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CD34\(^+\) mesenchymal cells are a major component of the intestinal stem cells niche at homeostasis and after injury

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Significance

Maintenance of stem cells in adult organs requires a specialized microenvironment called the niche, which provides structural cues and paracrine signals to ensure stemness. In the intestine, increasing evidence points toward a major role for the mesenchyme close to crypts to perform this function; however, such putative mesenchymal niche remains poorly characterized. Here, we identify nonmyofibroblastic CD34\(^+\) Gp38\(^+\) mesenchymal cells as a major component of the intestinal epithelial stem cells (IESCs) niche. We show that CD34\(^+\) Gp38\(^+\) mesenchymal cells develop after birth and contribute to the maintenance of IESCs at homeostasis and organization of intestinal inflammation after injury.


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contribute to host defense and tissue repair upon intestinal damage.

**Results**

**Coexpression of gp38 and CD34 Identifies Pericryptal \(\alpha SMA^-\) Mesenchymal Cells.** We showed that expression of gp38 (podoplanin) identifies a major fraction of mesenchymal cells of the intestinal lamina propria (19, 20). Because lymphatic endothelial cells (LECs) also express gp38 (21), we used a combination of gp38 and CD31, a marker for endothelial cells, to identify gp38\(^+\) CD31\(^-\) mesenchymal cells. We observed that gp38\(^+\) mesenchymal cells (gp38\(^+\) stroma) accounted for up to 73% of total non-hematopoietic (CD45\(^-\)) cells in the adult mouse colon (Fig. 1A, Left) and ileum (Fig. S1A, Left). A subpopulation of gp38\(^+\) stromal cells coexpressed CD34 (Fig. 1A, Right and Fig. S1A, Right), a marker also expressed by endothelial cells (22), mesenchymal stem cells (23) and human intestinal mesenchyme (24). Gp38\(^+\) CD34\(^+\) double positive stromal cells were localized near the crypts in colon (orange-yellow staining in Fig. 1B, Left) and ileum (Fig. 1B, Right) (thereafter called CD34\(^+\) CSCs for crypt stromal cells), in close contact with Lgr5\(^+\) stem cells (Fig. 1C), and were \(\alpha SMA^{low}\) (arrows in Fig. 1D and E, Top; analysis of \(\alpha SMA\) expression by intracellular FACS analysis is shown in Fig. S1B). In contrast, gp38\(^+\) CD34\(^-\) stromal cells were mainly found in the lamina propria of the upper villi and colon tops (Fig. 1B, green staining) and were mostly \(\alpha SMA^{high}\) (arrowheads in Fig. 1D and E, Bottom and Fig. S1B), a defining characteristic of \(\alpha SMA^+\) subepithelial myofibroblasts (15), and are thereafter called CD34\(^-\) MyoFs. Single positive CD34\(^+\) gp38\(^-\) cells in the crypts and villi were blood endothelial cells (Fig. S1C).

Subepithelial \(\alpha SMA^+\) myofibroblasts play a major role in promoting intestinal epithelial differentiation through secretion of BMPs (25, 26). Consistent with their pericryptal localization and low expression of \(\alpha SMA\), CD34\(^+\) CSCs isolated from the intestinal lamina propria expressed significantly lower levels of double positive stromal cells were localized near the crypts in colon (orange-yellow staining in Fig. 1B, Left) and ileum (Fig. 1B, Right) (thereafter called CD34\(^+\) CSCs for crypt stromal cells), in close contact with Lgr5\(^+\) stem cells (Fig. 1C), and were \(\alpha SMA^{low}\) (arrows in Fig. 1D and E, Top; analysis of \(\alpha SMA\) expression by intracellular FACS analysis is shown in Fig. S1B). In contrast, gp38\(^+\) CD34\(^-\) stromal cells were mainly found in the lamina propria of the upper villi and colon tops (Fig. 1B, green staining) and were mostly \(\alpha SMA^{high}\) (arrowheads in Fig. 1D and E, Bottom and Fig. S1B), a defining characteristic of \(\alpha SMA^+\) subepithelial myofibroblasts (15), and are thereafter called CD34\(^-\) MyoFs. Single positive CD34\(^+\) gp38\(^-\) cells in the crypts and villi were blood endothelial cells (Fig. S1C).
transcripts coding for Bmp2, Bmp7, Acta2 (coding for αSMA), and the Hedgehog receptor Patched (Pch1) (13, 25), compared with CD34+ MyoFs (Fig. 1F). These results show that CD34+ CSCs are localized near crypts, adjacent to Lgr5+ IESCs, and express significantly lower levels of transcripts coding for genes involved in epithelial differentiation compared with CD34+ MyoFs.

