Lactoferrin causes IgA and IgG2b isotype switching through betaglycan binding and activation of canonical TGF-β signaling

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Lactoferrin (LF), a pleiotropic iron-binding glycoprotein, is known to modulate the humoral immune response. However, its exact role in Ig synthesis has yet to be elucidated. In this study, we investigated the effect of LF on Ig production by mouse B cells and its underlying mechanisms. LF, like transforming growth factor (TGF)-β1, stimulated B cells to produce IgA and IgG2b, while downregulating other isotypes. Using limiting dilution analysis, LF was shown to increase the frequency of IgA-secreting B-cell clones. This was paralleled by an increase in Ig germ-line α (GLα) transcripts, indicating that LF plays a role as an IgA switch factor. Interestingly, LF directly interacted with betaglycan (TGF-β receptor III, TβRIII) and in turn induced phosphorylation of TβRI and Smad3 through formation of the TβRIII/TβRII/TβRI complex, leading to IgA isotype switching. Peroral administration of LF increased intestinal/serum IgA production as well as number of IgA plasma cells in lamina propria. Finally, we found that LF has an adjuvant activity when nontoxigenic *Salmonella typhimurium* was inoculated perorally, conferring protection against intragastrical infection of toxigenic *S. typhimurium*. These results suggest that LF has an important effect on the mucosal/systemic IgA response and can contribute to protection against intestinal pathogens.

INTRODUCTION

Lactoferrin (LF), retinoids, and transforming growth factor (TGF)-β, which are abundant in colostrum, milk, and mucosal secretions, are well-known multifunctional molecules involved in innate and adaptive immunity.[1,2] Related to the humoral immune response, TGF-β1 is best characterized as an IgA isotype switch factor. TGF-β1 induces both Ig germ-line (GL)α and GLγ2b transcription and subsequent class switch recombination (CSR) to IgA and IgG2b.[3,4] Despite the fact that TGF-β1 also induces IgG2b CSR, TGF-β1 is considered a physiological mediator of IgA CSR because it enhances IgA isotype switching at the clonal level and because deletion of the TGF-β or TGF-β receptor type-II (TβRII) genes results in severe loss of IgA expression.[5–7] Herein, IgA CSR has been attributed to the Smad-dependent canonical TGF-β pathway.[8,9]

Retinoic acid (RA), a vitamin A metabolite, is synthesized from precursor molecules such as retinol in GALT DCs.[10] RA also contributes to the intestinal IgA Ab response. It has been shown that RA enhances IgA expression in the presence of IL-5.[11,12] Recently, we have demonstrated that RA alone can enhance IgA CSR and that this activity of RA is more selective than that of TGF-β1.[13]

LF is an 80 kDa iron-binding glycoprotein that is released from mucosal epithelial cells and neutrophils during inflammation.[14] LF is regarded as an important immunomodulator in innate and adaptive immunity, bridging the two immune responses.[15] As for B-cell differentiation, a pepsin hydrolysate of LF induces proliferation, activates murine B cells, and increases total IgA production by PP cells.[16] These investigators also show that perorally delivered LF enhances Ag-specific IgA secretion in the intestine. Similarly, peroral administration of

LF into mice increases the intestinal secretion of total IgA and IgG isotypes but not serum IgG.[17] Further, LF increases resistance to *Salmonella typhimurium* infection and the production of total/Ag-specific intestinal IgA and serum IgG.[18] Together, these results suggest that LF may facilitate IgA-mediated B-cell differentiation, leading to protection against intestinal pathogens. Although the roles of TGF-β and RA in IgA-mediated B-cell commitment are relatively well characterized, research has yet to elucidate whether LF also produces such an effect, and if so, what the underlying mechanisms are. In the present study, limiting dilution analysis demonstrated that LF has a significant role as an IgA isotype switching factor. This LF activity was confirmed at the molecular level by measuring Ig germ-line transcripts. Subsequent study of the underlying mechanisms revealed that LF binds to betaglycan (TβRIII) and activates the canonical TGF-β pathway to induce IgA CSR.

RESULTS

LF directly modulates B cells to produce IgA and IgG2b isotypes

Although LF has been shown to be multifunctional in the immune system,[15] none of the studies have explored its direct effect on Ig synthesis by lipopolysaccharide (LPS)-stimulated mouse B cells in detail. Therefore, we first examined the effect of LF on Ig production by purified mouse splenic B cells. In this experiment, TGF-β1 was used as a positive control because it is a known IgA and IgG2b class switching factor.[3–5] Similar to TGF-β1, LF significantly increased production of IgA and IgG2b isotypes and concurrently downregulated IgM production (**Figure 1a**). Three forms of LF exist in man and cow according to its iron saturation level, namely apo LF (Fe^{3+} 0%), holo LF (Fe^{3+} 100%), and native LF (Fe^{3+} 50%),[19] with each exerting different biological activities. We compared the ability of these three forms to stimulate Ig synthesis alongside the LF-digested form, LF pepsin hydrolysate (LFH). As shown in **Figure 1b**, the three forms of LF except LFH increased IgA and IgG2b production, suggesting that the structural integrity of LF, not its iron content ratio, is relevant to this activity. There are two distinguishable B-cell populations in the spleen, follicular (FO) B cells and marginal zone (MZ) B cells. MZ B cells were mainly responsive to LF for IgA production, whereas both B-cell populations responded almost identically for IgG2b production (**Figure 1c**).

Enhancement of IgA/IgG2b expression by LF is not associated with TGF-β activity

As LF enhanced IgA and IgG2b production similar to that of TGF-β1, it was necessary to define the specificity of the purified LF used in this study. First, we examined the possibility that any residual TGF-β retained in the purified LF was responsible for the LF-induced response. Pretreatment with an anti-pan TGF-β Ab at the concentration that completely abolished TGF-β1-induced IgA/IgG2b responses had no effect on the LF-induced IgA/IgG2b enhancement (**Figure 2a**). In fact, the anti-pan TGF-β Ab also completely abolished TGF-β2-induced IgA/IgG2b responses (**Supplementary Figure 1** online). These

results indicate that the LF used in this study was not contaminated with TGF-β or that it was present at a level far below that which affects IgA and IgG2b production. Subsequently, we examined the effect of pretreating LF with anti-LF Ab before adding it to B-cell cultures. As shown in **Figure 2b**, anti-LF Ab not only abrogated LF-induced IgA/IgG2b production completely but also restored the LF-repressed IgM/IgG1 production. These results indicate again that the LF used in this study was not contaminated with any substance, including TGF-β. To further characterize the B-cell phenotype targeted by LF, resting CD43^{−} B cells were cultured in the presence of LF. Overall, IgA and IgG2b production by CD43^{−} resting B cells were the same as whole B cells, whereas IgM, IgG1, and IgG3 production were significantly diminished by LF (**Figure 2c**). In addition, we found that IL4-induced IgG1 production was downregulated by LF (**Figure 2d**).

There is a possibility that LF may stimulate B cells to secrete TGF-β, which in turn modulates B cells in an autocrine manner. However, LF did not stimulate B cells to express TGF-β1 or TGF-β2 (**Supplementary Figure 2**). Together, these results indicate that LF exhibits inherent activity on B-cell differentiation without any link to TGF-β.

LF, like TGF-β1, possesses anti-proliferation and IgA isotype switching activity

We previously demonstrated that the IgA enhancing activity of TGF-β1 is related, in part, to its ability to inhibit cell growth.[5] Therefore, it was necessary to determine the effect of LF on cell growth and proliferation. Every concentration of LF and TGF-β1 that increased IgA secretion also inhibited cell growth (**Figure 3a**) and cell proliferation (**Figure 3b**), suggesting that LF, like TGF-β1, partially contributes to enhancing IgA and IgG2b production by reducing the proliferation activity of B cells. On the other hand, LF ($20 \sim 540\,\mu g\,ml^{-1}$) did not affect B-cell viability in the absence of LPS (**Supplementary Figure 3A**). However, LF enhanced CD138 expression (reached a plateau at $60\,\mu g\,ml^{-1}$ of LF, **Supplementary Figure 3B**) and moreover augmented the number of membrane IgA^{+} (mIgA^{+}) CD138^{+} cells (**Supplementary Figure 3C**), indicating that LF enables B cells to differentiate into IgA plasma cells.

