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Transcription factor Spi-B–dependent and –independent pathways for the development of Peyer's patch M cells

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Although many of the biological features of microfold cells (M cells) have been known for many years, the molecular mechanisms of M-cell development and antigen recognition have remained unclear. Here, we report that *Umod* is a novel M-cell–specific gene, the translation products of which might contribute to the uptake function of M cells. Transcription factor Spi-B was also specifically expressed in M cells among non-hematopoietic lineages. Spi-B–deficient mice showed reduced expression of most, but not all, other M-cell–specific genes and M-cell surface markers. Whereas uptake of *Salmonella* Typhimurium via M cells was obviously reduced in Spi-B–deficient mice, the abundance of intratissue cohabiting bacteria was comparable between wild-type and Spi-B–deficient mice. These data indicate that there is a small M-cell population with developmental regulation that is Spi-B independent; however, Spi-B is probably a candidate master regulator of M-cell functional maturation and development by another pathway.

INTRODUCTION

Mucosa-associated lymphoid tissues, represented in the small intestine by Peyer's patches (PPs), are the inductive sites of the mucosal immune system.[1] Unlike other peripheral lymphoid tissues, mucosa-associated lymphoid tissues do not have afferent vessels; instead, they have a direct antigen-sampling system that takes antigens into the tissue from the mucosal surface. This antigen uptake is managed mainly by microfold cells (M cells), which are located within the follicle-associated epithelium (FAE) of mucosa-associated lymphoid tissues and are regarded as professional antigen-sampling cells.[1] M cells have unique morphological features, such as relatively short, irregular microvilli on their apical surfaces and a pocket structure on their basolateral side that enfolds lymphocytes and antigen-presenting cells. These distinctive features are considered to contribute to M-cell functions: the short microvilli enable easy contact with luminal antigens, and the ability to hold antigen-presenting cells, such as dendritic cells, in their pockets is valuable for the prompt transfer of antigens to dendritic cells for speedy initiation of antigen-specific mucosal immunity. Because M cells are principal targets for effective peroral vaccination, elucidation of the molecular mechanisms behind their antigen-uptake functions and differentiation is important for the development of new-era oral vaccines.

We have reported that glycoprotein 2 (GP2) is specifically expressed on M cells,[2] and a progressive study has demonstrated that GP2 acts as a binding receptor for FimH-expressing bacteria, inducing effective uptake of, and specific immune responses to, such bacteria.[3] Although several other M-cell–specific molecules, such as MARCKS-like protein,[2] peptidoglycan recognition protein S,[4] and fucosyltransferase 1 (FUT1),[5] have been reported, the functions of these molecules in contributing to antigen uptake have not yet been revealed. In addition, the mechanisms of M-cell differentiation are poorly understood.

Recently, it has been reported that the intestinal epithelial crypt–villus structure can be assembled from a single Lgr5-positive stem cell in the absence of a non-epithelial-cell niche.[6] It has also been reported that almost all small intestinal epithelial cell (EC) lineages develop from intestinal epithelial

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stem cells. First, the expression of Math1 transcription factor determines the destiny of stem cells for the secretory lineage.[7] Further differentiation of secretory lineage cells, such as goblet cells, Paneth cells, and endocrine cells, requires other factors such as Klf4, Sox9, and Ngn3, respectively.[8–11] On the other hand, if the stem cells express Hes1, which inhibits Math1 expression, these cells adopt a non-secretory lineage and become, for example, enterocytes.[12] Because M cells belong to a non-secretory lineage, at least one unknown factor might be expressed and activated downstream of Hes1 in precursor cells for differentiation into M cells.

Here, we report the existence of two additional M-cell–specific genes among mouse intestinal ECs, as determined from the data obtained in our previously reported DNA gene chip analysis.[2] One is *Umod*, which encodes the cell-surface expression molecule Tamm–Horsfall protein (THP). The other one, *Spib*, encodes Spi-B, which is a transcription factor belonging to the Ets family. In focusing on the function of Spi-B, our investigations revealed that M-cell–intrinsic Spi-B controls the expression of other, but not all, M-cell–specific genes. In addition, Spi-B regulates the ultrastructural development and GP2-mediated antigen-uptake functions of most PP M cells, whereas a translocation of the majority of *Alcaligenes* spp., which are commensal bacteria resident in PPs and isolated lymphoid follicles preferentially through M cells, was observed independently of Spi-B. We propose that M-cell–intrinsic Spi-B is an essential transcription factor for the functional and structural differentiation of M cells, although a small population of M cells may mature independently of Spi-B. These M cells, which were found within the FAE of Spi-B–deficient mice, might still be present in sufficient numbers to contribute to the migration of mucosa-associated lymphoid tissue-resident bacteria.

RESULTS

Spib and *Umod* are expressed in M cells but not other intestinal ECs

We previously reported the discovery of a series of PP M-cell–specific genes by DNA gene chip analysis.[2] To search for other M-cell–specific molecules—especially a key transcription factor for M-cell development or function—we used real-time polymerase chain reaction (PCR) to screen candidate genes from gene chip analysis data. Whole ECs and lymphocytes were prepared from well-trimmed duodenal PPs as "PP ECs" and "PP lymphocytes," respectively. PP M cells and non-M cells were then purified from PP ECs as NKM 16-2-4 antibody (NKM)/*Ulex europaeus* agglutinin 1 (UEA-1) double-positive and double-negative cells, respectively.[13] Among several candidates, we confirmed that *Spib* and *Umod* were preferentially expressed in PP M cells (**Figure 1a**). We next performed an *in situ* hybridization analysis of *Spib* and *Umod* expressions in PPs to confirm the real-time PCR data. An antisense cRNA probe for *Spib* specifically stained part of the FAE region of the PP among the intestinal ECs (**Figure 1b**) and (as was expect from the previous studies[14,15]) in the lymphocyte region of the PPs, especially in the germinal centers (**Figure 1b,c**, dotted circles). Similarly, *Umod* expression was observed within the FAE region. A negative control experiment using each sense probe gave no signals for either *Spib* or *Umod*. In addition, expression of both *Spib* and *Umod* was well colocalized with UEA-1–positive cells (**Figure 1c**). These findings demonstrated that *Spib* and *Umod* were expressed in PP M cells but not in other intestinal ECs.

