The Ets transcription factor Spi-B is essential for the differentiation of intestinal microfold cells

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Intestinal microfold cells (M cells) are an enigmatic lineage of intestinal epithelial cells that initiate mucosal immune responses through the uptake and transcytosis of luminal antigens. The mechanisms of M-cell differentiation are poorly understood, as the rarity of these cells has hampered analysis. Exogenous administration of the cytokine RANKL can synchronously activate M-cell differentiation in mice. Here we show the Ets transcription factor Spi-B was induced early during M-cell differentiation. Absence of Spi-B silenced the expression of various M-cell markers and prevented the differentiation of M cells in mice. The activation of T cells via an oral route was substantially impaired in the intestine of Spi-B-deficient (SpiB−/−) mice. Our study demonstrates that commitment to the intestinal M-cell lineage requires Spi-B as a candidate master regulator.

The mucosal surface of the mammalian gut is continuously exposed to a variety of foreign proteins and microorganisms, some of which are potentially harmful to the host. To protect the host from these dangers, the intestinal mucosa has evolved specialized organized lymphoid tissues. Gut-associated lymphoid tissue (GALT), including Peyer’s patches (PPs) and isolated lymphoid follicles, is the inductive site for intestinal immunity. Different from other peripheral lymphoid tissues, GALT lacks afferent lymphatics and directly samples mucosal antigens across the epithelial barrier to initiate immune responses. This task is accomplished by specialized epithelial cells, known as ‘microfold cells’ (M cells), in the follicle-associated epithelium (FAE) covering the lymphoid follicles of GALT. M cells have a large capacity for phagocytosis and transcytosis; these functions allow the rapid transport of antigens to underlying lymphoid tissues, especially antigen-presenting cells. Antigens are then presented to T cells that support B cell activation, which ultimately results in the generation of plasma cells that produce immunoglobulin A. Thus, M cell–mediated transport of antigen is an important step in the initiation of mucosal immune responses¹–³.

M cells have characteristic morphological features that set them apart from other subsets of intestinal epithelial cells. They have shorter and irregular microvilli on their apical surface and a pocket-like basolateral invagination of the plasma membrane that houses lymphocytes and antigen-presenting cells³. These morphological features enable the recognition of M cells by transmission or scanning electron microscopy. Although the functional and morphological features of M cells were initially described nearly 40 years ago⁴,⁵, many basic questions about M-cell differentiation and function remain unsolved. Gene-expression profiling of M cells has been used as one approach to learn more about M cells, and several M cell–specific molecules have been identified, including GP2, Marcksl1, M-Sec, Sgne-1, annexin V and CCL9 (refs. 6–11). Among those, GP2 mediates the uptake of bacteria that express FimH, a component of type I pili on the bacterial outer membrane, to initiate subsequent bacteria-specific immune responses⁶.

M cells are a small subset of intestinal epithelial cells derived from intestinal epithelial stem cells¹²,¹³. Because of the restricted localization of M cells in the FAE, it has been postulated that the cells of the immune system in GALT are involved in M-cell differentiation. Among those, the contribution of B cells to M-cell differentiation has been demonstrated in vivo and in vitro¹⁴–¹⁶. In addition to the contribution of hemopoietic cells, mesenchymal cells in the subepithelial dome of PPs have an essential role in M-cell differentiation by producing RANKL, a cytokine of the tumor necrosis factor superfamily¹⁷. Indeed, RANKL-deficient mice have considerably fewer M cells, and exogenous administration of recombinant RANKL restores the number of M cells in these mice. Furthermore, treatment with RANKL can induce ectopic differentiation of cells of the villous epithelium (VE) into M cells in wild-type mice¹⁸.
Many transcription factors are involved in the cell-fate ‘decisions’ made as intestinal epithelial stem cells in the crypt differentiate into one of the recognized types of terminally differentiated intestinal epithelial cells. Math1 (Atoh1), which is repressed by the Notch effector Hes1, is essential for the commitment of epithelial progenitor cells into the secretory lineage, including goblet cells, Paneth cells, enteroendocrine cells and tuft cells\(^{19,20}\). Downstream of Math1, the specification of the individual secretory cell lineages requires at least one additional transcription factor: KLF4 is required for the maturation of goblet cells\(^{21}\); Sox9 is required for the maturation of Paneth cells\(^{22,23}\); and neurogenin 3 is required for the maturation of enteroendocrine cells\(^{24}\). By analogy, M-cell differentiation is hypothesized to require regulation by one (or more) distinct transcription factor(s), although its (or their) identity is yet to be determined.