**CD34+ CSCs Promote IESCs Maintenance.** The localization of CD34+ CSCs close to Lgr5+ IESCs (Fig. 1C) suggests a role in supporting IESC function. To test this hypothesis, we used a 3D Matrigel culture system that allows for the growth of intestinal crypts into organoids (27). In the absence of mesenchymal cells, crypts containing IESCs spontaneously formed self-organizing organoids when EGF and Rspo1 were added to the medium (Fig. 24, Left). These structures contained budding crypts and epithelial cell shedding into the lumen. The addition to intestinal crypts of CD34+ CSCs isolated from mouse colon or ileum, but not of CD34+ myoFs, led to the formation of spherical structures, termed spheroids, with no visible crypts nor epithelial cell shedding (Fig. 2 and Fig. S24). Fully differentiated intestinal organoids contained Ki-67+ proliferating cells restricted to the crypts (Fig. 2B, Left), whereas spheroids contained a significantly higher proportion of Ki-67+ cells that were homogenously distributed throughout the structure (Fig. 2 B, Right; and C), indicating that CD34+ CSCs promote epithelial proliferation. Moreover, spheroids harbored fewer wheat germ agglutinin (WGA)-binding cells, such as Paneth cells and goblet cells, compared with organoids (Fig. 2D), suggesting that CD34+ CSCs impair epithelial differentiation.

To determine whether CD34+ CSCs affect the activity of Lgr5+ IESCs, normally restricted to crypts in fully differentiated mice and grown as indicated; CSCs did not require supplementation with Fig. (coding for

**CD34+ CSCs Maintain IESCs Through Secretion of Grem1 and Rspo1.** The Wnt agonists Wnt2b and Rspo1, and the BMP antagonist Grem1, play a pivotal role in the maintenance of IESCs (7, 10, 11). We observed that CD34+ CSCs expressed significantly higher levels of Grem1. Wnt2b and Rspo1 compared with lamina propria CD34+ MyoFs, gp38+ CD34+ stromal cells (DCNs), endothelial cells (ECs), or leukocytes (Fig. 3 and Fig. S2B). Spheroid formation induced by CD34+ CSCs was markedly reduced in the presence of neutralizing anti-Grem1 antibodies (Fig. 3B), indicating that Grem1 production by CD34+ CSCs play a critical role in this process. Furthermore, crypts grown in the presence of CD34+ CSCs did not require supplementation with Rspo1 (Fig. 3C), necessary for the growth of intestinal crypts, suggesting that CD34+ CSCs are an important source of Rspo1. Finally, transwell experiments confirmed that soluble factors were sufficient for the progeness function of CD34+ CSCs (Fig. 3D and Fig. S24), and that other stromal cells, such as skin fibroblasts, cannot compensate for the lack of CD34+ CSCs (Fig. 3D). Remarkably, maintenance of IESCs by CD34+ CSCs required their continuous presence, because spheroids rapidly differentiated into organoids when removed from CD34+ CSCs-containing medium (Fig. 3E). These results show that continuous supply of soluble niche factors by CD34+ CSCs is necessary and sufficient to promote IESC expansion. In agreement with these results, CD34+ CSCs express the transcription factor Foxl1, which was recently used as a marker to deplete intestinal mesenchymal cells with an essential role in maintenance of the IESC niche (28), and do not express Myh11, which identifies intestinal myofibroblasts with dispensable Wnt activity for the niche (18) (Fig. S3).