Expression of M-cell–specific surface markers is severely diminished in Spi-B–deficient mice

Expression of *Spib* in PP M cells was high—at levels comparable to that of *Gp2* and *Marcksl1*, which were previously reported to be M-cell–specific genes.[2] In addition, because Spi-B is a transcription factor belonging to the Ets family, it is probable that it controls the expression of genes encoding molecules that contribute to M-cell function or development, or both. Therefore, we decided to focus our efforts on investigating an *in vivo* role of Spi-B in M cells. Spi-B–deficient ($Spib^{-/-}$) mice[15] were thus employed and the characteristics of their PP M cells were compared with those of wild-type ($Spib^{+/+}$) mice.

GP2 is expressed on M cells and not on other intestinal ECs;[2,3] therefore, we first examined GP2 expression by using whole-mount staining. In wild-type mice, GP2 expression was clearly and specifically detected in the FAE region (**Figure 2a**), as demonstrated previously.[2,3] In contrast, $Spib^{-/-}$-derived PPs completely lost their GP2 expression, as found in $Gp2^{-/-}$-derived PPs[3] (**Figure 2b**). More classically, mouse PP M cells were defined as NKM antibody (and/or UEA-1)–positive and wheat germ agglutinin–negative ($NKM^{+}WGA^{-}$) FAE cells,[13] as seen in the PPs from $Spib^{+/+}$ mice (**Figure 2c**). $Spib^{-/-}$-derived PPs showed reduced numbers of $NKM^{+}WGA^{-}$ cells (**Figure 2d–f**); these cells are considered to have the hallmarks of M cells. Notably, although GP2-positive FAE cells were completely abolished in $Spib^{-/-}$ mice (**Figure 2b**), a few $NKM^{+}WGA^{-}$ cells were found in $Spib^{-/-}$-derived PPs (**Figure 2e,f**; about 10 per FAE region vs. about 350 per FAE region in the case of $Spib^{+/+}$ cells).

Gp2 is a target gene of Spi-B

Because GP2 is a highly glycosylated protein and the anti-mouse GP2 antibody used in this research (clone 2F11-C3) was raised against recombinant mouse GP2-Fc fusion protein produced by mammalian cells,[3] it was possible that recognition of GP2 by 2F11-C3 antibody depended on glycosylation. Hence, we next checked the expression level of *Gp2* transcript in the FAE region by using quantitative real-time PCR. Consistent with the whole-mount staining data, *Gp2* expression was dramatically suppressed in $Spib^{-/-}$ mice-derived FAE (**Figure 3a**), indicating that *Gp2* expression is exclusively regulated by Spi-B.

UEA-1 and NKM antibody recognize α (1,2)-fucose, which specifically binds the terminal galactose residues of some surface membrane proteins.[13] Given that terminal fucosylation on PP M cells is catalyzed by FUT1, which is coded by the M-cell–specific gene *Fut1* (Terahara *et al.*[5] and **Supplementary Figure S1** online), the cause of the decrease in numbers of $NKM^{+}WGA^{-}$ cells in the FAE of $Spib^{-/-}$ mice might have

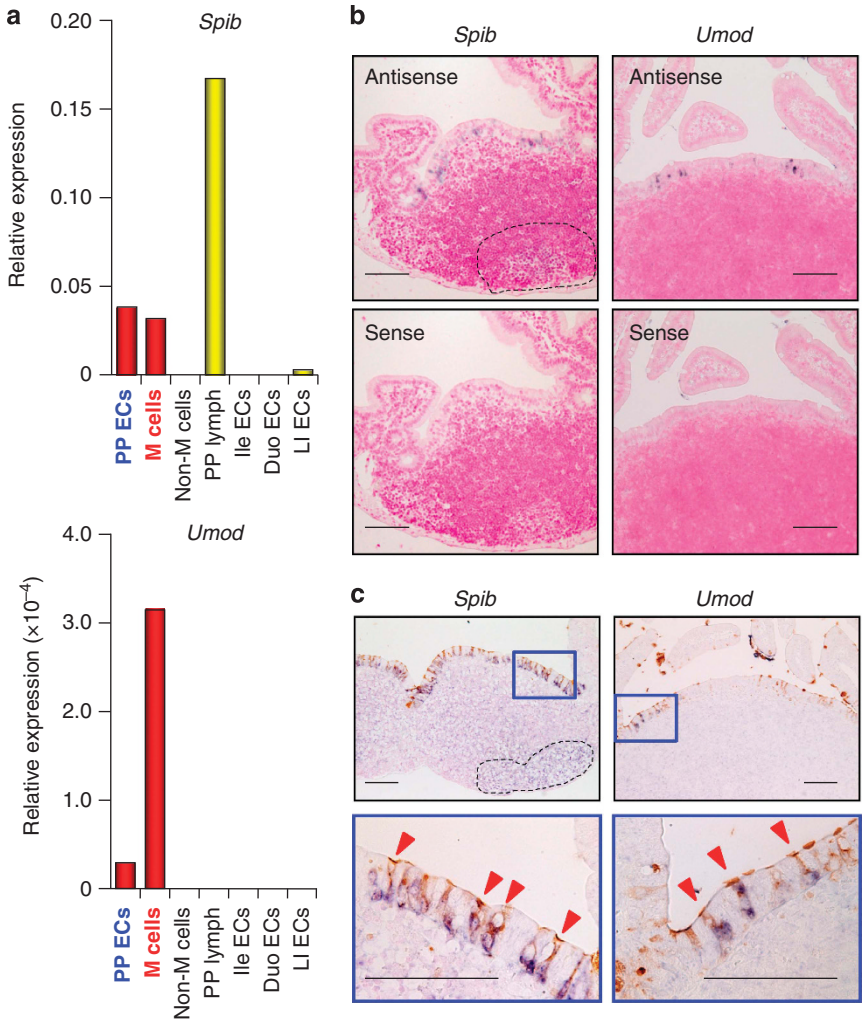

Figure 1 M cells are unique among intestinal epithelial cells in showing expression of *Spib* and *Umod*. (**a**) Real-time PCR analysis of the expression of newly identified M-cell–specific genes in the cell fractions indicated. Each result was normalized against the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and is representative of three independent experiments. Lymph, lymphocytes; Ile, ileal; Duo, duodenal; LI, large intestinal. (**b**) *In situ* hybridization assay to detect *Spib* and *Umod* mRNA in a duodenal PP. Each antisense, but not sense, cRNA probes reacted with FAE regions to give a blue stain. Bar = 100 μm. (**c**) *Spib* and *Umod* signals were merged with brown UEA-1 staining in the FAE region. Each bottom panel is a higher magnification of the blue square in each top panel. Dotted circles found in **b** and **c** indicated germinal center region of PPs. Data are representative of two independent experiments. Bar = 100 μm.

