Probiotics-derived metabolite ameliorates skin allergy by promoting differentiation of FOXP3$^+$ regulatory T cells

To the Editor:

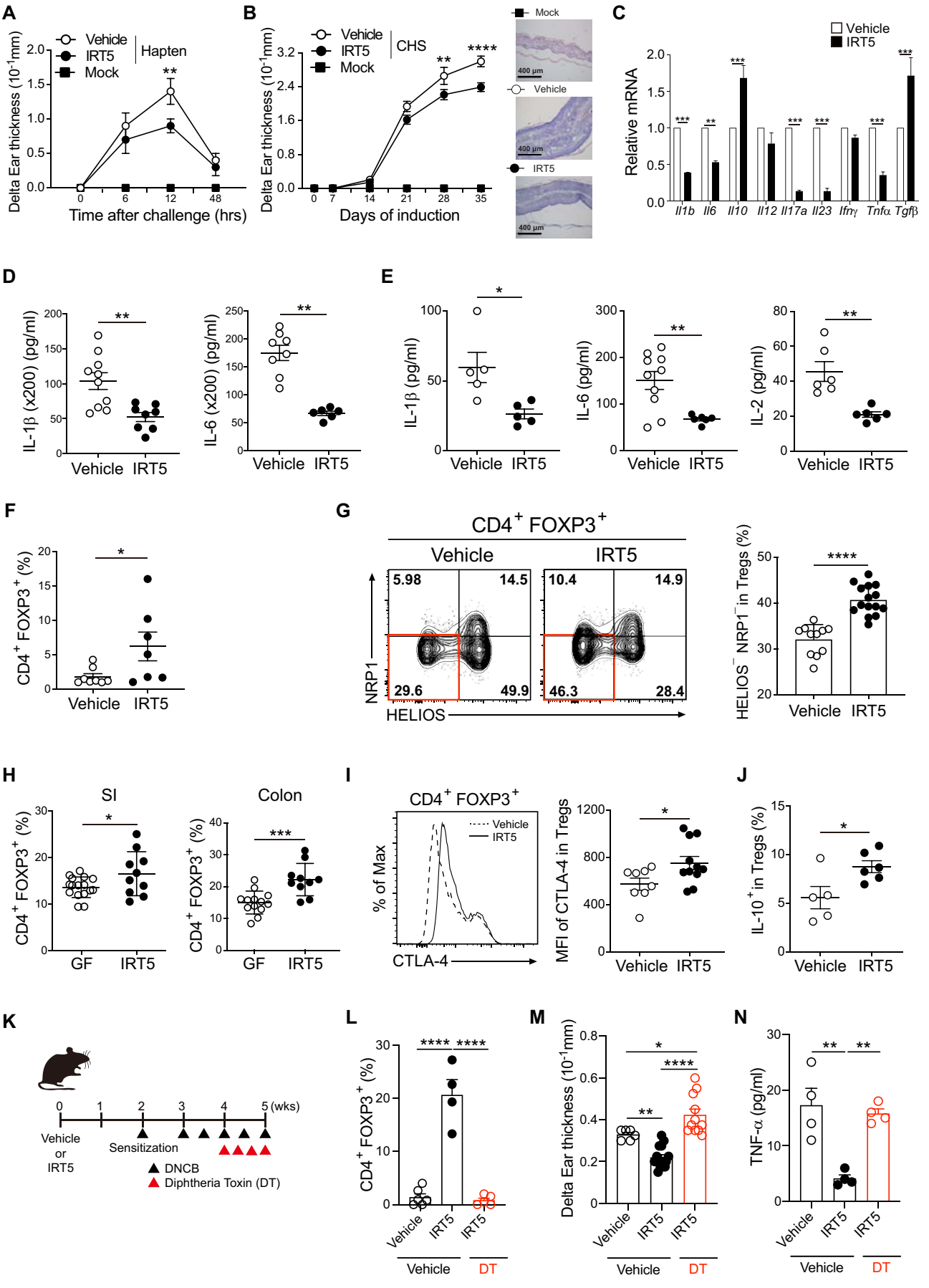
Probiotics have shown their potent immunomodulatory effects and are considered a promising alternative for the prevention and treatment of inflammatory disorders.[1] However, lack of precise action mechanisms of probiotics restrained their application, especially for the treatment of non-gastrointestinal diseases such as skin allergies including atopic dermatitis and allergic contact dermatitis.[2] Previously, we have shown that the combination of rationally selected 5 probiotic bacteria strains (known as IRT5) has persuasive therapeutic effects on autoimmunity.[3] However, it is still unclear how oral supplementation of IRT5 modulates the immune system and eventually has therapeutic efficacy in skin allergies such as contact dermatitis and atopic dermatitis.