**CD34+ CSCs Develop After Birth and Expand Around Crypts After Weaning.** Crypts isolated from fetal mouse intestine form spontaneously spheroids, a potential that is rapidly lost after birth (29), suggesting that postnatal IESCs become dependent on external factors. Accordingly, crypts mature between embryonic day (E) 16.5 and the first weeks after birth (30). Whereas E16.5 embryos displayed clusters of gp38+ CD34+ stromal cells at sites of intestinal villus formation (Fig. 4A, Left and Fig. S4), gp38+ CD34+ stromal cells were absent from fetal or neonatal intestines. Gp38+ CD34+ stromal cells were detected in the first weeks after birth in the submucosa underlying colon crypts (Fig. 4A, Center) and were accounting for nearly one-half of the total gp38+ intestinal stromal cells 2 wk after birth (Fig. 4B). Submucosal gp38+ CD34+ cells at 2 wk expressed similar levels of Grem1 than their adult counterparts, whereas expression of Rspo1 and Wnt2b were still low (Fig. 4C), suggesting that these cells are immature CD34+ CSCs. During the third week after birth, the percentage of gp38+ CD34+ cells among total intestinal
stromal cells increased markedly (Fig. 4C). CSCs near regenerating crypts Published online January 10, 2017

(ECs, endothelial cells (CD31 CD34)); ECs, endothelial cells (CD31 CD34). CSCs isolated from the colon supported T-cell ± CSCs of most proinflammatory molecules was transient, (3 from two independent experiments. Values are expression

CSCs isolated from acutely (3 from two independent experiments. (Fig. 5D). Inflamed CD34+ CSCs also strongly up-regulated Vcam1 (Fig. 5C), an adhesion molecule expressed by mesenchymal stromal cells in lymphoid organs, which is essential for the recruitment of immune cells. In addition, CD34+ CSCs isolated from acutely inflamed colon significantly overexpressed, compared with CD34+ CSCs at steady state, transcripts coding for Ccl2 (MCP-1) and Csf1 (M-CSF); essential for the recruitment of monocytes and macrophages to inflamed tissues and for macrophage maintenance, respectively; the neutrophil chemotactic factors Cxcl1 and Cxcl2; the proinflammatory cytokine Il6; as well as Ccl19, Ccl3, Icam1, Trance (Rankl), and Ifngr1, all involved in the organization of adaptive immune responses; and the lymphangiogenic growth factors Vgf, Vegfd, and Flg2 (Fig. 5D). Finally, CD34+ CSCs up-regulated a number of growth factors for epithelial cells such as Ang (Amphiregulin), Fgfr, Fgfr10, as well as Ptg2, which modulates epithelial proliferation through effects on prostaglandin synthesis (31), and Colla1 (coding for type I collagen), suggesting a direct role in epithelial proliferation and tissue repair. Overexpression by CD34+ CSCs of most proinflammatory molecules was transient, peaking during the acute phase of inflammation, with a few notable exceptions including the T cells and DCs chemotactic factor Ccl19, and the lymphocyte survival factor Il7, which are key factors of adaptive immunity (Fig. 5D). Consistent with a role in adaptive immunity, CD34+ CSCs isolated from the colon supported T-cell survival in vitro (Fig. S6). Finally, a number of transcripts were overexpressed in CD34+ MyoFs compared with CD34+ CSCs, including, in addition to BMPs, transcripts coding for genes involved in vasculature growth and remodeling such as Angpt1, Angpt2, and Vegfa, as well as Wnt3a, a noncanonical Wnt ligand that plays an essential role in intestinal regeneration (32), suggesting that intestinal homeostasis and repair require the coordinated action of functionally distinct subsets of mesenchymal cells (a scheme is shown in Fig. S7).