In this study, we took advantage of the exogenous administration of recombinant RANKL as a means of synchronously inducing M-cell differentiation throughout the small intestinal epithelium\(^{18}\) to identify genes induced during the process of M-cell differentiation. We found substantially more transcripts encoding the Ets family transcription factor Spi-B at an early stage of RANKL-induced M-cell differentiation and these were expressed specifically by naturally occurring villous M-like cells. Of note, the kinetics of expression of these M-cell markers after treatment with RANKL were distinct; the expression of Marcksl1, CCL9 and GP2 peaked after 1, 2 and 3 d, respectively, both as mRNA (Fig. 1) and protein (Supplementary Fig. 1\(^{b}\)). Genes encoding all of these markers were upregulated in the VE of distal ileum after treatment with glutathione S-transferase–RANKL (Fig. 1\(^{a}\) and Supplementary Fig. 2\(^{b}\)), which suggested that RANKL-induced ectopic M cells in the villi more closely resembled FAE M cells than the rare, naturally occurring villous M-like cells. Of note, the kinetics of expression of these M-cell markers after treatment with RANKL were distinct; the expression of Marcksl1, CCL9 and GP2 peaked after 1, 2 and 3 d, respectively, both as mRNA (Fig. 1\(^{a}\)) and protein (Fig. 1\(^{b}\)). Furthermore, the localization of these M-cell markers moved from the crypt zones toward the tips of the villi from day 1 to day 3 after the treatment with RANKL, with Marcksl1 limited to the crypt-villus junction at day 1 and GP2 expression restricted to the upper part of villi on day 3 (Fig. 1\(^{b}\)). Given that the position of cells along the
The identification of M-cell lineage–specific transcription factors expressed early in M-cell differentiation is key in elucidating the molecular mechanisms of M-cell differentiation. Whole-genome expression profiling of mouse VE showed considerable upregulation of the gene encoding Spi-B shortly after treatment with RANKL (Fig. 2a). Real-time PCR analysis confirmed high expression of Spi-B mRNA in PP FAE but not in VE (Fig. 2b). In situ hybridization (ISH) also demonstrated the distribution of Spi-B mRNA after treatment with RANKL. We observed Spi-B mRNA in the crypt as early as 6 h after treatment. By day 1 after treatment, Spi-B+ cells were concentrated in the transit-amplifying cell compartment in the mid-crypt and migrated further up the crypt-villus axis at later time points (Fig. 2c). In the PPs, Spi-B mRNA was expressed in a subset of FAE cells that also bound UEA-1, a lectin that recognizes M cells (Fig. 2d).

We detected Spi-B protein in the nuclei of crypt cells at 18 h after treatment with RANKL and in the GP2+ M cells of PP FAE stained with DAPI. Dotted lines indicate FAE; arrows indicate Spi-B+GP2+ cells. Scale bars, 20 µm. (f) ISH of Spi-B mRNA in a PP follicle from an untreated mouse (top), and higher magnification of the area outlined at top after restaining of the section with UEA-1 (bottom three images): dotted lines indicate FAE; arrows indicate Spi-B+UEA-I+ cells; asterisks indicate higher magnification of the area outlined from an untreated mouse (top), and (RANKL (below). Scale bars, 50 µm. (e) Immunostaining (immunohistochemistry) of Spi-B (red) in the small intestine of a mouse 18 h after treatment with RANKL (top row) and dual staining (below) of Spi-B (red) and GP2 (green); all sections were counterstained with DAPI. Dotted lines indicate FAE; arrows indicate Spi-B+GP2+ cells. Scale bars, 20 µm. (f) ISH of Spi-B mRNA in a PP from a wild-type mouse at embryonic day 18.5 (top), and of Spi-B mRNA in other GALT, such as isolated lymphoid follicles (ILF), colonic patches and cecal patches, from a wild-type adult mouse (bottom three images). Scale bars, 50 µm. Data are representative of three independent experiments (error bars a,b, s.d.).

Figure 2 ‘Preferential’ expression of transcripts encoding Spi-B in mouse M cells. (a) Quantitative PCR analysis of the kinetics of Spi-B expression after treatment of mice with RANKL (presented as in Fig. 1a). (b) Quantitative PCR analysis of Spi-B mRNA expression in VE and FAE from untreated mice (n = 3); results were normalized to Gapdh expression and are presented relative to expression in VE cells from untreated mice. (c) ISH analysis of Spi-B mRNA in the small intestines of an untreated mouse (Day 0; top) and mice at various times (left margin) after treatment with RANKL (below). Scale bars, 50 µm. (d) ISH of Spi-B mRNA in a PP follicle from an untreated mouse (top), and higher magnification of the area outlined at top after restaining of the section with UEA-1 (bottom three images): dotted lines indicate FAE; arrows indicate Spi-B+UEA-I+ cells; asterisks indicate higher magnification of the area outlined from an untreated mouse (top), and (RANKL (below). Scale bars, 50 µm. (e) Immunostaining (immunohistochemistry) of Spi-B (red) in the small intestine of a mouse 18 h after treatment with RANKL (top row) and dual staining (below) of Spi-B (red) and GP2 (green); all sections were counterstained with DAPI. Dotted lines indicate FAE; arrows indicate Spi-B+GP2+ cells. Scale bars, 20 µm. (f) ISH of Spi-B mRNA in a PP from a wild-type mouse at embryonic day 18.5 (top), and of Spi-B mRNA in other GALT, such as isolated lymphoid follicles (ILF), colonic patches and cecal patches, from a wild-type adult mouse (bottom three images). Scale bars, 50 µm. Data are representative of three independent experiments (error bars a,b, s.d.).