Here, we have investigated the action mechanisms of IRT5 for the induction of FOXP3$^+$ regulatory T (Treg) cells and the resolution of skin inflammations in hapten-induced contact hypersensitivity (CHS) as well as house dust mite–induced atopic dermatitis (AD) as type 1 and type 2 inflammatory skin allergic models, respectively.[4] Consistent with known immunopathology of CHS, the topical single (Fig 1, *A*) or repeated challenges (Fig 1, *B*) of hapten (2,4-dinitrochlorobenzene) provoked severe skin swelling accompanied with intensive infiltration of mononuclear cells (Fig 1, *B*). Prophylactic treatment of IRT5 significantly mitigated severity of CHS via sensitization (Fig 1, *A*) along with elicitation phase (Fig 1, *B*) by suppressing the infiltration of inflammatory innate immune cells (see Fig E1, *A* in this article's Online Repository at www.jacionline.org) and pathogenic cytokines (IL-1β, IL-6, IL-17A, IL-23, and TNF-α) in inflamed lesion (Fig 1, *C* and *D*) as well as in serum (Fig 1, *E*). Consistently, oral administration of IRT5 significantly suppressed IFN-ɣ and IL-17A production in CD4$^+$ (Fig E1, *B*) and CD8$^+$ (Fig E1, *C*) T cells indicating subdued pathogenic T_H1/T_H17 type inflammation by IRT5 administration.

As was previously observed,[3] oral administration of IRT5 in CHS-induced mice significantly increased Treg cells in inflamed tissue (Fig 1, *F*), draining lymph nodes, and small intestine (see Fig E2, *A* and *B* in this article's Online Repository at www.jacionline.org). Interestingly, IRT5 administration seems to specifically enhance generation of peripherally induced Treg cells (HELIOS$^-$NRP1$^-$FOXP3$^+$ Treg cells) in CHS-induced mice in specific-pathogen-free (SPF) (Fig 1, *G*). Single administration of IRT5 in germ-free (GF) mice significantly enhanced NRP1$^-$HELIOS$^-$ peripheral induced Treg (pTreg) cells in small intestine and colon (Fig 1, H; see Fig E3 in this article's Online Repository at www.jacionline.org). To further prove that IRT5 administration induces pTreg cells, we have performed 2 different experiments. Indeed, we found that adoptively transferred naive CD4$^+$ T cells could be converted into Treg cells by IRT5 administration in GF mice (see Fig E4, *A* and *B* in this article's Online Repository at www.jacionline.org). To further prove whether IRT5 could convert naive T cells into RORγt$^+$FOXP3$^+$ Treg cells, we performed a cotransfer experiment in which allelically marked naive CD4$^+$ T cells from wild-type or *Rorc*^{flox}/*FoxP3*^{cre} mice, lacking microbes-induced pTreg cells,[5] into *Rag1*$^{-/-}$ host. Indeed, we found that exclusive conversion of naive T cells into pTreg cells from wild-type but not from *Rorc*^{flox}/*FoxP3*^{cre} mice by IRT5 treatment (Fig E4, *C* and *D*). These data collectively indicate that IRT5 administration enhances the generation of pTreg cells. Together with quantitative expansion of Treg cells, IRT5 qualitatively modulated Treg cells by enhancing Treg cells' effector molecules such as cytotoxic T lymphocyte–associated antigen 4 (Fig 1, *I*) and IL-10 (Fig 1, *J*) compared with the numbers for the control group. Intriguingly, we found that transient depletion of Treg cells during the course of CHS induction completely abrogated the therapeutic potency of IRT5 in reducing ear thickness and serum TNF-α level, indicating the bona fide protective role of Treg cells in allergic contact dermatitis and AD (Fig 1, *K-N*; see Fig E5 in this article's Online Repository at www.jacionline.org). Furthermore, likewise CHS, treatment of IRT5 has shown potent therapeutic effects in AD by suppressing pathogenic T_H2 cell inflammation and infiltrating inflammatory monocytes and neutrophils while promoting expansion of Treg cells, which may not be as the result of long-term colonization of IRT5 (see Fig E6, *G* in this article's Online Repository at www.jacionline.org). Collectively, these indicate potent therapeutic effects of IRT5 as the intrinsic pTreg cells augmenter in skin allergies.

