

Fig. 8. A schematic model of signaling pathways involved in CXCL13 production in stromal cells. Engagement of LTβR by LTα1β2 transmits a signal required for CXCL13 induction through the cooperation of at least three different pathways, including NF-κB canonical, non-canonical and PKCs. TNFR engagement that strongly activates PKCs and NF-κB canonical pathways, but not the non-canonical pathway, is unable to induce CXCL13 expression.

In summary, the maturation of SLO stromal cells mediated by LTβR and/or TNFR is accomplished by the cooperation of multiple signaling pathways, not only NF-κB canonical and non-canonical pathways but also additional signaling components (Fig. 8). PKC activity is a critical mediator in addition to NF-κBs.

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Abbreviation

BIM-1	bisindolylmaleimide-1
cDNA	complementary DNA
CXCL	CXC chemokine ligand
DMSO	dimethyl sulfoxide
IκB	inhibitor of NF-κB
LT	lymphotoxin
LTβR	lymphotoxin-beta receptor

NF-κB	nuclear factor-kappa B
NIK	NF-κB-inducing kinase
PKC	protein kinase C
PS	pseudosubstrates
RIPA	radio immuno protein assay
SLO	secondary lymphoid organ
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor

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Requirement for Runx Proteins in IgA Class Switching Acting Downstream of TGF- β 1 and Retinoic Acid Signaling

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IgA is a specific isotype required for mucosal immunity and is the most abundant Ab produced in vivo. Recently, several inductive signals for IgA class switch recombination have been identified; however, the molecular details of the action of these signals and the specific factors acting in B cells remain elusive. In this study, we show that combination of retinoic acid (RA) and TGF- β 1 with other factors induced a much higher frequency of IgA-switched cells than reported previously. In addition, IgA production is severely impaired in Runx2-Runx3 double-deficient mice. In Runx2-Runx3-deficient B cells, both RA- and TGF- β 1-dependent inductions of α germline transcription are completely blocked. These data suggest that Runx proteins play an essential role in IgA class switching acting downstream of RA and TGF- β 1 signaling. *The Journal of Immunology*, 2010, 184: 2785–2792.

Immunoglobulin A is the most abundantly produced Ig in vivo and is predominantly secreted in the gastrointestinal tract. In addition to protection against mucosal pathogenic microorganisms, neutralizing toxins and protection from the penetration of microorganisms into the epithelium, IgA controls the size and species of appropriate bacterial communities in the intestine (1–3). In germfree mice, the level of IgA is very low but normalizes within a few weeks after intestinal bacterial colonization (1). Thus, IgA production depends on bacterial stimulation. In sensing bacteria, TLR signals on dendritic cells (DCs) play key roles (1, 4, 5). Accordingly, skewed IgA class switch recombination (CSR) takes place in GALTs, called Peyer's patches (PPs) and isolated lymphoid follicles, and the lamina propria (LP) of the gut (1–3, 6, 7).

Previous studies have identified many critical factors governing IgA CSR (1–3, 8, 9). Among them, retinoic acid (RA) and TGF- β 1 are the strongest candidates for region-specific factors because DCs from LP, but not the spleen, have enzymes for the production of RA, called *raldh* (4, 5, 10, 11), and GALT and LP are rich in TGF- β 1.

Accumulating evidence indicates the special role of TGF- β 1 in IgA CSR. TGF- β 1-deficient mice and B cell-specific TGF- β 1 receptor II-deficient mice have low levels of IgA (12, 13). In addition, inducible NO synthase (iNOS)-deficient B cells lose their TGF- β 1 receptor II expression and consequently show a defect in IgA production (4, 5, 10, 11); however, the actual effect of RA on IgA CSR is limited. In vitamin A-deficient mice, reduced IgA-producing cells were observed in the LP of the small intestine, but the level of IgA in the sera was not affected (9). Moreover, the RA effect on IgA CSR was determined mostly under conditions without TGF- β 1 in vitro; therefore, the specific roles of TGF- β 1 and RA in IgA CSR and the relationship between them have not yet been resolved. In this regard, one experiment demonstrated that TGF- β 1 inhibits IgA CSR induced by RA, indicating the antagonistic effect of TGF- β 1 and RA on IgA CSR in some experimental settings (9). Thus, the complicated regulation of IgA CSR in vivo and the mutual antagonizing functions of TGF- β 1 and RA on IgA CSR are suggested (9).