preferential' expression of Spi-B by intestinal M cells

The identification of M-cell lineage–specific transcription factors expressed early in M-cell differentiation is key in elucidating the molecular mechanisms of M-cell differentiation. Whole-genome expression profiling of mouse VE showed considerable upregulation of the gene encoding Spi-B shortly after treatment with RANKL (Fig. 2a). Real-time PCR analysis confirmed high expression of Spi-B mRNA in PP FAE but not in VE (Fig. 2b). In situ hybridization (ISH) also demonstrated the distribution of Spi-B mRNA after treatment with RANKL. We observed Spi-B mRNA in the crypt as early as 6 h after treatment. By day 1 after treatment, Spi-B+ cells were concentrated in the transit-amplifying cell compartment in the mid-crypt and migrated further up the crypt-villus axis at later time points (Fig. 2c). In the PPs, Spi-B mRNA was expressed in a subset of FAE cells that also bound UEA-1, a lectin that recognizes M cells (Fig. 2d).

We detected Spi-B protein in the nuclei of crypt cells at 18 h after treatment with RANKL and in the GP2+ M cells of PP FAE from untreated mice (Fig. 2e). In addition, we observed Spi-B mRNA in a subset of cells in the PP FAE of mouse embryos at embryonic day 18.5 (Fig. 2f). The parallel upregulation of the gene encoding Spi-B during both natural M-cell development in the PP during ontogeny and after RANKL-induced M-cell differentiation in the VE suggested a pivotal role for Spi-B in the induction of intestinal M-cell differentiation. We also examined Spi-B expression in various GALT in addition to PPs, such as isolated lymphoid follicles, colonic patches and cecal patches, and found that M cells in these tissues also expressed Spi-B mRNA (Fig. 2f). To assess the possibility that human M cells also express Spi-B, we examined the expression of Spi-B in human PP by ISH and found that Spi-B mRNA was ‘preferentially’ expressed in human M cells (Supplementary Fig. 4).

To determine if Spi-B is required for normal M-cell differentiation, we examined Spi-B-deficient (Spib−/−) mice. Whole-mount staining

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and immunohistochemistry of PPs demonstrated that GP2+ or CCL9+ M cells were completely absent from the PP FAE of Spib−/− mice (Fig. 3a,b). Real-time PCR analysis also showed a nearly complete loss of transcripts for GP2, CCL9, M-Sec and Sgne-1 in these mice (Fig. 3c and Supplementary Fig. 5). We also observed loss of the expression of these M-cell markers in Spib−/− mice during RANKL-induced M-cell differentiation (Fig. 4 and Supplementary Fig. 6). In contrast, the expression of mRNA for Marcksl1 and annexin V in both natural PP M cells and RANKL-induced M cells from Spib−/− mice was maintained at an amount equivalent to that in cells from wild-type mice (Figs. 3c and 4b and Supplementary Figs. 5 and 6).

On the basis of these observations, we propose that the expression of GP2, CCL9, M-Sec and Sgne-1 in M cells is dependent on Spi-B, whereas the expression of Marcksl1 and annexin V is independent of Spi-B. Of note, we found cells expressing Marcksl1 and annexin V (data not shown) near the apex of PP FAE from Spib−/− mice, which indicated that Spi-B was required for the expression of late markers of M-cell differentiation but was dispensable for the survival of the Marcksl1+ annexin V–positive cells, which allowed them to migrate toward the top of the FAE before their death.