How does IRT5 promote the expansion of pTreg cells? Because we could not observe a significant alteration of overall microbiome composition by IRT5 (see Fig E7 in this article's Online Repository at www.jacionline.org), we hypothesized IRT5 might directly promote the expansion of pTreg cells. Recent studies have shown a pivotal role of gut commensal to produce short chain fatty acids (SCFAs), mainly from microbial fermentative activity, that have potent immunomodulatory activities especially on Treg cells.[6-8] However, there is still no direct evidence showing that enhanced SCFAs by administration of specific bacteria could upregulate Treg cells in allergic disorders. Intriguingly, we observed that oral administration of IRT5 specifically promoted the relative production of propionate but not acetate and butyrate in gut of CHS mice compared with their vehicle-treated counterparts (Fig 2, *A*). Furthermore, gnotobiotic colonization of IRT5 in GF mice was sufficient to promote propionate (Fig 2, *B*) but not butyrate production (see Fig E8, *A* and *B*), indicating intrinsic capacity of IRT5 to produce propionate. Then, to identify the major contributor for propionate production by IRT5, we monocolonized each strain of IRT5 and monitored alteration of major SCFAs in gut of GF mice. Consistent with SPF setting, none of bacteria induced butyrate production, which is well-known for the expansion/generation of Treg cells[6,7] (Fig E8, *A*). Interestingly, *Lactobacillus reuteri* (*L reuteri*) monocolonization specifically enhanced propionate level in gut indicating the key role of *L reuteri* in propionate production by IRT5 (Fig E8, *B*).