Discussion
Here, we identify CD34+ CSCs as the major intestinal source for the IESC niche factors Wnt2b, Grem1, and Rspo1. CD34+ CSCs are closely adjacent to Lgr5+ IESCs, both in colon and ileum, and are sufficient to promote expansion of Lgr5+ IESCs in organoids, an effect mainly dependent on Grem1. CD34+ CSCs are topographically and functionally distinct from aSMA+ subepithelial myofibroblasts, and develop after birth in the intestinal submucosa, independently of the microbiota. We further show that, after intestinal damage and inflammation, CD34+ CSCs remain in close proximity to regenerating crypts and up-regulate Grem1 and Rspo1, as well as a number of chemokines, cytokines, and growth factors with key roles in intestinal immunity and tissue repair. CD34+ CSCs are therefore an essential component of the IESC niche at homeostasis and contribute to intestinal inflammation and repair after damage.

Wnt signaling is required to maintain IESCs (4). Paneth cells are the main source for the Wnt ligand Wnt3 and play an important role in the IESC niche (5). However, mice lacking Wnt activity in epithelial cells or myofibroblastic cells have normal intestinal homeostasis, and ablation of Paneth cells does not affect the function of IESCs (16–18), suggesting redundant or compensatory mechanisms involving nonepithelial and non-myofibroblastic sources. In agreement with this hypothesis, CD34+stromal cells increased markedly (Fig. 4B), and such an increase coincided with appearance of gp38+ CD34+ cells around the crypts (Fig. 4A, Right). Expansion and pericryptal localization of CD34+ CSCs was independent of intestinal colonization by microbiota and recognition of MAMPs, which in mice increase massively around weaning age (Fig. S5A), suggesting that development of CD34+ CSCs is a noncanonical Wnt ligand that plays an essential role in intestinal regeneration (32), suggesting that intestinal homeostasis and repair require the coordinated action of functionally distinct subsets of mesenchymal cells (a scheme is shown in Fig. S7).

Intestinal Injury Induces Remodeling and Activation of CD34+ CSCs.
Intestinal injury induces an inflammatory response and repair process to control infection and regenerate the damaged epithelial layer. To determine the role of CD34+ CSCs during intestinal inflammation, we analyzed the intestine of adult mice subjected to DSS-mediated colitis. DSS induced damage and loss of colonic epithelial layer integrity (blue asterisk in Fig. 5A, Left), triggering regeneration of the epithelial layer in the following weeks (white asterisk in Fig. 5A, Right). Seven days after initiation of DSS treatment, damaged zones were filled with an abundant scar tissue containing CD34+ CSCs near regenerating crypts (arrow in Fig. 5A, Left), whereas CD34+ MyoFs expanded in colon tops (arrowhead in Fig. 5A, Left). The proportion of CD34+ CSCs among total gp38+ stromal cells decreased during inflammation, reaching preweaning levels (Fig. 5B). CD34+ CSCs isolated from DSS-inflamed colon overexpressed niche factors Grem1 and Rspo1, compared with CD34+ CSCs at steady state, suggesting that their prostenness function is maintained during intestinal inflammation (Fig. 5D). Inflamed CD34+ CSCs also strongly up-regulated Vcam1 (Fig. 5C), an adhesion molecule expressed by mesenchymal stromal cells in lymphoid organs, which is essential for the recruitment of immune cells. In addition, CD34+ CSCs isolated from acutely inflamed colon significantly overexpressed, compared with CD34+ CSCs at steady state, transcripts coding for Ccl2 (MCP-1) and Csf1 (M-CSF); essential for the recruitment of monocytes and macrophages to inflamed tissues and for macrophage maintenance, respectively; the neutrophil chemotactic factors Cxcl1 and Cxcl2; the proinflammatory cytokine Il6; as well as Ccl19, Ccl3, Icam1, Trance (Rankl), and Ifngr1, all involved in the organization of adaptive immune responses; and the lymphangiogenic growth factors Vgf, Vegfd, and Flg2 (Fig. 5D). Finally, CD34+ CSCs up-regulated a number of growth factors for epithelial cells such as Ang (Amphiregulin), Fgfr, Fgfr10, as well as Ptg2, which modulates epithelial proliferation through effects on prostaglandin synthesis (31), and Colla1 (coding for type I collagen), suggesting a direct role in epithelial proliferation and tissue repair. Overexpression by CD34+ CSCs of most proinflammatory molecules was transient, peaking during the acute phase of inflammation, with a few notable exceptions including the T cells and DCs chemotactic factor Ccl19, and the lymphocyte survival factor Il7, which are key factors of adaptive immunity (Fig. 5D). Consistent with a role in adaptive immunity, CD34+ CSCs isolated from the colon supported T-cell survival in vitro (Fig. S6). Finally, a number of transcripts were overexpressed in CD34+ MyoFs compared with CD34+ CSCs, including, in addition to BMPs, transcripts coding for genes involved in vasculature growth and remodeling such as Angpt1, Angpt2, and Vegfa, as well as Wnt3a, a noncanonical Wnt ligand that plays an essential role in intestinal regeneration (32), suggesting that intestinal homeostasis and repair require the coordinated action of functionally distinct subsets of mesenchymal cells (a scheme is shown in Fig. S7).

