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IL-33 induces murine intestinal goblet cell differentiation indirectly via innate lymphoid cell IL-13 secretion

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Abstract

Regulation of the intestinal mucus layer by goblet cells is important for preventing inflammation and controlling infection. IL-33, a cytokine upregulated in inflammatory bowel disease and helminth infection, induces intestinal goblet cells, but the mechanism remains unclear. Enteroids are three dimensional structures of primary small intestinal epithelial cells that contain all differentiated intestinal epithelial cell types. We developed an enteroid-immune cell co-culture model to determine the mechanism through which IL-33 affects intestinal goblet cell differentiation. We report that IL-33 does not directly induce goblet cell differentiation in murine enteroids; however, IL-13, a cytokine induced by IL-33, markedly induces goblet cells and gene expression consistent with goblet cell differentiation. When enteroids are co-cultured with $CD90^+$ mesenteric lymph node cells from IL33-treated mice, IL-33 then induces IL-13 secretion by group 2 innate lymphoid cells and enteroid gene expression consistent with goblet cell differentiation. In co-cultures, IL-33-induced *Muc2* expression is dependent on enteroid *Il4ra* expression demonstrating a requirement for IL-13 signaling in epithelial cells. In vivo, IL-33-induced intestinal goblet cell hyperplasia is dependent on IL-13. These studies demonstrate that IL-33 induces intestinal goblet cell differentiation not through direct action on epithelial cells, but indirectly through IL-13 production by goup 2 innate lymphoid cells.

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AUTHOR CONTRIBUTIONS

AW – obtained funding, experimental design, execution of experiments, analysis and interpretation of data, and drafting of the manuscript; JEV – experimental design, execution of experiments, analysis and interpretation of data, and drafting of the manuscript; AH – execution of experiments, critical review of the manuscript for important intellectual content; TA – contributed critical reagents; MJR – obtained funding, study concept and supervision, experimental design, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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Keywords

interleukin-33; epithelial cells; goblet cells; interleukin-13

INTRODUCTION

Goblet cells generate the first line of defense at the intestinal mucosa, primarily through secretion of the mucin MUC2, which generates the intestinal mucus layer (1). The mucus layer prevents the luminal contents, particularly intestinal microbiota, from coming in contact with intestinal epithelial cells. However, when the mucus becomes penetrable, this leads to colitis in mice and is associated with disease in ulcerative colitis patients (2, 3). In fact, rare variants in *MUC2* were recently found to be associated with ulcerative colitis (4). Mucins are also upregulated during helminth infections and are important for their expulsion (5).

IL-33 is a member of the IL-1 family of cytokines that signals through the IL-33 receptor (IL-33R, also called ST2) (6). Many cells types express IL-33R, including immune cells, epithelial cells and stromal cells (7–10). Mucosal IL-33 is increased during helminth infection and colitis. IL-33 augments type 2 cytokine (IL-4, IL-5, IL-13) production from T cells and innate lymphoid cells (ILCs), which is important for helminth expulsion (11–13). Injection of supraphysiological levels of IL-33 induces goblet cells in the healthy mouse intestine (6). We and others have demonstrated that IL-33 and IL-33R are protective in murine models of ulcerative colitis, in part through preservation of goblet cells (14, 15). However, the mechanism through which IL-33 regulates intestinal goblet cells, whether direct or indirect, and the important secreted intermediaries, remains under debate (8, 14).

Enteroids are structures of primary small intestinal epithelium grown from isolated crypt stem cells that contain the full complement of differentiated intestinal epithelial cell types, including goblet cells (16). The application of enteroids to the study of the intestinal epithelium overcomes many of the limitations of conventional transformed cell lines, which may behave differently than primary cells and have limited capacity for differentiation. The refinement of techniques to co-culture enteroids with other cell types holds promise for advancing studies of the interaction between the intestinal epithelium and other cellular compartments, such as mucosal-associated immune cells (17, 18).

In this study we modeled intestinal immune-epithelial interactions by co-culturing murine enteroids with mesenteric lymph node (MLN) cells enriched for group 2 ILCs (ILC2s) to demonstrate that IL-33 induces epithelial goblet cell differentiation through stimulation of ILC2s to produce IL-13. IL-13, but not IL-33, directly induced goblet cell differentiation in enteroids cultured alone. IL-33 induction of goblet cell differentiation was dependent on the presence of ILC2-enriched MLN cells and enteroid IL-13 signaling in vitro, and on IL-13 in vivo.

MATERIALS AND METHODS

Mice and In Vivo Treatment

Il13$^{-/-}$ (Balb/C), *Il4ra*$^{-/-}$ (Balb/C) (Jackson Labs strain 003514), *Il1rl1* (IL33-R)$^{-/-}$ (C57BL/6), Lgr5-EGFP-IRES-creERT2 (Jackson Lab strain 008875) and wild type (WT) C57BL/6 and Balb/C mice were bred at CCHMC and under specific pathogen free conditions and maintained on a standard laboratory chow diet in a half-day light cycle exposure and temperature-controlled environment. Male and female strain-matched mice were used and were age 6–12 weeks at the start of the experiments. The generation of the *Il13*$^{-/-}$ mice and *Il1rl1*$^{-/-}$ mice was previously described (19, 20). Mice were given phosphate-buffered saline or 0.4 ug rIL-33 daily i.p. for 4 days. The study was carried out following recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The CCHMC Institutional Animal Care and Use Committee approved the protocol.

Generation of murine enteroids and co-cultures

Mouse ileum was dissected and flushed with ice cold PBS. The ileum was opened longitudinally, cut into 1 cm pieces and incubated in 2 mM EDTA for 30 minutes at 4°C with rocking. The tissue was transferred into a new tube containing 5 mL Shaking buffer (PBS:43.3 mM Sucrose:54.9 mM Sorbitol) and shaken gently by hand for 2 minutes. Dispersed crypts were plated overnight in Matrigel (Corning, Corning, NY) with enteroid growth media (Advanced DMEM:F12, 2 mM GlutaMax, 10 mM HEPES, 100 U/mL penicillin, 100 ug/mL streptomycin, 1X N2 supplement, 1x B27 supplement (all from Invitrogen)) containing EGF (50ng/mL, Sigma, St. Louis, MO), 20% L-WRN conditioned media (L-WRN cells from ATCC, Manassas, VA). The following day, 100 ng/mL IL-33 (Peprotech, Rocky Hill, NJ) was added daily to the cultures for up to 5 days. Growth factors were replenished every other day. For co-cultures, enteroids were plated on hanging transwells (Corning). MLN cells from IL-33-treated or naïve mice (400,000 cells) were plated in the well and were stimulated with IL-2 (Peprotech), IL-7 (Peprotech) and anti-IFN-γ (Biolegend, San Diego, CA) with or without IL-33 for 4 days. WEHI-YH2 cells (kindly provided by Antony Burgess) (21) were plated to confluency in 24-well plates. Primary murine colon myofibroblasts (CMF) were generated using the methods previously described (22). In brief, mouse colons were treated with EDTA followed by digestion with collagenase and single cells were plated onto tissue culture plates for 3 hours before non-adherent cells were removed. Passage 2 was plated to confluency in 24-well plates for co-culture experiments. For each experiment, a separate mouse was used to generate enteroids, and each experiment was performed in triplicate.