To validate whether propionate, induced by IRT5, promotes expansion/generation of Treg cells, we pretreated mock (PBS) or propionate on CD11c$^+$ dendritic cells (DCs), cocultured them with naive CD4$^+$ T cells. Intriguingly, compared with PBS-treated DCs, propionate-treated DCs preferentially differentiated naive CD4$^+$ T cells into FOXP3$^+$ Treg cells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Il1b Il6 Il10 Il12 Il17a Il23 Ifnγ Tnfα Tgfβ
D
IL-1β (x200) (pg/ml)
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E
IL-1β (pg/ml)
IL-6 (pg/ml)
IL-2 (pg/ml)
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CD4+ FOXP3+ (%)
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CD4+ FOXP3+
5.98
14.5
29.6
49.9
10.4
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46.3
28.4
NRP1
HELIOS
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Sensitization
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(Fig 2, *C*). Because both DCs and T cells expressed G protein–coupled receptor 43 (GPR43), the receptor for propionate (see Fig E9 in this article's Online Repository at www.jacionline.org), we directly treated propionate in naive $CD4^+$ T cells and confirmed significantly enhanced Treg cells differentiation on TGF-β_1 stimulation (Fig 2, *D*). Consistently, treatment of propionate significantly enhanced histone acetylation on FoxP3 promoter as well as conserved non-coding sequence (CNS) 1 locus, which are important for the peripheral induction of FOXP3 (Fig 2, *E*; see Fig E10 in this article's Online Repository at www.jacionline.org).[9] However, cotreatment of A-485, the histone acetyltransferase inhibitor, with propionate significantly abolished Treg cells induction as well as histone acetylation on FoxP3 promoter (Fig 2, *D* and *E*), indicating the causality of histone acetylation on propionate treatment for Treg cell expansion. Altogether, these suggest that propionate directly modulates DCs as well as $CD4^+$ T cells to intensify pTreg cell differentiation. Interestingly, propionate specifically curtailed inflammatory responses by T cells (Fig 2, *F*) but not by other innate cells such as neutrophil and monocyte (see Fig E11 in this article's Online Repository at www.jacionline.org), indicating cell type–specific effects of individual SCFA. Furthermore, as with CHS, propionate treatment potently suppressed pathogenic cytokines (IL-4, IL-5, and TNF-α in $CD4^+$ T cells from AD-induced mice (see Fig E12 in this article's Online Repository at www.jacionline.org). Altogether, these indicate potential mechanisms of propionate to suppress skin inflammations by directly acting on T cells. To further test whether propionate could recapitulate the protective effect of IRT5 *in vivo*, we orally gavaged mice with vehicle or *L reuteri*, the major propionate inducer in IRT5, under CHS and AD progression. However, *L reuteri* alone failed to mimic the therapeutic potency of IRT5 (see Fig E13 in this article's Online Repository at www.jacionline.org), suggesting that an immunological synergism among bacterial strains in IRT5 may mediate therapeutic potency of IRT5. Then, we decided to use orphan G protein–coupled receptor 43, one of major receptor for propionate, -deficient mice (*Gpr43* knockout [KO]).[8] Discordant with the wild-type mice shown in Fig 1, oral supplementation of IRT5 in *Gpr43* KO mice failed to suppress pathogenesis of hapten-induced CHS (Fig 2, *G*) accompanied with impaired expansion of pTreg cells (Fig 2, *H*), resulting in uncontrolled IFN-ɣ production in $CD4^+$ (see Fig E14, *A* in this article's Online Repository at www.jacionline.org) and $CD8^+$ T cells (Fig E14, *B*). Thus, these results indicate that propionate is the key immunomodulatory metabolite induced by IRT5 for the Treg cells expansion and mitigating skin inflammation in CHS.

Taken together, our study has given the answer for the fundamental question how IRT5 (multistrain probiotics) can be the alternative therapeutics for the prevention and treatment of skin allergies. Together with our recent studies,[3] we describe a series of novel anti-inflammatory cascades triggered by oral administration of IRT5: (1) IRT5 preferentially induces the production of propionate in the gut. (2) Propionate directly acts on naive $CD4^+$ T cells to promote pTreg cell differentiation or indirectly impacts on $CD11c^+$ DCs to endow regulatory function to promote the conversion of naive $CD4^+$ T cells into pTreg cells. (3) In addition to pTreg cell expansion, propionate directly influences effector T cells to suppress allergen-induced inflammations. Importantly, all of these beneficial effects by IRT5 were disappeared by depletion of Treg cells or in *Gpr43* KO mice. In short, this study enlightens novel cellular and molecular pathways involved in the regulation of skin allergies by Treg cells inducing multiprobiotic strains and has shown their potentials as alternative therapeutics for the treatment of a broad spectrum of skin allergies.