Detailed analysis of TGF- β 1 and bone morphogenetic protein (BMP) signaling revealed that Runx transcription factors act in concert with Smad transcription factors in response to TGF- β family signaling (14). Several researches have demonstrated that Smad2, 3 and Runx3 complex worked on the promoter of α germline transcription (GLT) using reporter and biochemical assays (15–18). Accordingly, Runx3 is believed to work as an activator of IgA CSR (15). In this regard, Groner et al. (19) demonstrated that Runx3-deficient B cells showed impaired IgA CSR in an in vitro class switching assay, whereas the levels of IgA in bronchoalveolar lavage from Runx3-deficient mice were not decreased but instead increased significantly. These observations suggest several possibilities. First, Runx proteins are only essential under specific conditions for the induction of IgA CSR in vitro but not in vivo. In other words, Runx function is not so important in IgA CSR, as previously speculated. Second, if Runx proteins play a significant role downstream of TGF- β 1 signaling in IgA CSR, TGF- β 1 is not the only major signal for IgA CSR in vivo; however, serum and intestinal IgA levels are very low in TGF- β 1-deficient mice, and this possibility will therefore be excluded, rather indicating the existence of Runx-dependent and -independent pathways in TGF- β 1 signaling. Alternatively, TGF- β 1 and Runx proteins act in concert to induce IgA CSR, but Runx proteins are not absolutely required for TGF- β 1 signaling in IgA CSR. Third,

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Abbreviations used in this paper: BMP, bone morphogenetic protein; CSR, class switch recombination; DC, dendritic cell; FO, follicular; GLT, germline transcription; iNOS, inducible NO synthase; LP, lamina propria; LPL, lamina propria lymphocyte; MLN, mesenchymal lymph node; MZ, marginal zone; NF, newly formed; PP, Peyer's patch; RA, retinoic acid; RAR, retinoic acid receptor.

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redundant functions of other Runx proteins compensate for the effect of Runx3 deficiency in IgA CSR *in vivo*. To address these matters, we first tested whether Runx3 influenced the efficiency of IgA CSR *in vitro*; however, we could not confirm the defect of IgA CSR in Runx3-deficient B cells as demonstrated previously. Thus, many investigators have suggested the involvement of Runx3 in IgA CSR by reporter assays, but there is no conclusive evidence that Runx proteins play a key role in IgA CSR.

In this study, we first established an effective induction system for *in vitro* IgA CSR using splenic B cells by only adding soluble factors and found that TGF- β 1 and RA act in synergy to induce IgA CSR. Using this system, the involvement of Runx proteins in IgA CSR was assessed, and it was found that Runx protein will work as a positive regulator of IgA CSR by inducing α GLT. We generated RAG2-deficient mice receiving runx2-runx3 double-deficient fetal liver cells to analyze the functions of these factors in IgA class switching *in vivo*. As expected, RAG2^{-/-} mice bearing Runx2-Runx3-deficient lymphocytes have lower IgA in the sera and intestine. These B cells were further analyzed *in vitro*, and it was found that the defect in IgA production observed in these mice correlates with IgA class switching deficiency. We further demonstrated that Runx2 and Runx3 are involved in both TGF- β 1- and RA-dependent pathways for the induction of α GLT. These data suggest that Runx proteins are required to sense extracellular signals and play an essential role in IgA CSR.

Materials and Methods

Mice

Wild-type mice, Runx2^{+/-} mice, Runx3^{+/-} mice, and Rag2^{-/-} mice against the C57BL/6 genetic background were maintained under specific pathogen-free conditions. Procedures involving animals and their care were conducted according to the guidelines for animal treatment at the Institute of Laboratory Animals (Kyoto University).

Fetal liver transfer

Single-cell suspensions of 2–4 \times 10⁶ whole fetal liver mononuclear cells harvested from Runx2^{-/-}, Runx3^{-/-}, Runx2^{-/-} Runx3^{-/-}, and wild-type embryos at E14.5 were injected *i.v.* into irradiated male Rag2^{-/-} recipient mice. At least 8 wk after transplantation, the mice were sacrificed, and cell compartments were analyzed by flow cytometry or used for *in vitro* culture. All Rag2^{-/-} mice used for experiments comparing the Runx effect were age-matched, and fetal liver cells were from littermates.

Cell preparation

Splenic B cells were prepared by forcing spleen fragments through a mesh wire screen. After hypotonic lysis of RBCs for 10 min at room temperature, the cell suspensions were washed and incubated for 60 min on ice with anti-Thy-1 (30H12), anti-CD4 (RL172.5), and anti-CD8 (3.155). The cells were then pelleted and resuspended in 10% rabbit complement (Lo-Tox M; Cedarlane Laboratories, Hornby, Ontario, Canada) diluted in RPMI 1640 medium (Wako Pure Chemical, Osaka, Japan) containing penicillin, streptomycin, and 2-ME and 10% FCS. Cells were incubated with complement for 60 min at 37°C.

To prepare LP lymphocytes (LPLs), the small intestines were removed, and PPs and mesenchymal lymph nodes (MLNs) were isolated (4). The intestines were opened longitudinally and washed with PBS and then cut into 5-mm pieces. To remove endothelial cells and intraepithelial lymphocytes, the pieces were shaken in HBSS⁺ containing 5 mM EDTA for 20 min at 37°C and through a cell strainer twice. The remaining tissue was cut into smaller pieces and digested with RPMI 1640 medium containing 4% FCS, 1 mg/ml collagenase II, 1 mg/ml Dispase (Life Technologies, Grand Island, NY) and 40 μ g/ml DNase I (TaKaRa, Kusatsu, Japan) at 37°C with stirring for 20 min. Whole-intestinal-cell suspensions, which included LPLs, were passed through a cell strainer and placed on a 40/80% discontinuous Percoll gradient (GE Healthcare, Little Chalfont, U.K.), and cells at the interface between 40 and 80% were collected and used as LPLs.