simply been impairment of fucosylation on some M-cell–specific surface molecules. To test this possibility, we assessed the expression of *Fut1* mRNA in the FAE region. Unexpectedly, the levels of *Fut1* expression were comparable in $Spib^{+/+}$ and $Spib^{-/-}$ mice (**Figure 3b**). We further examined the expression of other M-cell–specific genes, including *Umod*, in $Spib^{+/+}$ and $Spib^{-/-}$ mice. Although *Umod*, like *Gp2*, was poorly expressed in $Spib^{-/-}$ FAE, the expression levels of *Marcksl1* (which encodes MARCKS-like protein) and *Pglyrp1* (which encodes peptidoglycan recognition protein S) in the FAE region of $Spib^{-/-}$ mice were similar to those in wild-type mice (**Figure 3c**). These results indicated that M-cell–specific expression molecules could be divided into two groups according to their dependence on Spi-B for gene regulation.

Abundance of cells with irregular and short microvilli is diminished in $Spib^{-/-}$ PPs

M cells are easily distinguished from the neighboring columnar ECs by their morphologically unique surface microvilli, which are sparser and shorter. It is likely that these irregular microvilli are convenient for the effective uptake of ingested antigens from luminal sites in the digestive tract. Therefore, this feature is another hallmark of M cells. We prepared PPs from $Spib^{+/+}$ and $Spib^{-/-}$ mice and then observed and counted hollow cells by scanning electron microscopy. M cells were preferentially found at the edge of the FAE region in wild-type mice as "sunken" cells (**Figure 4a,b**; about 35 sunken cells per 0.01-mm^2 field). In contrast, these sunken cells were rarely detected (about 3 sunken cells per 0.01-mm^2 field) in the corresponding region of $Spib^{-/-}$ mice. Notably, however, a pocket structure

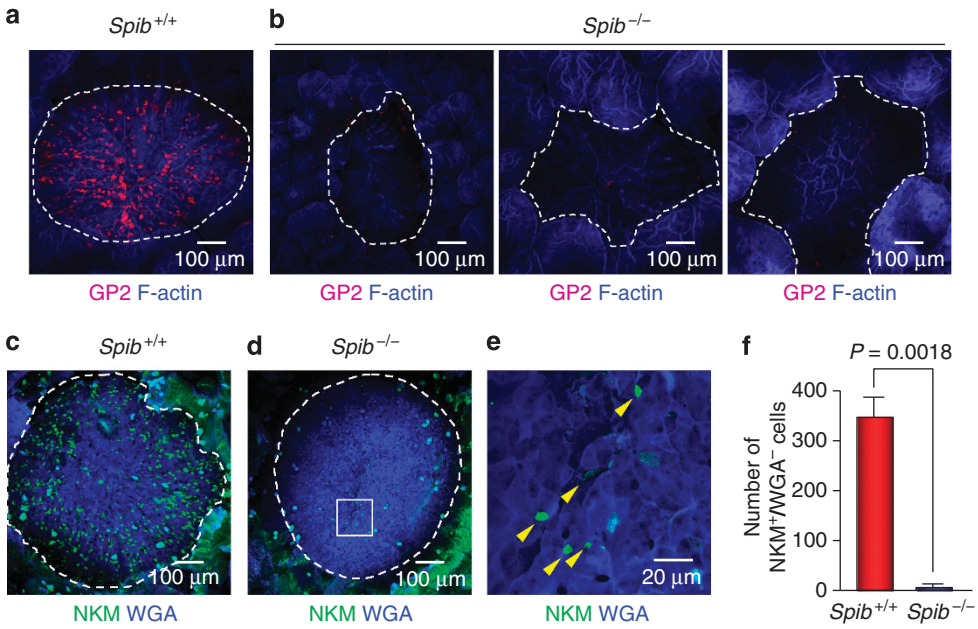

Figure 2 Abundance of surface markers on microfold cells (M cells) is severely diminished in Spi-B–deficient mice. Whole-mount staining analysis using anti-GP2 antibody (red; **a** and **b**) and NKM 16-2-4 antibody (green; **c**–**e**) in PPs from wild-type (**a** and **c**) and *Spib*$^{-/-}$ (**b** and **d**) mice. (**e**) High-magnification image of the white square in **d**. Data are representative of at least three independent experiments. (**f**) The number of NKM^{+} WGA^{-} cells found in one FAE region. Data are mean ± s.e.m. of one representative from three independent experiments. *P*-values were determined by Mann–Whitney *U*-test.

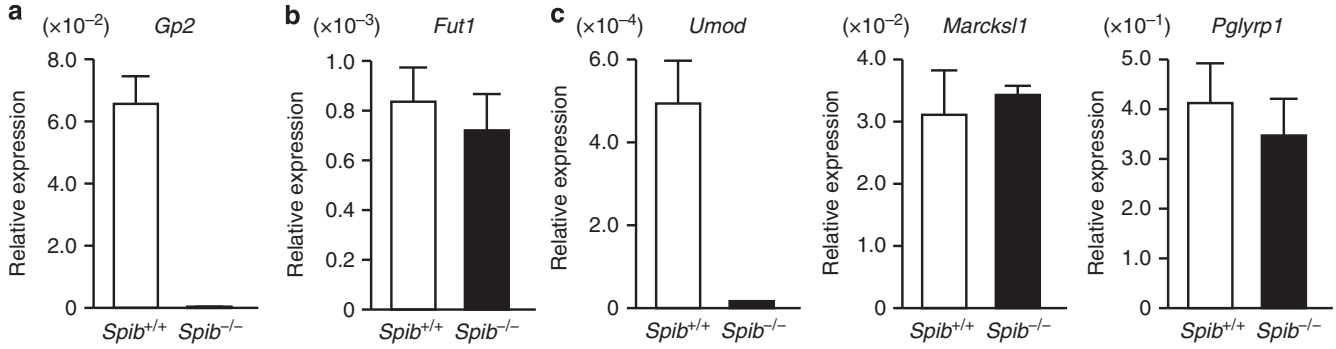

Figure 3 Spi-B–dependent and –independent expression of M-cell–specific genes. Real-time PCR analysis of the expression of several M-cell–specific genes (**a**, *Gp2*; **b**, *Fut1*; and **c**, *Umod*, *Marcksl1*, and *Pglyrp1*) in FAE prepared from *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice. Each result was normalized against the expression of *Gapdh* and is shown as the mean ± s.d. of one representative from three independent experiments. *P*-values were determined by Student's *t*-test.