Next, it was necessary to investigate whether LF enhanced IgA production by increasing the total number of IgA-secreting cells or simply by increasing the amount of IgA secreted per cell. As determined by an ELISPOT assay, we found an increase in the number of IgA-secreting cells in LF- or TGF-β1-stimulated cultures (**Figure 3c**). We subsequently performed a limiting dilution analysis to distinguish whether the observed increase in the number of IgA-secreting cells was due to: (i) an increase in the frequency of B cells that switch to express the IgA isotype after LF stimulation or (ii) an increase in proliferation of B cells that are already committed to expressing the IgA isotype. mIgA[−] splenic B cells were cultured in limiting dilution with LF in parallel with TGF-β1. As shown in **Figure 3d**, LF stimulated an approximately nine-fold increase in the frequency of IgA-secreting B-cell clones among populations of LPS-activated mIgA^{−} B cells with slightly increasing the
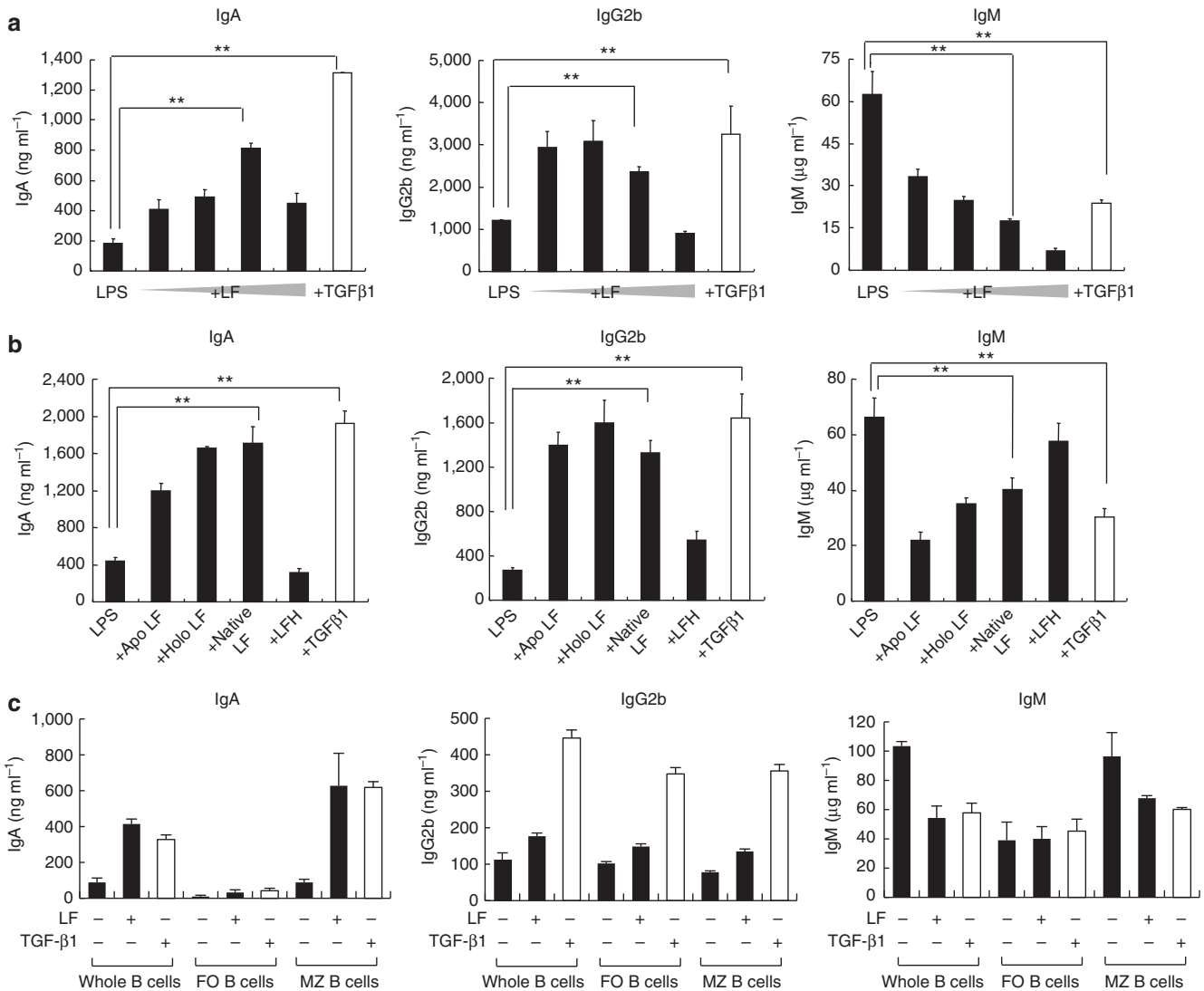
Figure 1 Native LF stimulates mouse splenic B cells to produce IgA and IgG2b. Normal splenic B cells (2×10^5) were cultured with (**a**) LPS ($12.5 \mu g\,ml^{-1}$), native LF (15, 30, 60, and $120\,\mu g\,ml^{-1}$), or TGF-β1 ($0.2\,ng\,ml^{-1}$), as well as (**b**) $60\,\mu g\,ml^{-1}$ of apo LF, holo LF, native LF, and LFH. (**c**) Whole splenic B cells (B220$^+$CD43$^-$), follicular B cells (B220$^+$CD43$^-$CD23^{high}), and marginal zone B cells (B220$^+$CD43$^-$CD23^{low}) were stimulated with LPS ($12.5\,\mu g\,ml^{-1}$), native LF ($60\,\mu g\,ml^{-1}$), and TGF-β1 ($0.2\,ng\,ml^{-1}$). After 7 days of culture, supernatants were collected and Ig production was determined by isotype-specific ELISA. Data represent the mean of triplicate samples ± s.e.m. **, $P<0.01$. LF, lactoferrin; LFH, LF hydrolysate; LPS, lipopolysaccharide; TGF, transforming growth factor.

number of IgA-secreting B cells per clone. This effect of LF was quite similar to that of TGF-β1. These data document the activity of LF as an IgA switch factor.

This result was further confirmed by analyzing Ig germ-line transcription. When Ig isotype switching occurs, transcription of an unrearranged C_H gene produces the corresponding germ-line transcripts (GLTs) and continues to be active and generates post-switch transcripts (PSTs) and circle transcripts (**Figure 3e**).[20] Studies have shown that TGF-β1 induces IgA and IgG2b transcripts including GLTs, PSTs, and circle transcripts.[3,4] As shown in **Figure 3f**, LF increased the levels of GLTα/GLTγ2b but not GLTγ1/GLTγ3. Similar to IgA and IgG2b secretion shown in **Figure 2**, pretreatment with anti-pan TGF-β Ab had little effect on the increased expression of GLTα

and GLTγ2b by LF. In contrast, pretreatment with anti-LF Ab virtually abolished LF-induced GLTα/PSTα/GLTγ2b expression (**Figure 3g**). However, LF did not enhance the expression of AID, an essential enzyme for Ig CSR,[21,22] even in the suboptimal doses of LPS (**Supplementary Figure 4**). These results clearly indicate that LF selectively causes IgA and IgG2b isotype switching at the genetic level.