**Spi-B is critical for M-cell differentiation**

In the PP FAE of Spib−/− mice, cells expressing only Marcksl1 and annexin V were present but lacked other M-cell markers that are normally acquired at later stages of differentiation. As GP2 is a functional antigen-uptake receptor on M cells6 and is acquired at a late stage of M-cell differentiation, this molecule is considered a marker for terminally differentiated, fully functional M cells. We therefore hypothesized that the residual Marcksl1+ annexin V–positive cells in the PP FAE of Spib−/− mice were not functionally mature M cells. To explore that possibility, we examined transcytotic activity, a critical function of M cells that enables the rapid delivery of particulate luminal antigens across the epithelial barrier. We used oral administration of fluorescent nanoparticles, an established assay of M-cell transcytosis10, to Spib−/− and wild-type mice. In wild-type mice, particles were abundant in the subepithelial dome area of PPs, whereas very few particles were taken up into the PPs of Spib−/− mice (Fig. 5a, b).

We further investigated the loss of M-cell function in the PPs of Spib−/− mice by examining the translocation of orally administered bacteria. There was much less uptake of *Salmonella enterica* serovar Typhimurium (S. Typhimurium) in PPs of Spib−/− mice than in the PPs of wild-type mice. This result was consistent with the absence of GP2+ M cells in Spib−/− mice because the uptake of S. Typhimurium is considerably impaired in GP2-deficient mice6. In addition, PPs from Spib−/− mice had a significantly smaller bacterial load of *Yersinia enterocolitica* after oral administration of these bacteria (P = 0.0003; Supplementary Fig. 7), which suggested that GP2-dependent bacterial uptake was also impaired in the PPs of Spib−/− mice. Thus, the residual Marcksl1+ annexin V–positive cells in Spib−/− mice were defective in their ability to take up luminal antigens.

Consistent with the absence of transcytotic ability of the residual Marcksl1+ annexin V–positive cells in Spib−/− mice, analysis of fluorescent nanoparticles in the PPs of Spib−/− mice further indicated that GP2 expression was reduced in these mice (Fig. 6).

**Transcytosis is dependent on Spi-B in M cells**

We further examined transcytotic activity in M cells from wild-type and Spib−/− mice in vitro (Fig. 7a). In wild-type M cells, fluorescent nanoparticles were internalized and transcytosed within 30 min, whereas no internalization of these particles was observed in M cells from Spib−/− mice. This result suggests that the transcytotic ability of M cells is dependent on Spi-B.
of the ultrastructure of the FAE showed an absence of cells with typical M-cell morphology. PP M cells have unique morphological characteristics, including irregular and sparse microvilli and a pocket-like invagination of the basolateral plasma membrane4,5.

Scanning and transmission electron microscopy showed that the FAE of Spib−/− mice completely lacked cells with these morphological features (Fig. 5c,d). In contrast, we observed no obvious defect in the development of goblet cells, Paneth cells or enteroendocrine cells in Spib−/− mice (Supplementary Fig. 8), which indicated that Spi-B was not involved in the differentiation of these secretory intestinal epithelial cell lineages. On the basis of these observations, we propose that Spi-B has a crucial role in the differentiation of morphologically and functionally mature M cells and that its role in cellular differentiation is specific to M cells among the known types of intestinal epithelial cells.

M-cell differentiation requires intrinsic Spi-B

In the hematopoietic system, Spi-B is known to be involved in the function and development of B cells31. In addition, it has been reported that B cells contribute to M-cell differentiation14–16. On the basis of those studies, the possibility that absence of Spi-B in these cells of the immune system, but not in M cells themselves, was responsible for the observed defects in M-cell differentiation could not be excluded. To address this issue, we prepared reciprocal bone marrow chimeras with Spib−/− and wild-type mice and characterized the differentiation of M cells in the PP FAE. The transfer of wild-type bone marrow cells into irradiated Spib−/− mice failed to restore the expression of M-cell markers and the establishment of normal M-cell morphology in the FAE, whereas the transfer of Spib−/− bone marrow cells into wild-type mice did not interfere with normal M-cell differentiation in the PPs of wild-type recipient mice (Fig. 6). Together these data indicate that M-cell–intrinsic expression of Spi-B was critical for the expression of M-cell–specific genes encoding CCL9, GP2, M-Sec and Sgne-1 as well as the formation of M cells with typical morphological features.

Impaired mucosal immune response in Spib−/− mice

The defect in functionally mature M cells in Spib−/− mice raised the possibility that Spi-B is essential for the induction of adaptive immune responses initiated by the delivery of antigen by M cells in the mucosal immune system. As the expression of Spi-B in cells of the immune system25,26 could affect the immune responses of Spi-B–deficient mice, we examined the activation of antigen–specific T cells in PPs after oral infection of mice with S. Typhimurium in a model of the adoptive transfer of SM1 T cells, which have transgenic expression of a T cell antigen receptor specific for S. Typhimurium flagellin32,33. This is a well-established model for the analysis of antigen–specific immune response in PPs, and the biological importance of M cells in efficient mucosal immune responses after oral challenge with S. Typhimurium has been shown6. We transferred SM1 T cells into Spib−/− or wild-type mice and left the mice uninfected or orally infected them with S. Typhimurium, then collected PPs for the analysis of S. Typhimurium–specific T cell activation. We detected SM1 T cells in the PPs of