enfolding lymphocytes was found in the *Spib*$^{-/-}$ M cells (**Supplementary Figure S2** online), as in the wild-type M cells.[13] These data are consistent with the whole-mount staining data we obtained with NKM/WGA, but not with anti-GP2 antibody (**Figure 2f,b**); they suggest that although the unique surface morphology of most M cells is also regulated by Spi-B, there is a small population of M cells (about 10 per FAE region; **Figure 2f**) of which differentiation is independent of Spi-B.

B-cell–intrinsic Spi-B is dispensable in the induction of M-cell–specific markers

Spib$^{-/-}$ mice showed a severe reduction in M-cell features, but Spi-B is expressed mainly in hematopoietic cells—particularly in B cells and plasmacytoid dendritic cells.[14–16] Moreover, one report has indicated that a certain B-cell subset residing in the subepithelial dome of the PP mucosa contributes to M-cell differentiation.[17] Therefore, we next investigated whether B cells (or T cells, or both) were responsible for the expression of GP2 on M cells. We used bone marrow (BM) chimeric mice, created by transplanting wild-type or *Spib*$^{-/-}$ BM cells into recombination activating protein 1 (RAG1)-deficient mice, which have no mature B and T lymphocytes.[18] The number of GP2^{+} cells in RAG1-deficient mice that received *Spib*$^{-/-}$ BM cells was comparable to the number in those that received wild-type BM cells (**Figure 5**), indicating that Spi-B expressed in B and T cells was not important in inducing or maintaining M-cell surface markers.

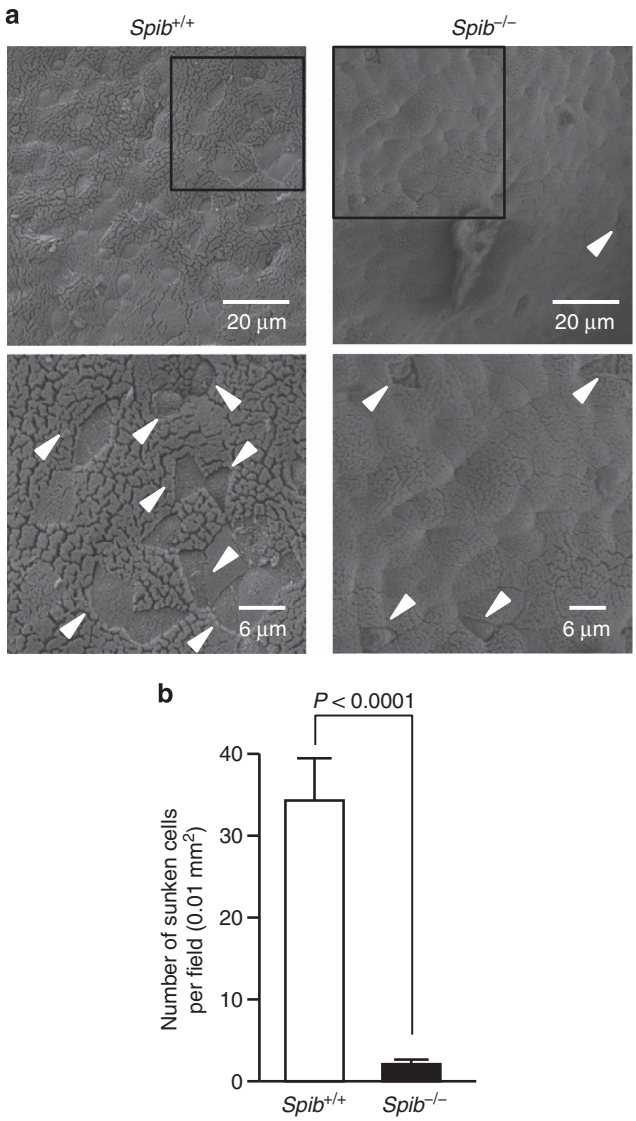

Figure 4 Number of sunken cells in FAE regions is decreased in Spi-B–deficient mice. (**a**) Scanning electron microscopic analysis of duodenal Payer's patch PPs from *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice. Typical M cells are indicated by arrowheads. Each bottom panel is a higher magnification of the box in each top panel. Data are representative of two independent experiments. (**b**) Calculated numbers of sunken cells per field. Data are means ± s.e.m. of one experiment representative of two independent experiments. *P*-values were determined by the Mann–Whitney *U*-test.