### Smad3 mediates LF-induced GLTα expression and IgA production

We and others[9,23] have shown that Smad3/4 and Runx3 mediate TGF-β1-induced GLTα expression leading to IgA production. As shown earlier, LF and TGF-β1 revealed similar activity for IgA and IgG2b expression. Therefore,
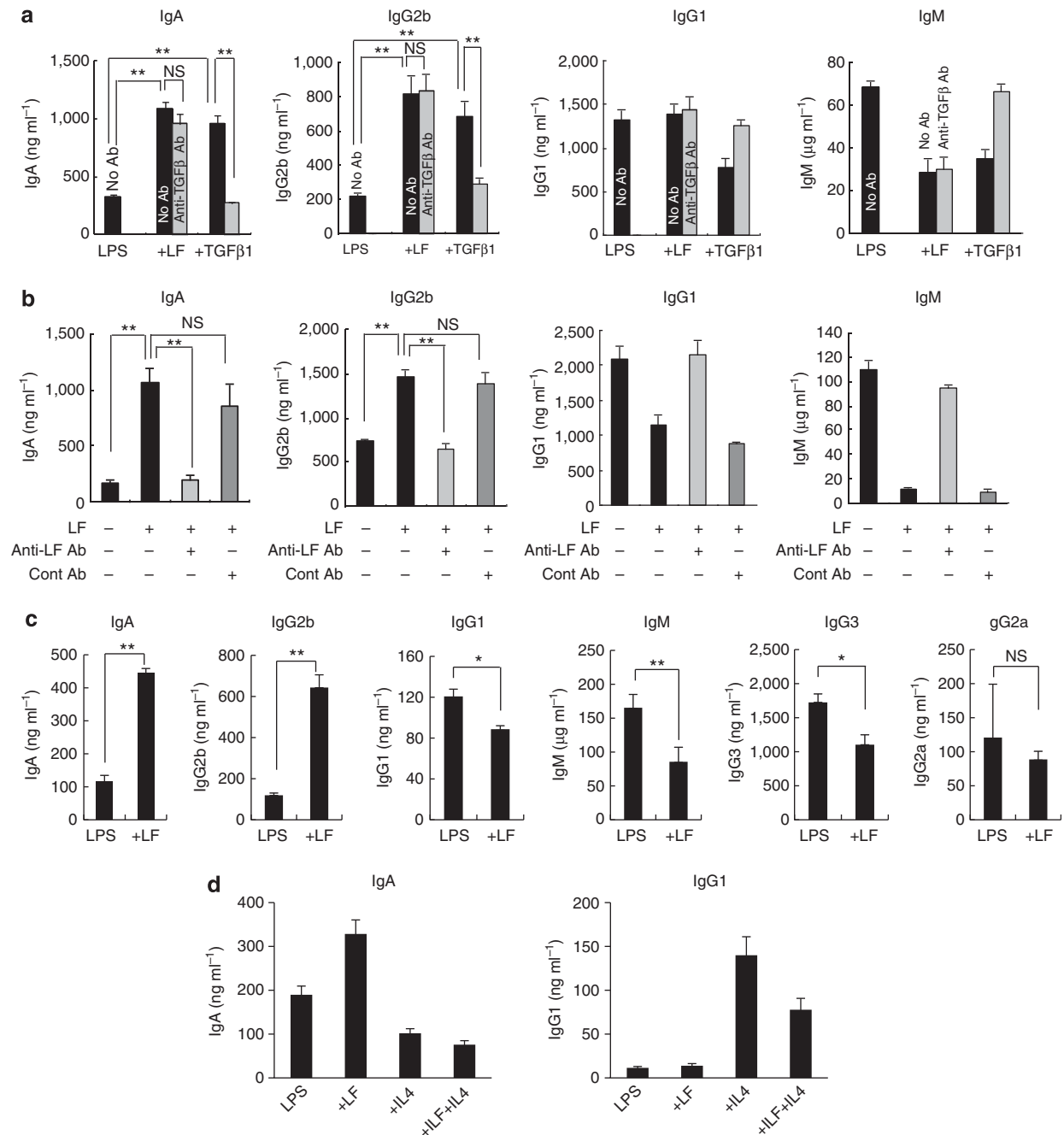
Figure 2 Effect of LF on Ig production is not associated with TGF-β1. (**a**) Normal mIgA⁻ splenic B cells were cultured with LPS (12.5 μg ml⁻¹), LF (60 μg ml⁻¹), or TGF-β1 (0.2 ng ml⁻¹) after pretreatment with a neutralizing anti-pan-TGF-β Ab (5 μg ml⁻¹) for 1 h. (**b**) Normal IgA⁻ splenic B cells were cultured with LPS (12.5 μg ml⁻¹) or LF (60 μg ml⁻¹) after treatment with anti-LF serum (1:8) to neutralize LF. Normal goat serum (1:8) was used as a control. (**c**) CD43⁻ resting B cells were cultured with LPS (12.5 μg ml⁻¹) and LF (60 μg ml⁻¹). (**d**) Culture conditions were the same as in panel (**a**). IL-4 (10 ng ml⁻¹) was added to LPS-stimulated B cells. After 7 days of culture, supernatants were collected and Ig production was determined by isotype-specific ELISA. Data represent the mean of triplicate samples ± s.e.m. *, $P < 0.05$; **, $P < 0.01$. LF, lactoferrin; LPS, lipopolysaccharide; N.S, not significant; TGF, transforming growth factor.

we hypothesized that Smad3 and Smad4 may be involved in LF-induced IgA expression. Indeed, LF stimulated B cells to phosphorylate Smad3 (**Figure 4a**) and promoted its nuclear translocation from the cytoplasm (**Figure 4b**). In addition, LF increased GLα promoter activity. This increase was further augmented by the overexpression of Smad3/4 (**Figure 4c**) but abrogated by overexpression of a dominant negative form of Smad3 (**Figure 4d**). Consistently, LF-induced GLTα expression and IgA production were abolished by knockdown of Smad3 using retroviruses encoding Smad3 shRNA (**Figure 4e**).
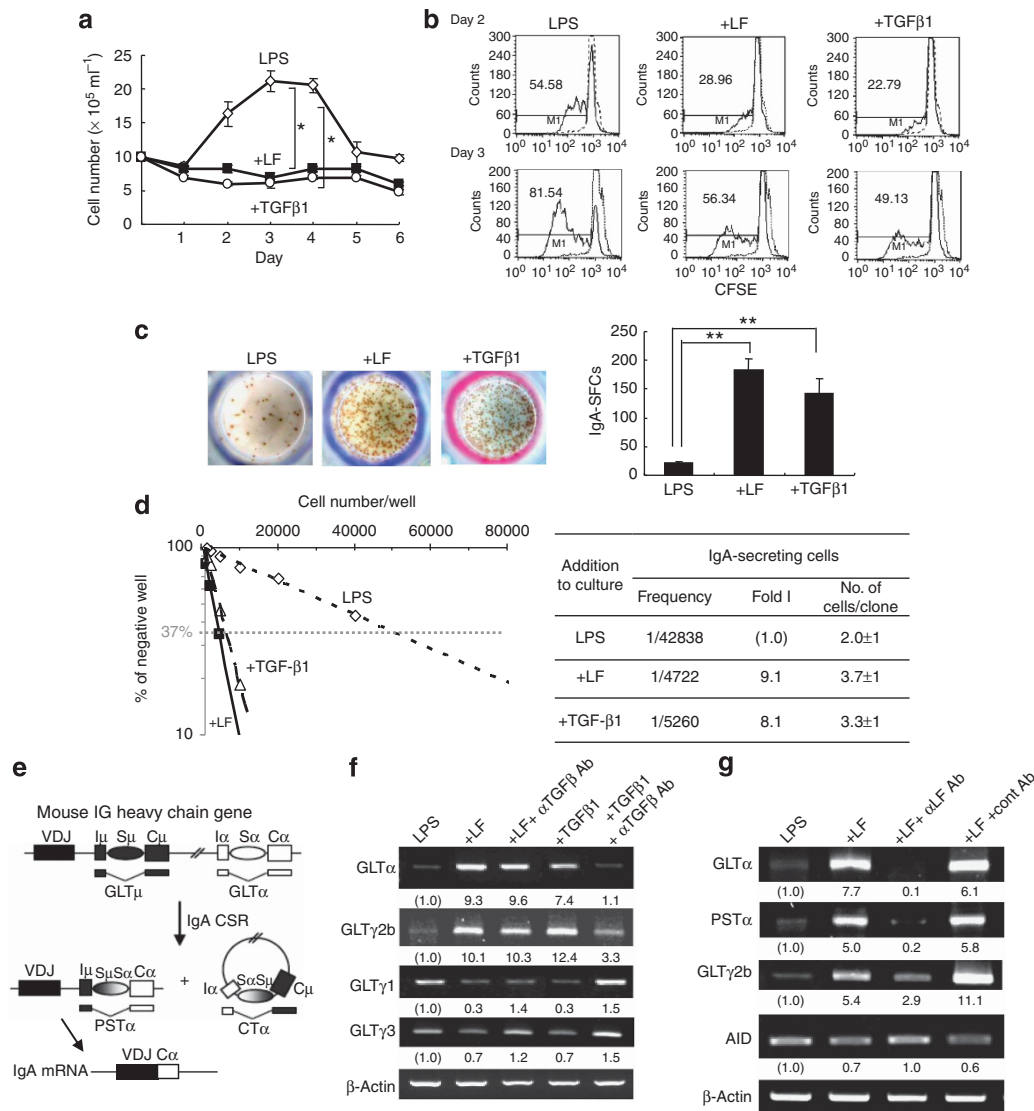
Figure 3 LF induces IgA production at the clonal and transcriptional level. (**a**) Effects of LF on the growth kinetics of splenic B cells. Normal splenic B cells (2×10^5) were cultured with LPS (12.5 µg ml^{-1}), LF (60 µg ml^{-1}), or TGF-β1 (0.2 ng ml^{-1}). Numbers of viable cells were enumerated by trypan blue exclusion. Data represent the mean ± s.e.m. (vertical bars) of triplicate culture wells. (**b**) Isolated splenic B cells were labeled with a CFSE kit and cultured as in (**a**). B-cell proliferation was assessed after 48 and 72 h by analyzing CFSE dilution in the same number of viable cells by flow cytometric analysis. Dotted line indicates CFSE-labeled B cells at day 0. (**c**) Effects of LF on IgA secretion at the clonal level. LPS-stimulated mIgA$^-$ spleen B cells were cultured for 6 days with LF (60 µg ml^{-1}) or TGF-β1 (0.2 ng ml^{-1}). The number of IgA-secreting cells was determined by ELISPOT assay. (**d**) For limiting dilution analysis, LPS-stimulated mIgA$^-$ splenic B cells were seeded into 96-well plates at various cell densities and measured SFCs. Culture conditions were the same as (**a**), and the presence or absence of IgA-secreting cells in culture wells, in addition to the number of IgA-secreting cells in each culture well, were determined by ELISPOT assay. (**e**) A diagram for DNA recombination occurring during IgA switching. Rectangles and ovals represent exons and S regions, respectively. RNA transcripts are indicated beneath the DNA diagrams. (**f** and **g**) Freshly isolated mIgA$^-$ splenic B cells were treated as in **Figures 2a** (**f**) or **Figures 2b** (**g**). After 2 days of culture, total RNA was isolated and levels of GLTs, PSTα AID, and β-actin were measured by RT-PCR. *, $P < 0.05$; **, $P < 0.01$. Fold I, Fold increase; GLTs, germ-line transcripts; LF, lactoferrin; LPS, lipopolysaccharide; PSTα, α post-switch transcript; SFC, spot-forming cells; TGF, transforming growth factor.