Cell cultures

Before starting the culture, the percentages of IgA-positive cells were assessed by flow cytometry. In all experiments, the percentages of IgA-positive cells were <0.01%. Splenic B cells (1 \times 10⁶ cells/ml) were

cultured in medium in the presence of LPS (10 μ g/ml; Sigma-Aldrich, St. Louis, MO), recombinant human APRIL (180 ng/ml), mouse IL-5 (5 ng/ml), human TGF- β 1 (1 ng/ml) (all from R&D Systems, Minneapolis, MN), RA (10 nM; Sigma-Aldrich), Ro41-5253 (10 nM; BIOMOL, Plymouth Meeting, PA), LE135 (10 nM; Tocris Bioscience, Ellisville, MO), GW6471 (10 nM; Sigma-Aldrich), GW9662 (10 nM; Cayman Chemical, Ann Arbor, MI), and Am80 (10 nM; Wako Pure Chemical). Cells were analyzed by RT-PCR (day 3) and flow cytometry (day 5), and the concentrations of IgA in culture supernatants were measured by ELISA (day 7).

Flow cytometric analysis

The following Abs were used for staining: FITC anti-mouse B220 (BD Pharmingen, San Diego, CA); biotin anti-mouse IgA (Southern Biotechnology, Birmingham, AL); and allophycocyanin-streptavidin (Molecular Probes, Eugene, OR). All analyses were performed with FACSCalibur or FACSAria (BD Biosciences, Mountain View, CA).

Retroviral infections

Retrovirus was produced by transfecting the ecotropic Plat-E packaging cell line (20) with pMSCV-IRES-GFP retroviral vector. After stimulation with LPS plus APRIL for 8 h, purified splenic B cells were spin-infected with the virus-containing supernatant in the presence of 4 μ g/ml polybrene for 1.5 h at 2500 rpm and 32°C and cultured for 14.5 h. After removing virus-containing supernatants, cells were recultured in medium in the presence of LPS (10 μ g/ml), APRIL (180 ng/ml), IL-5 (5 ng/ml), TGF- β 1 (1 ng/ml), and RA (10 nM). After 5 d, cells were analyzed by flow cytometry.

ELISA

ELISA plates were coated with goat-anti-mouse IgA (10 mg/ml; Southern Biotechnology Associates) in PBS and blocked with 2% PBS/BSA. After washing, serial dilutions of serum, fecal, culture supernatants, and IgA standard (Zymed, San Francisco, CA) were added for 1.5 h. Plates were washed, and goat-anti-IgA-HRP was added for 1 h. After washing, the reaction was developed with 0.5 mg/ml *o*-phenylenediamine (Sigma-Aldrich) in 0.0003% H₂O₂ and 0.1 M citrate phosphate buffer (pH 5.0) for 15 min, and plates were read at a wavelength of 490 nm with a reference wavelength of 620 nm.

RT-PCR

Total RNAs were extracted from splenocytes and cultured splenic B cells with TRIzol (Life Technologies), according to the manufacturer's instructions, and then quantitated at A260. Oligo(dT)-primed cDNAs were prepared with reverse transcriptase. For semiquantitation, 50 ng of cDNAs were serially diluted and subjected to PCR amplification with the following primer pairs: all PCR products were resolved electrophoretically in 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Values of *p* were calculated by Student *t* test.

Results

RA and TGF- β 1 synergistically activate IgA class switching *in vitro*

Although markedly skewed IgA CSR takes place in GALT and LP *in vivo*, there is no good experimental setting to induce IgA CSR at high frequency *in vitro*. In contrast, various inducing factors for IgA CSR have been identified, but their exact functions have not been explored extensively. We therefore investigated combinatorial signaling effects on the regulation of IgA CSR.

In CSR, target specificity is determined through the induction of GLT (21), which recruits activation-induced cytidine deaminase to the target loci, and the recruited activation-induced cytidine deaminase deaminates the transcribed Switch region (S region) to initiate a recombination reaction (8, 22–25). Thus, the induction of α GLT is the most important property for specific factors of IgA. Depending on this criterion, TGF- β 1 and RA are thought to be IgA inducers, as suggested previously.