Immunofluorescence

Enteroids were plated in a very thin layer of Matrigel or myofibroblasts were plated on IBIDI 4 well chamber slides. Enteroids were stimulated for 5 days with rIL-33 (100 ng/mL) or rIL-13 (10 ng/ml). Enteroids or myofibroblasts were fixed with 4% paraformaldehyde and permeabilized in PBS containing 0.1% Tween. Enteroids or myofibroblasts were stained using rabbit anti-ST2 (1:100, AB25877 from AbCam), chicken anti-GFP (1:1000 GFP-1010, Aves labs), rabbit anti-IL4r (1:100, PAS-38615, Invitrogen), rabbit anti-IL13ra1 (1:100

Pas-50989, Invitrogen) or rabbit anti-vimentin (1:100, ab45939, AbCam) followed by donkey anti-rabbit AF594 (1:200, Jackson Immuno) or donkey anti-Chicken AF488 (1:200, Jackson Immuno). Cells were also stained with FITC-UEA I (Vector Labs, Burlingame, CA), Phalloidin:AF647 (A22287, ThermoFisher) and nuclei were counterstained with Hoechst (1:1000, B2261, Sigma Aldrich). Enteroids were visualized using a Nikon A1 inverted confocal microscope. 5 um confocal optical sections were opened in NIS Viewer (Nikon), and nuclei and goblet cells were counted.

Histopathology

Mouse colon and jejunum sections were stained with periodic acid Schiff (PAS) and staining was quantified (3.14 ± 0.21 mm^2 of distal colon and 1.67 ± 0.15 mm^2 of jejunum) using a modified nuclear algorithm with Aperio Imagescope software (Buffalo Grove, IL) as previously described (15).

RNA Expression

RNA was isolated from tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA) per the manufacturer's instructions. RNA (100 ng) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). Real-time PCR was performed with TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA) for *Spdef (*Mm00600221_m1)*, Atoh1* (Mm00476035_s1)*, Muc2* (Mm00524818_m1), *Areg* (Mm01354339_m1), *Il13* (Mm00434204_m1), *Il1rl1 (*Mm01233982_m1), *Retnlb* (Mm00445845_m1) and *Gapdh* (Mm99999915_g1). All reactions were performed on a StepOnePlus real-time PCR system (ThermoFisher Scientific). Relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method with *Gapdh* as the reference.

Western Blot

Membranes were blocked with 5% nonfat dry milk in TBS-Tween (0.05%) for 1 hour and incubated overnight at 4°C with primary antibodies against Actin (Seven Hills Bioreagents, Cincinnati, OH) and Phospho-p44/42 MAPK (ERK1/2) (Cell Signaling, Danvers, MA). Membranes were incubated with HRP-linked secondary antibodies, anti-rabbit- or anti-mouse and visualized using ECL Prime and FujiFilm LAS-4000 Gel Documentation system (GE Healthcare, Piscataway, NJ). Band densitometry was determined using Image J (NIH), and the ratio of the intensity of Phospho-p44/42 MAPK to Actin was used to determine fold activation of signaling.

Flow cytometric analysis and cell sorting

For co-culture experiments, MLN cells from IL-33-treated mice were stained with PerCp-Cy5.5-conjugated CD90.2 (30-H12) and APC-Cy7-conjugated CD45 (30-F11, Biolegend, San Diego, CA) followed by cell sorting with a FACSAria II (BD Biosciences, San Jose, CA) For intracellular cytokine analyses, after restimulation with phorbol 12-myristate 13-acetate/ionomycin and treatment with Golgi blocker, LP and MLN cells were stained with biotinylated anti–T1/ST-2 (DJ8, MD Biosciences, Oakdale, MN) followed by Streptavidin-BV650, APC-conjugated anti-CD4 (GK1.5), FITC-conjugated anti-CD3 (17A2) and PerCp-Cy5.5-conjugated B220 (RA3–6B2), FCεRI (MAR-1), CD11c (N418), NK1.1 (PK136) and

CD11b (M1/70) (Biolegend). Stained cells were fixed and permeabilized for intracellular cytokine staining by using PE-conjugated anti–IL-13 (eBio13A, eBioscience, Waltham, MA). MLN and LP cells were then analyzed with an LSR II (BD Biosciences, San Jose, CA).

Statistical Analysis

For all data from experiments with three or more groups, non-parametric Kruskal-Wallis test was performed followed by two-stage step-up method of Benjamini, Krieger, and Yekutieli for false discovery rate. Data from experiments with two groups was analyzed using the non-parametric Mann-Whitney test. Individual data points and medians are plotted on all graphs. The analysis was performed on Prism software (version 7.0b, GraphPad Software, La Jolla, CA).

RESULTS

IL-33 does not directly induce goblet cells in murine enteroids

We first tested whether IL-33 directly induces goblet cells in primary murine enteroids. Fluorescence microscopy demonstrated that IL-33R is expressed on murine enteroids derived from mouse ileal crypts (Fig. 1A). Both goblet cells, labeled by UEA-1 staining of mucins, and stem cells, labeled by anti-GFP in Lgr5-EGFP-IRES-creERT2 reporter miceLgr5, expressed IL-33R, as well as IL-4RA and IL-13RA1 (Fig 1A-C and Supplemental Fig. 1A-C). We then stimulated enteroids with rIL-33 (10 ng/mL, data or shown or 100 ng/ml) for up to 5 days to determine the effect of IL-33 on goblet cell differentiation. By real-time RT-PCR we did not detect alteration in the expression of the transcription factors *Atoh1* and *Spdef,* which direct commitment to the secretory and goblet cell lineages, respectively, nor *Muc2,* the primary mucin produced by intestinal goblet cells, or *Retnlb* and *Tff3*, secretory products of goblet cells (Fig. 1D, data not shown for *Tff3*).

Since IL-33 induces IL-13 production by T cells and ILCs (6, 11), and others have shown that IL-13 induces intestinal goblet cell differentiation (19, 23), we tested whether IL-13 induces goblet cell differentiation in our primary murine enteroid cultures. As expected, IL-13 significantly induced expression of *Atoh1, Spedf, Muc2* and *Retnlb* (Fig. 1E). However, *Tff3* was not induced (data not shown). We performed confocal microscopy analysis of goblet cells using UEA1 staining, which confirmed that IL-13, but not IL-33, directly induced goblet cells in enteroids (Fig. 1F and G).

IL-33 induction of goblet cells is dependent on intestinal-associated CD45^{+}CD90^{+} immune cells in vitro

Since we did not observe a direct effect of IL-33 on enteroid goblet cells, we hypothesized that IL-33 affects goblet cells indirectly through action on either myofibroblasts or lymphoid cells. We chose to examine both intestinal myofibroblasts and immune cells, since both have been shown to respond to IL-33 and can make mediators that could affect goblet cell differentiation and mucus production (6, 9, 12, 14, 24, 25). In order to examine the role of secreted factors from an intermediate cell type, we developed a co-culture system whereby murine enteroids were suspended on a semipermeable membrane over other cell types and

both exposed to IL-33 (Fig. 2A). We first demonstrated that MLN cells, the colonic subepithelial myofibroblast cell line, WEHI-YH2 cells, and primary CMF all expressed *Il1rl1* (Fig. 2B). Furthermore, immunofluorescence analysis demonstrated that CMF expressed IL-33R, and purity of this population was confirmed with vimentin staining (Fig. 2C). To determine whether myofibroblasts are required for IL-33 to affect epithelial goblet cells, we treated enteroids co-cultured with WEHI-YH2 cells or CMF with IL-33 for 4 days. We observed no change in enteroid *Muc2* expression (Fig. 2D).

