reg} cells after bacterial colonization (**Fig. 1a**). Proliferating (EdU^+) T_{reg} cells were much more abundant in the cLP of exGF mice than in that of GF mice (**Fig. 1b**). Differences in the expression of neuropilin-1 (Nrp1) has been proposed as a marker for distinguishing natural T_{reg} cell subsets from peripherally induced T_{reg} cell subsets[20,21]. We observed that both the $Nrp1^-Foxp3^+$ subset (T_{reg} cells that arose in the periphery) and $Nrp1^+Foxp3^+$ subset (T_{reg} cells that arose in the thymus) displayed the proliferative response, although it was more prominent in the $Nrp1^-$ population (**Fig. 1c**). This proliferative response was confined to colonic T_{reg} cells and was minimal in $CD4^+Foxp3^-$ conventional T cells (T_{conv} cells) and splenic $CD4^+Foxp3^+$ T cells (**Fig. 1b** and **Supplementary Fig. 1a**). Similarly, we observed the rapid T_{reg} cell population expansion in the cLP but not the spleen of specific pathogen–free (SPF) mice before weaning, a time during which the intestinal microflora is established (**Supplementary Fig. 1b,d**). To gain further evidence showing that the proliferation of T_{reg} cells was occurring locally in the colon, we blocked the influx of extraintestinal

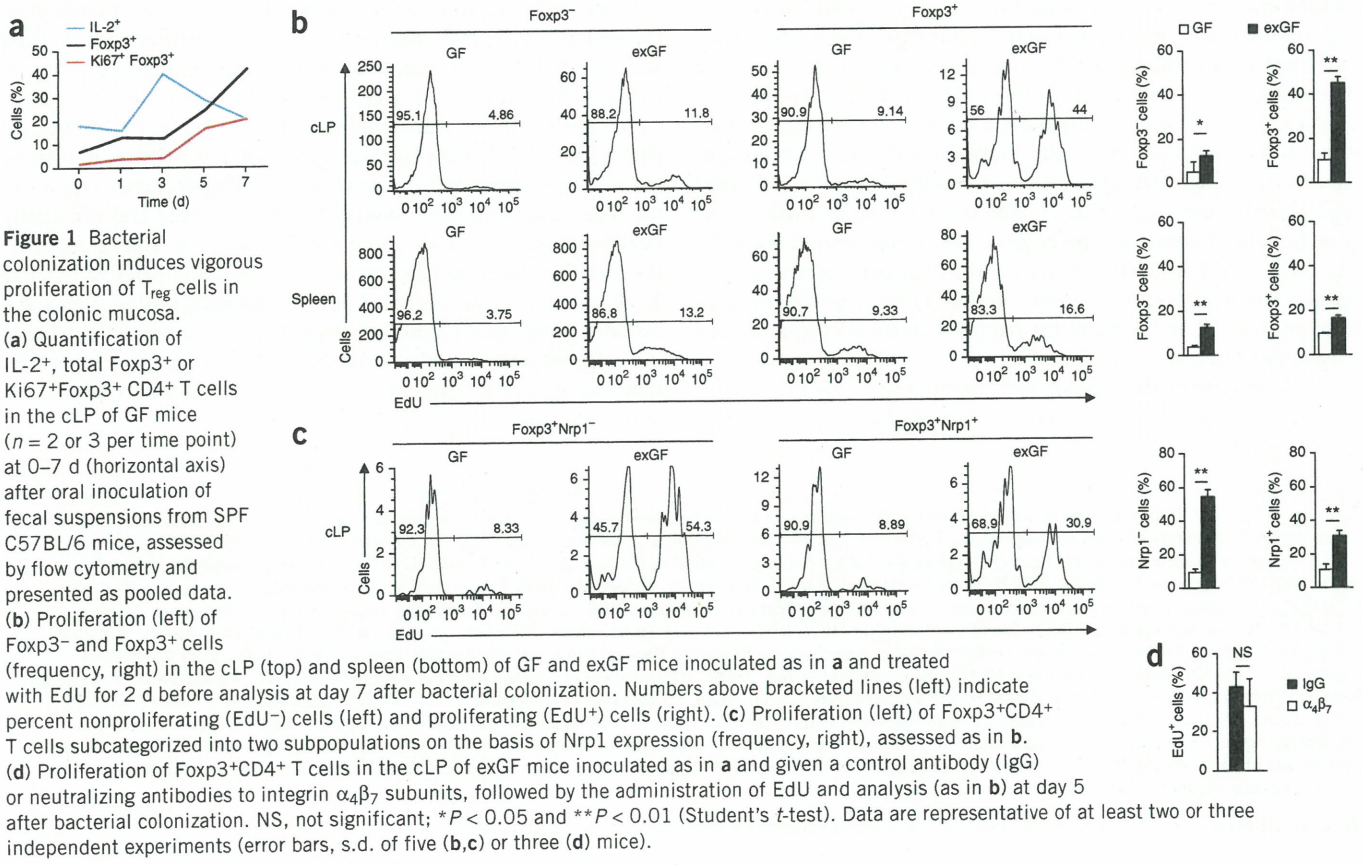

Figure 1 Bacterial colonization induces vigorous proliferation of T_{reg} cells in the colonic mucosa. (**a**) Quantification of $IL\text{-}2^+$, total $Foxp3^+$ or $Ki67^+Foxp3^+$ $CD4^+$ T cells in the cLP of GF mice ($n = 2$ or 3 per time point) at 0–7 d (horizontal axis) after oral inoculation of fecal suspensions from SPF C57BL/6 mice, assessed by flow cytometry and presented as pooled data. (**b**) Proliferation (left) of $Foxp3^-$ and $Foxp3^+$ cells (frequency, right) in the cLP (top) and spleen (bottom) of GF and exGF mice inoculated as in **a** and treated with EdU for 2 d before analysis at day 7 after bacterial colonization. Numbers above bracketed lines (left) indicate percent nonproliferating (EdU^-) cells (left) and proliferating (EdU^+) cells (right). (**c**) Proliferation (left) of $Foxp3^+CD4^+$ T cells subcategorized into two subpopulations on the basis of Nrp1 expression (frequency, right), assessed as in **b**. (**d**) Proliferation of $Foxp3^+CD4^+$ T cells in the cLP of exGF mice inoculated as in **a** and given a control antibody (IgG) or neutralizing antibodies to integrin $\alpha_4\beta_7$ subunits, followed by the administration of EdU and analysis (as in **b**) at day 5 after bacterial colonization. NS, not significant; $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of at least two or three independent experiments (error bars, s.d. of five (**b**,**c**) or three (**d**) mice).