To determine whether RA can act as a strong inducer of IgA class switching, naive B cells stimulated with LPS and April were cultured with various combinations of TGF- β 1, RA, and IL-5. As shown in Fig. 1, RA induces IgA-positive cells as efficiently as

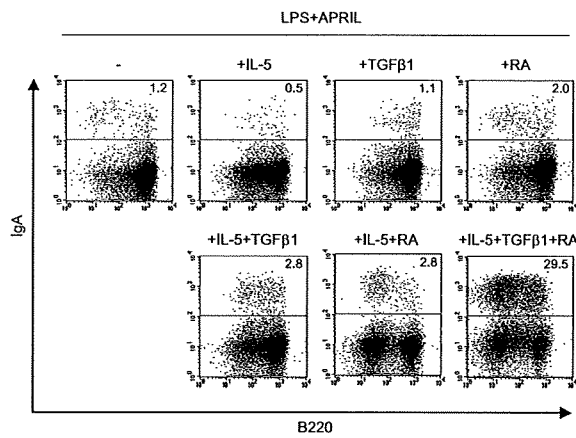


FIGURE 1. RA and TGF- β 1 synergistically induce IgA CSR in vitro. Splenic B cells were cultured in the presence of LPS and APRIL in combination with various cytokines, as indicated. On day 5, surface IgA expressions were determined by FACS. Numbers indicate the percentages of IgA-positive cells. Data are representative of three independent experiments.

TGF- β 1. In addition, RA and TGF- β 1 synergistically induce IgA class switching (29.5% IgA-positive cells in a population stimulated with TGF- β 1, IL-5, and RA as compared with 2.8% in

a population with TGF- β 1 plus IL-5 or RA plus IL-5). Such synergistic action might occur in vivo, because robust IgA production was observed in mucosal immune tissues, LP is rich in TGF- β 1, and RA-producing DCs exist in LP (5, 10, 11).

Runx proteins act as positive regulators for IgA class switching

TGF- β 1 signaling regulates the promoter activity of α GLT (α GL promoter). This observation further confirmed the existence of Smad binding sites within the α GL promoter. In addition, Runx binding sites were found near Smad sites, and the involvement of Runx3 in IgA CSR was suggested by reporter assay and biochemical assays, indicating the physical interaction between Runx3 and R-Smad (15, 16, 18). In contrast, IgA production in Runx3-deficient mice was not affected, and the effect of forced expression of Runx proteins on IgA CSR has not been shown so far. Thus, the functional importance of Runx factors in IgA CSR remains unclear. To address these questions, we used a retrovirus-mediated approach to introduce Runx1 into primary B cells. Runx proteins are known to repress or enhance target gene expression, depending on the cell type and the context of the regulatory element. Functional domains of Runx proteins in transcriptional regulation have also been determined. Domain VWRPY is required for repression by recruiting Groucho family members. Domain ID is involved in the inhibitory function of the activator domain. Runt is a DNA-binding domain. Runx proteins with

