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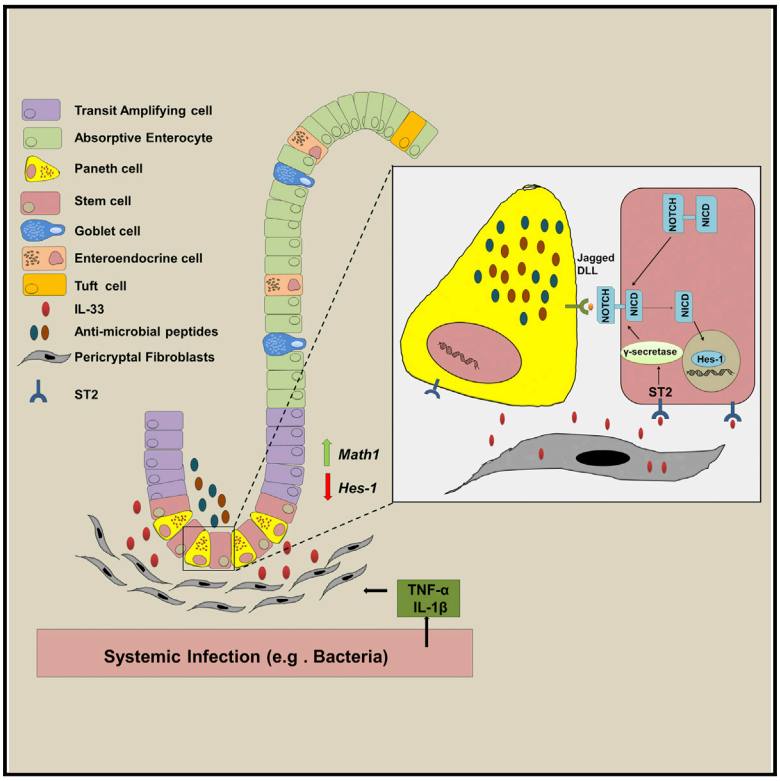
Cell Reports

Programming of Intestinal Epithelial Differentiation by IL-33 Derived from Pericryptal Fibroblasts in Response to Systemic Infection

Graphical Abstract



Highlights

- IL-33 signaling to intestinal epithelial cells protects against *Salmonella* infection
- IL-33 is released by pericryptal fibroblasts in the small intestine
- IL-33 directly stimulates intestinal stem and progenitor cells via the ST2 receptor
- IL-33 inhibits Notch signaling, promoting the differentiation of secretory IEC

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In Brief

Mahapatro et al. find a role for pericryptal fibroblast-derived IL-33 in the maintenance of the intestinal barrier during *Salmonella* infection. They show that IL-33 can program epithelial progenitor cells toward the secretory IEC lineage via the ST2 receptor on intestinal progenitor cells by downregulating the Notch-signaling pathway.

Mahapatro et al., 2016, Cell Reports *15*, 1743–1756
May 24, 2016
http://dx.doi.org/10.1016/j.celrep.2016.04.049

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Programming of Intestinal Epithelial Differentiation by IL-33 Derived from Pericryptal Fibroblasts in Response to Systemic Infection

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http://dx.doi.org/10.1016/j.celrep.2016.04.049

SUMMARY

The intestinal epithelium constitutes an efficient barrier against the microbial flora. Here, we demonstrate an unexpected function of IL-33 as a regulator of epithelial barrier functions. Mice lacking IL-33 showed decreased Paneth cell numbers and lethal systemic infection in response to *Salmonella typhimurium*. IL-33 was produced upon microbial challenge by a distinct population of pericryptal fibroblasts neighboring the intestinal stem cell niche. IL-33 programmed the differentiation of epithelial progenitors toward secretory IEC including Paneth and goblet cells. Finally, IL-33 suppressed Notch signaling in epithelial cells and induced expression of transcription factors governing differentiation into secretory IEC. In summary, we demonstrate that gut pericryptal fibroblasts release IL-33 to translate bacterial infection into an epithelial response to promote antimicrobial defense.

INTRODUCTION

Interleukin 33 (IL-33) is an IL-1-like cytokine acting both as a conventional cytokine through activation of the ST2 cell surface receptor complex and as an intracellular nuclear factor with transcription-factor-like properties (Haraldsen et al., 2009). Depending on the physiological context, IL-33 has been described to be host protective or pathogenic in humans and mice (Bamias et al., 2012; Lopetuso et al., 2013). IL-33 is expressed in many cell types like endothelial, bone-marrow-derived dendritic cells, epithelial cells, and synovial fibroblasts (Oboki et al., 2010). It has been demonstrated to act as a signaling molecule with dichotomous functions in the gut. Where on the one hand it is able to activate many cells of the immune system, notably eosinophils and type 2 innate lymphoid cells to drive inflammation, on the other hand, it has been demonstrated to promote epithelial integrity (Pastorelli et al., 2013; Polumuri et al., 2012). In recent years, IL-33 has emerged as a classical activator of type 2 immunity along with cytokines such as IL-4, IL-9, and IL-13 (Pastorelli et al., 2013; Schmitz et al., 2005). Besides its association with multiple diseases including asthma, fibrosis, and dermatitis, the IL-33/ST2 axis has also been shown to be vital in gut-related disorders like ulcerative colitis (UC) and Crohn's disease (Beltrán et al., 2010; Kobori et al., 2010; Pastorelli et al., 2010; Sedhom et al., 2013). However, the detailed functional role of IL-33 as a signaling molecule in the intestine is still in ambiguity.

Intestinal stem cells, like LGR5^{+} crypt base columnar cells (CBCs), are responsible for maintaining differentiated cells, which are broadly classified into two distinct lineages: absorptive enterocytes and cells of the secretory type (Gracz and Magness, 2014). It has been established that the master transcriptional regulators *Hes1* and *Math1* can guide the stem cell in committing to either of these cell types (Yang et al., 2001). Secretory cells like Paneth, goblet, and enteroendocrine (EE) cells have specific immune and tissue regulatory functions such as secretion of antimicrobial peptides (AMPs), mucus production, and maintenance of gut-hormonal balance, respectively (Vereecke et al., 2011). Perturbations in these crucial functions lead to barrier defects and increased bacterial translocation.

Cell intrinsic signaling like Notch ensures integrity in the intestinal epithelium by controlling early differentiation markers such as *Hes1* and *Math1* (Obata et al., 2012). Inhibition of the Notch-signaling pathway has been associated with goblet cell hyperplasia (Fre et al., 2005). Goblet cell expansion in parasite-infected models has been attributed to IL-33 (Humphreys et al., 2008). Additionally, the expression of the Notch ligands Dll1 and Dll4 is vital for intestinal homeostasis and maintenance of stem cells (Pellegrinet et al., 2011; VanDussen et al., 2012). A recent study has indicated a regulation of the Notch ligands Jag1, Dll1, and Dll4 in IL-33-treated animals during chemically induced colonic inflammation (Imaeda et al., 2011). In contrast, another report demonstrated that Notch signaling is upstream of IL-33 expression in cultured endothelial cells (Sundlisaeter et al., 2012). Collectively, IL-33 is a highly pleiotropic cytokine. Its regulation and function in the gut is currently not well understood and might depend on the target cell and the immunological challenge.