We then sought to determine whether intestinal-associated T cells or ILCs are required for IL-33 to affect enteroid goblet cells by treating enteroids co-cultured with $CD45^+CD90^+$ mesenteric lymph node (MLN) cells from IL-33-treated mice (IL-33 i.p.) with additional IL-33 in vitro (IL-33 i.p. + IL-33). IL-33 treatment in vitro significantly induced *Atoh1, Spdef* and *Muc2* expression in enteroids co-cultured with MLN cells (Fig. 2E). Furthermore, increases in goblet cell markers were dependent on IL-33R expression on $CD90^+$ MLN cells. *Il1rl1*$^{-/-}$ $CD90^+$ MLN cells treated with IL-33 and co-cultured with enteroids did not induce *Atoh1, Spdef* or *Muc2* expression compared to media alone (Fig. 2F). Collectively, these data show that IL-33R-expressing T cells and/or ILCs are required for IL-33 to induce epithelial goblet cells.

IL-13 produced by primarily ILC2s is required for IL-33-induced goblet cell differentiation in vitro

Since IL-33 induces IL-13 production by T cells and ILCs, and we observed IL-13 directly induces goblet cell differentiation in murine enteroids (Fig. 1 and 2), we tested the role of IL-13 signaling in the interaction between IL-33, intestinal immune cells, and epithelial cells. IL-33 significantly induced *Il13* expression, as detected by real-time RT-PCR (Fig. 3A), and IL-13 protein production, as detected by ELISA (Fig. 3B), in MLN cells co-cultured with enteroids. IL-4 was undetectable at baseline, and IL-33 induced minimal IL-4 production (0.45 ± 0.26 pg/mL IL-4 compared to 4,297 ± 698 pg/mL IL-13).

In order to identify the MLN cells producing IL-13, flow cytometry analysis was performed for IL-13 and markers for T cells and ILC2s. All cells were negative for lineage markers B220, CD11b, NK1.1, FCεRI and CD11c (data not shown). Both $CD4^+$ and $CD4^-$ cells expressed IL-13 (Fig. 3C). However, IL-33 treatment *in vitro* only induced IL-13 production in $CD4^-$ cells, and 94 ± 3.8% of the IL-13-expressing cells after IL-33 treatment were $CD4^-$ (Fig. 3D). Both $CD4^+$ and $CD4^-$ IL-13-producing cells also expressed IL-33R, which in known to be expressed on Th2 cells and ILC2s (26) (Fig. 3C). As expected**,** $CD4^+IL\text{-}13^+$ cells also expressed CD3, while $CD4^-IL\text{-}13^+$ cells were $CD3^-$, consistent with an ILC2 phenotype ($Lineage^-CD90^+CD4^-CD3^-IL\text{-}33R^+$, Fig. 3C). We confirmed the finding from C57BL/6 mice in Balb/C mice, since Balb/C mice are known to be Th2-prone and were required for other experiments (Fig. 3E) (27). In Balb/C mice, we utilized mice without IL-33 i.p. (naïve) since IL-33 i.p. leads to high levels of IL-13 secretion in vitro even without additional IL-33 added in vitro (Fig. 4A). Similarly to C57BL/6 mice, Balb/C mice exhibited a large increase in ILC2s with IL-33 stimulation in vitro, but there was not a significant increase in $IL\text{-}13^+CD4^+$ cells from naïve to IL-33 i.p. or IL-33 i.p. + IL-33 (Fig. 3E).

To confirm that epithelial cell-intrinsic IL-13 signaling is required for IL-33 induction of *Muc2* expression, we generated enteroids from *Il4ra*$^{-/-}$ Balb/C mice (IL-4Rα is a component of the IL13 receptor-α−1 heterodimer). CD45^{+}CD90^{+} MLN cells were isolated from naïve or IL-33 i.p. mice, cocultured with enteroids derived from WT or *Il4ra*$^{-/-}$ mice, and, in some conditions, stimulated with additional rIL-33. MLN cells from IL-33 i.p. mice secreted IL-13 in vitro (5.9 ± 1.5 ng/mL in the media from IL-33 i.p. mice versus undetectable in that of naïve mice), which was further augmented by the addition of IL-33 to the culture media (45.7±13.6 ng/mL, Fig. 4A). Comparatively very small amounts of IL-4 were secreted by the MLN cells from the IL-33 i.p. mice (0.006±0.001 ng/mL), and this was not increased with additional IL-33 *in vitro* (Fig. 4A).

Muc2 and *Atoh1* expression were significantly increased in WT enteroids co-cultured with MLN cells from IL-33 i.p. mice both with and without IL-33 in culture (Fig. 4B). There were numerical but not statistically significant increases in *Spedf* in co-cultures with MLN cells from IL-33 i.p. mice both with and without IL-33 in culture. In contrast, no increase in *Atoh1*, *Spdef* or *Muc2* was observed in *Il4ra*$^{-/-}$ enteroids co-cultured with MLN cells from IL-33 i.p. mice with or without IL-33 (Fig. 4C). These studies indicate that goblet cell differentiation induced by IL-33 in enteroids is dependent on IL-13 signaling in the enteroids.

MLN cells cultured with IL-33 also expressed increased levels of mRNA for the epidermal growth factor ligand amphiregulin (*Areg)* (Fig. 5A), which has been shown to be important for maintaining goblet cells during DSS-induced colitis (14). While amphiregulin (50 ng/mL) did induce phopho-ERK 1/2 in enteroids (Fig. 5B), it did not alter *Atoh1, Spdef* or *Muc2* expression (Fig. 5C), further supporting that IL-13 secreted predominantly from ILC2s is primarily responsible for the effect of IL-33 on intestinal goblet cells.

Induction of goblet cells by IL-33 in vivo is dependent on IL-13

Although is known that IL-13 induces goblet cell hyperplasia in vivo (19) and that IL-33 potentiates IL-13 secretion by T cells and ILCs, it is not known whether IL-13 is required for IL-33 to induce goblet cell hyperplasia *in vivo*. We administered IL-33 i.p. for 4 days to WT and *Il13*$^{-/-}$ mice and sacrificed mice to examine intestinal goblet cells. In the colon, IL-33 significantly increased the number of PAS^{+} goblet cells in WT mice but had no effect on goblet cell number in *Il13*$^{-/-}$ mice (Fig. 6A and 6B). In line with this finding, colon mucosal *Atoh1, Spdef,* and *Muc2* expression were increased in IL-33-treated WT mice, but not in *Il13*$^{-/-}$ mice (Fig. 6B).

We observed similar effects in the small intestine, with IL-33 inducing increased goblet cells in WT but not *Il13*$^{-/-}$ mice (Fig. 6C and 6D). There were also numerical increases in mean small intestinal mucosal *Atoh1, Spdef,* and *Muc2* expression with IL-33 treatment in WT but not *Il13*$^{-/-}$ mice in vivo. These differences did not reach statistical significance, likely due to high variability in the expression of these mRNAs in the small intestine (Fig. 6D). Interestingly, there was some signal, albeit inconsistent, for increased goblet cell markers in *Il13*$^{-/-}$ mice at baseline compared to WT, which decreased with IL-33 treatment. These numerical differences were only statistically significant in the cases of increased small

intestinal *Spdef* expression in *Il13*$^{-/-}$ mice compared to WT mice, and decreased *Atoh1* expression in IL-33-treated compared to untreated *Il13*$^{-/-}$ mice.