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CSCs express high levels of Wnt2b, which is sufficient to compensate for the loss of Paneth cells-derived Wnt3 (7); Rspo1, which supports canonical Wnt signaling in crypts by binding to Lgr4/5 receptors (8–10) and synergizes with secreted Wnt proteins (8, 33); and Grem1, which favors Wnt signaling by antagonizing BMPs (12). These data are consistent with the previously reported expression of Wnt2b and Grem1 by intestinal mesenchyme (7, 11), and of an ill-defined intestinal mesenchyme supporting IESC growth in vitro (34). We further show that inhibition of Grem1 significantly reduces the generation of CD34+ CSC-induced spheroids, indicating that secretion of Grem1 by CD34+ CSCs plays a predominant role in maintenance of IESCs. Consistent with this hypothesis, forced expression of Grem1 in epithelial cells is sufficient to disrupt intestinal morphogenic gradients, promoting the persistence or reacquisition of stem cells properties by differentiated epithelial cells (35). The massive epithelial proliferation observed in CD34+ CSC-induced spheroids is therefore consistent with a global activation of Wnt signaling, normally restricted to the crypts (36). In the colon, which lacks Paneth cells, CD34+ CSCs production of Wnt2b, Rspo1, and Grem1 may therefore represent the major source of niche signals supporting IESCs.

We further show that CD34+ CSCs are topographically and functionally distinct from αSMA<sup>+</sup> subepithelial myofibroblasts, identified as gp38<sup>+</sup> αSMA<sup><sub>high</sub></sup> (CD34<sup>+</sup> MyoFs), which are located beneath epithelial cells in the ileum villi and colon tops. CD34<sup>+</sup> CSCs develop after birth, in contrast to CD34<sup>−</sup> MyoFs that are already present in fetal and neonatal intestine. Postnatal gp38<sup>+</sup> CD34<sup>+</sup> cells are first detected in the intestinal submucosa, then around the crypts starting at 3 wk after birth. Because they express high levels of Grem1, postnatal CD34<sup>+</sup> cells may play a role in intestinal crypt maturation during the first few weeks after birth, characterized by the rapid increase in crypt numbers (37) and Paneth cells maturation (38), two processes that require Wnt signaling. The increase in number and pericryptal localization of CD34<sup>+</sup> CSCs is independent of the microbial colonization of the gut, which massively increases at 3 wk of age in mice, suggesting that postnatal IESC mesenchymal niche is developmentally regulated. By occupying the pericryptal niche in the lamina propria, CD34<sup>+</sup> CSCs might prevent BMP-producing CD34<sup>−</sup> MyoFs from expanding into the crypts and disrupting homeostasis of stem cells. Thus, development and specific positioning of two distinct mesenchymal populations in the intestinal villi and crypts might be critical to maintain the pool of IESCs, and to promote directional differentiation of villous epithelial cells.