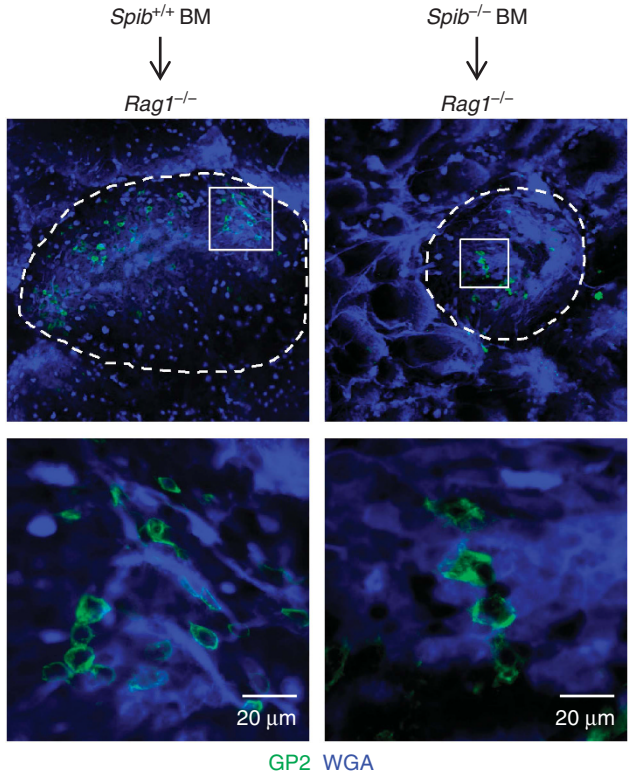

Figure 5 Spi-B expressed in B and T cells is not important for the expression of GP2 on M cells. Bone marrow (BM) cells were prepared from *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice and then transferred to RAG1-deficient mice. At 8 weeks after the transfer, GP2-positive FAE cells were visualized by whole-mount staining using anti-GP2 antibody (green). WGA was used for epithelial-cell counterstaining (blue). Each bottom panel is a higher magnification of the white square in each top panel. Data are representative of two independent experiments.

M cells differentiated by an Spi-B–independent pathway can transcytose PP-resident bacteria

M cells are specialized for antigen uptake.[1] Although small numbers of M cells were observed in the FAE region in *Spib*$^{-/-}$ mice (fewer than 5% of those in the wild type), it seemed that most M-cell features, such as expression of surface markers and apical morphology, were diminished in these mice. These findings raise the possibility that *Spib*$^{-/-}$ mice have diminished uptake of pathogenic or opportunistic bacteria, or both. Consistent with the loss of GP2 expression, uptake of *Salmonella* Typhimurium, which depends on host GP2 for binding to M cells, was markedly lower in *Spib*$^{-/-}$ mice than in wild-type mice (**Supplementary Figure S3** online).

We further examined whether PPs of *Spib*$^{-/-}$ mice possessed *Alcaligenes* spp., which, as we recently discovered, are opportunistic bacteria that inhabit inside PPs;[19] *Alcaligenes* spp. were moved from the lumen of the small intestine to the inside of the PPs preferentially via PP M cells (**Figure 6a**). Consistent with our previous reports,[19,20] *Alcaligenes* spp. had colonized the inside of the PPs well in wild-type mice (**Figure 6b**). Interestingly, intratissue *Alcaligenes* spp. were also detected inside the PPs from *Spib*$^{-/-}$ mice at levels almost comparable to those found in wild-type PPs (**Figure 6b**). Next, we examined *Alcaligenes* uptake capability in the PPs of *Spib*$^{-/-}$ mice by ligated-intestinal loop assay using culturable *A. faecalis*. Although *Spib*$^{-/-}$ mice showed a tendency toward reduced uptake, there was no significant difference between *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice in the translocation of *Alcaligenes* to the inside of the PPs (**Figure 6c**). These data indicated that *Alcaligenes* spp. could move to, and inhabit, the inside of the PPs, even if the host lacked Spi-B–dependent M cells.

DISCUSSION

In this study, we identified the novel M-cell–specific expression genes *Umod* and *Spib*. Spi-B-deficient mice showed complete

loss of GP2-positive M cells. However, these mice possessed small numbers of M cells, as defined by their pattern of reactivity to NKM/WGA and the ultrastructure of the apical surface, indicating that there are at least two nonredundant pathways for the development of PP M cells. Interestingly, although Spi-B regulated many other M-cell–specific genes, such as *Gp2*, expression of *Fut1*, *Marcksl1*, and *Pglyrp1* on M cells was observed to be independent of Spi-B (**Figure 3**). GP2 acts as a scaffold protein on M cells to transcytose FimH-positive type-I–piliated bacteria such as *S.* Typhimurium and *Escherichia coli*.[3] Therefore, GP2- and Spi-B–deficient mice showed low levels of uptake of these strains via M cells (Hase *et al.*[3] and **Supplementary Figure S3** online). Given that *Gp2* expression is under the control of Spi-B transcriptional activity, Spi-B has a critical role in M cells in regulating the uptake of FimH-positive bacteria.

Interestingly, although the expression of *Fut1* was independent of Spi-B, terminal fucosylation of PP M cells was severely diminished in $Spib^{-/-}$ mice. *Fut1* is specific to M cells among intestinal ECs, and terminal fucosylation on M cells is catalyzed by FUT1 (Terahara *et al.*[5] and **Supplementary Figure S1** online). Therefore, one possible explanation for our contradictory finding is that the expression of some molecules that are specifically expressed on M cells and are fucosylated by FUT1 is regulated by Spi-B. If a substrate for the fucosylation mediated by FUT1 was absent, M cells would not be detectable by NKM antibody and UEA-1. GP2 is a candidate for such a molecule, but both NKM antibody and UEA-1 react well with the M cells of GP2-deficient mice (**Supplementary Figure S4** online). The explanation presented above is compatible with our data in which M cells differentiated independently of Spi-B were fucosylated but did not express GP2 on their apical surfaces. Therefore, it may be of interest to look for differences in the FUT1-positive cells of wild-type and $Spib^{-/-}$ mice.

Several lines of evidence have indicated that non-intestinal ECs are involved in M-cell development. These cells include $CCR6^{hi}CD11c^{int}$ B cells and RANKL-positive stromal cells, both of which are located in the subepithelial dome region under the FAE.[17,21] Although Spi-B is expressed mainly in lymphoid cells,[14,15] our results have shown that Spi-B is also expressed, and regulates the expression of some genes, in non-hematopoietic cells. In addition, our finding that $Spib^{-/-}$ B cells were sufficient for the induction or maintenance of M-cell surface markers showed that M-cell–, but not