Since others have demonstrated that Areg is required for IL-33-induction of goblet cells during inflammation, we examined both *Il13* and *Areg* expression in the large and small intestine following IL-33 i.p. As expected, *Il13* was significantly increased in both the colon and the jejunum (Fig. 6E and 6F). However, there was no increase in *Areg* in the large or small intestine with IL-33 i.p. compared to control (Fig. 6E and 6F).

DISCUSSION

We have applied an enteroid-immune cell co-culture system as a model of epithelial-immune cross-talk in the intestine to demonstrate that IL-33 induces goblet cell differentiation indirectly by stimulating primarily ILCs to produce IL-13, rather than through direct action on epithelial cells. Although intestinal epithelial cells express the IL-33R and activate ERK 1/2 in response to IL-33, IL-33 did not directly induce goblet cell differentiation, while IL-13 did. In co-cultures with CD90^{+} MLN cells from IL-33-treated mice, IL-33 induced *Muc2* and *Atoh1* expression, indicating increased goblet cell differentiation. CD3^{-} CD90^{+}ST2^{+} ILC2s from MLN of IL-33-treated mice produce large amounts of IL-13 in co-cultures. The IL-33-induced *Muc2* response in vitro was dependent on enteroid *Il4ra* expression. Furthermore, IL-33 induction of goblet cells in mice in the large and small intestine was dependent on IL-13.

Large doses of IL-33 administered to mice leads to goblet cell hyperplasia, both in the lungs and the intestines (6), but the mechanisms by which IL-33 induces goblet cells in the intestines have not been fully delineated. IL-33 increases production of the Th2 cytokine IL-13 both in vitro and in vivo in T cells and ILC2s (6, 11, 12), and IL-13 is able to induce intestinal and lung goblet cells (19, 23, 28, 29). We now tie together this circuit by demonstrating the dependence of IL-33-induced goblet cell differentiation on primarily IL-13 producing ILC2s and epithelial IL-13 signaling in vitro and IL-13 in vivo.

Since IL-33-treated mice have goblet cell hyperplasia, for co-cultures with immune cells, we used MLN from IL-33-treated mice as a relevant source of immune cells for the intestine, as other studies have previously done (14). Since IL-33 is known to induce a type 2 immune response in both T cells and innate lymphoid cells (6, 11, 12), we isolated CD90^{+} cells from the MLN to capture both cell types. When stimulated further with IL-33 in vitro, CD90^{+} MLN cells made substantial amounts of IL-13. Importantly, in *Trichuris muris* (13)and *N. brasiliensis* (12) infections, nanogram amounts of IL-13 are produced, which is what we saw following IL-33 stimulation of MLN cells *in vitro*. Although both ILCs and T helper cells produced IL-13, over 90% of the IL-13-producing cells were ILC2s. This is consistent with the findings of others that IL-33 treatment leads to accumulation of ILC2s in the MLN (14). Furthermore, in the setting of *Nippostrongylus brasiliensis* infection, ILC2s are the predominant cell type producing IL-13 and T cells are not required for IL-13 production to be induced (12, 30). However, a limitation of this study is that in an unchallenged mouse, the majority of T cells in the MLN are naïve, and there are only a minority of IL-33R-expressing Th2 cells able to respond to IL-33 (31). We now show that IL-33-exposed MLN

cells can increase goblet cell differentiation in murine enteroids in vitro. Furthermore, we used *Il4ra*$^{-/-}$ enteroids to show that this effect is dependent on epithelial intrinsic IL-13 signaling.

This study is one of a small number of emerging reports of intestinal epithelial organoids co-cultured with other cell types as a more complex model of the intestinal mucosa. Intestinal subepithelial myofibroblasts support the growth of enteroids and colon organoids (colonoids) when co-cultured together (21, 32, 33). Macrophages co-cultured on the basolateral surface of enteroid monolayers enhance epithelial barrier function and maturation, and phagocytose apical bacteria (17). Co-cultures of T lymphocytes and enteroids have also been reported. One group co-cultured group 3 ILCs (ILC3s) from *Il22*-deficient mice in matrigel with enteroids to demonstrate that ILC3 augmentation of enteroid growth is IL-22-dependent (18). Our approach differed in that enteroids in matrigel were on a standing semipermeable insert so that ILC2-enriched lymphoid cells were physically separated from the enteroids. This approach further supported that an ILC2 secreted factor rather than direct intercellular interactions were responsible for enteroid goblet cell differentiation in our system.

Several studies have begun to dissect the role of IL-33 in regulating intestinal secretory cell differentiation, including goblet cells (8, 34). With the discovery of IL-33, it was demonstrated that IL-13 is required for IL-33-induced goblet cell hyperplasia in the lung in vivo (6). A more recent study demonstrated a positive feedback circuit in vivo whereby intestinal tuft cells produce IL-25, which potentiates IL-13 production by ILC2s, which then stimulates the further differentiation of epithelial tuft cells (34). The same study showed that IL-33 similarly induces tuft cells through ILC2 IL-13 production. Tuft cells branch off from a common secretory progenitor cell as goblet cells and require *Atoh1*, but not *Spdef* (35). We now build on these findings by demonstrating a the requirement of ILC2s and IL-13 for induction of goblet cell differentiation by IL-33 using an in vitro model of epithelial-immune crosstalk.

Interestingly, we show that in IL-13-deficient mice, there is a signal for increased goblet cell markers in mouse intestine, which decreased following IL-33 treatment. These differences were, for the most part, not statistically significant. It is possible that at baseline, in the chronic absence of IL-13, other cytokines known to induce goblet cells, such as IL-22 could be increased (36). Furthermore, without the strong induction of IL-13 by IL-33 in IL-13-deficient mice, other cytokines induced by IL-33, such as IFN-gamma (37), which is known to decrease goblet cells, may take the lead in regulating goblet cells (38, 39).

In contrast to our findings, others have proposed that IL-33 directly induces goblet cell differentiation in the intestinal epithelium. One group of investigators similarly treated murine enteroids with IL-33 and observed marked increases in Paneth cell numbers and the Paneth cell marker *Ang4*, with more modest increases in goblet cells and *Muc2* expression (8). They went on to show that the induction of Paneth cell differentiation was independent of enteroid *Il4ra* expression. Using the same IL-33 concentration and exposure duration, we did not observe any effect of IL-33 alone on enteroid goblet cell numbers, the secretory lineage differentiation marker *Atoh1*, or the goblet cell differentiation markers *Spdef* and

Muc2 across multiple experiments. Only with the addition of MLN cells enriched for IL-13 producing ILC2s did we observe effects of IL-33 on enteroid goblet cell numbers and marker expression, leading us to conclude that IL-33 primarily acts on ILCs to indirectly induce goblet cell differentiation. IL-33 may directly activate other important pathways in intestinal epithelial cells. Recently is has been demonstrated that IL-33 acts directly on the Caco2 intestinal epithelial cell line to increase proliferation through upregulation of miR-320a (40), and further studies are warranted to explore direct effects of IL-33 in intestinal epithelial cells.