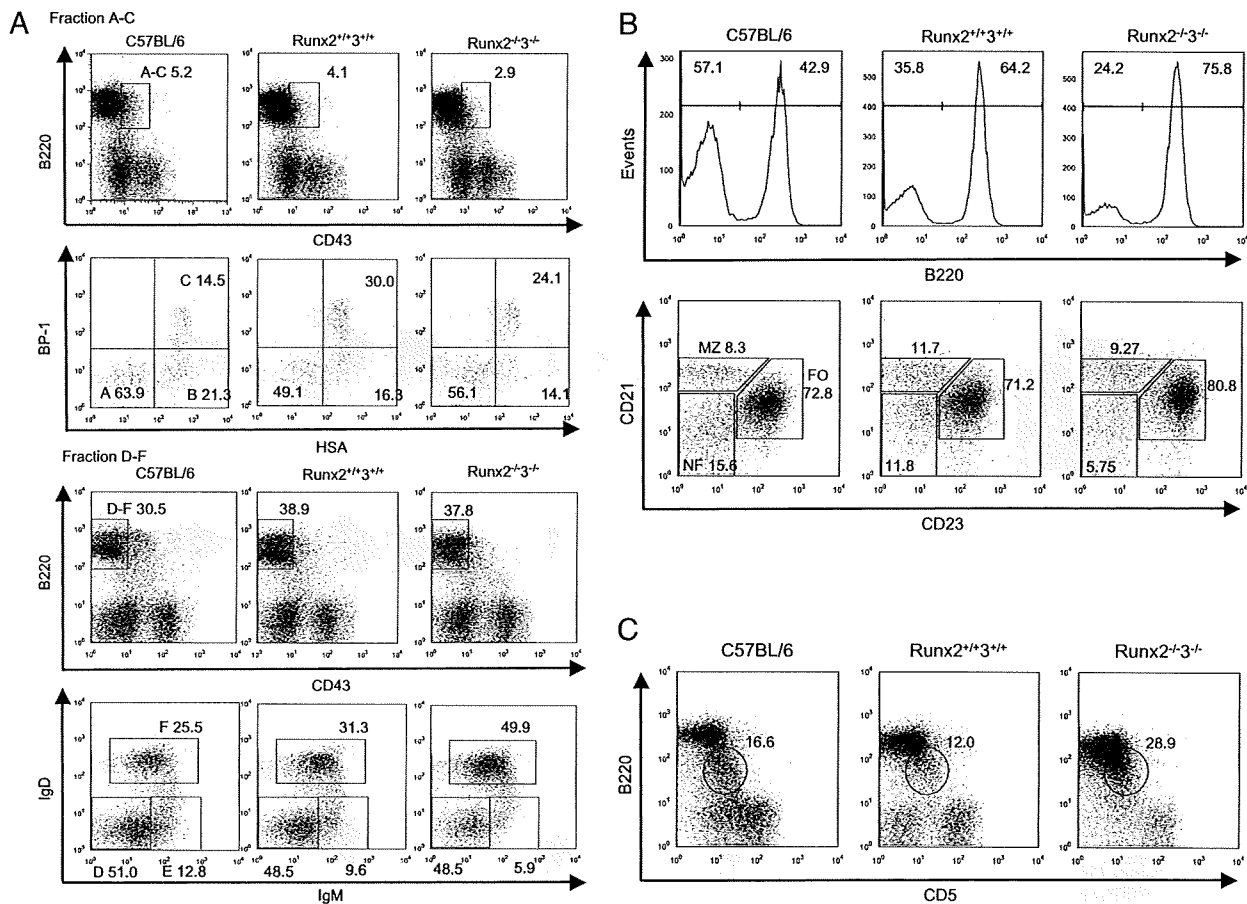


FIGURE 2. B cell development of Runx2^{-/-}Runx3^{-/-} fetal liver cells in RAG2^{-/-} mice. *A*, Flow cytometry of bone marrow cells from control and Runx2^{-/-}Runx3^{-/-} fetal liver-derived cells to identify B cell developmental subsets. Fractions A–F indicate Hardy’s nomenclature for murine B cell development. B cell developments from wild-type bone marrow are shown. *B*, Flow cytometry of splenocytes for subset analysis (*left panel*). B220-positive cells were subdivided into NF, MZ, and FO B cells. B cell developments from wild-type spleen cells are shown. *C*, Flow cytometry of peritoneal cavity cells (*left panel*). Circles indicate B1a B cells. Numbers in the figure indicate the percentages of the indicated populations. Data are representative of two independent experiments. FO, follicular; MZ, marginal zone; NF, newly formed.

a point mutation K83E in the runt domain lose their DNA-binding functions (26). Accordingly, Runx1-VWRPY and Runx1AD work as constitutive activators at target loci. In contrast, Runx1-runt and Runx1K83E is dominant negative to antagonize both positive and negative effects on transcription (Supplemental Fig. 1A). Various mutant forms of the Runx1 gene were cloned into the MSCV-IRESGFP vector, which allowed Runx expression to be monitored by GFP. B cells were infected with the virus by spin infection and analyzed for the cell surface expression of IgA after 5 d of culture with LPS, TGF- β 1, IL5, RA, and April. As shown in Supplemental Fig. 1B, Runx1-, Runx1-VWRPY-, and Runx1AD-transduced GFP-positive cells showed enhanced numbers of IgA-positive cells (29% IgA-positive cells in the vector-transduced population as compared with 37% in Runx1-expressed, 37% in Runx1VWRPY-expressed, and 47% in Runx1AD-expressed populations). In contrast, Runx1runt- and Runx1K83E-transduced GFP-positive cells showed substantially reduced numbers of IgA-positive cells (29% IgA-positive cells in the control compared with 18% in Runx1runt-expressed or 20% in Runx1K83E-expressed populations). Essentially the same results were obtained using Runx2 and Runx3 retrovirus (data not shown), indicating that Runx proteins will act as positive regulators of IgA CSR.

Reduced IgA production in Runx2-Runx3 double-deficient mice

All three Runx proteins are expressed in B cells (data not shown). Because Runx1 is essential for hematopoietic cell differentiation, all hematopoietic lineages are absent in Runx1^{-/-} mice; therefore, we focused on estimating the functions of Runx2 and Runx3 in IgA CSR. Runx2-deficient mice and Runx3-deficient mice die within a short period after birth (27–29). We therefore generated

Runx2, 3-deficient lymphocytes within RAG2^{-/-} mice by infusing fetal liver cells from Runx2^{-/-}, Runx3^{-/-}, and Runx2^{-/-}Runx3^{-/-} embryos to analyze the effects of Runx proteins in IgA production (called Runx2^{-/-}, Runx3^{-/-}, and Runx2^{-/-}Runx3^{-/-} mice in this study). Two months after fetal liver transfer, we examined the B cell differentiation status in RAG2^{-/-} mice. As shown in Fig. 2 and Supplemental Table I, B cell differentiation in bone marrow and the periphery is not so affected by loss of Runx proteins. In Runx2^{-/-}Runx3^{-/-} mice, a slight increase of B1a cells in the peritoneal cavity was observed, and then the levels of IgA in sera and feces were determined by ELISA. IgA levels in serum were ~50-fold lower in Runx2^{-/-}Runx3^{-/-} mice but not in Runx2^{-/-} or Runx3^{-/-} mice (Fig. 3A) than in control mice. With a fecal extract, ~24-fold reduction of the levels of secreted IgA was observed in Runx2^{-/-}Runx3^{-/-} mice compared with control mice (Fig. 3B). These data support our idea that Runx proteins act as a master regulator of IgA production in vivo. The amount of Runx protein has a threshold in IgA regulation, because there is no significant effect on IgA production in Runx2 or Runx3 single-knockout mice. These results were further confirmed by estimating the proportion of IgA-positive cells in MLNs, PPs, and LP lymphocytes (LPLs). As shown in Fig. 3C, the percentages of IgA-positive cells in all tissues examined were reduced in Runx2^{-/-}Runx3^{-/-} mice compared with control mice. To further determine whether IgA deficiency observed in Runx2^{-/-}Runx3^{-/-} mice is a consequence of the defect in B cell activation, germinal center reaction in the PPs were assessed by staining with GL7 Ab (30). As shown in Fig. 3D, GL7-positive cells exist in the PP from Runx2^{-/-}Runx3^{-/-} mice, suggesting that a defect in B cell activation is not the cause of IgA deficiency. In addition, IgG1