Likewise, LF-induced GLTγ2b expression and IgG2b production were reduced (**Supplementary Figure 5**). To gain further evidence for Smad3 involvement in LF-induced GLTα expression, we examined the effect of LF on the promoter activity of a GLα reporter possessing a mutation in the Smad binding element (pGLα(mSBE)).[24] Unlike the WT GLα promoter, LF did not enhance the promoter activity of pGLα(mSBE) (**Figure 4f**), implying that the Smad-binding element (SBE) is essential for LF-inducible GLα promoter activity. This result prompted us to examine by EMSA the binding of nuclear proteins from LF-treated B cells to a probe containing the SBE. LF treatment enhanced the formation of DNA–protein complexes. These complexes were eliminated by preincubation with an excess of unlabeled specific probe containing the SBE sequence but not with an excess of unlabeled non-specific AP-1 probe (**Figure 4g**). Taken together, these
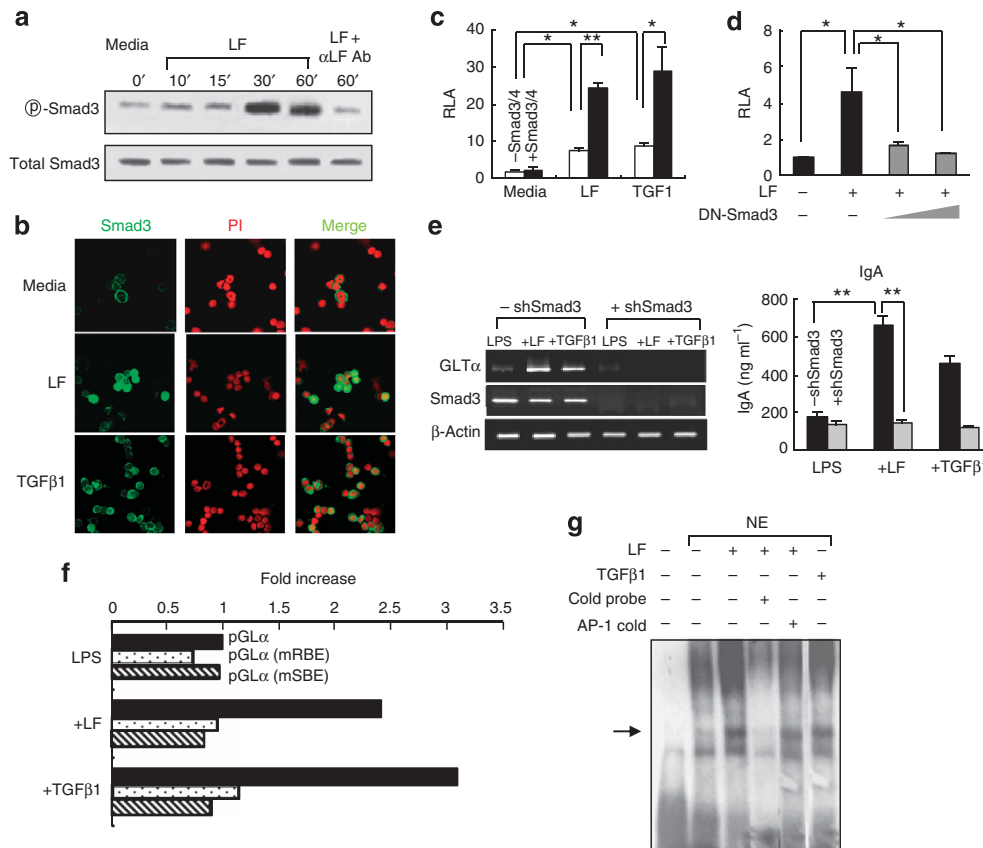
Figure 4 LF induces GLα transcription through a Smad3-dependent signal pathway. (**a**) Normal splenic B cells were stimulated with LF for the indicated times. The anti-LF treatment was the same as in **Figure 2b**. Phosphorylated Smad3 (℗-Smad3) and total Smad3 were examined by western blot analysis. (**b**) Normal splenic B cells were treated with LF (60 μg ml^{-1}) or TGF-β1 (0.2 ng ml^{-1}) for 2 h and then stained with anti-Smad3, anti-mouse IgG-FITC, and PI. The cellular distribution of Smad3 was determined by immunocytochemistry. (**c** and **d**) CH12F3-2A B lymphoma cells were transfected with expression vectors for Smad3/4 (2 μg each, **c**) or DN-Smad3 (10 μg or 20 μg, **d**) and GLα promoter (10 μg) prior to treatment with LF (60 μg ml^{-1}) and TGF-β1 (0.2 ng ml^{-1}) for 16 h. Promoter activity were determined by luciferase activity. (**e**) Smad3 knockdown abrogates LF-induced IgA expression. Normal splenic B cells were transduced with control or Smad3-specific shRNA retroviruses, and then cultured with LF (60 μg ml^{-1}) or TGF-β1 (0.2 ng ml^{-1}). After 7 days of culture, supernatants were collected and IgA production was determined by isotype-specific ELISA (right panel). After 2 days of culture, total RNA was isolated, and levels of GLTα, Smad3, and β-actin were measured by RT-PCR (left panel). (**f**) Functional analysis of RBE and SBE in LF-induced S-GLα promoter activity. For the luciferase assay, CH12F3-2A B lymphoma cells were transfected with 15 μg each of vectors expressing S-GLα (WT), S-GLα (mRBE), or S-GLα (mSBE). Cells were then stimulated with LF (60 μg ml^{-1}) or TGF-β1 (0.2 ng ml^{-1}), and luciferase activity were measured 16 h later. (**g**) EMSA reveals LF-induced nuclear protein complex formation at the SBE of the GLα promoter. Nuclear extracts from normal splenic B cells was prepared after 16 h incubation with LF (60 μg ml^{-1}) or TGF-β1 (0.2 ng ml^{-1}). *, $P < 0.05$; **, $P < 0.01$. LF, lactoferrin; RBE, Runx-binding element; SBE, Smad-binding element; TGF, transforming growth factor.

results consistently indicate that Smad3 mediates LF-induced GLα transcription leading to IgA expression.