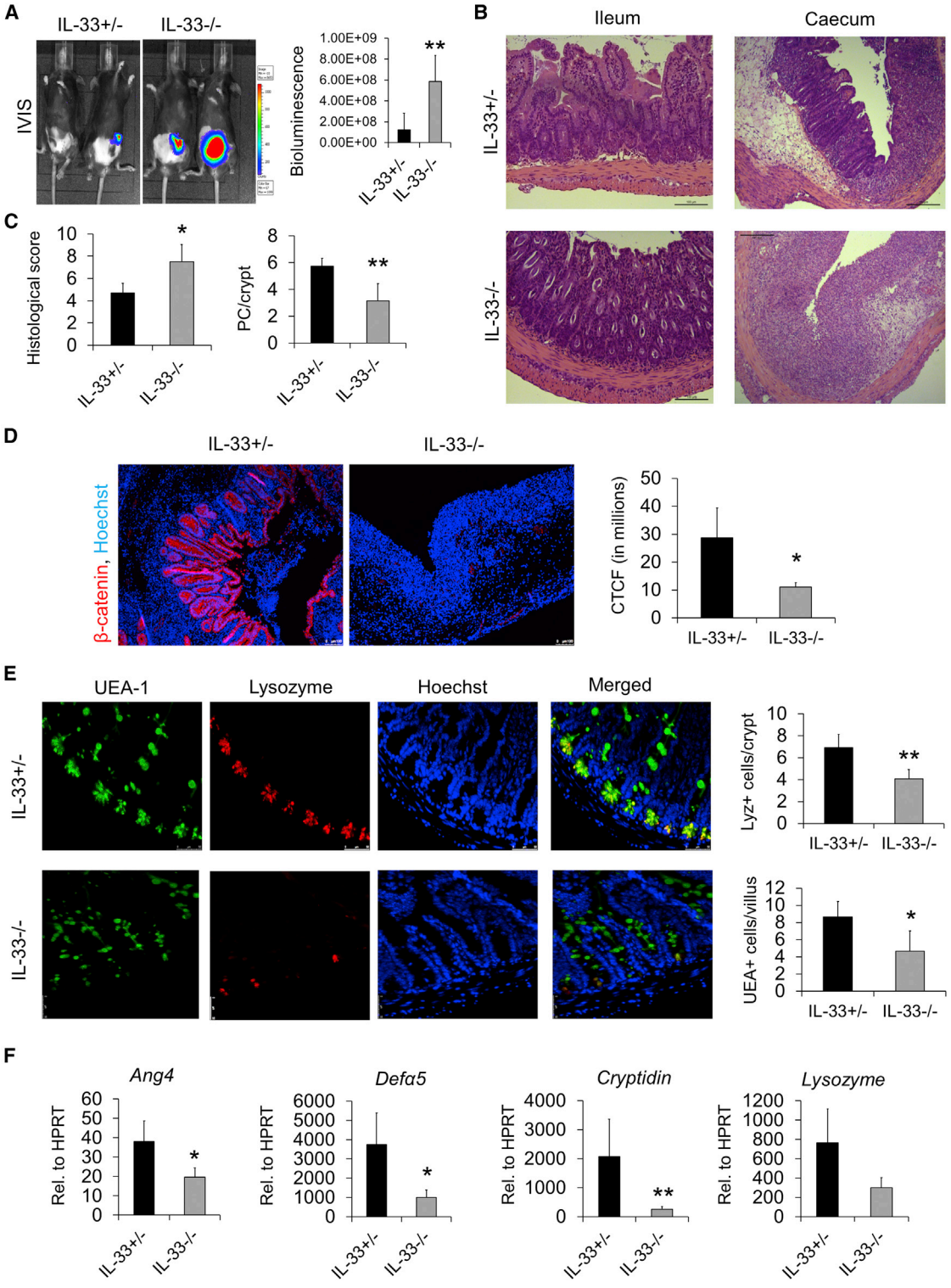

Figure 1. IL-33$^{-/-}$ Mice Are Highly Susceptible to *Salmonella Typhimurium* Infection

(A) In vivo imaging of IL-33$^{+/-}$ and IL-33$^{-/-}$ mice orally infected with bioluminescent *S. Typhimurium* for 5 days. Graph depicts quantification of bioluminescence signals measured as counts/s/cm^2/sr (n = 5; mean ± SD).

(B) Representative pictures of H&E stainings of ileum and caecum cross-sections of IL-33$^{+/-}$ and IL-33$^{-/-}$ mice after infection (scale bars represent 100 μm and 200 μm).

(C) Histological scoring of tissue damage in the caecum of infected animals (n = 5; mean ± SD) and ileal Paneth cell (PC) counts (n = 5; mean ± SD).

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We now describe that IL-33 directly influences intestinal stem cells in a ST2-dependent manner. IL-33 was expressed by a distinct population of pericryptal fibroblasts in spatial vicinity to the stem cell niche. IL-33 signaling led to a specific expansion of cells of the secretory type, including Paneth and goblet cells by downregulating Notch-signaling pathway. This pathway is functionally relevant as mice lacking IL-33 showed decreased Paneth cell (PC) numbers, increased infection, and severe tissue destruction in response to *Salmonella Typhimurium* challenge.

RESULTS

IL-33 Provides Protection against the Pathogen *Salmonella Typhimurium*

Previous studies have clearly demonstrated the involvement of the IL-33/ST2 axis in helminth expulsion and neutrophil influx during sepsis (Alves-Filho et al., 2010; Rostan et al., 2015). However, although IL-33 is known to be particularly expressed at barrier tissues such as the gut, its role in the context of bacterial infections remains to be fully determined. To access more precisely the role of IL-33 in intestinal pathology, we employed an established *Salmonella* infection model in IL-33$^{-/-}$ and littermate control mice that were backcrossed to homozygosity for the natural resistance-associated macrophage protein 1 (Nramp1) wild-type alleles (Vidal et al., 1995). Interestingly, after infection with a virulent *Salmonella* strain, as compared to IL-33$^{+/-}$ littermates, IL-33$^{-/-}$ mice showed an increased *Salmonella* burden 5 days postinfection (Figure 1A). This was accompanied by increased immune cell infiltration and more-severe intestinal tissue damage in both caecum and ileum and a striking loss of PCs in ileum of infected IL-33$^{-/-}$ mice (Figures 1B and 1C). Staining with the epithelial marker β-catenin on cecal cross-sections confirmed areas of almost complete epithelial loss in IL-33$^{-/-}$ mice as compared to controls (Figure 1D). Based on these findings, we speculated that the epithelial antimicrobial defense might be compromised in mice lacking IL-33. Indeed, *Salmonella*-infected IL-33$^{-/-}$ mice showed reduced lysozyme and UEA-1 (*Ulex europeus agglutinin-1*) staining, markers for Paneth and goblet cells, respectively (Figure 1E). Paneth and goblet cells are important secretory epithelial cells with innate immune functions and defense against enteric pathogens (Liévin-Le Moal and Servin, 2006). Additionally, in previous studies, goblet cell depletion in the course of *Salmonella* infection has been suggested as a strategy of the pathogen to reside within the host (Barthel et al., 2003). Our finding was further supported by qPCR analyses of AMPs produced by PCs such as angiogenin 4, defensin α5, cryptidin, and lysozyme, which were also downregulated in IL-33$^{-/-}$ mice as compared to controls (Figure 1F). Collectively, our data suggest that IL-33 signal transduction protects from *S. Typhimurium* infection and promotes epithelial antimicrobial defense. Notably, 16S-based next generation sequencing analysis of the intestinal microbiota of IL-33$^{-/-}$ and littermate control mice in the steady state revealed no significant alterations in the microbial composition, ruling out that microbiota changes in IL-33$^{-/-}$ mice account for their increased susceptibility (Figure S1). We next performed infection experiments using the non-invasive AroAΔ *S. Typhimurium* strain that establishes chronic gut infections in C56BL/6 wild-type mice, but not significant lethality. Interestingly, compared to unchallenged control mice, infected mice displayed a strong increase in intestinal IL-33 expression (Figure S2A). Moreover, IL-33$^{-/-}$ mice showed higher bacterial burden and increased tissue damage upon *Salmonella* infection as compared to control mice, confirming our previous findings that IL-33 is important to control *Salmonella*-dependent pathogenesis (Figures S2B and S2C). Similar to the results obtained with the wild-type (UK-1) strain, AroAΔ *Salmonella*-treated IL-33$^{-/-}$ mice showed decreased numbers of PCs and AMP expression (Figures S2D–S2F). Indeed, we observed a significant reduction of goblet cells and lower transcripts of its marker *Muc2* in infected IL-33$^{-/-}$ mice as compared to controls (Figures S2G and S2H). Collectively, these data suggest that IL-33 triggers essential epithelial changes during danger- or infection-associated challenge to the host.

IL-33 Induces Specific Expansion of Cells of the Secretory Lineage in the Small Intestine

To better characterize the role of IL-33-dependent signal transduction in the gut epithelium in vivo, we generated mice conditionally expressing IL-33 in the intestine (IL-33Vcre; Figure 2A). Histological analysis of the terminal ileum of mice constitutively expressing IL-33 in the gut indicated the presence of pronounced goblet cell hyperplasia, confirming previous observations on the role of IL-33 in helminth infection models (Koyasu and Moro, 2013). Interestingly, more-detailed histological analyses using H&E and PAS (periodic acid-Schiff) staining demonstrated that IL-33 overexpression not only led to an increment in goblet cells but also to significantly increased numbers of PCs within small intestinal crypts (Figure 2B). Our findings therefore indicated that IL-33 might have broader functions on epithelial cells than previously reported. In fact, immunohistochemical (IHC) staining of UEA-1, lysozyme, and chromogranin A (ChgA), which serve as gut-specific markers for goblet, Paneth, and EE cells, respectively, also demonstrated increased numbers of these cells after IL-33 overexpression. In contrast, cells of the absorptive lineage were not increased as indicated by staining for the enterocyte marker alkaline phosphatase (AP) (Figure 2C). To further confirm the high numbers of secretory cells in IL-33VCre mice, we analyzed the expression of a panel of genes preferentially produced by these cell types by qPCR. We

(D) Immunohistological staining for the epithelial marker β-catenin on cecal cross-sections from IL-33$^{+/-}$ and IL-33$^{-/-}$ mice infected with *S. Typhimurium* (scale bars represent 100 μm). Graph shows quantification of fluorescence intensity calculated by ImageJ from the β-catenin staining (CTCF, corrected total cell fluorescence).