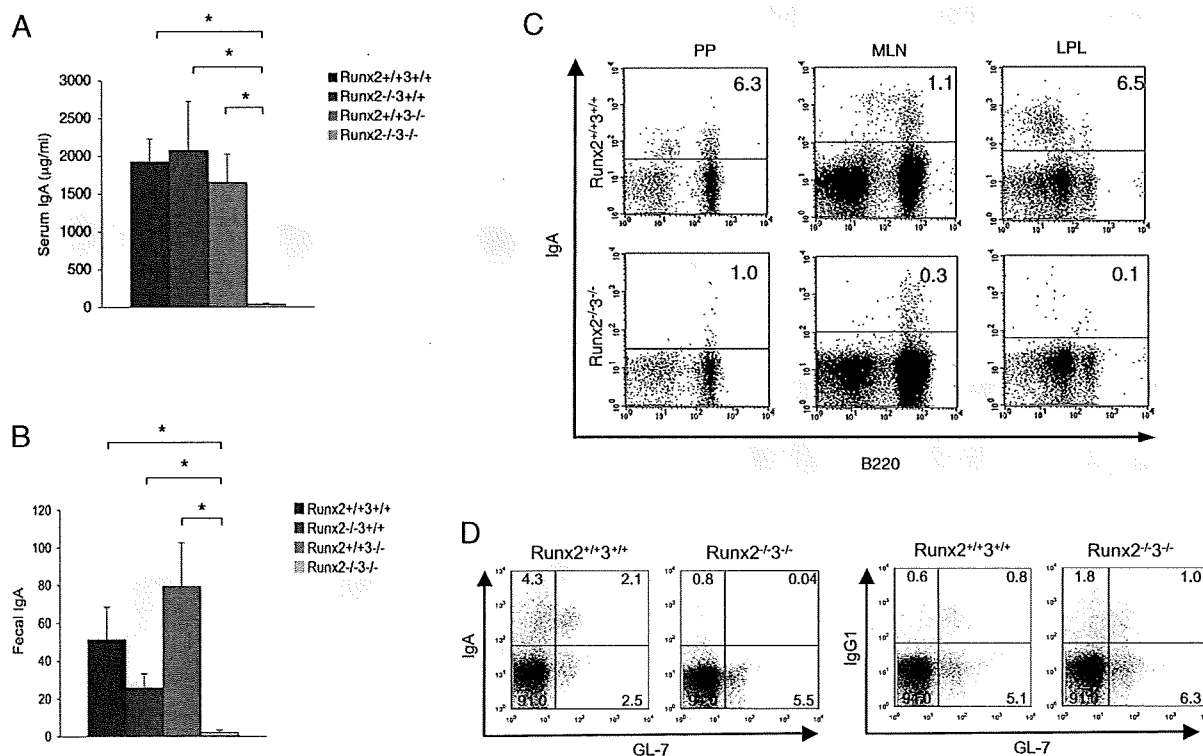


FIGURE 3. Defects in IgA productions in Runx-deficient mice. *A*, IgA levels in serum from individual mice were determined by ELISA. **p* < 0.01; *n* = 4. *B*, IgA levels in feces from individual mice were determined by ELISA and compensated for with the weight of feces. **p* < 0.01; *n* = 4. Arbitrary unit = concentrations of IgA (µg/ml)/concentrations of protein (µg/ml). *C*, Flow cytometric analysis of IgA expression in PPs, MLN, and LP cells. Cells were stained for B220 and IgA. Numbers indicate the percentages of IgA-positive cells among all cells. Data are representative of three independent experiments. *D*, Flow cytometric analysis of IgA or IgG1 expressions in combination with Germinal center marker, GL7, in PPs.

class switching was observed in $Runx2^{-/-}Runx3^{-/-}$ mice, indicating that class switching to other classes is not affected in these mice (Fig. 3D, Supplemental Fig. 2). Therefore, IgA production is specifically reduced.