LF induces IgA isotype switching through binding to betaglycan (TGF-β type III receptor, TβRIII)

Our findings reveal that LF, like TGF-β activates the canonical Smad3-dependent signaling pathway. Thus, it was important to determine its major receptor in the context of IgA B-cell commitment. Upon binding TGF-β1, the TGF-β type II receptor (TβRII) recruits and activates the TGF-β type I receptor (TβRI), resulting in the phosphorylation of TβRI.[25] We first examined whether TβRII is involved in LF signaling by blocking this receptor with anti-TβRII Ab. Our data demonstrate that the anti-TβRII Ab eliminated LF-induced IgA production as well as TGFβ1-induced IgA production

(**Figure 5a**). In contrast, the pretreatment of LF with soluble TβRII (sTβRII) as a decoy receptor did not abrogate LF-induced IgA production (**Figure 5b**). As expected, pretreatment of TGF-β1 with sTβRII virtually abolished TGFβ1-induced IgA production. These results suggest that LF requires TβRII in its signal transduction without binding to TβRII itself.

TβRIII (also called betaglycan) is a non-signaling coreceptor for TGF-β[26] and possesses two glycosaminoglycan attachment sites for binding heparan sulfate and chondroitin sulfate domains.[27] As LF is known to bind heparan sulfate chains of proteoglycan,[28] we hypothesized that LF may also bind TβRIII. This possibility was assessed using soluble TβRIII (sTβRIII). Pretreatment of LF with sTβRIII eliminated LF-induced IgA/IgG2b production (**Figure 5c** and **Supplementary Figure 6C**), indicating that LF triggers canonical Smad3-dependent
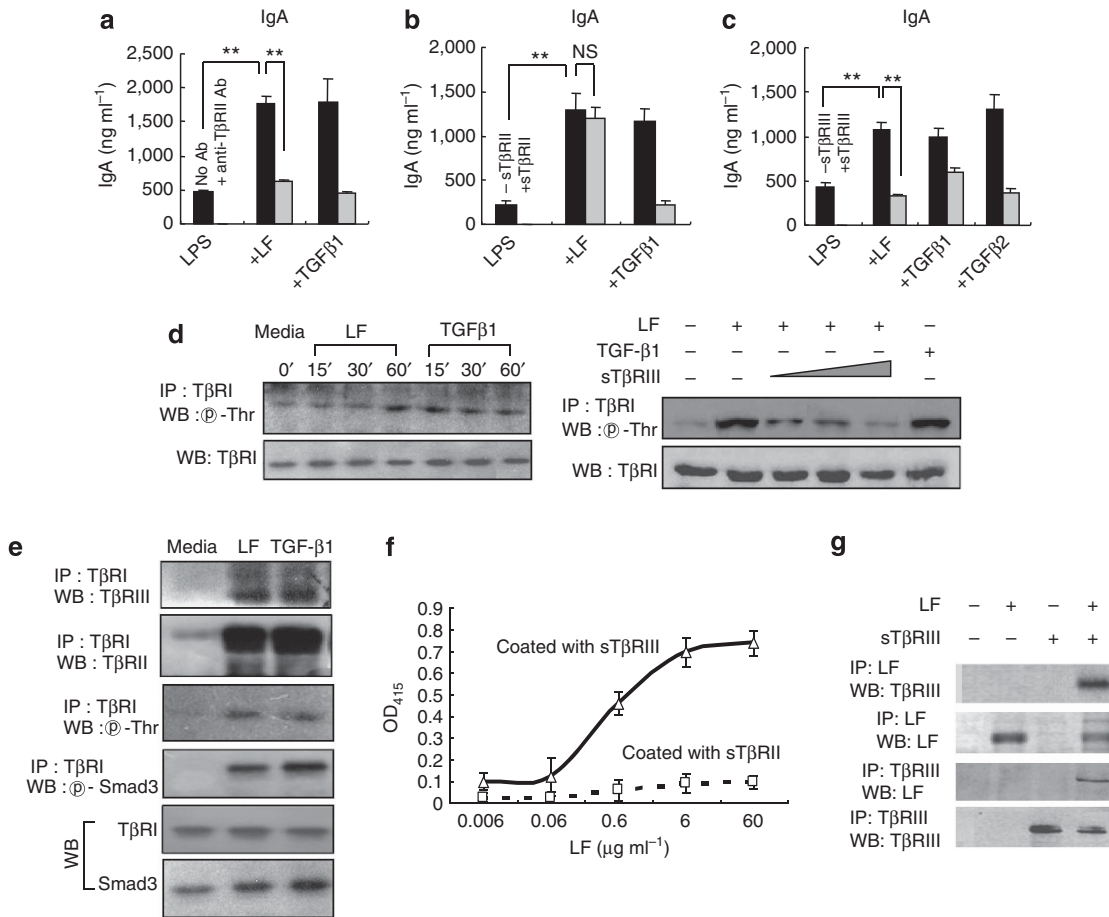
Figure 5 LF binds to betaglycan and activates the canonical TGF-β1 pathway for IgA isotype switching. (a–c) Splenic mIgA$^-$ B cells were cultured with LF (60 μg ml^{-1}) after pre-incubation with TβRII Ab (10 μg ml^{-1}) (a), soluble TβRII (50 ng ml^{-1}) (b), or soluble TβRIII (1 μg ml^{-1}) (c). Cells were treated with 0.2 ng ml^{-1} TGF-β1 (0.2 ng ml^{-1}) or TGF-β2 (1 ng ml^{-1}) as controls. IgA production was determined by isotype-specific ELISA after 7 days of culture. B cells were treated with LF (60 μg ml^{-1}) or TGF-β1 (0.2 ng ml^{-1}) for the indicated times (d) and 1 h (e) and then subjected to immunoprecipitation followed by western blot analysis as indicated. Cells were treated with 0.25, 0.5, or 1 μg ml^{-1} soluble TβRIII (d, right panel). (f and g) The direct interaction between LF and TβRIII was determined by receptor ELISA (f) and immunoprecipitation (g). **, $P < 0.01$. LF, lactoferrin; N.S, not significant; TGF, transforming growth factor.

signaling by binding TβRIII. This activity of LF is similar to that of TGF-β2, which binds TβRIII as the major TGF-β receptor. The TGF-β2:TβRIII complex consecutively recruits TβRII and TβRI.[29] As such, we assessed whether LF activates TβRI. LF induced TβRI phosphorylation within 1 h. This activation was abrogated by treatment of sTβRIII in a dose-dependent manner (**Figure 5d**). In addition, LF induced TβRI:TβRIII and TβRI: TβRII coupling (**Figure 5e**). Under the same conditions, TβRI and Smad3 were phosphorylated. These results reveal that LF signaling actually initiates TβRIII:TβRII:TβRI complex formation. Indeed, ELISA showed that LF binds specifically to TβRIII but not TβRII (**Figure 5f**). Physical interaction between LF and TβRIII was further confirmed by reciprocal immunoprecipitation and western blot analysis for the two proteins as shown in **Figure 5g**. Together, these results indicate that LF binds primarily to TβRIII, recruits TβRII and TβRI successively, and activates Smad3 to induce IgA/IgG2b class switching.