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FIG 1. Probiotics ameliorate hapten induced contact hypersensitivity. In vehicle or IRT5-treated CHS-induced mice, ear thickness on **(A)** single or **(B)** repeated treatment of 2,4-dinitrochlorobenzene (*DNCB*) and histological changes (hematoxylin and eosin staining) were measured. Total cells from inflamed tissue were used to analyze **(C)** mRNA and **(D)** protein expression of cytokines. **E,** Key pathogenic cytokines of CHS pathogenesis in serum from vehicle- or IRT5-treated CHS mice. **F,** Frequency of Treg cells at inflamed tissue and **(G)** HELIOS/NRP1 expression in colonic Treg cells from vehicle- or IRT5-treated CHS mice. **H,** Frequency of Treg cells in small intestine (*SI*) and colon in vehicle- or IRT5-treated germ-free mice. **I,** Cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) expression and **(J)** IL-10 production in draining lymph nodes of Treg cells from vehicle- or IRT5-treated CHS mice. **K,** Experimental scheme for Treg cell depletion experiment. On vehicle or IRT5 treatment with or without Treg cell depletion in CHS-induced mice, **(L)** Treg cell proportion, **(M)** ear thickness, and **(N)** TNF-α in serum were analyzed. Each symbol represents individual animal, and at least 3 independent experiments were performed. Graphs show mean ± SEM. $*P < .05$, $**P < .01$, $***P < .005$, and $****P < .0001$ calculated by Student *t* test. *DT*, Diphtheria toxin; *MFI*, mean fluorescence intensity.