Nevertheless, whether CD34<sup>+</sup> MyoFs and CD34<sup>+</sup> CSCs have a common progenitor, or whether one population is generated from the other, remains to be investigated.

Intestinal damage induces a coordinated inflammatory response and tissue repair to regenerate the intestinal epithelial...
barrier and restore homeostasis. In lymphoid organs, specialized subsets of mesenchymal cells termed lymphoid stromal cells (mostly expressing gp38) are essential for leukocyte recruitment, cross-talk, and survival (19). Our results indicate that, during intestinal inflammation, CD34^+ CSCs acquire characteristics of lymphoid stromal cells, such as expression of Vcam1, Icam1, and LtbR, and the lymphocyte survival factor Il-7. The formation of clusters of LTβR^+ Vcam1^+ Icam1^+ lymphoid stromal cells initiates lymphoid tissue genesis, because these adhesion molecules are essential to establish cell-cell contact between stromal cells and lymphotoxin-expressing lymphocytes (39, 40). Consistent with their production of Il-7, we found that CD34^+ CSCs are sufficient to support T cells survival in vitro. In addition, CD34^+ CSCs up-regulate expression of several chemokines involved in leukocyte trafficking and immune regulation, including Ccl2, which has a pivotal role in recruiting monocytes and macrophages to inflamed tissues, and Csf1, essential for macrophage maintenance. Because macrophages are necessary for crypt regeneration after injury (31), it is possible that CD34^+ CSCs contribute to the maintenance and restoration of the IESC niche by several mechanisms, including secretion of niche factors at homeostasis and attraction of additional components of the IESC niche, such as macrophages, after injury. Expression by CD34^+ CSCs of PtgS2 after injury, necessary for regenerative responses after DSS-induced colitis (41), further supports this hypothesis.

Finally, given their central role in promoting proliferation of IESCs and inflammation, CD34^+ CSCs may play a role in the pathogenesis of intestinal diseases such as inflammatory bowel disease and colon cancer. Grem1 is overexpressed in carcinoma-associated fibroblasts of several human carcinomas, including colon carcinomas, and promotes tumor cell proliferation (42). Cocultures of intestinal organoids with CD34^+ CSCs may therefore provide a relevant model to decipher the stromal cross-talk that is dysregulated in cancer or inflammatory diseases and to develop novel therapies targeting stromal cells. Conversely, manipulation of the endogenous mesenchymal niche might be beneficial to improve stem cells engraftment and survival, opening new avenues for regenerative medicine.

Materials and Methods

Mice. C57Bl/6J wild-type (WT) mice were purchased from Charles River. Lgr5^+egfp mice (B6.129P2-Lgr5^tm6Trай1/Traj1), were purchased from Jackson Laboratories. Myd88^−/−;Trif^−/− mice were obtained from C. Werts, Institut Pasteur. All mice were kept in specific pathogen-free (SPF) conditions. Germ-free (GF) mice of the C57/Bl6J background (CDTA) were bred and maintained at the Institut Pasteur. To induce intestinal colitis, we treated 8- to 12-wk-old mice with 2.5% (wt/vol) dextran sodium sulfate (DSS) in the