(E) Representative pictures of UEA-1 and lysozyme double staining on terminal ileum cross-sections of IL-33$^{+/-}$ and IL-33$^{-/-}$ animals after *S. Typhimurium* infection (scale bars represent 50 μm). Graph shows quantification of positive cells from above stainings (n = 5; mean ± SD).

(F) qPCR analysis of the antimicrobial peptides angiogenin-4, defensin α5, cryptidins, and lysozyme in *Salmonella*-treated IL-33$^{+/-}$ and IL-33$^{-/-}$ mice (n = 5; mean values relative to HPRT ± SD).

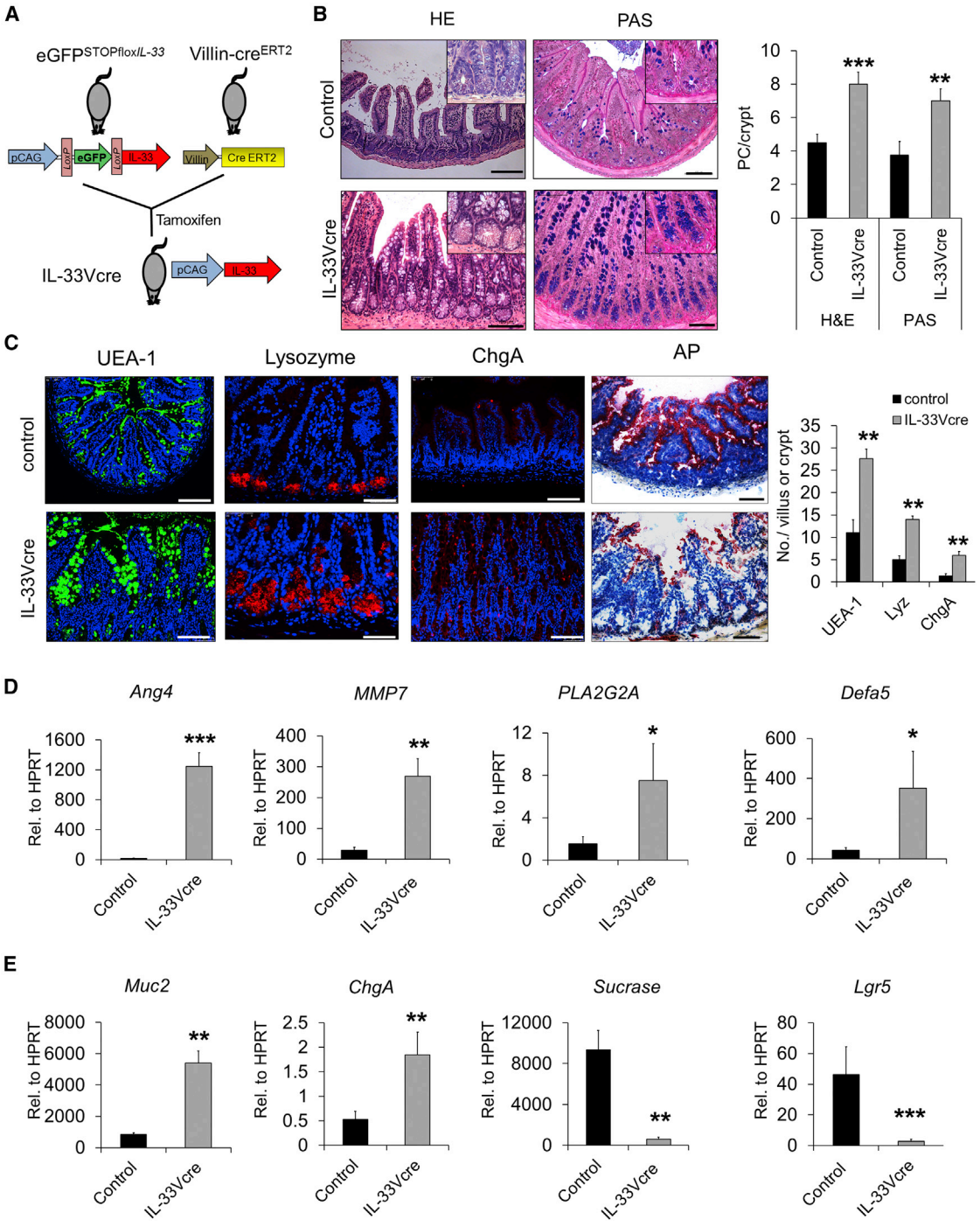

Figure 2. Gut-Specific IL-33 Expression Induces Expansion of Secretory-type Intestinal Epithelial Cells

(A) Schematic illustration of the generation of IL-33VCre mice.

(B) Terminal ileum cross-sections of control and IL-33Vcre mice were stained with H&E and PAS (scale bar represents 100 μm). Insets show magnified crypt areas. Graph shows quantification of PCs present per crypt from above stainings.

(C) Terminal ileum cross-sections from control and IL-33VCre mice stained with UEA-1, lysozyme, chromogranin A (ChgA), and alkaline phosphatase (AP) (scale bar represents 50 μm).The graph on the right shows quantification of the stainings (n = 3; mean ± SD).

(D) qPCR analysis for PC markers in small intestines of control and IL-33VCre mice (n = 9; mean values relative to HPRT ± SD).

(E) qPCR for markers of other small intestinal cell types: goblet (*Muc2*); enteroendocrine (*ChgA*); absorptive enterocyte (*Sucrase*); and stem cell (*Lgr5*) in control and IL-33Vcre mice (n = 9; mean values relative to HPRT ± SD).

also observed a marked increment in transcripts of the AMPs *Ang4*, *MMP7*, *PLA2G2A*, and *Defα5* in IL-33Vcre mice as compared to controls (Figure 2D), indicating increased number of PCs in these mice. Moreover, the markers for goblet and EE cells *Muc2* and *ChgA*, respectively, were also significantly upregulated in IL-33Vcre mice as compared to controls (Figure 2E). In striking contrast, transcripts of *Sucrase*, a marker for absorptive enterocytes, and *Lgr5*, a marker for intestinal stem cells, were significantly downregulated, indicating that IL-33 affects differentiation rather than proliferation of epithelial progenitors (Figure 2E). Hence, our analyses showed a marked and specific IL-33-dependent increment in gut epithelial cell types of the secretory lineage.

Recently, the role of IL-33 as a signaling molecule has been discussed in many chronic and auto-immune diseases like arthritis, liver injury, and multiple sclerosis (Palmer and Gabay, 2011). Hence, our experimental approach did not exclude other strategies of IL-33 signaling, importantly where the cytokine can be perceived by the epithelium through external sources as an alarmin. To investigate the physiological relevance of the cytokine when secreted in a systemic manner and not when it is restricted to be expressed intracellularly, we employed a strategy to systemically overexpress IL-33 via minicircle DNA (mcIL-33; Figure S3A). In support of our previous conclusions, similar abundance of cells of the secretory lineage as in IL-33Vcre mice was observed in mcIL-33-injected mice by histological staining (Figure S3B). Quantitative mRNA analyses of the markers of these secretory cell types further supported these findings (Figure S3C). Taken together, our data indicate that intestinal epithelial progenitor cells are targets of IL-33, skewing their differentiation toward secretory IEC, cells with essential functions in barrier integrity and intestinal homeostasis.

Pericryptal Fibroblasts in the Small Intestine Are the Primary Source of IL-33 Expression upon Systemic Infection

Because IL-33 has been reported to be expressed by various cell types including mast cells, epithelial cells, fibroblasts, and macrophages (Liew et al., 2010; Sponheim et al., 2010), we next investigated the primary cellular source of intestinal IL-33 by staining cross-sections from healthy humans and mice guts. Interestingly, the staining patterns suggested that IL-33 is expressed specifically in a distinct population of subepithelial cells neighboring the crypt base. In contrast, no positive signal could be observed in intestinal epithelial cells (Figure 3A). This finding was further supported by stainings in IL-33$^{LacZ/LacZ}$ reporter mice where a marked β-galactosidase activity was also found in pericryptal cells (Figure 3B). Moreover, double staining for β-galactosidase and the myofibroblast marker α-smooth muscle actin (α-SMA) demonstrated co-localization of IL-33 to pericryptal myofibroblasts (Figure 3C). In addition, isolated primary myofibroblasts from IL-33$^{LacZ/LacZ}$ reporter mice stained positive for X-gal, further confirming our above findings (Figure 3C). Thus, our data indicate that pericryptal fibroblasts act as a major source of IL-33 in the steady state gut as opposed to epithelial localization observed in samples from the skin or stomach (data not shown). Collectively, these data suggest a cross talk of pericryptal fibroblasts with epithelial progenitor cells via IL-33.