Goblet cell depletion is a pathologic hallmark of inflammatory bowel disease. We and others previously demonstrated that in the setting of colitis IL-33 and IL-33R reduce histopathologic severity and preserve goblet cells (14, 15). Here, we show that in unchallenged mice and primary intestinal epithelial cells, IL-33 induces goblet cell differentiation through IL-13 produced mainly by ILC2s. Others have shown that in the setting of epithelial injury and acute colitis induced by dextran sodium sulfate, IL-33 protection and preservation of goblet cells is dependent on ILC-intrinsic Areg (14). Although we corroborate that ILC2s in culture expressed *Areg*, direct stimulation of enteroids with Areg did not increase goblet cells, despite activation of signaling. We found that IL-33 robustly increased intestinal mucosal *Il13* expression, but did not affect *Areg* expression in vivo and that IL-33 induced goblet cell hyperplasia was IL-13-dependent. Together these studies indicate that although Areg is important for epithelial repair and preservation of goblet cells during colitis, IL-13, and not Areg, induced by IL-33 directly stimulates goblet cell differentiation in unchallenged primary epithelium.

IL-33 induction of goblet cells through promoting ILC2s and IL-13 expression may be important for immune regulation in multiple settings. IL-33 expression is increased in the colon of patients with ulcerative colitis as well as in various mouse models of colitis and parasitic infection (13, 15, 41–44). We acknowledge that the levels of IL-33 we used in vitro and in vivo were higher than reported serum levels in humans during health or disease; however, it is difficult to ascertain the levels of IL-33 that cells are exposed to in the tissue microenvironment. IL-13 is required for expulsion of *N. brasiliensis* and *H. polygyrus* adult parasites from the intestinal lumen by inducing intestinal epithelial cells to differentiate into goblet cells that secrete resistin-like molecule (RELM) β (28). IL-33 induction of IL-13-producing ILC2s is important for worm expulsion during *N. brasiliensis* infection (12). Loss of mucus leads to colitis in mice and goblet cell depletion is associated with disease in human ulcerative colitis and necrotizing enterocolitis (2, 3, 45). We have previously shown that IL-33 limits goblet cell depletion during oxazolone colitis (15). Furthermore, we have also reported that increased mucosal *Il13* expression at diagnosis is associated with superior outcomes in pediatric ulcerative colitis patients (46). Recently, ILC2s have been shown to help promote resolution of inflammation in a mouse model of arthritis (47). IL-33-induction of ILC2s, IL-13 and goblet cells is an important protective mechanism in the intestine and could be targeted for future therapeutics to promote healing following inflammation.

In conclusion, our data in a relevant primary murine enteroid culture system demonstrate that IL-33 indirectly induces goblet cell differentiation through IL-13 produced predominantly by ILC2s. Furthermore, our study demonstrates that IL-13 is required for

IL-33-induced goblet cell hyperplasia *in vivo* in mice. We have successfully developed an enteroid-immune cell co-culture system, which can be leveraged to assess other roles for immune cells in epithelial cell functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this manuscript:

CMF	colon myofibroblast
ILC	innate lymphoid cell
ILC2	group 2 innate lymphoid cell
MLN	mesenteric lymph node
WT	wild-type

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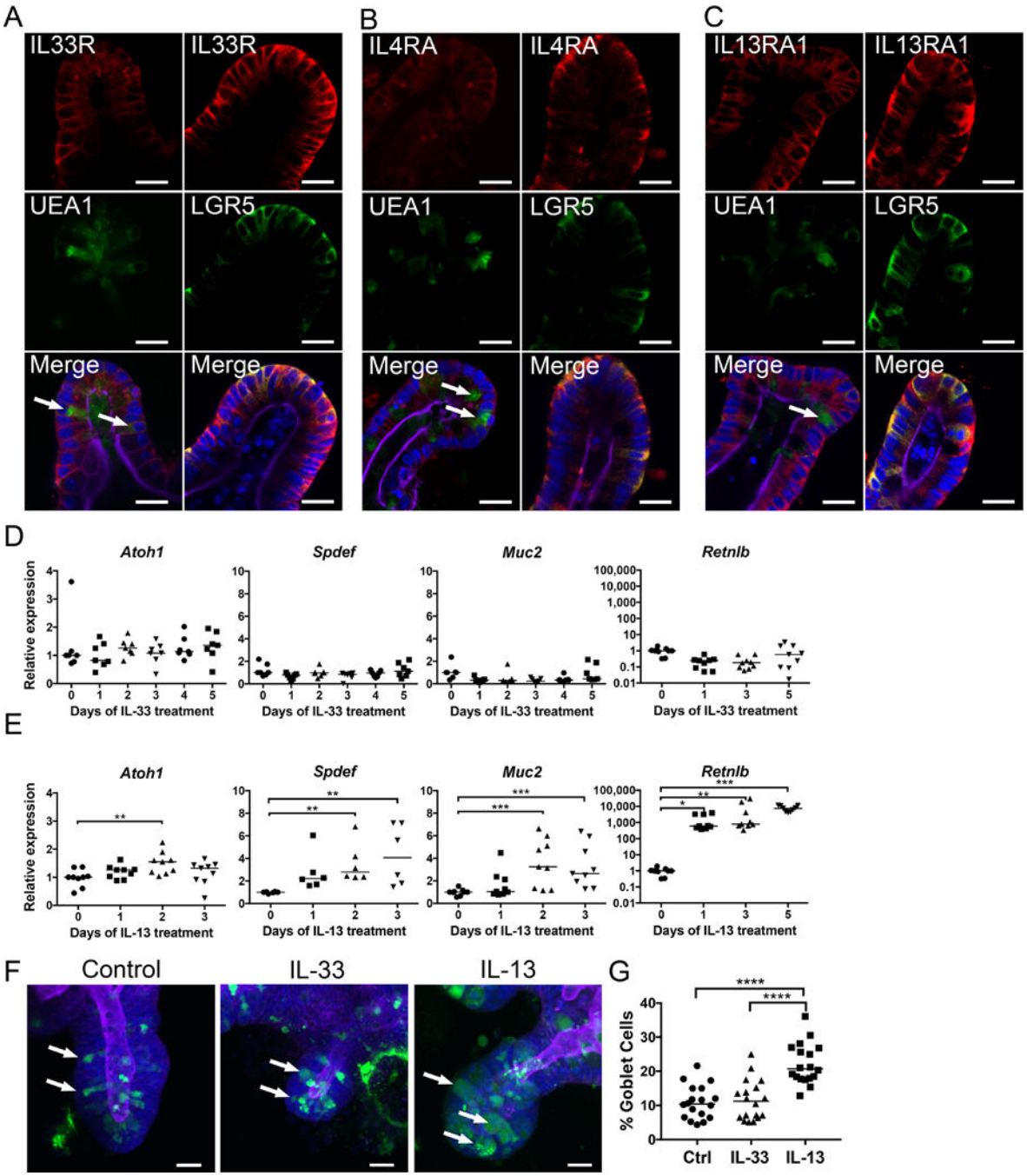

FIGURE 1.

IL-33 and IL-13 effects on goblet cell differentiation in murine enteroids. Immunofluorescence microscopy for (**A**) IL-33R (**B**) IL-4RA and (**C**) IL-13RA1 (red) in enteroids counterstained with Hoecht (blue), Phalloidin (pink) and UEA1 or LGR5 (green). Scale bars = 20 μM (**D**-**E**) Graphs of real-time RT-qPCR analysis of enteroids treated with (**D**) IL-33 (100 ng/mL) or (**E**) IL-13 (10 ng/mL). (**F**) Representative photomicrographs of UEA-1 FITC (green) immunofluorescence microscopy in enteroids treated for 4 days with PBS, IL-33 or IL-13. Enteroids are counterstained with DAPI (blue) and Phalloidin (pink)

(G) Quantification of goblet cells. Scale bars = 20 μM. White arrows indicate goblet cells. 7–9 wells per condition pooled from 3 independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$.