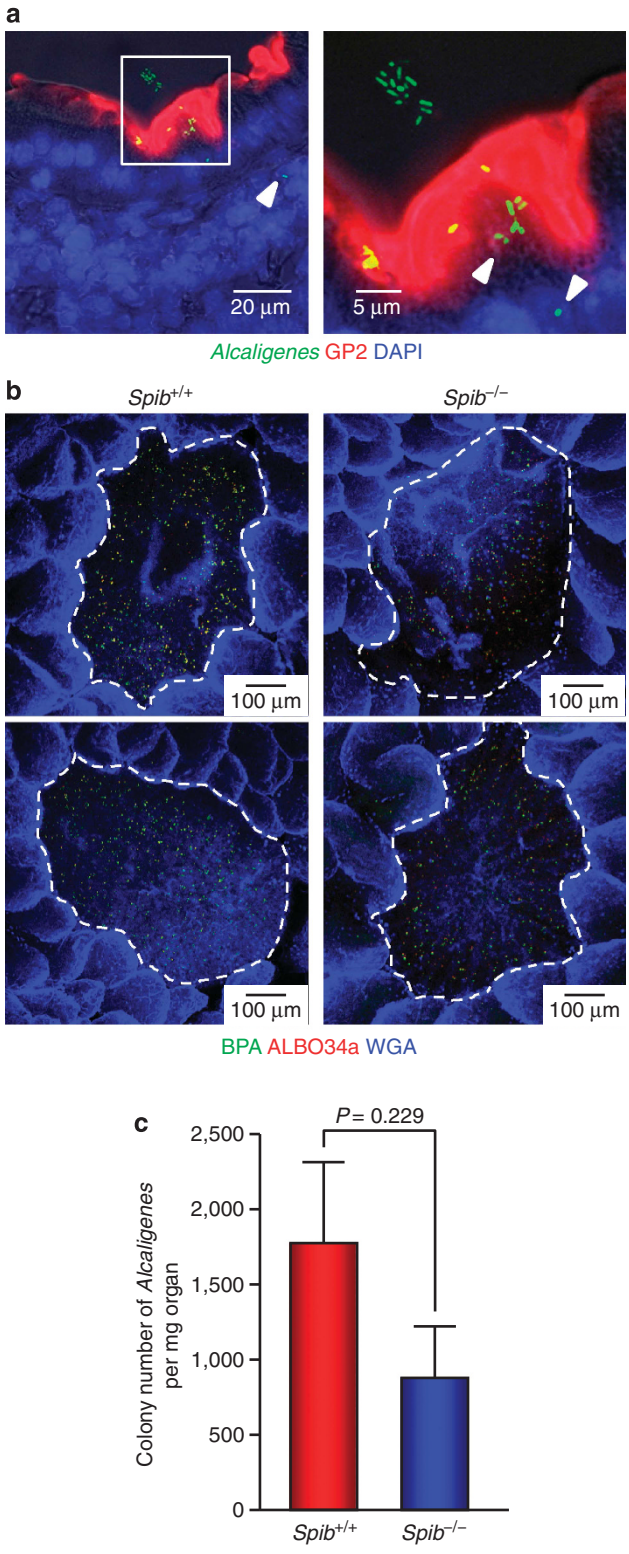

Figure 6 PP-resident commensal bacteria cohabit, and translocate within, the PPs preferentially through M cells. (**a**) GFP-expressed *Alcaligenes faecalis* were injected into ligated intestinal loops included in PPs. At 4 h after the injection, mice were euthanized and the PPs were dissected out. Fixed samples were sectioned and then stained with anti-GP2 antibody (red) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). M cells ($GP2^+$ cells) and GFP^+ *Alcaligenes* (green) were visualized under a fluorescence microscope (BZ-9000; Keyence). Transcytosed *Alcaligenes* are indicated by arrowheads. Data are representative of two independent experiments. (**b**) PPs from $Spib^{+/+}$ and $Spib^{-/-}$ mice were analyzed by fluorescent *in situ* hybridization (FISH) using probes to identify *Alcaligenes* spp. as BPA (green) and ALBO34a (red) doubly positive yellow dots. WGA was used for epithelial-cell counterstaining (blue). Data are representative of three independent experiments. (**c**) Uptake of culturable *Alcaligenes* on ligated-intestinal loop assay was quantified by counting colony numbers on streptomycin-containing culture plates. Results were normalized against PP weight, and data are means ± s.e.m. of one experiment representative of two independent experiments. *P*-values were determined by the Mann–Whitney *U*-test.

B-cell–, intrinsic Spi-B contributed to the development of M cells. Furthermore, the levels of RANKL expression in the PPs were similar in wild-type and *Spib*$^{-/-}$ mice (**Supplementary Figure S5** online). Taken together, these data indicate that M-cell–intrinsic Spi-B has a critical role in the functional development of Spi-B–dependent M cells.

Alcaligenes spp. are Gram-negative bacteria that reside within the PPs of not only mice but also humans and nonhuman primates.[19] Although we could not exclude the possibility that M-cell–independent uptake of *Alcaligenes* spp. occurred, our findings suggest that *Alcaligenes* spp. are preferentially transcytosed by M cells into the PPs (**Figure 6a**). The *Alcaligenes* spp. are then contained within the PPs by interleukin-22–producing innate lymphoid cells.[20] Although it is still not known whether *Alcaligenes* spp. are habituated to this environment and undergo a growth cycle there, or whether they are continuously supplied from outside, we observed this bacterial strain in the PPs of *Spib*$^{-/-}$ mice at levels comparable to those in the PPs of wild-type mice (**Figure 6b**). Given that *Spib*$^{-/-}$ mice possess fewer than 5% of the M cells of the wild-type, the few M cells that are present might be enough for the translocation and cohabitation of *Alcaligenes* spp. in the PPs. In fact, the M cells of *Spib*$^{-/-}$ mice showed the capability for preferential transcytosis of *Alcaligenes* (**Supplementary Figure S6** online). Indeed, our *ex vivo* ligated-loop assay showed that there was no significant difference between wild-type mice and *Spib*$^{-/-}$ mice in terms of the level of *Alcaligenes* transcytosis into the PPs, although the level tended to be lower in the latter (**Figure 6c**). Even though most of the cells found in the FAE of *Spib*$^{-/-}$ mice did not have M-cell features, it remains to be determined whether the FUT1-positive cells within the *Spib*$^{-/-}$ FAE, which in our study were putative "M cells," had really lost their transcytosis activity. Again, it will be important to investigate and compare the transcytosis abilities of FUT1-positive cells within the FAEs of wild-type and *Spib*$^{-/-}$ mice.