IL-33 has been reported to act in response to cellular stress including necrosis and infectious challenges (Liu and Turnquist, 2013). Accordingly, peritoneal macrophages upregulate IL-33 at the mRNA level upon TLR ligand stimulation (Espinassous et al., 2009). In order to investigate whether pericryptal fibroblasts upregulate IL-33 expression in response to microbial challenges, we intraperitoneally treated C57BL/6 mice with a sub-lethal dose of lipopolysaccharide (LPS) or fecal bacteria. IL-33 gene expression was strongly induced in the small intestine (Figure 3D) of LPS-challenged animals. Moreover, stainings of ileal sections of control, LPS-treated, or septic IL-33$^{LacZ/LacZ}$ reporter mice demonstrated increased activity of β-galactosidase in crypt subepithelial myofibroblasts (Figure 3E). Quantification of specific β-galactosidase activity showed increased IL-33 expression in LPS-treated and septic animals as compared to controls. Our data indicate that IL-33-mediated signaling from subepithelial cells to epithelial progenitor cells might be a defense mechanism by which the organism responds to an infectious challenge at barrier tissues. IL-33 might therefore serve to translate signals derived from systemic infections into a local organ-specific response, which serves to enforce barrier function in order to reduce further bacterial translocation. To further support this hypothesis, cultured primary myofibroblasts from small intestines of C57BL/6 mice were stimulated with serum collected from either control or LPS-treated IL-33$^{-/-}$ mice. IL-33 was detected in the supernatants of fibroblasts by ELISA with significantly higher concentrations in cultures treated with serum from LPS-treated animals (Figure 3F) as compared to mock-treated control serum. IHC stainings of LPS-serum-stimulated primary fibroblasts confirmed the presence of IL-33 within the cells (data not shown).

Systemic infection in humans, as mimicked in our experimental model, is associated with an acute phase response and the expression of various proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), IL-6, and IL-1β (Papadakis and Targan, 2000). Previous studies reported human intestinal fibroblasts to respond to various proinflammatory cytokines released during systemic infection (Jobson et al., 1998). We therefore reasoned whether inflammatory cytokines would also induce pericryptal fibroblasts from human gut to synthesize IL-33. Indeed, stimulation of human gut biopsies with inflammatory cytokines was sufficient to induce IL-33 expression in vitro (Figure 3G). Moreover, myofibroblasts isolated from human ileum significantly upregulated IL-33 expression when stimulated with the proinflammatory cytokines TNF-α or IL-1β (Figure 3H). Finally, IL-33 upregulation in stimulated ileal fibroblasts could be confirmed at the protein level using immunofluorescence staining (Figure 3I). Collectively, our data suggest that IL-33 can be induced and secreted by subepithelial fibroblasts in response to microbial challenges in proximity to the stem cell niche, thereby indicating an important regulatory role of the cytokine.

IEC-Intrinsic IL-33 Signaling Drives the Expansion of the Secretory Lineage

Recent studies have shown that IL-33 stimulates the release of type 2 cytokines like IL-4, IL-13, and IL-5 by various immune cell types to carry out its biological functions (Yang et al., 2013).

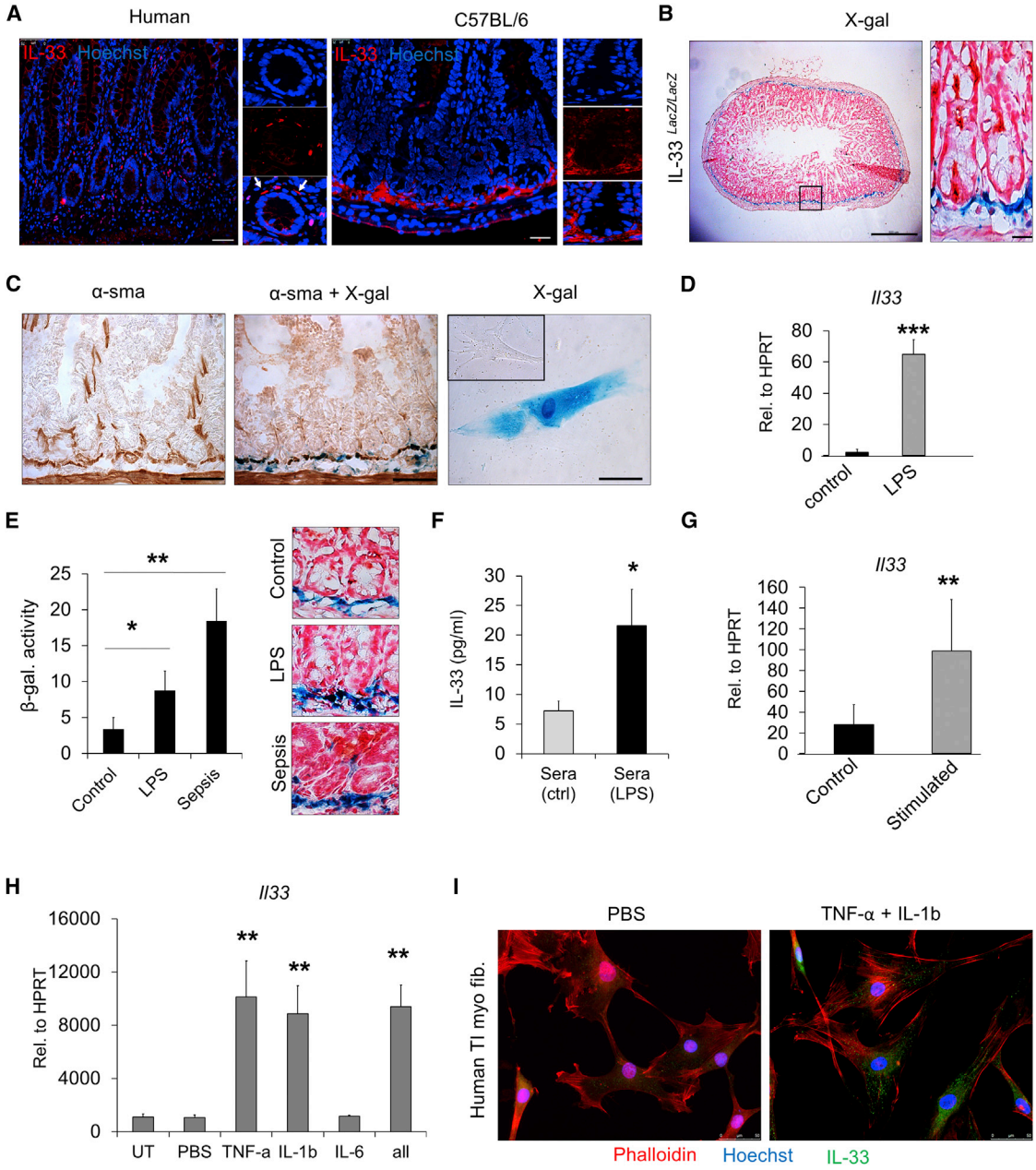

Figure 3. Intestinal IL-33 Expression Is Localized to Pericryptal Fibroblasts and Increased in Response to Systemic Infection

(A) Terminal ileum cross-sections from healthy patients and C57BL6 mice stained for IL-33 (scale bar represents 75 μm). Insets show magnified crypt areas with positive staining in subepithelial cells.

(B) X-gal staining of terminal ileum sections from untreated IL-33^{LacZ} reporter mice showing β-galactosidase activity at the crypt bottom (scale bar represents 500 μm). The right side picture shows a magnified crypt bottom (scale bar represents 50 μm).

(C) Terminal ileum sections from IL-33^{LacZ} reporter mice stained with anti-αSMA (scale bar represents 100 μm) for myofibroblasts. Next picture shows co-localization of αSMA and X-gal staining (scale bar represents 100 μm). The last picture shows X-gal staining of a single terminal ileum myofibroblast from a primary culture (scale bar represents 50 μm). Inset shows negative control.

(D) qPCR analysis of IL-33 expression in the ileum of LPS-treated animals as compared to control C57BL/6 (nine mice per group; relative expression to HPRT ± SD).

(E) Ileum cross-sections stained with X-gal from control, LPS-challenged, and septic IL-33 reporter mice (scale bar represents 50 μm). Graph depicts quantification of β-galactosidase activity (n = 6; relative to 1 μg of total protein ± SD).

(F) IL-33 measured via ELISA in supernatants of primary ileal myofibroblasts stimulated with serum from either control or LPS-challenged IL-33$^{-/-}$ mice.

(G) Expression levels of IL-33 in human ileal biopsies stimulated with a combination of the inflammatory cytokines TNF-α, IL-6, and IL-1β (n = 8; mean values relative to HPRT + SD).