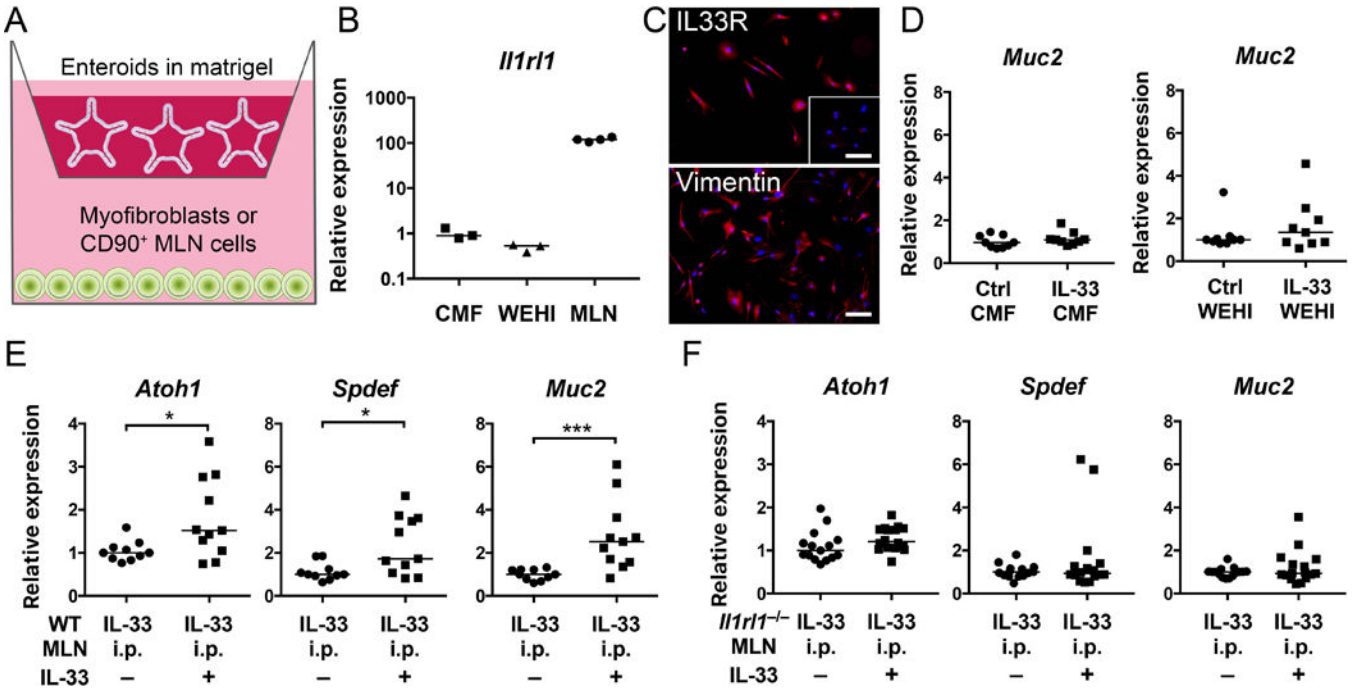

FIGURE 2.

IL-33 induced goblet cells in enteroids co-cultured with immune cells, but not those with myofibroblasts. (**A**) Diagram of in vitro model wherein enteroids in matrigel were suspended in transwells in co-culture over either CD90$^+$ MLN cells from IL33-treated mice, WEHI-YH2 myofibroblast cell or primary CMF with or without IL-33 (100 ng/mL). (**B**) *Il1rl1* expression in CMF, WEHI-YH2 and MLN cells. (**C**) Immunofluorescence analysis of IL-33R and vimentin in primary CMF counterstained with Hoecht. Scale bars = 25 μM *Atoh1, Spdef* and *Muc2* expression in enteroids co-cultured with (**D**) primary CMF or WEHI-YH2 cells, (**E**) WT CD90$^+$ or **(F)** *Il1rl1*$^{-/-}$ CD90$^+$ MLN cells was assessed by real time RT-qPCR. Data is graphed as medians. $P < 0.001$, n = 8–16 wells pooled from 3 or 4 independent experiments.

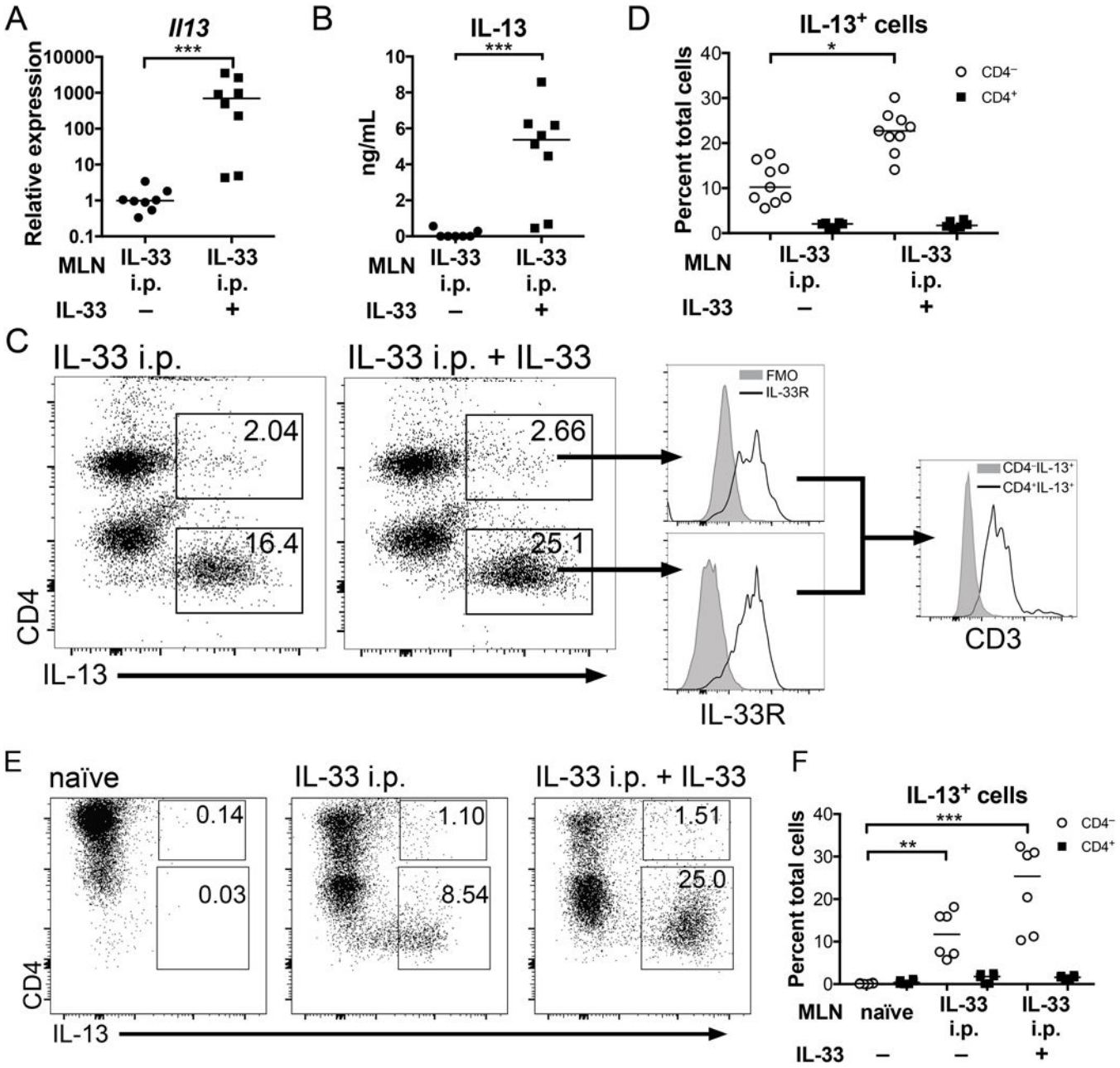

FIGURE 3.