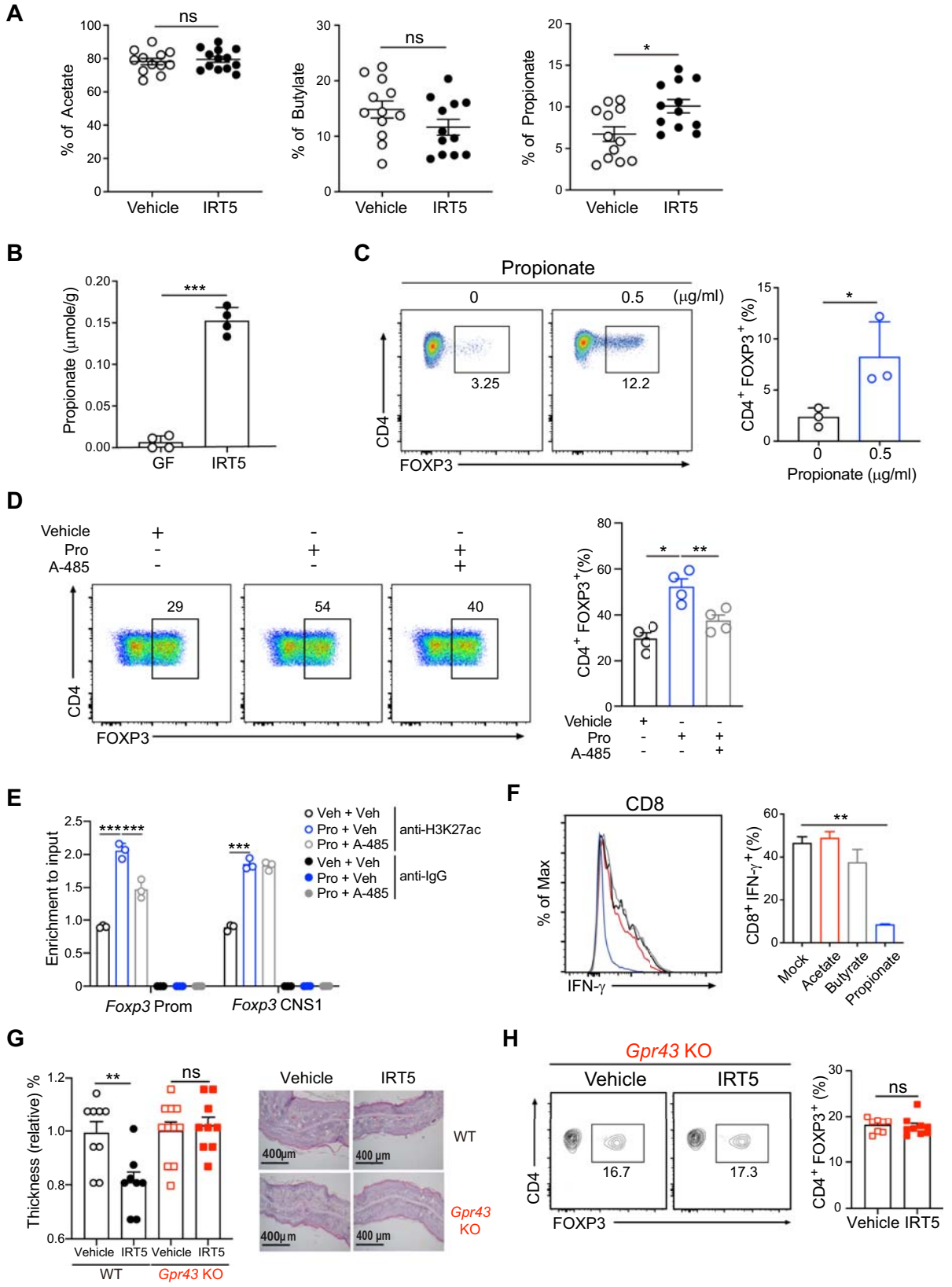

FIG 2. Probiotics derived propionate suppresses hapten induced skin inflammation. **A,** Relative levels of SCFA in fecal contents after treatment of vehicle or IRT5 in CHS mice. **B,** Propionate level in GF mice after treatment of vehicle or IRT5. **C,** Treg cell differentiation of naive T cells by vehicle (left) or propionate (right) pretreated DCs. **D,** TGF-β induced Treg cell differentiation and **(E)** H3K27 acetylation at *FoxP3* promoter and CNS1 using chromatin immunoprecipitation–quantitative PCR on propionate treatment in the absence or presence A-485. **F,** IFN-γ production in $CD8^+$ cells by treatment of various SCFAs. **G,** Ear thickness and **(H)** frequency of Treg cells at inflamed tissue in CHS-induced *Gpr43* KO mice treated with vehicle or probiotics. Each symbol represents individual animal, and at least 3 independent experiments were performed. Error bars denote mean ± SEM. Graphs show mean ± SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .005$, and **** $P < .0001$ calculated by Student *t* test. *ns*, Not significant; *Pro*, propionate; *Prom*, promoter; *Veh*, vehicle; *WT*, wild type.

These authors declare no conflicts of interest for this paper as the research was conducted without any commercial or financial relationships. The rest of the authors declare that they have no relevant conflicts of interest.

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Human basophils release the anti-inflammatory cytokine IL-10 following stimulation with α-melanocyte–stimulating hormone

To the Editor:

Basophils play a crucial role in T_H2 cell–type immune responses, including allergy, atopy, and asthma. Additionally, autoimmune skin diseases (eg, chronic spontaneous urticaria and bullous pemphigoid) are at least partially driven by basophils because of their capacity to readily release IL-4 and IL-13, as well as histamine, leukotriene C_4, and various other proinflammatory cytokines and chemokines. We previously reported that the neuropeptide α-melanocyte–stimulating hormone (α-MSH) has anti-inflammatory effects on basophil function.[1] This neuropeptide, which was originally discovered in the brain, is widely expressed in various organs and immunocompetent cells. In the skin it is expressed by keratinocytes, melanocytes, fibroblasts, and endothelial cells after stimulation with proinflammatory cytokines or UV light. Although α-MSH is notably involved in cutaneous pigmentation, it has a broad spectrum of antimicrobial, anti-inflammatory, and immunomodulatory functions in the skin, lung, and gut. Interestingly, α-MSH has potential antiallergic properties and suppresses allergic airway inflammation in an IL-10–dependent manner.[2] Because α-MSH downregulates proinflammatory basophil functions and its immunomodulatory effects *in vivo* depend on IL-10 production, we wished to address whether basophils generate IL-10 following α-MSH stimulation.