Requirement of Runx2 and Runx3 for IgA CSR in B cells

To assess whether Runx action in B cells is responsible for this phenomenon, IgA class switching abilities were estimated in vitro. As shown in Fig. 4A, IgA class switching was completely blocked in $Runx2^{-/-}Runx3^{-/-}$ B cells in all conditions. These observations were further supported by quantifying the levels of IgA in the supernatant (Fig. 4B). The supernatants from $Runx2^{-/-}Runx3^{-/-}$ B cells contain almost no IgA. To discriminate whether the effect on IgA class switching is specific, we examined IgG2b class switching, which is another TGF- β 1-inducible isotype, using the same cultured cells as shown in Fig. 4A. In this case, there was no significant difference in the proportion of IgG2b-positive cells between genotypes (Fig. 4C). Thus, Runx will work specifically on IgA production in B cells; however, slight inhibition of IgG2b class switching was reproducibly observed in culture conditions with LPS, TGF- β 1, and APRIL in $Runx2^{-/-}Runx3^{-/-}$ B cells. In this culture condition, Runx proteins will contribute to induce IgG2b class switching, as demonstrated previously (31). In addition, no inhibitory effect of IL-5 in IgG2b class switching was observed in $Runx2^{-/-}Runx3^{-/-}$ B cells. These data suggest that IL-5 inhibits IgG2b class switching by inhibiting Runx activities. We next ex-

amined whether Runx acts on the induction of α GLT to regulate IgA class switching. As expected, Runx proteins are required for appropriate induction of α GLT in response to TGF- β 1 and RA (Fig. 4D and data not shown).

We further examined the relationship between IgA class switching and cell proliferation using CFSE-labeled B cells, because class switching is strongly correlated with cell division (32). As shown in Fig. 5, the majority of B cells in $Runx2^{-/-}Runx3^{-/-}$ mice proliferate more than wild-type-derived cells in both conditions (with or without TGF- β 1), indicating that cell proliferation defects are not the cause of IgA deficiency in $Runx2^{-/-}Runx3^{-/-}$ mice. Accordingly, Runx proteins act as master regulators of IgA production in B cells by inducing α GLT in response to RA and TGF- β 1.

Retrovirus-mediated transfer of Runx proteins in $Runx2^{-/-}Runx3^{-/-}$ B cells rescues the defect in IgA class switching

To determine whether the reintroduction of Runx proteins can rescue the reduced class switching to IgA observed in $Runx2^{-/-}Runx3^{-/-}$ B cells, splenic B cells were infected with the virus by spin infection and analyzed for the cell surface expression of IgA after 5 d of culture. As shown in Fig. 6A, Runx-transduced GFP-positive cells showed significantly increased numbers of IgA-positive cells (~30% IgA-positive cells in the Runx-expressing population as compared with 2.0% in the control population). In Runx-transduced cells, inhibition in α GLT was also restored (data not shown). Thus, Runx expression is required to induce α GLT

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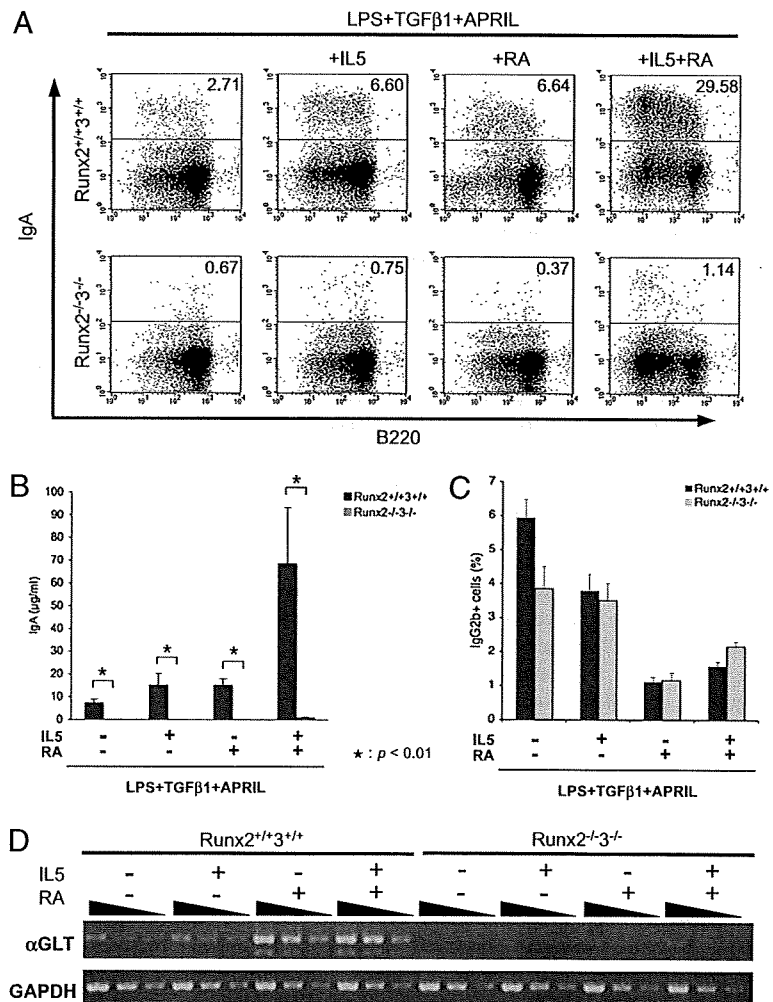