IL-33 induced IL-13 from ILC2s in MLN:enteroid co-cultures. CD90$^+$ MLN cells from C57BL/6 mice were co-cultured with enteroids for 4 days with or without 100 ng/mL IL-33 and IL-13 was assessed. (**A**) *Il13* real-time RTq-PCR (**B**) IL-13 ELISA. (**C**) Representative flow plots for CD4$^+$ and CD4$^-$ IL-13$^+$ cells from co-cultured MLN cells from C57BL/6 mice and expression of IL-33R and CD3 on CD4$^+$ and CD4$^-$ IL-13$^+$ cells. (**D**) Quantification of IL-13$^+$ cells from (C). (**E**) Representative flow plots for CD4$^+$ and CD4$^-$ IL-13$^+$ cells from co-cultured MLN cells from Balb/C mice. (**F**) Quantification of IL-13$^+$ cells from (E). *P < 0.05 ***P < 0.001. n = 6–9 wells pooled from 3 independent experiments.

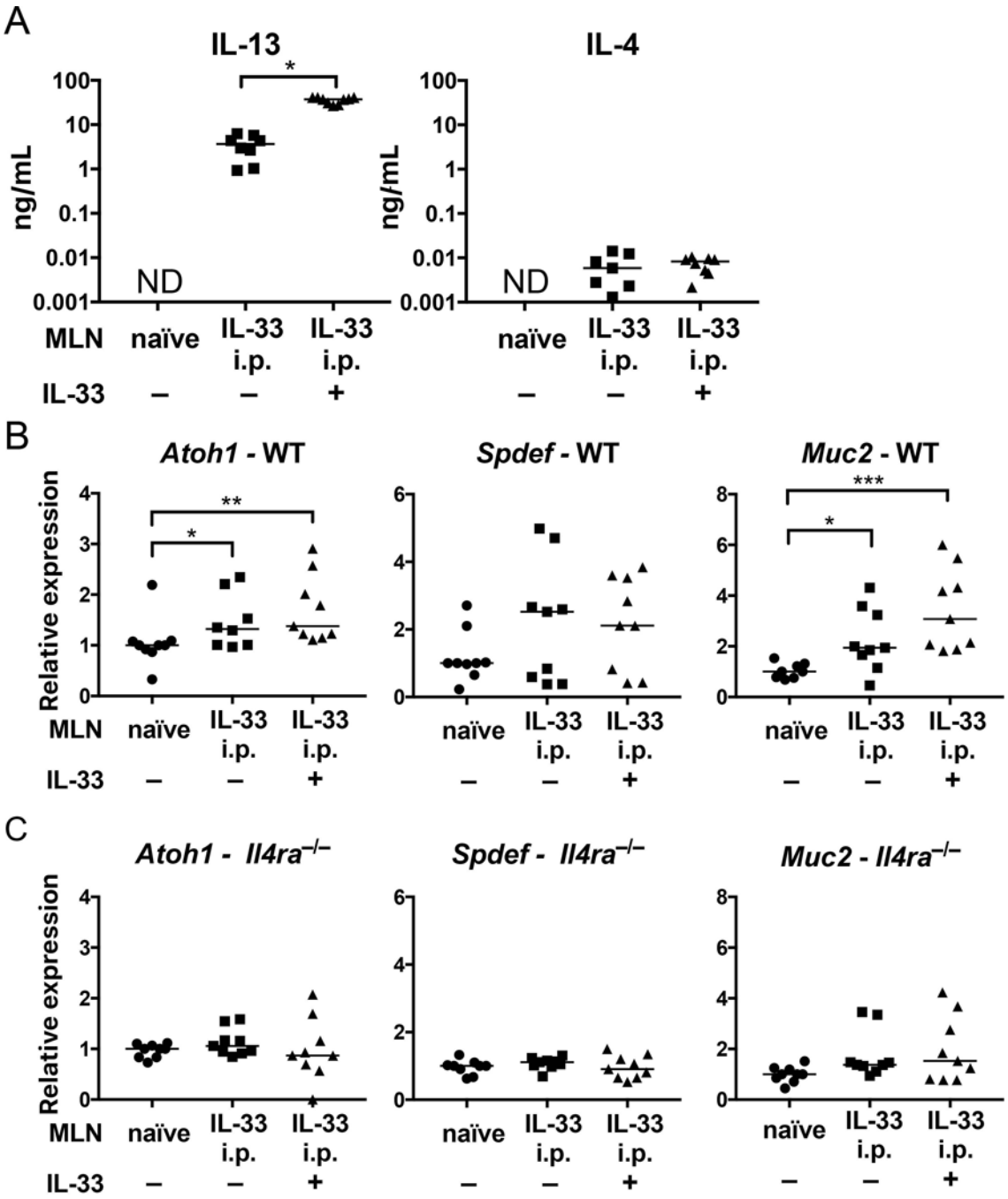

FIGURE 4.

IL-33-exposed CD90$^+$ MLN cells induce gene expression consistent with goblet cell differentiation in WT but not *Il4ra*$^{-/-}$ enteroids. CD90$^+$ MLN cells were purified from naïve mice or mice given IL-33 (0.4 ug) i.p. daily for 4 days. (**A**) IL-13 and IL-4 secretion in co-cultures was determined by ELISA, ND, not detected. MLN cells were co-cultured with (**B**) WT or (**C**) *Il4ra*$^{-/-}$ enteroids with 100 ng/ml IL-33 added *in vitro* to some cultures, and enteroid mRNA expression was assessed by real time RT-qPCR *P < 0.05, ***P < 0.001; n = 9 wells pooled from 3 independent experiments