Fig. 5. Intestinal inflammation induces remodeling and activation of CD34^+ CSCs. (A) Immunofluorescence analysis of gp38 (green) and CD34 (red) expression by intestinal stromal cells after acute or chronic DSS-induced colitis. Insets show bigger magnification of CD34^+ CSCs (arrow) or CD34^−MyoFs (arrowhead) in acute DSS. BV, blood vessels; C, crypts; L, lumen. (Scale bar: 50 μm.) (B) Percentage of CD34^+ and CD34^− cells among total gp38^+ stromal cells (gated CD45^−CD31^−) obtained by FACS at steady state, or after acute DSS and chronic DSS. (C) Mean fluorescence intensity (MFI) of Vcam1 expression by CD34^+ CSCs and CD34^− MyoFs in the indicated conditions. (D) Clustered gene expression analysis, as measured by qRT-PCR, for CD34^+ CSCs and CD34^− MyoFs in the indicated conditions. n = 4–6 for each condition from two to three independent experiments. DAPI stains nuclei. In B and C, values are mean ± SD. ****P < 0.0001, ***P < 0.001, **P < 0.005, *P < 0.05.
drinking water for 7 d (acute model) or with one cycle of 2% (wt/vol) DSS during 7 d, followed by 7 d of water, one cycle of 2% (wt/vol) DSS during 7 d, and 7 d of water (chronic model). All experiments were approved by the committee on animal experimentation of the Institut Pasteur and by the French Ministry of Agriculture.

Antibodies. The following monoclonal antibodies were purchased from eBioscience: V500-conjugated CD45.2 (104), eFluor 605-conjugated Ki-67 (Goa15), eFluor 605-conjugated CD31 (390), and eFluor 660-conjugated CD34 (RAM34). Cy3-conjugated α-SMA–specific antibody (1A4) was purchased from Sigma. Purified rabbit GFP-specific antibody (A11122), Alexa 555-conjugated WGA, and Alexa 647-conjugated anti-rat IgG (H+L) were purchased from Invitrogen. Cy3-conjugated and Alexa 647-conjugated anti-syrian hamster antibody were purchased from Jackson ImmunoResearch. Syrian hamster antibody to gp38 was a gift from A. Farr, University of Washington, Seattle.

Histology. Tissue processing and staining were performed as described (43). Briefly, the entire colon and ileum were washed in PBS, fixed overnight at 4 °C in 4% paraformaldehyde (Sigma-Aldrich), embedded in OCT compound (VWR Chemicals), and stored at −80 °C. Frozen blocks were cut at 8 μm thickness, and sections were collected onto Superfrost Plus slides (VWR International). Sections were processed for antibody staining: After blocking with 10% (vol/vol) bovine serum in PBS, slides were incubated with primary antibodies overnight at 4 °C, washed three times for 5 min, incubated with secondary antibodies for 1 h at room temperature, washed once, incubated with DAPI (Sigma) for 5 min, washed three times for 5 min, and mounted with Fluoromount-G (Southern Biotechnology Associates). We examined slides with an Axiolimger M1 or A2 (Apotome) fluorescence microscope (Zeiss) equipped with a CCD camera and processed images with AxioVision or Zen software (Zeiss).

Isolation of Intestinal Stromal Cells. To isolate intestinal stromal cells, we processed mouse ileum or colon as described (20). Briefly, after removal of Peyer’s patches (from the ileum), epithelial cells were removed by incubating gut fragments in calcium and magnesium-free DMEM (Gibco) containing 10 mM EDTA for 20 min at 37 °C with gentle agitation. Gut pieces were washed and incubated in DMEM (Gibco) containing Liberase TL (1 unit/ml), Rock Excell and DNase I (1 unit/ml; Invitrogen) at 37 °C. After 20 min, the digested fraction was collected and put on ice. This cycle was repeated two additional times for a total digestion time of 60 min. The remaining intestinal fragments were collected, filtered through a 100-μm mesh, and mixed with the collected supernatants.