LF exhibits a potent mucosal adjuvant activity

To determine the role of LF in Ig production *in vivo*, LF was administered into mice perorally and ELISA was performed to measure Ig synthesis. Administration of LF resulted in higher levels of IgA in fecal pellets and IgA/IgG2b in sera along with decreased IgM (**Figure 6a**). Consistent with our observation, peroral administration of LF significantly increased IgA/IgG2b production by PP B cells (**Figure 6b** and **Supplementary Figure 7A**) and also IgA-bearing plasma cells (B220$^-$, intracellular IgA$^+$) in the lamina propria (**Figure 6c**). To test the adjuvant effect of LF on Ag-specific Ig production, mice were pre-administered with LF perorally three times a week for 3 weeks and then inoculated intragastrically with 10^8 CFU of non-toxigenic *S. typhimurium* and LF on days 8 and 15. *S. typhimurium*-specific Igs were measured by ELISA on day 21. LF treatment significantly enhanced the level of Ag-specific IgA, IgG2b, and IgG1 in fecal pellets and sera (**Figure 6d** and **Supplementary Figure 7B**). We also tested the protective effect
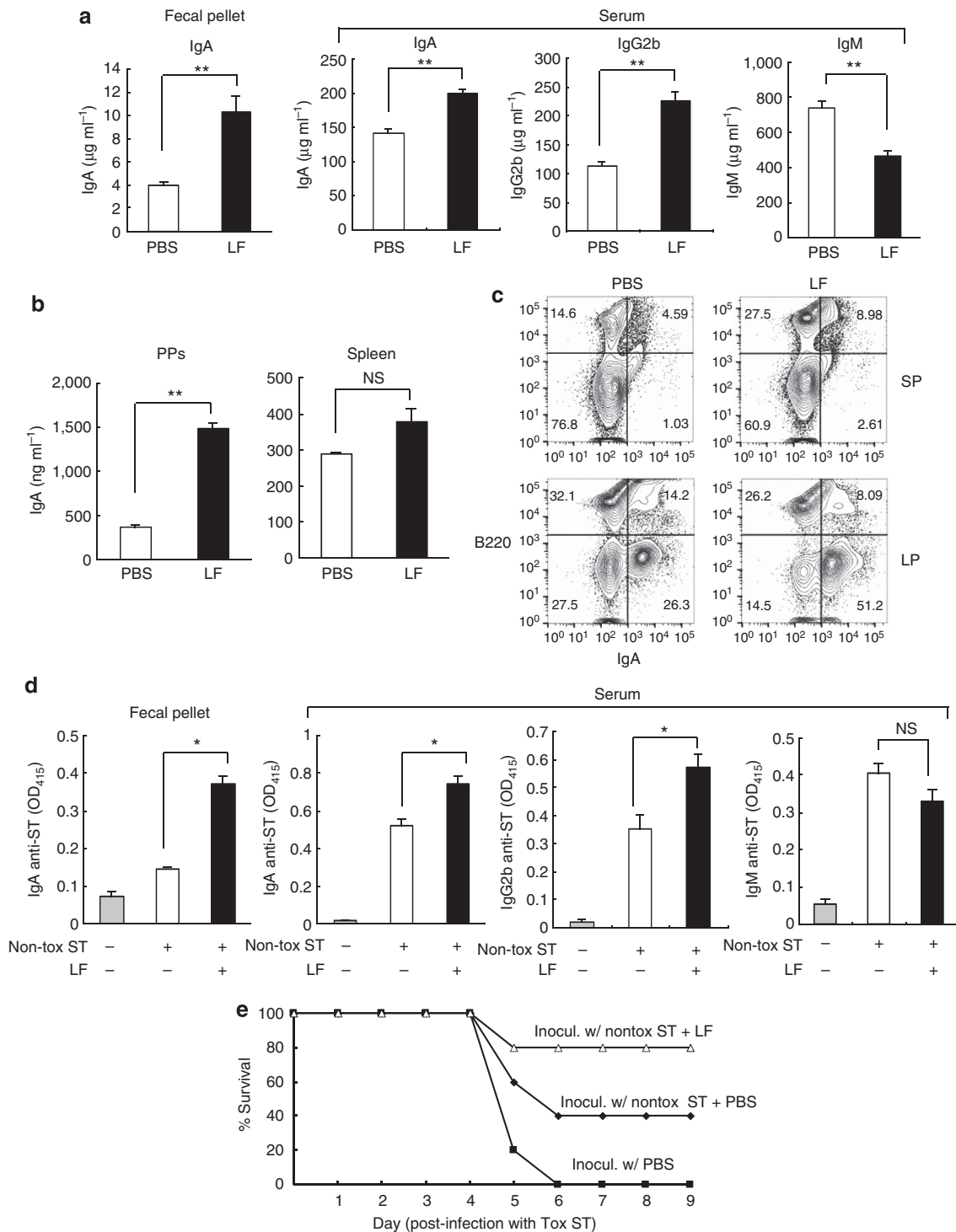
Figure 6 *In vivo* effect of LF on Ig production. (**a**) Effect of peroral administration of LF on intestinal and systemic Ig production. BALB/c mice were perorally administered with LF (5 mg kg^{-1}) in PBS (100 μl) three times a week for 10 weeks. Fecal pellets and sera were collected, pooled, and assayed in triplicate using isotype-specific ELISA. (**b**) PP, and splenic B cells from the treated mice were isolated, and splenic B cells were stimulated with LPS (12.5 μg ml^{-1}). After 7 days of culture, supernatants were collected and Ig production was determined by isotype-specific ELISA. (**c**) Effect of peroral administration of LF on the expression of surface B220 and intracellular IgA in SP and LP. Mice were treated as in (**a**). After 15 weeks later, SP and LP cells were isolated and analyzed for surface B220 and intracellular IgA expression by FACS. (**d**) Effect of peroral administration of LF on *S. typhimurium*-specific IgA production by B cells. BALB/c mice were perorally administered with LF (5 mg kg^{-1}) in PBS (100 μl) three times a week for 2 weeks. On days 7 and 14 of LF treatment, mice were intragastrically infected with 10^8 CFU of attenuated *S. typhimurium*. On day 21, fecal pellets and sera were collected, pooled, and *S. typhimurium*-specific IgA production was determined by modified isotype-specific ELISA. Data are expressed as absorbance values measured at 415 nm. (**e**) At day 14, three groups of five mice were treated as in (**d**). On day 21, mice were intragastrically infected with 10^8 CFU of toxigenic *S. typhimurium*. Mortality was recorded daily up to 9 days post infection. *, $P<0.05$; **, $P<0.01$. LF, lactoferrin; LP, lamina propria; LPS, lipopolysaccharide; N.S, not significant; PBS, phosphate-buffered saline; SP, spleen.

of LF against toxigenic *S. typhimurium* infection. Mice were first inoculated with non-toxigenic *S. typhimurium* with or without LF, then infected with toxigenic *S. typhimurium* intragastrically (**Figure 6e**). Inoculation with non-toxigenic *S. typhimurium* plus LF significantly enhanced the survival of infected mice compared with those administered non-toxigenic *S. typhimurium* alone. These findings indicate that LF exhibits mucosal adjuvant activity by protecting against intestinal infection.