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To investigate whether IL-33 directly or indirectly acts on intestinal epithelial cells, we used a pure epithelial organoid culture system in vitro (Figure S4A) as previously described (Sato et al., 2009). Isolated small intestinal crypts were cultured to epithelial organoids in the presence or absence of recombinant IL-33. In agreement with our in vivo studies, a significantly higher number of PCs per crypt could be observed in cultures treated with IL-33 for 5 days (Figure 4A). This finding was confirmed by qPCR for markers of secretory cells like *Ang4* and *Muc2*, suggesting a direct effect of IL-33 on secretory cell expansion. To ultimately exclude an indirect effect via residual contaminating lymphoid cells, such as ILC2, in the crypt culture, organoids were grown from the small intestine of NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/*SzJ* (NSG) mice. IL-33-dependent PC expansion in organoids generated from these mice was indistinguishable from wild-type mice (Figure 4B). In line with this observation, transcripts of the marker *PLA2G2A* were significantly increased in NSG organoid cultures stimulated with IL-33 in comparison to controls (Figure 4C). Notably, administration of IL-33 to NSG mice in vivo also increased the number of PCs and relative expression of *Ang4*, a specific marker for this cell type (Figure S4B).

To further exclude a potential role of ILC2, we generated organoids from Ror$\alpha^{-/-}$ mice that are deficient in the differentiation of these cells (Wong et al., 2012). Again, a significantly higher number of PCs and increased lysozyme expression was observed upon IL-33 stimulation, confirming that functional ILC2 is not relevant for this effect (Figure S4C). Finally, to exclude an indirect effect of IL-33 via autocrine IL-4 and/or IL-13 produced by IEC or potentially contaminating cells, we generated crypt organoids from gut tissue of IL-4R$\alpha^{-/-}$ mice, which lack signaling of the latter two cytokines. IL-33-treated IL-4R$\alpha^{-/-}$ organoids again showed increased PC numbers indistinguishable from IL-33-treated wild-type organoids, indicating that neither IL-4 nor IL-13 was necessary for this phenomenon and that IL-33 has a direct effect on epithelial cells within the crypt niche (Figure S4D). Collectively, our data suggested that IL-33 can directly signal to the intestinal epithelium, promoting the expansion of secretory IEC.

The ST2 Receptor Is Expressed on Epithelial Progenitor Cells and Is Essential for IL-33-Induced Secretory Cell Expansion

The above data indicated that intestinal epithelial cells can directly respond to extracellular IL-33. To ascertain the expression of the IL-33 receptor ST2 in IEC, we first stained ileal cross-sections of healthy humans and unchallenged C57BL/6 mice for ST2. Membranous staining was confirmed throughout the crypt-villous axis. The specificity of the staining was reaffirmed by using corresponding sections from ST2$^{-/-}$ mice as controls (Figure 5A). To validate the above findings, we analyzed ST2 expression on cultured epithelial cells derived from Lgr5-eGFP reporter mice, in which stem cells can be traced by GFP expression. Of note, the expression of ST2 on differentiated intestinal cells (Lgr5^{neg}) was low whereas stem (LGR5^{high}) and progenitor (Lgr5^{int}) cells showed high ST2 expression, providing evidence that stem and progenitor cells can be direct targets of IL-33 (Figure 5B). Strikingly, no increase in PC counts or expression of secretory cell markers was observed when small intestinal organoids were generated from ST2$^{-/-}$ mice and were stimulated with IL-33 (Figures 5C and 5D), indicating that IL-33 signaling functionally depends upon ST2 expression on the crypt epithelium. Finally, to exclude indirect IL-33 signaling in vivo via ST2 receptor expressed on hematopoietic cells, we generated chimeric mice, which received bone marrow cells from either C57BL/6 or ST2$^{-/-}$ mice (Figure S5A). IL-33 expression in chimeric mice resulted in a comparable secretory cell phenotype among the two groups. This was confirmed by IHC stainings for various markers of secretory cells (lysozyme, ChgA, and UEA-1), which were equally upregulated in IL-33-treated mice that received wild-type or ST2-deficient BM cells, respectively (Figure S5B). This was also highlighted by quantitative mRNA analysis for the PC marker *Ang4* and the goblet cell marker *Muc2* (Figure S5C). Collectively, our data indicated that ST2 receptor expression on intestinal epithelial cells is functional and transmits the IL-33 signal into epithelial progenitors in a direct manner.

IL-33 Regulates Intestinal Cell Fate Decisions via Diminished Notch Signaling

Early expression of key transcription factors (TFs) like *Hes1* and *Math1* are known to direct intestinal epithelial differentiation into the two major lineages: the absorptive and the secretory lineage, respectively (Gazit et al., 2004; Jensen et al., 2000). Because our model showed a specific increase in cells of the secretory lineage, we hypothesized that IL-33 might regulate expression of these differentiation factors. Indeed, we observed an increased expression of the early differentiation markers *Math1*, *Sox9*, and *Ngn3* and, at the same time, a lower expression of *Hes1* in IL-33VCre mice when compared to controls (Figure 6A). Similar results were obtained when IL-33 was expressed systemically (data not shown). The latter finding was confirmed by *Hes1* immunostaining showing abundant *Hes1* protein expression in enterocytes and CBCs in control mice whereas, in IL-33VCre mice, expression was significantly downregulated (Figure 6B). Collectively, our data indicate that IL-33 signaling directly affects cell fate decisions at very early stages of intestinal epithelial cell fate determination.

Recent studies have provided evidence for a major role of the Notch-signaling pathway in determining intestinal cell fate decisions and expression of the respective master TFs (Noah and Shroyer, 2013). Notch signaling is activated when a signal-receiving cell bearing the Notch receptor binds to Delta (Dll1 and -4) or Jagged (Jag1 and -4) transmembrane proteins on the signal-sending cell. The receptor-ligand complex when activated is cleaved by the γ-secretase complex, resulting in the release of the Notch intracellular domain (NICD), which then can regulate gene expression as part of TF complexes in the nucleus. Because Notch signaling has been demonstrated to

(H) Expression levels of IL-33 in cultured human primary ileal myofibroblasts stimulated with TNF-α, IL-6, IL-1β, or in combination (n = 3; mean values relative to HPRT + SD).

(I) Representative pictures of primary human ileal myofibroblasts stained for IL-33 (green), phalloidin (red), and Hoechst (blue) after stimulation with inflammatory cytokines (scale bar represents 50 μm).

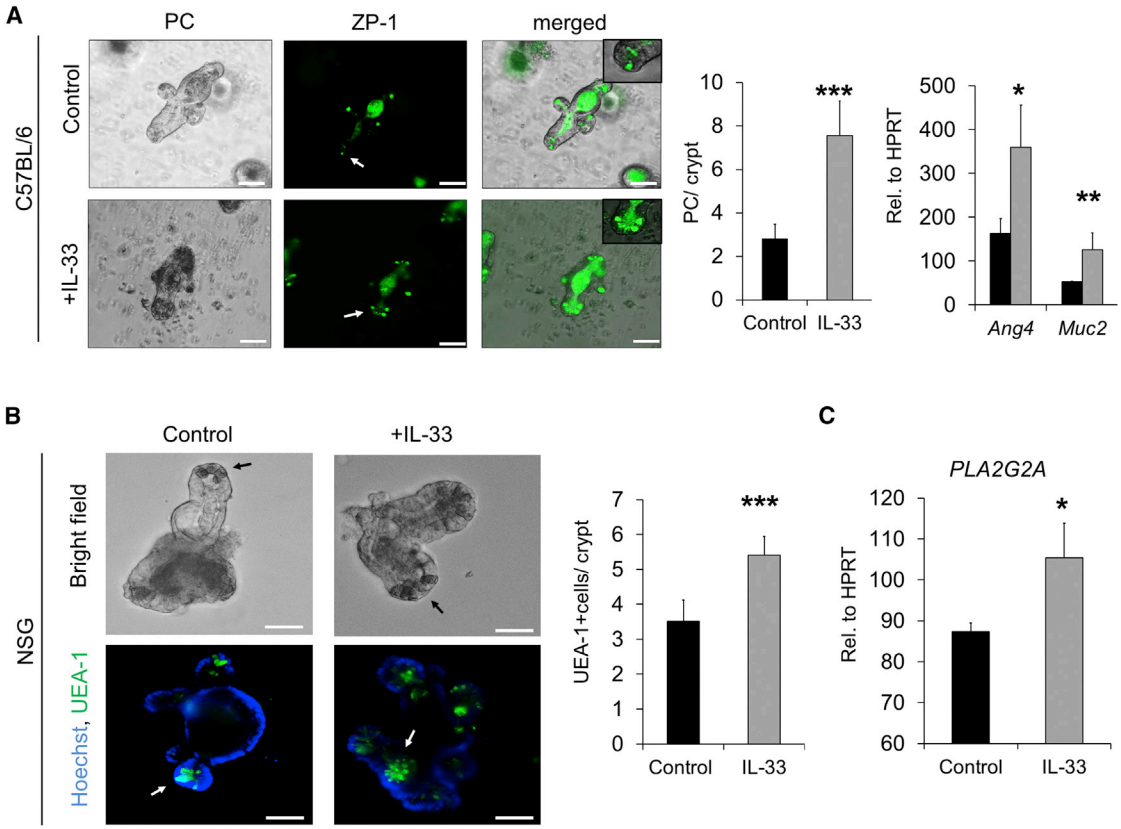

Figure 4. IL-33 Drives Intestinal Epithelial Cell Responses in a Direct Manner Independent of ILC2

(A) Representative pictures of small intestinal crypt organoids of C57BL/6 mice (scale bar represents 100 μm) stimulated with IL-33 over a period of 5 days. PC granules are stained with Zinpyr-1 (ZP-1). Arrows indicate PCs. Insets show single crypts from organoids bearing PCs at higher magnification. First graph depicts quantification of PCs from the above experiment (n = 30; mean values ± SD) and second graph depicts real-time analyses for secretory cell markers (*Ang4* and *Muc2*) from control and IL-33-stimulated small intestinal crypt organoids (n = 9; mean values relative to HPRT ± SD).