Figure 5 Disappearance of functional M cells from Spib−/− mice. (a) Microscopy of PPs from wild-type and Spib−/− mice given oral administration of green fluorescent nanoparticles (200 nm in diameter), followed by isolation of the distal three PPs 3 h later for staining with anti-GP2 (red) and counterstaining with DAPI (blue). Dotted lines indicate FAE. Scale bars, 10 µm. (b) Quantification of nanoparticles in mice (n = 9 per genotype) treated as in a (20 serial sections per PP). Each symbol represents an individual mouse; small horizontal lines indicate the mean. *P = 0.0269 (Student’s t-test). (c) Scanning electron microscopy of PPs from wild-type and Spib−/− mice (left), and higher magnification (right) of the areas outlined at left. Scale bars, 20 µm. (d) Transmission electron microscopy of PPs from wild-type and Spib−/− mice. Scale bars, 5 µm. Arrows (c,d) indicate typical M cells, with sparse and irregular microvilli and basolateral pocket-like structure. Data are representative of two independent experiments.

Figure 6 Maturation of M cells in bone marrow chimeras. (a) Microscopy of PPs obtained from irradiated Spib−/− (CD45.2+) mice 8 weeks after transfer of bone marrow cells from wild-type (CD45.1+) mice (WT→Spib−/−) or from irradiated wild-type mice 8 weeks after transfer of bone marrow cells from Spib−/− mice (Spib−/−→WT), followed by immunostaining of the M-cell markers GP2, CCL9 and Marcksl1. Scale bars, 50 µm. (b) Scanning electron microscopy of PPs from bone marrow chimeras as in a (top), and higher magnification (bottom) of the areas outlined above. Scale bars, 20 µm (top) and 6 µm (bottom). Data are representative of two independent experiments.
 Spi-B. SM1 T cells in the PPs of uninfected wild-type mice had the transferred SM1 T cells migrated normally into PPs in the absence of cells at left (right). (Numbers adjacent to outlined areas indicate percent CD90.1 + SM1 cells expression of CD69 and CD4 (right) on cells gated (outlined area) at left. By no infection (transfer only) or oral inoculation with naive phenotype of low surface expression of the activation marker Spi-B. SM1 T cells in the PPs of uninfected wild-type mice had the transferred SM1 T cells migrated normally into PPs in the absence of cells at left (right). (Numbers adjacent to outlined areas indicate percent CD90.1 + SM1 cells expression of CD69 and CD4 (right) on cells gated (outlined area) at left. By no infection (transfer only) or oral inoculation with

**Figure 7** Defect in S. Typhimurium-specific activation of T cells in Spib−/− mice. (a) Expression of CD4 and CD90.1 (left) on PP cells from wild-type or Spib−/− mice given adoptive transfer of SM1 T cells, followed by no infection (transfer only) or oral inoculation with S. Typhimurium at 24 h after transfer (infected), assessed 24 h after infection, and expression of CD69 and CD4 (right) on cells gated (outlined area) at left. Numbers adjacent to outlined areas indicate percent CD90.1 + SM1 cells among total cells (left) or activated CD69+ cells among the gated SM1 cells at left (right). (b) Frequency of CD69+ SM1 T cells in PPs from mice (n = 4) treated as in a. *P < 0.01 (one-way analysis of variance followed by Bonferroni post-hoc test). Data are representative of (a) or from (b) two independent experiments (error bars (b), s.d.).

uninfected mice of both genotypes (Fig. 7a), which indicated that the transferred SM1 T cells migrated normally into PPs in the absence of Spi-B. SM1 T cells in the PPs of uninfected wild-type mice had the naïve phenotype of low surface expression of the activation marker CD69, which increased considerably 24 h after oral infection with S. Typhimurium. In contrast, the induction of CD69 on SM1 T cells after infection was significantly impaired in Spib−/− mice (Fig. 7).