We found that, in addition to *Spib*, *Umod* is an M-cell–specific gene. *Umod* encodes THP (also known as uromodulin), which is a GP2 homolog and is expressed on the apical membrane of tubular ECs in the kidney. Membrane-bound THP is digested by the activity of phospholipase D or protease and released into the urine.[22] Secreted THP competitively binds to type-I–fimbriated *E. coli*, which can bind and internalize to the urothelium via FimH and host mannosylated uroplakins Ia and Ib.[23] THP thus helps to accelerate bacterial clearance. This information, together with our findings, suggests that THP contributes to the uptake of FimH-positive bacteria by PP M cells, although the level of *Umod* expression was very low compared with that of *Gp2* (**Figure 3a,c**).

During the preparation of this manuscript, Kanaya *et al.*,[24] using another line of Spi-B–deficient mice, and de Lau *et al.*,[25] using a "minigut" system, reported results similar to our data on Spi-B–dependent M-cell differentiation. Although there was a discrepancy between the results of these two groups in terms of the dependence of the expression of Annexin V (an M-cell–specific gene) on Spi-B, both groups reported that PP M cells were differentiated by RANKL stimulation via the induction of Spi-B in the M cells. They also reported that PP M cells were completely lost in Spi-B–deficient mice. In contrast to the above two reports, our study demonstrates that there is a population of PP M cells of which the differentiation mechanism is independent of Spi-B. Spi-B–deficient mice still possessed cells morphologically considered to be PP M cells (**Figure 4** and **Supplementary Figure S2** online). In addition, although the precise mechanism for the translocation of *Alcaligenes* spp. into PPs, including the nature of the host recognition receptor, has not been elucidated, substantial cohabitation of this opportunistic bacterium within, as well as its uptake into, PPs was observed in Spi-B–deficient mice (**Figure 6b,c**). Kanaya *et al.*[26] found much lower uptake of not only *S.* Typhimurium but also *Yersinia enterocolitica* (the host binding receptor of which is β1-integrin[26]) in the PPs of *Spib*$^{-/-}$ mice than in the wild type. Although they did not mention the levels of expression of β1-integrin, the reduced translocation of *Y. enterocolitica* might have been caused by a reduction in β1-integrin expression on the apical surfaces of the cells in *Spib*$^{-/-}$ PPs. Therefore, the difference in Spi-B dependency between bacterial species might be due to differences in the pathogenicity of the bacteria. The de Lau group[25] also proposed that RANKL induces the expression of other regulator genes for sufficient M-cell differentiation, because forced expression of Spi-B, but not RANKL, in the minigut system did not induce GP2 expression. These data raise the interesting possibility that other RANKL-induced regulators are involved in Spi-B–independent M-cell differentiation.

In conclusion, we discovered novel M-cell–specific genes, one of which, *Spib*, is a candidate master transcription factor for M-cell functional and structural maturation. Although M-cell–intrinsic Spi-B regulated most M-cell–specific gene expression, future studies are required to clarify in detail the mechanisms by which individual Spi-B target molecules contribute to M-cell function and maturation. In addition, the mechanisms of Spi-B–independent M-cell development and their contribution to the antigen-uptake function of these cells need to be demonstrated in future.

METHODS

Mice. RAG1-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Gp2*$^{-/-}$ and *Fut1*$^{lacZ/lacZ}$ mice were kindly provided by Dr Anson W. Lowe[27] and Dr Steven E. Domino,[28] respectively. Spi-B–targeted mice on a C57BL/6 background were kindly provided by Dr LeeAnn Garrett-Sinha, with permission from Dr M. Celeste Simon.[15] Heterozygous mice were crossed to obtain *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice. Littermates or age- and gender-matched *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice were used for all subsequent experiments. All animals were housed in specific pathogen–free conditions at the animal facility of the Institute of Medical Science, The University of Tokyo. All animal experiments were carried out with the approval of the Animal Research Committee of the Institute of Medical Science, The University of Tokyo (Tokyo, Japan).

Preparation of small intestinal epithelial fractions. Small intestinal ECs were isolated as described previously.[2] Briefly, PPs and PP-free small intestines were treated with 0.5 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) for 20 min at 37°C. Dissociated cells were filtered through a 40-μm nylon mesh, centrifuged,

and resuspended with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. ECs from PPs were stained with 5 μg ml^{-1} fluorescein isothiocyanate-conjugated NKM 16-2-4 monoclonal antibody,[13] 6 μg ml^{-1} rhodamine-labeled UEA-1 (Vector Laboratories, Burlingame, CA), and 1 μg ml^{-1} APC-Cy7-conjugated antimouse CD45 monoclonal antibody (BD Biosciences, Oxford, UK) for 40 min before being reacted with 7-aminoactinomycin D (BD Biosciences) for 10 min at 4°C. The stained cells were sorted with a flow cytometer FACSAria II (BD Biosciences); NKM$^+$/UEA-1$^+$/CD45$^-$ cells and NKM$^-$/UEA-1$^-$/CD45$^-$ cells were sorted as PP M cells and PP non-M cells, respectively. Dead cells were excluded on the basis of their positive reactivity to 7-aminoactinomycin D. To prepare FAE sheets, PPs were washed well with PBS and then soaked in 25 mM ethylenediaminetetraacetic acid in PBS for 15 min at room temperature. The FAE was peeled off by manipulation with fine needles under a stereoscopic microscope.

Real-time PCR. Total RNA was isolated from the cells by using an RNeasy Kit (Qiagen, Tokyo, Japan). Reverse transcription was performed with SuperScript VILO (Invitrogen, Carlsbad, CA). For quantitative PCR, cDNA products were amplified with a set of specific primers and LightCycler 480 SYBR Green I Master (Roche, Indianapolis, IN) on a LightCycler 480 System II (Roche). The mRNA expression level of each target was normalized against that of *Gapdh*. The sets of primers used were as follows: *Gapdh* (forward) 5′-TGTC CGTCGTGGATCTGAC-3′ and (reverse) 5′-CCTGCTTCACCAC CTTCTTG-3′; *Spib* (forward) 5′-GCCCACACTTAAGCTGTT TGTA-3′ and (reverse) 5′-CTGTCCAGCCCCATGTAGAG-3′; *Umod* (forward) 5′-GGATGGGGATCCCTTTGA-3′ and (reverse) 5′-GGCA TTCAGAACACCGTCTC-3′; *Gp2* (forward) 5′-GTGGGTTGTGA CCTGCTGT-3′ and (reverse) 5′-CTGGGCCTCCCATAACCT-3′; *Fut1* (forward) 5′-AAAGAATTCGCTTGCACCAC-3′ and (reverse) 5′-GAGAGCACACAGACCAACAGA-3′; *Marcksl1* (forward) 5′-GGC AGCCAGAGCTCTAAGG-3 and (reverse) 5′-TCACGTGGCCAT TCTCCT-3′; and *Pglyrp1* (forward) 5′-CAGTTCGCTACGTGGT GATCT-3′ and (reverse) 5′-TCTCCAATAAGGAAGTTGTAGG CTAC-3′.