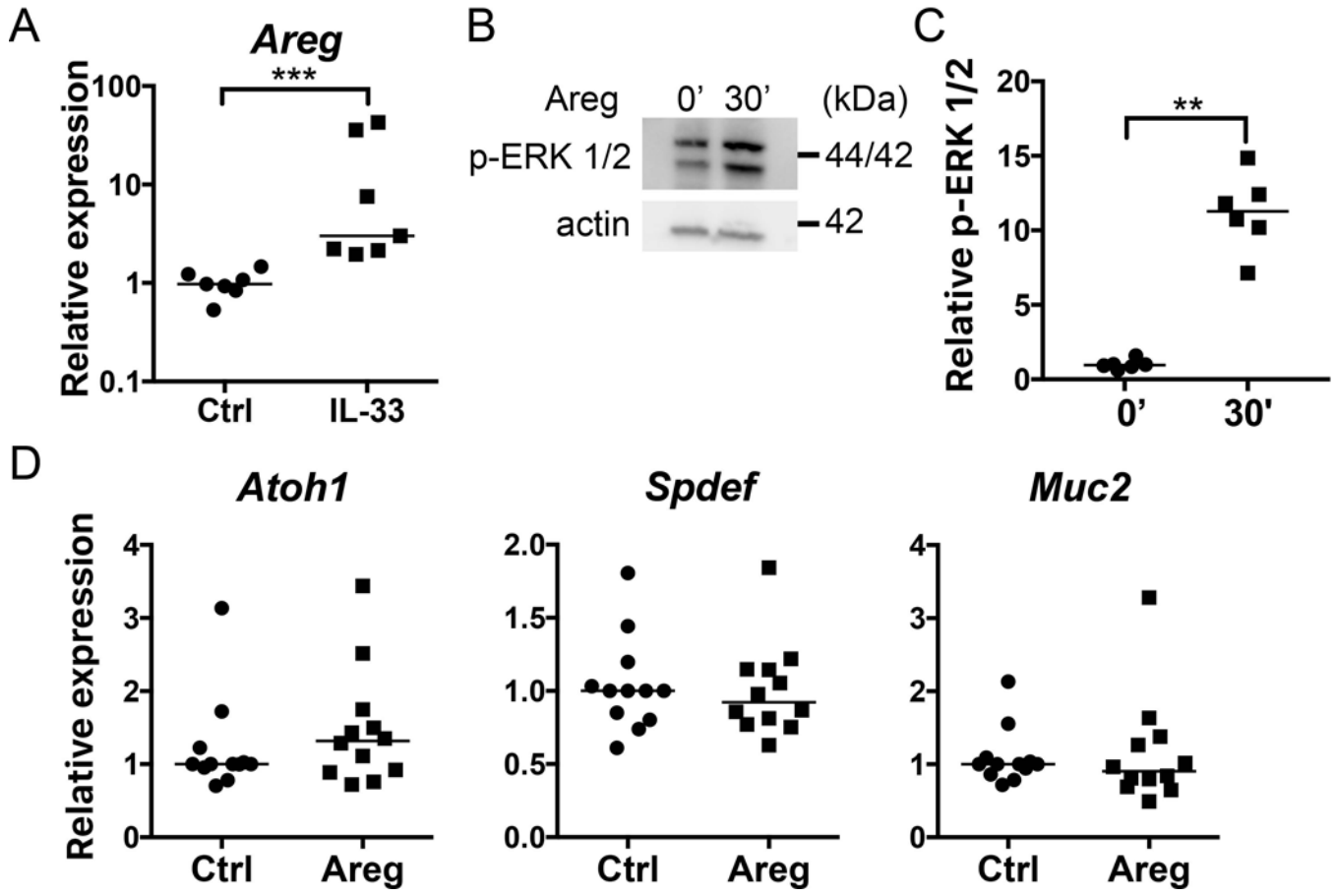

FIGURE 5.

Areg does not induce gene expression consistent with goblet cell differentiation in enteroids. (**A**) *Areg* expression in $CD90^{+}$ MLN co-cultured with enteroids with or without IL-33 (100 ng/mL) for 4 days. (**B**) Representative western blot and (**C**) quantification of ERK 1/2 activation following Areg stimulation for 30 minutes. (**D**) Real-time RTq-PCR analysis of enteroids stimulated with Areg (100 ng/mL) for 4 days. ***P < 0.001 n = 9 wells pooled from 3 independent experiments.

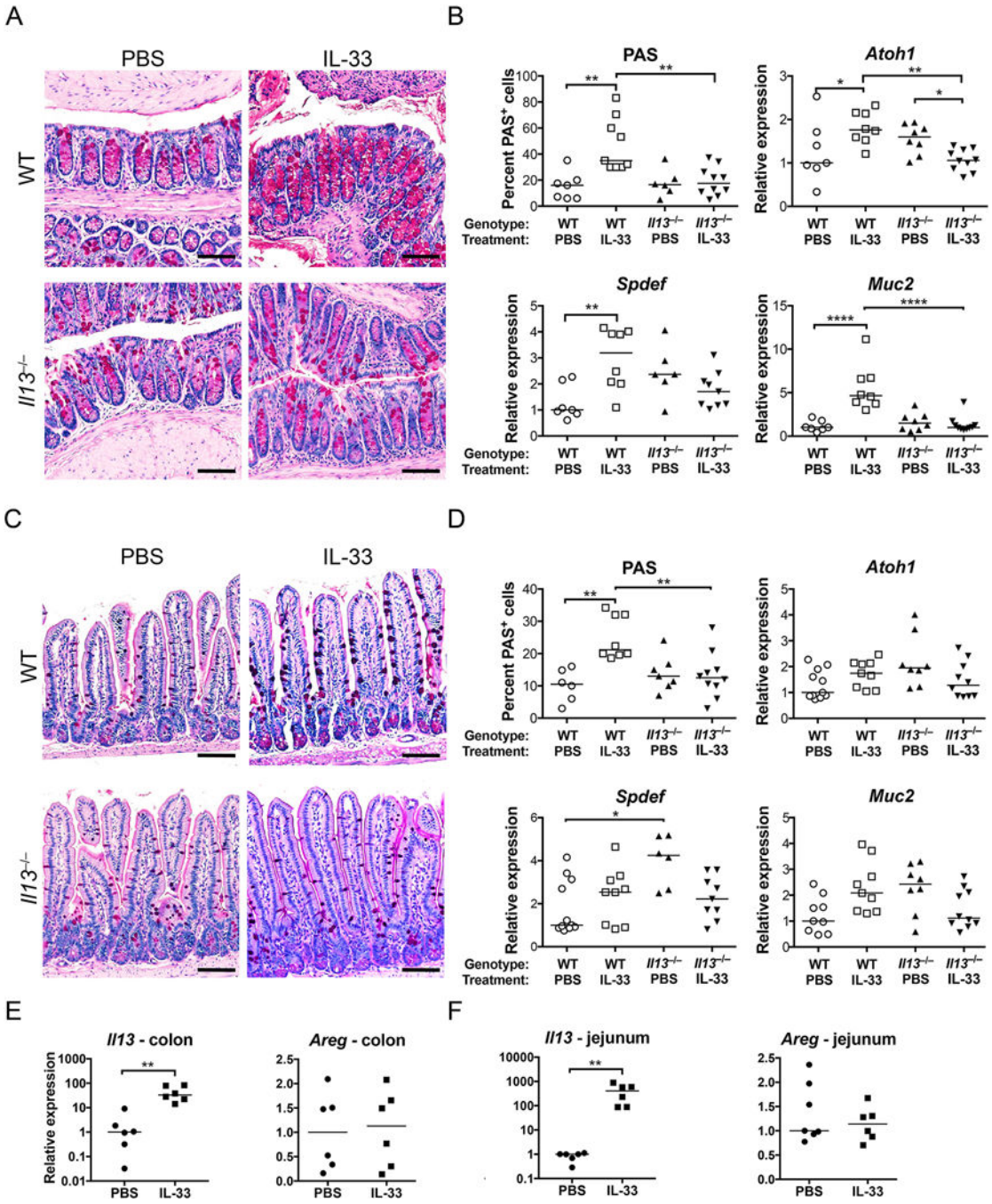

FIGURE 6.

IL-33-induced intestinal goblet cell hyperplasia is dependent on IL-13 in vivo. Representative photomicrographs of PAS-stained goblet cells in (**A**) colon and (**C**) small intestine. Quantification of PAS staining using a modified nuclear algorithm and real time RT-qPCR analysis of tissue RNA from the (**B**) colon and (**D**) small intestine. Real time RT-qPCR for *Il13* and *Areg* from the (**E**) colon and (**F**) small intestine. *P < 0.05, **P < 0.01, ***P < 0.001, n = 6–10 mice per group across 3 independent experiments.