The defect in T cell activation after oral antigen challenge was probably not a result of impaired antigen presentation due to the absence of Spi-B in hematopoietic cells, as we observed equivalent systemic activation of T cells directed against S. Typhimurium administered intraperitoneally in Spib−/− or wild-type mice given transfer of SM1 T cells (Supplementary Fig. 9). To further exclude the possibility of effects attributable to the absence of Spi-B in hematopoietic cells, we prepared bone marrow chimeras by transferring wild-type bone marrow into wild-type and Spib−/− recipients. After reconstitution, wild-type and Spib−/− bone marrow chimeras received SM1 T cells labeled with the cytosolic dye CFSE, and we subsequently infected them orally with S. Typhimurium. At 3 d after infection, we analyzed the proliferation of SM1 T cells in the bone marrow chimeras. At this time point, most SM1 T cells in wild-type recipients of wild-type marrow had undergone one (or more) division(s). In contrast, SM1 T cells in Spib−/− recipients of wild-type marrow showed a lower proliferative response (Supplementary Fig. 10). Together these results confirmed that the M cell-intrinsic expression of Spi-B was critical for the differentiation of M cells required for the host to initiate an efficient antigen-specific mucosal immune response.

**DISCUSSION**

Here we have reported that that Spi-B is a RANKL-induced transcription factor essential for the differentiation of intestinal M cells. The identification of Spi-B as a candidate ‘master regulator’ of M-cell differentiation resolves a longstanding question about the genesis of M cells and demonstrates a previously unknown and unanticipated function for Spi-B. Furthermore, the lack of M cells in Spi-B-deficient mice also provides a unique tool for elucidating the physiological and pathological functions of this enigmatic type of epithelial cell.

Spi-B is a member of the Ets family of transcription factors. Spi-B is reported to have a role in signaling via the B cell antigen receptor, antibody responses and germinal-center formation, as well as B cell development. In addition, Spi-B is required for development of human plasmacytoid dendritic cells. In this study, we found high expression of Spi-B in both RANKL-induced M cells and PP FAE M cells. This ‘preferential’ expression of Spi-B in intestinal M cells is the first demonstration, to our knowledge, of Spi-B expression in nonhematopoietic cells. Furthermore, we found that Spib−/− mice completely lacked M cells in PPs, which provides evidence that M-cell differentiation is regulated by a specific transcription factor, as has been shown for most other intestinal epithelial cell lineages. In addition, Spi-B expression in M cells was conserved in all mouse GALT, as well as M cells in human PPs, which suggests an essential role for Spi-B in M-cell differentiation.

Many transcription factors of the Ets family other than Spi-B are expressed in epithelial cells of the small intestine. However, identifying the function of individual Ets transcription factors in these cells has been challenging, in part because of the potential for compensatory activity by other Ets factors coexpressed in the absence of a single Ets transcription factor. Although nearly two-thirds of all Ets transcription factors have been inactivated genetically in mice, the absence of just two of these factors due to genetic inactivation has been reported to perturb intestinal epithelial cell differentiation. Mice with homozygous deficiency in the transcription factor Elf3 show abnormal morphogenesis of villi and terminal differentiation of enterocytes and goblet cells. In contrast, homozygous deletion of the gene encoding the transcription factor Spdef impairs the full maturation of both goblet cells and Paneth cells. The indispensable role of Spi-B in M-cell differentiation indicates that all other Ets transcription factors expressed in M cells are unable to replace Spi-B in orchestrating M-cell differentiation.

The relationship between the expression pattern of M-cell markers and the lack of M cells in Spib−/− mice has greatly improved the understanding of M-cell differentiation. The FAE in Spib−/− mice lacked several M cell–associated markers, including GP2, CCL9, M-Sec and Sgne-1, which indicated that induction of the genes encoding these in M cells required Spi-B expression. GP2 is thought to be a marker of terminally differentiated, functional M cells because of its role in the uptake of pathogenic and commensal bacteria. In contrast to the dependence on Spi-B for the expression of most M cell–associated markers and full functional maturation of M cells, we found that the expression of Marksl1 and annexin V was not lower in Spib−/− cells from either the PP FAE or the VE after treatment with RANKL. These Marksl1+ annexin V–positive cells in Spib−/− mice lacked not only GP2 expression but also the ability to take up macromolecules, as well as typical M-cell morphologies such as irregular microvilli and a pocket-like basolateral invagination. These observations suggested that Spi-B is essential for the morphological and

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functional maturation of M cells and that genes expressed under the transcriptional control of Spi-B are involved in the maturation and/or function of M cells. CCL9 is a chemokine involved in the recruitment of CD11b+ dendritic cells into the subepithelial dome region in PPs. Its expression preceded GP2 expression during both RANKL-induced and FAE M-cell differentiation. In addition, we found that Spi-B directly bound the promoter region of the gene encoding CCL9 in vitro (data not shown), which raised the possibility of a substantial role for CCL9 in M-cell maturation. CD11b+ dendritic cells attracted to the subepithelial dome by CCL9 may provide signals that contribute to the terminal differentiation of M cells. M-Sec was also dependent on Spi-B expression during the RANKL-induced differentiation of M cells. M-Sec has been shown to be involved in the formation of tunneling nanotubes, which indicates that the appearance of the tunneling nanotube structures observed in M cells is also a feature of fully mature M cells.