Flow cytometric analysis of healthy donor basophils revealed intracellular expression of IL-10 when stimulated with 10^{-8} M α-MSH and incubated in the presence of the secretion-blocking agent brefeldin A (Fig 1, *A*). To exclude nonbasophil cellular sources for IL-10 in our experiments, we analyzed the purity of the isolated basophils (Fig 1, *B*), which was at least 99%.

Basophils also released IL-10 at higher than basal levels, as detected by ELISA, following α-MSH stimulation over a range of concentrations (10^{-6} M to 10^{-12} M) for 24 hours (Fig 1, *C*). Further analysis of the kinetics of IL-10 release from basophils subsequently indicated that this cytokine was largely released following 4 hours of stimulation (not shown). We further verified the production of IL-10 in basophils by Western blot (WB) analysis following 4 hours of incubation with IL-3 (10 ng/mL), formyl-methionyl-leucyl-phenylalanin (fMLP) (1 μM), α-MSH (10^{-8} M and 10^{-10} M), or culture medium alone. We observed that although IL-3 or anti-IgE induced a moderate increase in IL-10 production, stimulation with either fMLP or 10^{-10} M α-MSH substantially enhanced production compared with that in unstimulated controls (Fig 1, *D*). However, stimulation of purified basophils with α-MSH in combination with anti-IgE, phorbol myristate acetate, fMLP, or IL-3 did not result in a significant increase of IL-10 release (see Fig E1 in the Online Repository at www.jacionline.org), potentially suggesting an important role of α-MSH in controlling basophil IL-10 production.

Next, we asked whether α-MSH–induced IL-10 release from basophils is mediated by the melanocortin 1 receptor (MC1R), which we previously showed to be expressed without other melanocortin receptor subtypes in basophils.[1] We utilized the IL-10 secretion assay to analyze whether agouti signaling protein (ASIP), an inverse agonist for the MC1R,[1] modulates IL-10 secretion in response to α-MSH stimulation. Preincubation of basophils with ASIP before overnight stimulation with α-MSH prevented α-MSH–induced IL-10 secretion in purified basophils, whereas ASIP alone had no effect (Fig 2, *A*).

Because α-MSH–dependent signal transduction and downstream transduction of the IL-10 receptor engage the Jak/STAT pathway,[3] we analyzed the expression of phosphorylated signal transducer and activator of transcription 3 (STAT3) by using a phosphorylated STAT3 ELISA (Fig 2, *B*) and by WB analysis (Fig 2, *C*). A further WB analysis of total STAT3 expression is shown in Fig E2 (in the Online Repository at www.jacionline.org). ELISA testing of protein lysates derived from purified basophil cultures following 24 hours of α-MSH stimulation revealed increased levels of STAT3 phosphorylation in α-MSH–stimulated basophils compared to unstimulated controls (Fig 2, *B*). WB analysis confirmed that α-MSH–stimulated basophils showed increased phosphorylation of STAT3 after 4 hours compared with the level in unstimulated controls (Fig 2, *C*). Basophil STAT3 phosphorylation was also significantly increased by IgE-dependent stimulation, but no enhanced effects were observed with a combination of α-MSH and IgE-dependent stimulation (Fig 2, *C*).

We previously showed that α-MSH inhibits IgE-dependent release of IL-4 and other basophil cytokines as well as allergen- and fMLP-induced degranulation.[1] The concomitant induction of IL-10 release from these cells, as observed in our present experiments, reveals that α-MSH not only serves to reduce the proallergic inflammatory responses of basophils but also actively induces an anti-inflammatory function through the production of IL-10. Because basophils constitute less than 1% of peripheral blood