DISCUSSION

The present study demonstrates that LF binds TβRIII and activates the canonical TGF-β1 pathway to induce IgA and IgG2b isotype switching. LF stimulates mouse B cells to produce IgA and IgG2b, and this LF activity is equivalent to that of TGF-β1. TGF-β1 induces IgA and IgG2b isotype switching by increasing the frequency of precursors of IgA-producing cells at the clonal level[5] and inducing GLTα and GLTγ2b expression.[3,4] Studies have shown that Smad3/4 and Runx3 mediate TGF-β1-induced GLα promoter activity while p300 cooperates with Smad3/4 and Runx3 to activate the GLα promoter.[30] In addition, deletion of TGF-β1 or TGF-β receptor type II results in severe loss of IgA expression.[6,7] Therefore, TGF-β1 is regarded as a physiological mediator of IgA and IgG2b isotype switching in mice. In the present study, LF causes IgA CSR as demonstrated by the observed increase in both GLTα/PSTα expression and IgA clonal frequency. This property of LF is thought to enable the enhancement of IgA production *in vitro* and *in vivo*. The same is also true for IgG2b expression because LF increases GLTγ2b transcription and IgG2b secretion *in vitro* and *in vivo*. However, whether LF actually increases IgG2b clonal frequency has yet to be determined. In addition, LF induces GLα promoter activity via a Smad3/4- and Runx3-dependent pathway. Taken together, our data strongly suggest that LF is an important mediator for IgA and IgG2b isotype switching comparable with TGF-β1. Furthermore, the anti-proliferative activity of LF (**Figure 3a** and **b**) contributes to IgA-mediated B-cell differentiation. Consistently, human LF inhibits the LPS-TLR4 signaling pathway by binding LPS and soluble CD14.[31] In addition, LF interacts with CpG-containing oligodeoxynucleotides and inhibits the effects of oligodeoxynucleotide on human B cells.[32] We demonstrated that a synthetic CpG-oligodeoxynucleotide, M6-395, can act as a murine polyclonal activator even though its strong mitogenic activity is unfavorable for Ig synthesis.[33] Thus, it is likely that LF partially contributes to Ig CSR by inhibiting the B-cell mitogenic activities of LPS and CpG similar to the anti-proliferative activity of TGF-β that facilitates IgA CSR to increase IgA production.[5]

The present study demonstrates that LF activates the Smad-dependent pathway to induce IgA isotype switching. More importantly, we report for the first time that this occurs through binding of LF to TβRIII (betaglycan). This seems to be the case because the TβRIII has glycosaminoglycan side chains. The extracellular domain of TβRIII contains two glycosami-noglycan attachment sites for binding heparan sulfate and

chondroitin sulfate.[27] As LF binds to the heparan sulfate chains of proteoglycan[28,34] and fibroblast growth factor binds to the heparin sulfate chains of TβRIII,[35] it is plausible that LF binds to the heparin sulfate chains of TβRIII and stimulates canonical TGF-β signaling to induce IgA and IgG2b CSR. TβRIII is traditionally thought to function by binding TGF-β2 via its core protein and then transferring the growth factor to its signaling receptor, TβRII, to enhance TGF-β signaling.[36] On the other hand, TβRIII can act negatively on TGF-β signaling. In some cell lines, the type and number of glycosaminoglycan chains attached to the TβRIII core protein sterically prevents the access of TGF-β to the type I and II receptors, thereby preventing its downstream signaling.[37] However, because LF enhances Smad-dependent signaling, our data demonstrate that LF binding to TβRIII must be favorable for subsequent signaling involving TβRII/TβRI/Smad, similar to TGF-β2. In fact, there is evidence that LF activates Smad through TβRII and TβRI. LF inhibits proliferation of Mv1Lu cells by increasing Smad2 nuclear translocation. This anti-proliferative activity is eliminated in TβRI- or TβRII-deficient Mv1Lu cells.[38] These results strongly suggest that TβRI and TβRII are required for LF-induced Smad activation even though the receptor for LF binding in Mv1Lu cells was not identified. Other putative LF receptors on immune cells include low-density lipoprotein receptor-related protein (LRP), proteoglycan, and surface nucleolin.[15] We were especially interested in LRP because LRP-1 is identical to TβRV[39] and critical for TGFβ1-mediated growth inhibition of CHO cells.[40] Further, it has been shown that LRP-1 mediates LF-induced activation of p38 and ERK1/2, as well as Smad2 nuclear translocation.[41] LRP-1 transcripts were detected in macrophages but not in mouse B cells (**Supplementary Figure 8**), suggesting that LRP-1 is not involved in LF-induced Ig CSR in mouse B cells. Altogether, our results reveal that TβRIII is mainly responsible for LF-stimulated IgA and IgG2b isotype switching. Establishment of a B-cell-specific TβRIII knockout mouse model will be necessary to determine the role of TβRIII in LF-induced isotype switching, as ablation of whole TβRIII expression in mice is embryonic lethal.[42]

The present study clearly shows that exogenously delivered LF strongly increases total mucosal IgA production in mice. Moreover, LF treatment further enhanced the *S. typhimurium*-specific IgA response and conferred better protection in mice against infection by pathogenic *Salmonella*. This demonstrates that exogenous LF can effectively stimulate a protective IgA response to *Salmonella* infection. We note that the present study does not address the role of endogenous LF in mucosal IgA production. Nevertheless, studies have demonstrated that LF can affect IgA production in the mucus.[43] LF is constitutively expressed as an iron-free form from epithelial cells of the developing digestive and respiratory tracts and, under the control of prolactin, a substantial amount of sIgA and LF is produced in the mammary gland.[44] These observations show that LF may stimulate B cells to produce sIgA in mucosal secretions. Neutrophils are the major source of LF during inflammation.[45] However, even in the absence of infection or

the inflammatory response, neutrophils reside in the peri-MZ area of the spleen to activate MZ B cells to induce Ig class switching, somatic hypermutation, and antibody production.[46] In this regard, it is feasible that neutrophil-derived LF in MALT can potentiate IgA CSR.

In conclusion, the current findings suggest that LF signals through a mechanism involving TβRIII, TβRII, TβRI, and Smad3, leading to IgA isotype switching. Further, because LF strongly enhances mucosal IgA response, and it is synthesized endogenously, it may be considered an effective and safe mucosal adjuvant.

METHODS

Animals. BALB/c mice were purchased from the Daehan Biolink. Co. (Seoul, Korea). Animals were fed Purina Laboratory Rodent Chow 5001 (Daehan Biolink, Seoul, Korea) *ad libitum*. Animal care was performed in accordance with the institutional guidelines set forth by Kangwon National University.

Cell preparation and reagents. Murine splenic B-cell suspensions were prepared as described previously.[47] This resulted in the removal of approximately 60% of total spleen cells with B cells comprising more than 90% of the residual population as assessed by the presence of surface Ig using flow cytometric analysis. mIgA$^-$ B cells were prepared using anti-mouse IgA Ab-coated tissue culture dish panning. This procedure resulted in greater than 95% depletion of mIgA$^+$ cells. To prepare splenic FO and MZ B cells, CD43$^-$ splenic B cells were stained with anti-CD23-conjugated microbeads for 15 min at 4 °C and sorted by MACS into the following populations: FO B cells, B220$^+$ IgM^{low}CD23^{high}; MZ B cells, B220$^+$ IgM^{high}CD23^{low}.[48] The murine B-cell lymphoma line, CH12F3-2A (surface μ +), was provided by Dr T. Honjo (Kyoto University, Japan). PP cells were prepared as described previously[49] and MLN cells were separated from intestinal fatty tissues using two forceps in a petri dish containing phosphate-buffered saline. MLN cells were isolated and harvested by centrifugation at 500 × g for 5 min. Cells were washed twice with HBSS and suspended in complete medium.

Bovine LF was supplied by Morinaga Milk Co., Ltd. (Zama, Japan), which contains less than 5.0 pg mg^{-g} of LPS (endotoxin).[50] Anti-bovine LF antiserum was purchased from Bethyl Laboratories (Montgomery, TX). Recombinant human TGF-β1, porcine TGF-β2, soluble TβRII, soluble TβRIII, TGF-β pan-specific Ab, anti-TβRIII Ab, and anti-TβRII Ab were purchased from R&D Systems (Minneapolis, MN).