Marsck1 was one of the earliest M-cell markers found to be expressed mainly in response to treatment with RANKL; it was expressed mainly at the crypt-villus junction but not by fully differentiated villous enterocytes shortly after such treatment. This suggests that the cells in the crypt, most probably undifferentiated transit-amplifying cells, can respond to the activation of RANKL signaling through RANK with the induction of Marsck1. Notably, the frequency of Marsck1+ annexin V–positive cells in the epithelium decreased at later time points after the onset of treatment with RANKL, and the remaining Marsck1+ annexin V–positive cells began to coexpress M-cell markers that appear later, such as CCL9 and GP2. Given that Spi-B is essential for the expression of CCL9, GP2, M-Sec and Sgne-1 in M cells, we hypothesize that only the cells that achieve successful induction of Spi-B by RANKL fully commit to M-cell differentiation, which allows them to maintain expression of Marsck1 and annexin V, progress through M-cell differentiation and express markers of later stages characteristic of mature M cells. In contrast, those cells that fail to express Spi-B do not sustain the expression of Marsck1 and annexin V and instead eventually commit to differentiation into other types of mature enterocytes. Lateral inhibition by committed M cells may be one mechanism that contributes to the suppression of the expression of Marsck1 and annexin V by the surrounding cells, as observed in cells of the secretory lineage. That is in agreement with the observation that in Spi-B−/− mice, residual Marsck1+ annexin V–positive cells reached the apex of the FAE, as well as the villus tips, after treatment with RANKL. The Spi-B-independent expression of Marsck1 and annexin V in the initial phase of RANKL-induced M-cell differentiation probably indicates that these proteins may be markers of RANKL-responsive intestinal epithelial cells.

In summary, our study has demonstrated that Spi-B is an essential transcription factor that regulates M-cell differentiation. Our investigation of M-cell differentiation in Spi-B−/− mice showed tight linkage between the acquisition of Spi-B–dependent expression of M-cell markers and the induction of fully functional M cells. In addition, we observed much less activation of antigen-specific T cells in Spi-B−/− mice, which confirmed the importance of M cells in the induction of adaptive immune responses in the intestinal GALT. Spi-B-deficient mice serve as a unique tool for elucidating the physiological and pathological functions of M cells.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE37861.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

I.R.W., K.Ha. and H.O. conceived of the study; T.Kan. designed and did the experiments, analyzed data and wrote the manuscript; K.Ha. contributed to adoptive-transfer experiments and data analysis; D.T. and Y.K. helped with flow cytometry; S.F. and T.J. helped with experiments involving infection with S. Typhimurium; K.N. and A.S. did expression analyses; K.Ho., I.S., H.H. and T.Kai. generated Spi-B−/− mice; K.A.K., N.K. and I.R.W. developed the protocol for treatment with RANKL; M.S. and K.T. helped with electron microscopy; O.Y. and T.Kai. provided human PP samples; S.I.M. provided SM1 mice; K.Ha. and I.R.W. edited the manuscript; and H.O. directed the research and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Animals.** BALB/c mice, C57BL/6J (CLEA) mice, Spib−/− mice on the C57BL/6J background (I.S., T.Kai, et al., data not shown), CD45.1+ congenic mice on the C57BL/6 background and recombination-activating gene 2–deficient SM1 C57BL/6 mice expressing CD90.1 (refs. 32,33) were used for experiments. All mice were maintained under specific pathogen-free conditions at the Research Center for Allergy and Immunology Animal Facility. All animal experiments were approved by the Animal Research Committees of RIKEN Yokohama Research Institute.

**RANKL injection.** The glutathione S-transferase–RANKL fusion protein was prepared as described18 and was administered intraperitoneally to mice twice a day for up to 4 d.

**Isolation of epithelium.** For the preparation of VE, small pieces of the ileum 35 cm from the pylorus were dissected from mice. For FAE, the epithelial layer was peeled off from lamina propria of mouse small intestines containing PPs, and the FAE regions were isolated from surrounding VE. Detailed procedures for the isolation of the epithelium have been published4.

**Histology.** For immunohistochemistry, small intestinal samples including PPs were fixed with zinc formalin fixative (Polysciences) and were embedded in paraffin. De-paraffinized sections were incubated overnight at 4 °C with primary antibodies (Supplementary Table 1). Specific binding of primary antibodies was followed with biotinylated secondary antibodies (Supplementary Table 1) and streptavidin–horseradish peroxidase (ABC Elite; Vector Laboratories) and was visualized with 3,3-diaminobenzidine (DakoCytomation).