Flow Cytometry. For FACS analysis, we first incubated cells with monoclonal antibody 2.4G2 to block Fcγ receptors, and then with indicated antibodies for 40 min in a total volume of 100 μL of PBS containing 2 mM EDTA and 2% (vol/vol) bovine serum, followed by appropriate secondary antibodies for 30 min when necessary. Cells were incubated for 1 min with DAPI (Sigma) before analysis to exclude dead cells. For intracellular staining of α-SMA and Ki-67, we fixed and permeabilized cells at room temperature (RT), then stained with antibodies in PBS-IF for 60 min at RT. Cells were analyzed with Fortessa (BD Biosciences) and FlowJo software (Tristat). Fluorescence intensity is expressed in arbitrary units on a logarithmic scale or on a linear scale for forward and side scatter. Cells were sorted with FACS Aria 3 (BD Biosciences) by using a single-cell sorting mask.

RNA Isolation and Quantitative RT-PCR. We performed RNA isolation and quantitative RT-PCR (qRT-PCR) as described (43). Briefly, we extracted total RNA from FACS-sorted cells by using RNasy Micro Kit (Qiagen) and assessed the quality of total RNA by using the 2100 Bioanalyzer system (Agilent Technologies). We used 250–500 pg of high-quality total RNA for one linear mRNA amplification cycle by using the MessageBooster kit for qRT-PCR (Epicentre Biotechnologies). Amplified mRNA (50–100 ng) was transcribed into cdNA by using SuperScript III reverse transcriptase (Invitrogen). All procedures were performed according to the manufacturers’ protocols. We performed qRT-PCR by using RT-qPCR primer sets (SABiosciences) and RT2 SYBR-Green master mix (SABiosciences), on a PTC-200 thermocycler equipped with a C1000 Touch thermal cycler (Bio-Rad Laboratories), and analyzed data by using Opticon Monitor software (Bio-Rad Laboratories). We normalized Ct values to the mean Ct values obtained for the housekeeping genes Hsp90ab1, Hprt, and Gapdh. Heat maps were generated by using Glocu software.

Stromal Cells and Organoid Coculture. We isolated intestinal crypts as described (27). Briefly, we isolated small intestines, flushed with cold PBS, opened longitudinally, and removed villi. We cut tissues into 1-cm pieces and incubated in PBS containing 10 mM EDTA on ice for 30 min. We collected crypts-containing supernatants and counted. We mixed a total of 250 crypts with 5 × 104 stromal cells isolated by FACS from mouse intestine and embedded in matrigel (Corning). After polymerization, we added crypt medium (CM) composed of DMEM/F12 supplemented with 10% (vol/vol) bovine serum, 100 U/mL penicillin/streptomycin, 10 mM Hepes, 1 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and 100 ng/mL R-spondin1 (R&D) to the medium. After 2 or 5 d, we counted intestinal organoids by using an inverted bright field microscope and imaged by using an Olympus IX81 with the CellSence software.

Transwell Assays. We isolated intestinal stromal cells by FACS and plated in complete medium at a concentration of 1 × 105 cells per well. When cells reached 50% confluency, we added a transwell filter containing intestinal crypts in Matrigel, as described above. After 5 d, we counted organoids by using an inverted bright field microscope and imaged by using an Olympus IX81 with the CellSence software.

Confocal Microscopy. For confocal microscopy analysis, we processed intestinal organoids as described (44). Organoids were sectionned with a HM650V vibratome (Thermo Fisher Scientific) and mounted with Prolong Gold Antifade reagent (Life Technologies). We acquired images by using a confocal microscope (sp5, Leica; or Cell Voyager CV1000, Yokogawa) and analyzed with ImageJ. We applied a 2-projection by using average intensity to the z stacks and binarized images by using the OTSU threshold.

T-Cell Survival. We plated intestinal stromal cells isolated by FACS 96-well plates in DMEM containing penicillin (50 U/mL), streptomycin (50 μg/mL), and 10% (vol/vol) FBS (Sigma). After 5 d, we added a 2 × 103 T cells purified by magnetic-activated cell sorting to stromal cells. Reconstitent IL-7 was used at 1 ng/mL in the absence of stromal cells. After 60 h of coculture, we collected lymphocytes to analyze living cells by FACS analysis.

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