Mammalian expression vectors for Smad3 and Smad4 were generously provided by Dr Masahiro Kawabata. The DN-Smad3 plasmid was provided by Dr M. Kato (Department of Biochemistry, The Cancer Institute, Tokyo, Japan). GLα promoter reporters for − 130 to + 14, − 448 to + 72 (S-GLα and M-GLα), and Smad3/Runx3 binding element-substituted S-GLα reporters were described previously.[24]

ELISA and ELISPOT assay. Isotype-specific ELISAs were performed as described previously.[9] The reaction products were measured at 405 nm with an ELISA reader (VERSAMAX reader, Molecular Devices, Sunnyvale, CA). To detect Ab present in the gut, fecal pellets were diluted in phosphate-buffered saline and centrifuged at 10,000 × g for 10 min before supernatants were collected. To detect IgA, IgG2b, or IgM antibodies against *S. typhimurium*, bacterial cells (10^3) were coated onto 96-well microplates and then isotype-specific ELISA was performed. Isotype-specific ELISPOT assay was performed as described previously.[5] Data are presented as the number of spot-forming cells per 2 × 10^5 cultured cells after background subtraction.

Limiting dilution analysis. mIgA$^-$ splenic B cells were cultured at various cell densities, ranging from 10^1 to 10^5 cells/well, in 96-well tissue culture plates (Costar, Cambridge, MA) in 200 μl per well. Forty-eight replicate wells were set up for each cell density. Cultures were stimulated for 6 days, and wells were assayed individually for the number of isotype-specific Ig-secreting cells by ELISPOT assay. Wells containing spot-forming cells were scored as positive. Calculations to determine the frequency of B-cell precursors that develop into IgA-secreting B cells were based on the Poisson distribution analysis described previously.[5]

Determination of direct interaction between LF and TβRIII by ELISA and immunoprecipitation. For the determination by ELISA, 2 μg ml^{-1} of soluble TβRIII or soluble TβRII was coated to microtiter wells. LF (0.006 μg ml^{-1}– 60 μg ml^{-1}) was added as samples and goat anti-LF antiserum (1:500) was used as the capture Ab. The remaining steps were the same as ELISA. For the determination by immuno-precipitation, purified LF (60 μg) and TβRIII (60 μg) were incubated for 1 h. This mixture, LF alone, and TβRIII alone were precipitated with 2 μg ml^{-1} of either anti-LF Ab or anti-TβRIII Ab. Blots were subsequently probed with 2 μg ml^{-1} of either anti-LF Ab or anti-TβRIII Ab.

RNA preparation and RT-PCR. RNA preparation, reverse transcription, and PCR were performed as described previously.[9] PCR primers were synthesized by Bioneer Corp. (Seoul, Korea): GLTα sense, 5′-CAA GAA GGA GAA GGT GAT TCA G-3′ and antisense, 5′-GAG CTG GTG GGA GTG TCA GTG-3′; GLTγ2b sense, 5′-GGG AGA GCA CTG GGC CTT-3′ and antisense, 5′-AGT CAC TGA CTC AGG GAA-3′; PSTα sense, 5′-GAG CTG GTG GGA GTG TCA GTG-3′ and antisense, 5′-CTC TGG CCC TGC TTA TTG TTG-3′; GLTγ1 sense, 5′-CAG CCT GGT GTC AAC TG-3′ and antisense, 5′-CTG TAC ATA TGC AAG GCT-3′; TβRIII sense, 5′-GCCAGACGGC TACGAAGATTT-3′, and antisense 5-AACACTACCACTCCA GC ACGG-3′; β-actin sense, 5′-CATGT TTGAG ACCTT CAACA CC CC-3′ and antisense, 5′-GCCAT CTCCT GCTCG AAGTC TAG-3′. All reagents for RT-PCR were purchased from Promega (Madison, WI). PCR reactions for β-actin were performed in parallel to normalize cDNA concentrations within each set of samples. Band intensities were quantified using Scion Image software (Scion Corp., Frederick, MD).

Transfection and luciferase assays. Transfection was performed by electroporation with a Gene Pulser II (Bio-Rad, Hercules, CA) as described.[8,9] Reporter plasmids were cotransfected with expression plasmids and pCMVβgal (Stratagene, La Jolla, CA), and luciferase and β-gal assays were performed as described.[8,9]

Retroviral transduction. The retroviral vector pRetrosuper-GFP Smad3 (Addgene plasmid 15723) was purchased from Addgene (Cambridge, MA). pRetrosuper-GFP Smad3 was co-transfected with pVPack vectors (Stratagene) into 293T cells using the CaCl$_2$ method to produce retroviral particles. Retrovirus-containing supernatants were collected 48 h after transfection, passed through a 0.45 μm filter, and then concentrated by centrifugation at 27,000 × g for 2 h. Mouse splenic B cells (8 × 10^6 cells) were resuspended in 4 ml of retroviral supernatant containing 6 μg ml^{-1} polybrene. One milliliter of cells (2 × 10^6 cells) was added to each well of a 24-well flat-bottom tissue culture plate and then spun for 90 min at 1,000 × g at 32 °C. After centrifugation, cells were resuspended in their wells and then 1 ml of fresh complete medium was added. After incubation for 16 h, the transduced cells were plated for various assays.

Immunoprecipitation and western blotting. Normal splenic B cells were stimulated with LF or TGF-β1, and then collected, lysed, and subjected to immunoprecipitation with an anti-TGF-β receptor I Ab (Cell Signaling Technology, Danvers, MA) using protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). For western blot analysis, total cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to PVDF membranes (Bio-Rad). Specific immuno-

detection was carried out by incubation with the primary Ab followed by peroxidase-conjugated goat anti-rabbit IgG secondary Ab (Pierce, Rockford, IL). Bands were visualized by chemiluminescence (Supersignal detection kit, Pierce).

Confocal microscopy. CH12F3-2A cells were incubated with LF or TGF-β1 for 2 h, and then fixed in 4% paraformaldehyde. The cells were blocked and permeabilized with PBS containing 10% FBS and 0.1% Triton X-100. Cells were cytospun and the slides were incubated with anti-Smad3 Ab (Cell Signaling Technology) and then stained with anti-mouse IgG-FITC (Southern Biotech, Birmingham, AL) and propidium iodide for nuclear staining. The relative distribution of fluorochromes was visualized and scanned using a Fluoview FV1000 confocal laser microscope (Olympus, Tokyo, Japan).

EMSA. For detection of Smad binding to the SBE, a nonradioactive EMSA kit was used according to the manufacturer's instructions (Panomics, Fremont, CA). Nuclear extracts were prepared using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA), and then incubated with biotinylated oligonucleotide containing the SBE of the GLα promoter (5′-CACAGCCAGACCACAGGCCAGA CATGACGT-3′). The biotinylated nucleotides were detected using alkaline phosphatase-conjugated streptavidin at a 1:1,000 dilution in $1 \times$ binding buffer (provided) and visualized using the Supersignal detection kit (Pierce) on film.

***Salmonella* infection studies.** Two groups of five BALB/c mice (7 weeks old) were perorally administered with native LF ($5 \, \text{mg kg}^{-1}$) dissolved in 100 μl of phosphate-buffered saline three times a week for 10 weeks. Sera and fecal pellets were collected and pooled and centrifuged at $10,000 \times g$ for 10 min before supernatants were collected for ELISA. To test the adjuvant effect of LF, two groups of five BALB/c mice (7 weeks old) were perorally administered with LF ($5 \, \text{mg kg}^{-1}$) three times a week for 3 weeks. Mice were inoculated orally with a 100 μl dose (10^8 CFU) of the avirulent strain of *S. typhimurium,* FB331 (provided by Dr Hahn Tae-Wook, College of Veterinary Medicine, Kangwon National University) on days 7 and 14 after the first LF treatment. Fecal pellets and sera were collected and pooled on day 21, and *S. typhimurium*-specific IgA production was determined by antigen-specific ELISA. To test the protective effect of LF against infection with *S. typhimurium*, mice were administered with 10^8 CFU of toxigenic *S. typhimurium* (ST198) intragastrically.

Statistical analysis. Statistical differences between experimental groups were determined by analysis of variance. Values of $P < 0.05$ by unpaired two-tailed Student's *t*-test were considered significant.

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

DISCLOSURE
The authors declared no conflict of interest.

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