(B) Representative pictures of small intestinal crypt organoids from NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) stimulated with IL-33 over a period of 5 days (scale bar represents 100 μm). Secretory cells are stained with UEA-1 (scale bar represents 100 μm). Graph shows UEA-1+ cells per organoid bud (n = 45; mean ± SD).

(C) qPCR analysis of the antimicrobial peptide *PLA2G2A* from control and IL-33-stimulated NSG-derived organoids (n = 3; mean values relative to HPRT ± SD).

promote expression of *Hes* family genes and to repress expression of *Math1* (Tsai et al., 2014), we reasoned that IL-33 might program epithelial differentiation by regulating Notch signaling. Notably, IHC staining of NICD1 on ileal sections of control and IL-33VCre mice showed marked differences at crypt bottom cells. Accordingly, NICD1 was detected in the cytoplasm of crypt epithelial cells in control mice whereas, in IL-33VCre mice, NICD1 was mostly downregulated, indicating impaired Notch signaling in the presence of IL-33 (Figure 6B). Moreover, expression of the notch ligand Dll4 known to be present exclusively on cells of the secretory lineage (Sato et al., 2011) was markedly decreased in IHC stainings of ileum sections in IL-33Vcre mice as compared to controls (Figure 6B). The above findings were further supported by western blot analysis showing decreased intensity for both NICD1 and Dll4 in IEC (Figure 6C). Finally, qPCR analyses showed lower expression of the Notch ligands *Dll1*, *Dll4*, and *Jag1* in IL-33VCre mice (Figure 6D) whereas *Jag2* expression was unaltered (data not shown). We also evaluated whether Notch ligands are also suppressed in *Salmonella*-infected animals. As expected, *Dll1* and *Dll4* were downregulated in controls infected with *Salmonella* as compared to uninfected controls. However, this was not observed in infected IL-33$^{-/-}$ animals (Figure S6). To investigate whether Notch signaling was functionally involved in the epithelial response toward IL-33, we generated crypt organoids from C57BL/6 mice and stimulated them with IL-33 with or without addition of the Notch ligand Jagged-1 or the Notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT). Whereas organoids treated with either IL-33 or DAPT developed more PCs as demonstrated above, the addition of Jagged-1 completely blocked IL-33-induced expansion of PCs and decreased expression of the secretory cell markers *Ang4* (Figures 6E and 6F). Collectively, our data demonstrate a role of IL-33-balanced Notch signaling in promoting the reprogramming of intestinal epithelial differentiation.

DISCUSSION

The prevailing information regarding IL-33 in the intestine has focused its role either as a mediator of type 2 ILC, as an alarmin,

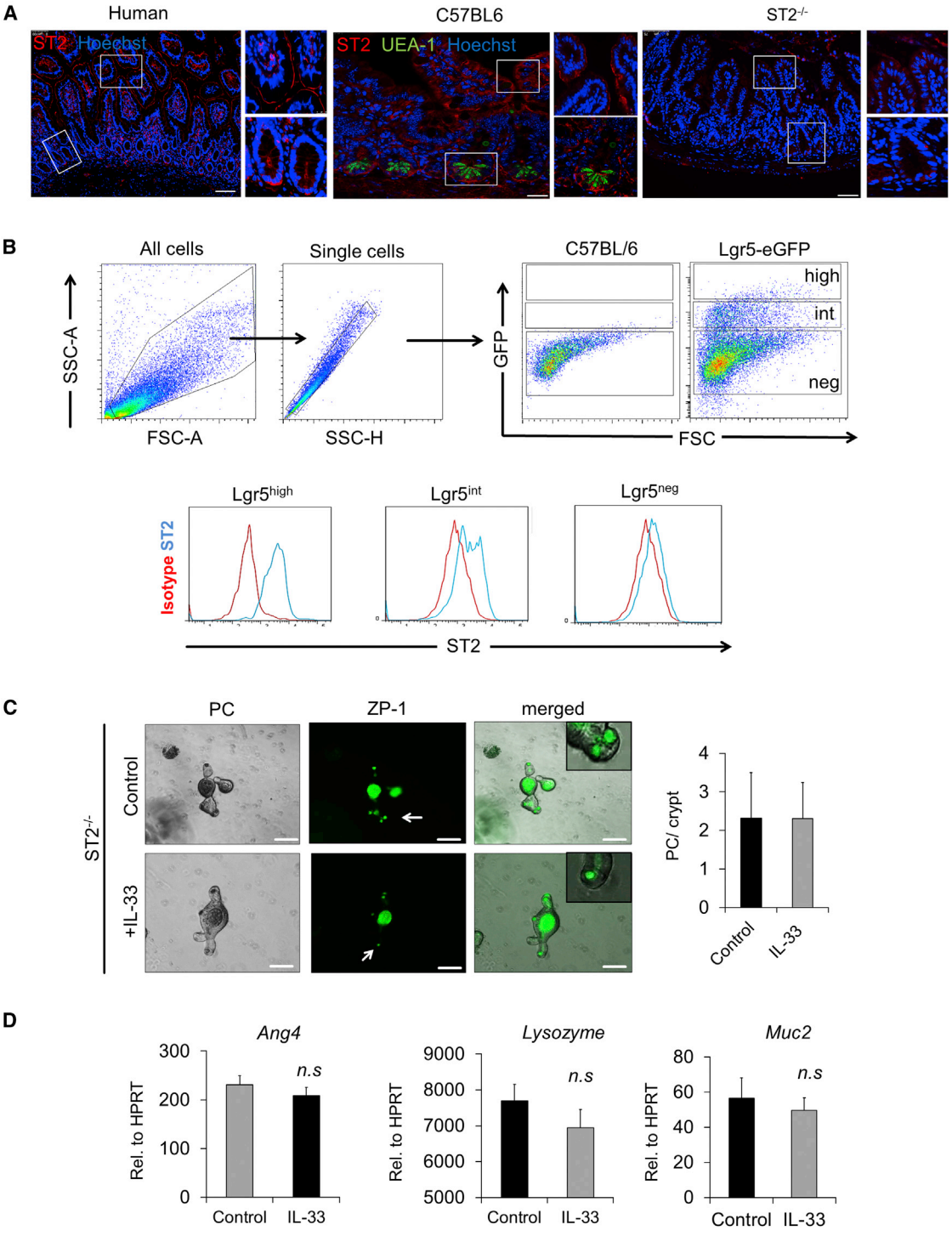

Figure 5. ST2 Is Highly Expressed on Epithelial Stem and Progenitor Cells and Is Essential for IL-33 Signal Transduction

(A) Terminal ileum cross-sections from healthy humans and C57BL/6 mice were stained for ST2 (red), Hoechst (blue), and UEA-1 (green; scale bars represent 100 μm and 75 μm). Highlighted sections show magnified villi and crypts with positive staining at crypt epithelial cells (scale bars represent 25 and 20 μm, respectively).

(B) FACS analysis of ST2 expression on intestinal epithelial cells derived from organoids. Upper panel shows the gating strategy in control and Lgr5-eGFP mice. Lower panel shows expression of ST2 gated on different Lgr5 populations.

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or as an activator of the nuclear factor κB (NF-κB) pathway, suggesting broad functions in immune defense (Seidelin et al., 2011). Moreover, recent publications have highlighted the role of IL-33 in establishing T-reg cells and indicated TH1 effector cells as cellular target of the cytokine (Schiering et al., 2014; Turnquist et al., 2011). Using various experimental approaches, we now provide evidence that, in the small intestine, IL-33 expression is confined to a distinct population of subepithelial myofibroblasts: (1) IL-33 reporter gene activity as well as IL-33 immunostaining was restricted to a population of spindle-like and α-sma+ cells directly underneath the epithelium and (2) isolated myofibroblasts from the small intestine of animals and humans expressed and released IL-33 in vitro. In contrast to a previously published study in a model of intestinal mucositis by treatment of a chemotherapeutic agent, we were not able to confirm epithelial IL-33 expression in the small intestine (Guabiraba et al., 2014). Hence, we speculate that IL-33 expression and secretion might be differentially regulated based on danger signals received. Although, our data indicate a specific function of pericryptal fibroblast released IL-33 in the small intestine, we do not rule out other sources of IL-33, which might be relevant in certain tissues and under certain circumstances.