***In situ* hybridization**. The DNA fragment encoding Spi-B (GenBank: NM_019866) or THP (GenBank: NM_009470) was amplified by PCR from BALB/c mouse embryo (14.5-day)–derived cDNA. The following primers were used: *Spib* (forward) 5′-CTCTGAACCACCATGC TTGCTCTGGAG-3′ and (reverse) 5′-GTACGGAGCATAAGCCA AGGAGCCCAG-3′; and *Umod* (forward) 5′-GTACCTAACCCATC CTTGCCTTTGGGC-3′ and (reverse) 5′-TCACAGGAGTAGCCCC ACACCATACTC-3′. The PCR products were subcloned into a pCR4-TOPO vector (Invitrogen). After the sequencing, digoxigenin-labeled sense and antisense RNA probes were transcribed *in vitro* with a digoxigenin RNA labeling mix (Roche). Paraffin-embedded sections of duodenal tissues (6 μm) from naïve BALB/c mice were obtained from Genostaff (Tokyo, Japan). The sections were fixed with 4% paraformaldehyde in PBS, and then treated with 20 μg ml^{-1} proteinase K in PBS for 30 min at 37°C. Hybridization was performed with 300 ng ml^{-1} probes at 60°C for 16 h. After hybridization, the bound probes were treated with anti-digoxigenin–AP conjugate (Roche) and visualized with NBT/BCIP solution (Sigma, St Louis, MO). After *in situ* hybridization, sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan) or reacted with 0.25 μg ml^{-1} biotin-conjugated UEA-1 after treatment with 0.3% H_2O_2. The sections were further reacted with HRP-conjugated streptavidin and then stained with diaminobenzidine (Vector Laboratories).

Whole-mount staining. PPs were excised from the duodenum and ileum and then washed with PBS. Isolated tissues were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) for GP2 staining, or just fixed with 4% paraformaldehyde for staining with NKM antibody. Specimens were then stained with anti-mouse GP2 antibody (MBL, Nagoya, Japan) or NKM 16-2-4 monoclonal antibody,[13] followed by secondary antibodies conjugated with Alexa Fluor 488 or Cy3 (Jackson ImmunoResearch, West Grove, PA). After being washed with PBS, the whole-mount specimens were further stained with Alexa Fluor 633-conjugated phalloidin (Invitrogen) or WGA (Invitrogen). All samples were mounted and then analyzed by DM IRE2/TCS SP2 confocal microscopy (Leica, Wetzlar, Germany).

To detect *Alcaligenes* spp., whole-mount fluorescent *in situ* hybridization analysis was performed as described previously.[19] X-gal staining to visualize FUT1 expression in tissues from *Fut1*$^{lacZ/+}$ mice was performed with a β-gal staining kit (Invitrogen) in accordance with the manufacturer's instructions.

Electron microscopy. PPs were fixed for 3 h at room temperature in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M of phosphate buffer (pH 7.5). After washes with 3% sucrose in 0.1 M of phosphate buffer, the tissues were fixed with 1% osmium tetraoxide on ice for 2 h and dehydrated with an ethanol series. For scanning electron microscopy, dehydrated samples were freeze-embedded in *t*-butyl alcohol and freeze dried, and then coated with osmium and observed under a scanning electron microscope (S-4200; Hitachi, Tokyo, Japan). For transmission electron microscopy, dehydrated samples were embedded in Epon 812 resin mixture (TAAB Laboratories, Aldermaston, UK) after treatment with propylene oxide. Ultrathin sections (90 nm) were stained with 2% uranyl acetate and Reynolds lead and examined under a Hitachi H-7500 electron microscope.

BM transfer. BM cells were prepared from *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice. The cells were intravenously injected into RAG1-deficient mice. The mice were analyzed 8 weeks after reconstitution.

Ligated-intestinal loop assay. Mice were anesthetized by using an isoflurane vaporizer. An intestinal loop containing a single PP was ligated off, and the lumen was then injected with 1×10^8 CFU/0.1 ml of *Alcaligenes*, which had been transformed with streptomycin resistance genes.[19] After 4 h, the mice were euthanized and the PP was excised from the ligated intestine. Specimens were extensively washed with PBS and then used to make a series of frozen sections or whole-mount stain to examine the uptake of *Alcaligenes*. To perform the colony-forming unit assay, washed tissues were incubated in 100 μg ml^{-1} gentamycin solution at room temperature for 30 min. After being washed with sterile PBS, all tissues were weighed, homogenized, and diluted with sterile PBS. The homogenates were then plated on LB agar plates containing 50 μg ml^{-1} streptomycin.

Colony-forming unit assay after *S.* Typhimurium oral infection. *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice were fed 1×10^8 CFU/0.1 ml of *S.* Typhimurium carrying a nalidixic acid resistance gene (strain χ3306, kindly provided by Dr Hidenori Matsui[29]). After 24 h, PPs were collected from the ileum region, extensively washed with PBS, and then incubated in 100 μg ml^{-1} gentamycin solution at room temperature for 30 min. After being washed with sterile PBS, all tissues were weighed, homogenized, and diluted with sterile PBS. The homogenates were then plated on LB agar plates containing 25 μg ml^{-1} nalidixic acid.

Statistical analysis. Results were compared by using Student's *t*-test and the Mann–Whitney *U*-test for parametric and nonparametric data, respectively. Statistical significance was established at $P < 0.05$.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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