**Electron microscopy.** PPs were fixed with 4% paraformaldehyde and 2% glutaraldehyde buffered with 100 mM sodium cacodylate (pH 7.4) and post-fixed with 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7.4). For scanning electron microscopy, samples were dehydrated and substituted with t-butyl alcohol. After being freeze dried, samples were coated with platinum and examined with a scanning microscope (JEOL JSM-6360LV; Jeol). For transmission electron microscopy, dehydrated samples were embedded in Epon812 resin (Taab). Ultrathin sections were stained with 4% uranyl acetate and then with lead citrate and were examined with a transmission electron microscope (JEM-1011; Jeol, SU1510; Hitachi).

**Whole-mount staining.** For whole-mount staining, mouse PPs were fixed with a Cytofix/Cytoperm kit (BD Biosciences). PP specimens were stained with monoclonal antibody to GP2 (2F11-3; produced in-house) and Alexa Fluor 488–conjugated antibody to rat immunoglobulin G (A-21208; Invitrogen). For counterstaining, F-actin was stained with Alexa Fluor 647–conjugated phallolidin (Invitrogen). Stained specimens were examined by confocal laser microscope. Projection images of whole-mount staining were edited with ImageJ v1.36b software (US National Institutes of Health).

**In vivo assessment of the uptake of fluorescent beads by M-cells.** Mice were given green fluorescent polylysine latex nanoparticles 200 nm in diameter (Fluoresbrite YG; Polysciences) by oral gavage (2 × 1011 particles in 200 µl PBS). After 3 h, PPs were isolated from mice and embedded in optimal cutting temperature compound. Frozen sections were incubated with monoclonal antibody to GP2 as described18 and then with DyLight 549–conjugated antibody to rat immunoglobulin G (712-506-150; Jackson ImmunoResearch). Incorporated fluorescent beads were quantified as described.

**Generation of bone marrow chimeras.** Bone marrow cells isolated from femora of wild-type (CD45.1+ ) mice and Spib−/− (CD45.2+ ) mice were injected intravenously (1 × 107 cells per mouse) into Spib−/− and wild-type mice, respectively. Before injection, recipient mice were irradiated with 8 Gy of gamma rays. Then, 8–10 weeks later, recipient mice were killed. For the evaluation of chimerism, PPs were immunostained with antibody to CD45.1 (A20; BioLegend) or antibody to CD45.2 (104; BioLegend).

**Adoptive transfer of SM1 T cells.** Cells from the spleen and mesenteric lymph nodes were collected from SM1 mice. Cell suspensions (3 × 105 to 4 × 106 per 104; BioLegend). SM1 T cells were stained with antibody to CD45.1 (17-2F1; BioLegend) or antibody to CD45.2 (104; BioLegend) before transfer.

**Bacterial infection.** S. Typhimurium carrying a gene encoding resistance to nalidixic acid (strain χ3306) was a gift from H. Matsui,43 Spib−/− and wild-type mice given transfer of SM1 T cells were inoculated orally with S. Typhimurium strain χ3306 (5 × 107 colony–forming units) 24 h after adoptive transfer. Then, 24 h later, PPs were collected for analysis. In some experiments, additional groups of mice were injected intraperitoneally with S. Typhimurium strain χ3306 (1 × 107 colony–forming units) and spleens were collected 24 h later.

**Assay of bacteria after oral infection.** Spib−/− and wild-type mice were fed 5 × 107 colony–forming units of S. Typhimurium strain χ3306 or 1 × 108 colony–forming units of Y. enterocolitica strain WA (27729; American Type Culture Collection)44. After 24 h, PPs were dissected and incubated for 30 min at 20 °C with gentle shaking in sterile PBS containing 500 µg/ml gentamicin. Pooled PP tissue was weighed and homogenized in sterile PBS. The homogenates were serially diluted in sterile PBS and plated on a Luria-Bertani agar plate containing 25 µg/ml nalidixic acid (for S. Typhimurium) or a Yersinia–selective agar plate (Oxoid) for quantification of colony–forming units.
Preparation of PP lymphocytes. PP lymphocytes were prepared as described.

Flow cytometry. PP lymphocytes were preincubated with monoclonal antibody to CD16-CD32 (93; eBioscience) in 2% FBS-PBS for blockade of the receptor FcγR and were stained with monoclonal antibodies to various differentiation cluster (DC) antigens (Supplementary Table 1). After being stained, cells were analyzed with a FACSCanto II (BD Biosciences). Data were analyzed with FlowJo software (TreeStar).

Statistical analysis. Student’s t test, the Mann-Whitney U-test and one-way analysis of variance followed by the Bonferroni post-hoc test were used. P values of less than 0.05 were considered statistically significant.

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