IL-33 expression and secretion by pericryptal fibroblasts was significantly increased as a response to microbial challenges and proinflammatory cytokines like TNF-α and IL-1β in both mouse and human. Thus, pericryptal fibroblasts, underneath the crypt base, might serve as mucosal sensors of local or systemic infections, translating danger signals to neighboring cells by releasing IL-33, which can directly induce epithelial progenitor cells to differentiate into specific epithelial subtypes. Indeed, forced expression of IL-33 within the gut epithelium led to selective differentiation of epithelial progenitors to cells of the secretory type (Paneth, goblet, and EE cells), cell types with important functions in innate immune and tissue defense. Importantly, similar findings were obtained when IL-33 was expressed in a gut extrinsic way, excluding the possibility that IL-33 acted epithelial cell intrinsically to affect progenitor cell differentiation. ILC2s have previously been shown to be major responders to IL-33 and have been demonstrated to promote secretory cell hyperplasia like goblet and tuft cells (Moro et al., 2010; von Moltke et al., 2016). However, our experimental evidence supports a direct IEC response toward extrinsic IL-33: (1) intestinal epithelial organoids, devoid of contaminating cells, responded to extrinsic stimulation of IL-33; (2) ST2$^{-/-}$ intestinal epithelial organoids were unable to promote secretory IEC differentiation; and (3) IL-4Rα, NSG-, and Rorα-deficient organoids responded to IL-33 comparably to wild-type organoids, ruling out indirect epithelial activation via lymphoid cells such as ILC2 or their related cytokines. However, our data do not rule out the possibility that, in vivo, the epithelial response by IL-33 might be further supported by IL-33 signaling into lamina propria cells. Chimeric C57BL/6 mice reconstituted with ST2$^{-/-}$ bone marrow cells showed partial impairment of secretory cell expansion following IL-33 expression. Hence, it is possible that, along with IL-33, lamina propria cells like ILC2s can contribute to epithelial differentiation and barrier maintenance.

FACS analysis of isolated epithelium from Lgr5-eGFP ileum crypt organoids showed co-localization of the ST2 receptor with the stem cell marker LGR5, suggesting that stem and progenitor cells are major targets of IL-33 in the intestinal epithelium. This conclusion is in line with our finding of differential Notch signaling and expression of TFs known to drive the differentiation of secretory IEC from intestinal epithelial progenitor cells. Although, it cannot be fully ruled out that LGR5- cells can be targeted by IL-33. Indeed, to directly attribute IL-33 signaling via intestinal stem cells, advanced mouse models with conditional deletion of ST2 in stem cell compartment are necessary.

Many parallel signaling pathways have been studied in the context of IL-33 functionality like MyD88, MAPK, and ERK signaling (Martin, 2013). Our data indicated an effect of IL-33 on early cell fate markers, as expression for *Hes1* was downregulated and *Math1* was significantly upregulated. Several studies have reported major signaling pathways like Wnt and Notch to direct stem cells into differentiation of these secretory cells (Tian et al., 2015). Accordingly, NICD, which functions as a TF for Hes1 (Tsai et al., 2014), was downregulated in an IL-33-proficient system. In agreement with these findings, IL-33 diminished Notch signaling in vivo and in vitro and forced Notch activation blocked IL-33-induced Paneth and goblet cell expansion in organoid cultures. Moreover, ablation of Notch activity in epithelial organoids by using a γ-secretase inhibitor mimicked IL-33-induced Paneth and goblet cell expansion. These data pointed toward an impairment of Notch signaling on the level of receptor-ligand interaction. In agreement with these previous reports, expression of *Dll1* and *Dll4* transcripts by Paneth and goblet cells (Shimizu et al., 2014) were suppressed by IL-33 stimulation. Taken together, our data indicate that IL-33-induced secretory cell hyperplasia occurs in a manner that is dependent upon Notch inhibition.

Previous studies have citied the imbalance of IL-33/ST2 axis in various infectious diseases like *Leishmania major*, *Toxoplasma gondii*, *Mycobacterium tuberculosis*, and *Schistosoma mansoni* (Rostan et al., 2015). Our data show compelling evidence of IL-33-induced secretory cell expansion, eventually resulting in copious amount of AMPs as a response to *Salmonella* infection. This phenomenon could give leverage in *Salmonella*-induced gut barrier disruption as IL-33$^{-/-}$ mice showed higher susceptibility to infection and severe gut tissue destruction in both an acute and chronic model of Salmonellosis. Expression of many essential AMPs including *Defensin α5*, a crucial factor for expelling pathogenic bacteria (Ouellette, 2011), was impaired in infected IL-33$^{-/-}$ mice, a finding that potentially contributes to their susceptibility. However, once the epithelial barrier is overcome by bacteria, type 2 immunity mediated by ILC2 or Th2 cells, which

(C) Representative pictures of small intestinal crypt organoids of control and IL-33-stimulated cultures from ST2$^{-/-}$ mice (scale bar represents 100 μm). PC granules are stained with fluorescent ZP-1. Insets show magnified buds of organoid-bearing PC. Arrows indicate PC. Graphs depict quantification of PC numbers per organoid (n = 50; mean ± SD).

(D) qPCR analysis of antimicrobial peptides from control and IL-33-stimulated organoids derived from ST2$^{-/-}$ mice (n = 9; mean values relative to HPRT ± SD).

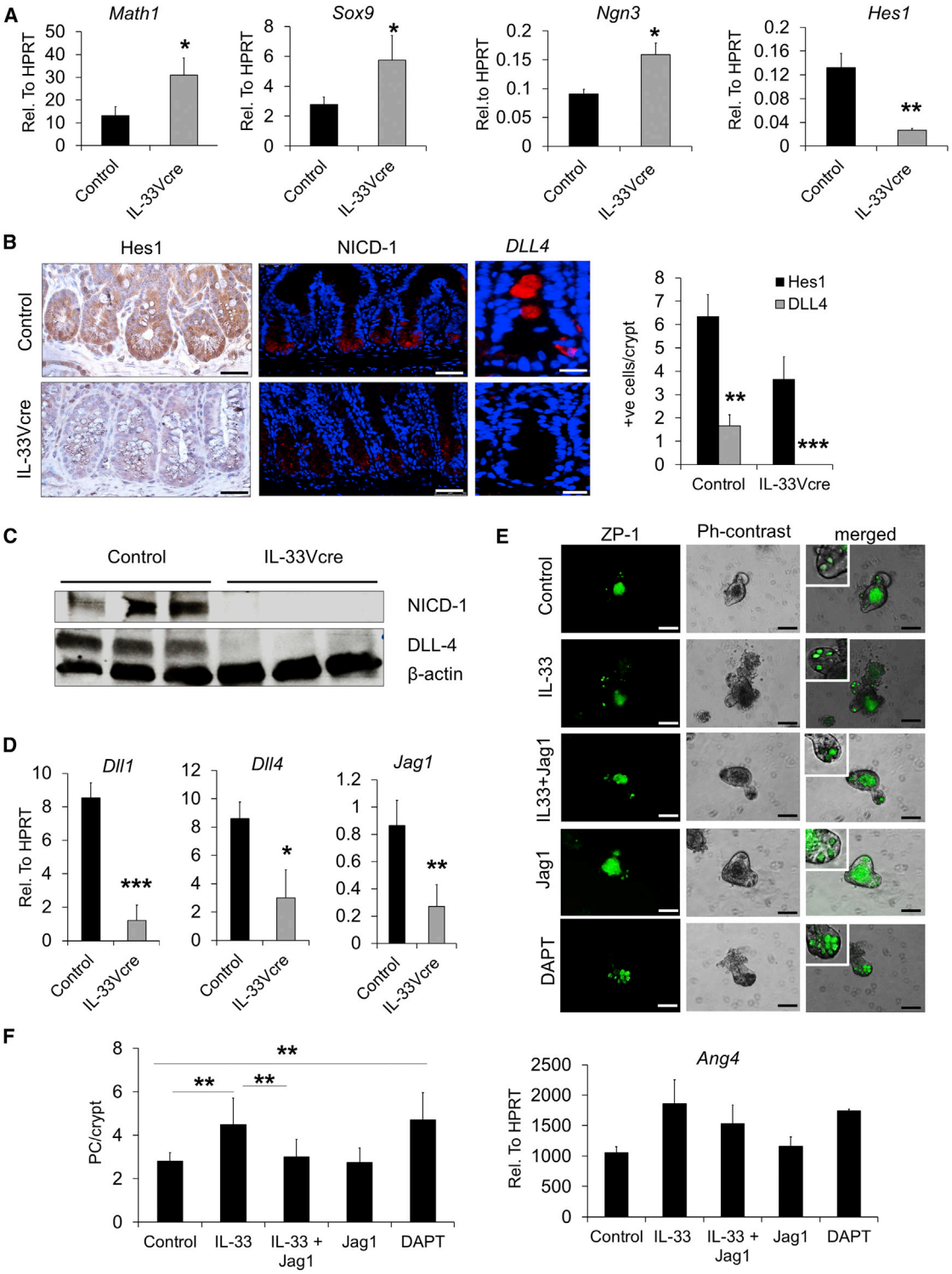

Figure 6. IL-33 Interferes with the Notch-Signaling Pathway and Expression of Master Regulators of Intestinal Epithelial Cell Differentiation

(A) qPCR analysis of early differentiation marker genes *Math1*, *Ngn3*, *Sox9*, and *Hes1* (n = 9; mean values relative to HPRT ± SD) from control and IL-33VCre mice.

(B) IHC staining for *Hes1*, NICD1, and Dll4 on terminal ileum sections of control and IL-33VCre mice (scale bar represents 50 μm). Graph depicts quantification of Hes-1-, NICD1-, and Dll4-positive cells from above staining (n = 3; mean ± SD).

(C) Western blot analysis of NICD1 and Dll4 expression in IEC lysates from control and IL-33VCre mice. The housekeeping gene β-actin was used as loading control.

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are IL-33 dependent, might be important in eradication of the pathogen.

In summary, our data show an unexpected role of IL-33 in the reprogramming of intestinal epithelial progenitor cells into cells of the secretory IEC lineage, cells with important innate immune functions. Our data indicate that pericryptal fibroblast are sensors of local and systemic infection, and their release of IL-33 can serve to translate these signals into a physiologic response to enforce barrier function in an effort to impede further microbial invasion.

EXPERIMENTAL PROCEDURES

Mice

Mice carrying a *loxp*-flanked GFP Stop cassette upstream of an IL-33 transgene (McHedlidze et al., 2013) were crossed with Villin-*cre*^{ERT2} mice to generate inducible gut-specific IL-33 transgenic mice (IL-33Vcre). Genotyping was performed by PCR on tail DNA. For induction of constitutive expression of IL-33 in the gut, mice were once injected intraperitoneally with 200 μl tamoxifen (5 mg/ml in ethanol; Sigma). To induce systemic release of soluble IL-33, hydrodynamic tail vein injection was performed using a minicircle-based plasmid vector containing IL-33 expression cassettes (mcIL-33). ST2$^{-/-}$ mice were described recently (McHedlidze et al., 2013). IL-33$^{LacZ/LacZ}$ reporter mice (C57BL/6 background) were generated as described previously (Skarnes et al., 2011). For some experiments, this strain was used after backcrossing to mice harboring an Nramp1^{G169} wild-type allele to overcome the Nramp1 deficiency of C57/Bl6 mice as previously described (Loomis et al., 2014). For the generation of bone marrow chimeras, 1 × 10^7 cells from femurs and tibias of donor mice (C57BL/6 or ST2$^{-/-}$) were intravenously injected into lethally irradiated (10 Gy) recipient mice. Mice were let to recover for 8 weeks followed by mcIL-33 injection into the tail veins. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG), IL-4Rα$^{-/-}$, and Lgr5-EGFP-IRES-*cre*^{ERT2} mice were obtained from Jackson Laboratory. Rorα$^{sg/sg}$ (Rora$^{tm1a(EUCOMM)Wtsi}$) were from EUCOMM. Animal experiments were approved by the governments of Rheinland-Pfalz and Mittelfranken.

Salmonella Typhimurium Infection

The *Salmonella Typhimurium* strain AroAΔ (Grassl et al., 2008) and bioluminescent UK-1 (Riedel et al., 2007) were routinely cultured in Luria-Bertani broth at 37°C with aeration and continuous shaking at 225 rpm with appropriate antibiotics. Mice were orally gavaged with 20 mg streptomycin 24 hr prior to infection. Food was removed for 8 hr before infection, after which mice were orally gavaged with ~1 × 10^6 colony-forming unit (CFU). Bacterial burden was monitored via IVIS Lumia II or plating of stool. Mice were sacrificed via CO_2 inhalation at indicated time points, and tissues were harvested for microscopy, histology, RNA extraction, and analysis of bacterial translocation.

Experimental Models of Septic Shock and Poly-microbial Sepsis

Mice were injected intraperitoneally with LPS (2.5 mg/kg body weight; Sigma-Aldrich) or stool suspensions from C57BL/6 mice. Viability was monitored by visual examination and by measuring body temperature and weight loss. Mice were sacrificed after 6–8 hr of injection.

Human Samples

Terminal ileum biopsies were collected from the endoscopy unit of the University Clinic Erlangen. The specimens were obtained during routine diagnostics procedures and immediately processed for stimulation with human cytokines TNF-α (100 ng/ml; Invitrogen), IL-1β (10 ng/ml; Sigma), and IL-6 (10 ng/ml; Life Technologies). The collection of samples was approved by the local ethical committee and the institutional review board of the University of Erlangen-Nuremberg with each patient giving their written informed consent.

Primary Myofibroblast Culture

Murine terminal ileum myofibroblasts were isolated and cultured as described previously (Lawrance et al., 2001). More details can be found in the Supplemental Experimental Procedures.

Histology, Immunohistochemistry, and *LacZ* Staining

Histopathological analyses were done with formalin-fixed paraffin-embedded tissue sections. After H&E and PAS staining, the sections were subjected to IHC staining using the TSA Cy3 (PerkinElmer) system for fluorescence or by addition of a diaminobenzidine (DAB) substrate (ABC staining kit; Santa Cruz Biotechnology) as recommended by the manufacturer. LacZ staining was performed by incubating frozen cryosections in X-gal solution O/N at 37° followed by nuclear staining using fast red solution. The sections were analyzed using fluorescence microscopy (Leica CTR6000) and confocal microscopy (Leica TCS SP5). A list of antibodies can be found in the Supplemental Experimental Procedures.

Crypt Isolation and Organoid Culture

Crypts were isolated from small intestines of 8- to 12-week-old mice and cultured for a minimum of 6 days as described previously (Sato et al., 2009). More details can be found in the Supplemental Experimental Procedures.

Flow Cytometry of LGR5-Positive Stem Cells

Intestinal crypts of LGR5-EGFP-IRES-CreERT2 mice (Barker et al., 2007) were isolated and cultured as described above and elsewhere (Jung et al., 2011). More details can be found in the Supplemental Experimental Procedures.

IEC Isolation and Immunoblotting

IECs were isolated and processed as described before (Martini et al., 2016). More details can be found in the Supplemental Experimental Procedures.

Gene Expression Analysis

Total RNA was extracted from whole-gut pieces, organoids, or isolated IECs using a RNA isolation kit (Nucleospin II; Machery Nagel). cDNA was synthesized by reverse transcription (iScript cDNA synthesis kit; Bio-Rad). Real-time PCR was performed using specific QIAGEN Quantitect primer assays. All experiments were normalized to the housekeeping gene HPRT.

ELISA and β-gal Assay

IL-33 production was measured by the specific Duoset ELISA kit from R&D systems according to manufacturer's instructions. Absorbance was read at 450 nm by a Tecan infinite M200 microplate reader. Specific activity of β-galactosidase in IL-33^{LacZ} reporter mice was measured using the β-gal assay kit (Invitrogen) following the manufacturer's protocol.

Statistical Analysis

Data were analyzed by Student's t test using Microsoft Excel and Graphpad Prism. Significance levels are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. All data are presented as mean values ± SD. ImageJ was used to perform statistics for IHC stainings. Investigators were blinded during data collection to eliminate potential bias during analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.049.

(D) Expression levels of Notch ligands *Dll1*, *Dll4*, and *Jagged-1* in control and IL-33VCre mice (n = 9; mean values relative to HPRT + SD).
(E) Representative pictures of in-vitro-cultured crypt organoids from C57BL/6 mice treated with recombinant IL-33, Jag-1, or Notch Inhibitor (DAPT; scale bar represents 100 μm). Insets show magnified crypt-bearing PCs.
(F) Quantification of PCs/crypt (+SD) and real-times analysis for *Ang4* from above experiment (n = 3; relative to HPRT ± SD).

AUTHOR CONTRIBUTIONS

M.M., S.W., and C.B. designed the research. M.M., S.F., M.H., G.-W.H., E.G.-V., T.M., M.K., and C.G. performed the experiments. S.V. and S.D. supplied materials for the study. M.M., M.F.N., S.W., and C.B. analyzed the data and wrote the paper.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the DFG clinical research unit KFO257 (TP1, TPZ). Furthermore, the work was supported by the Interdisciplinary Centre for Clinical Research (IZKF) of the University Erlangen-Nürnberg, the European Community's IMI initiative (acronym BTCure). The German Research Foundation DFG further supported this work within priority program SPP1656 (Intestinal Microbiota), the collaborative research centers SFB 1181 (A08, C05) and SFB796 (B07, B09), and individual grant BE3686/2. The authors thank Kristina Urbanova, Eva Podstawa, and Christina Lindner for excellent technical assistance.

Received: May 21, 2015
Revised: March 16, 2016
Accepted: April 8, 2016
Published: May 12, 2016

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