FIGURE 4. Defect in IgA CSR caused by Runx-deficient B cells. *A*, Class switching was analyzed by monitoring the surface expression of IgA. Numbers (*upper panels*) indicate percentages of IgA-positive cells. Data are representative of three independent experiments. *B*, Concentrations of IgA in culture supernatants on day 7 were estimated by ELISA. * $p < 0.01$. *C*, Class switching was analyzed by monitoring surface expression of IgG2b. *D*, RT-PCR analysis of α GLT in 3-d cultured B cells. GAPDH was used as an internal control for RT-PCR. Five-fold serial dilutions of cDNAs were amplified for the indicated transcripts.

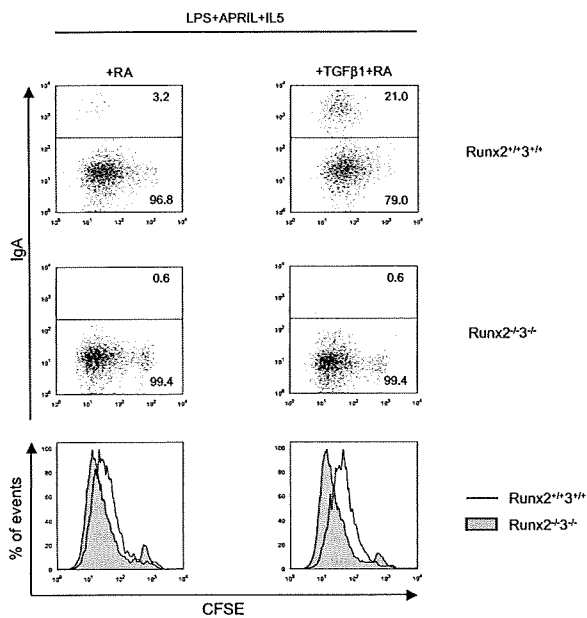


FIGURE 5. Tracking B cell division and IgA switching using CFSE. CFSE-labeled control and Runx2^{-/-}Runx3^{-/-} B cells were stimulated with LPS+APRIL+IL-5+RA in the presence or absence of TGF- β 1 for 5 d. Stimulated cells were stained for IgA. *Top and middle panels*, CFSE versus IgA expression. *Bottom panels*, CFSE intensity.

and IgA class switching in B cells. To examine further details of Runx functions in TGF- β 1 signaling, mutant constructs of Runx proteins with a defect in the interaction with Smad or TAZ were used for rescue experiments. RunxHTY mutants, which disrupt Runx-Smad interaction, have previously been shown to lose the ability to integrate BMP signal in differentiating osteoblasts using Runx2-deficient cells (33). Unexpectedly, the RunxHTY mutant can rescue the defect in IgA class switching (Fig. 6B). These data indicate that direct interaction of Runx and Smad proteins is not required for the induction of α GLT. Next, we examined whether physical interaction between Runx proteins and TAZ is required for IgA class switching. TAZ is a transcriptional regulator acting differentially with Runx2 and peroxisome proliferator-activated receptor and enhances the transcriptional activity of Runx but inhibits that of peroxisome proliferator-activated receptor; therefore, various amounts of TAZ are involved in the cell fate determination of mesenchymal stem cells toward osteoblasts or adipocytes. In addition, TAZ is required to maintain self-renewing capacity in response to TGF- β 1 signaling in human embryonic stem cells. In IgA class switching, RunxPPXY mutants that lose their ability to bind TAZ can also rescue the defect observed in Runx2^{-/-}Runx3^{-/-} B cells (Fig. 6B). These results indicate that direct interaction between TAZ and Runx proteins is not required to induce α GLT. In summary, Runx expression per se is required to induce α GLT; however, the more precise roles of Runx, Smad, retinoic acid receptor (RAR), and TAZ in the regulation of α GLT in vivo remain to be clarified.

In conclusion, these findings provide functional evidence that Runx has an important role in IgA CSR under the control of TGF- β 1 and RA signals in our culture conditions. Future studies should help define the exact functional links among Runxs, Smads, and RARs under various immunological conditions.

Discussion

Intestinal IgA exerts immune protection, neutralizing microbial toxins and pathogens, and immune exclusion, preventing the penetration of commensal bacteria into the mucosal epithelium

without inducing inflammatory reactions. To achieve this, it is proposed and generally believed that IgAs with various affinities and differences in their expression time courses contribute differentially to these processes (1–3, 34). In this regard, we cannot directly estimate their relationship, and accumulating evidence supports this concept. In PPs, high-affinity IgA is produced in a T cell-dependent manner. In isolated lymphoid follicles, IgA is produced more rapidly in a T cell-independent manner (7). T cell-independent IgA CSR also occurs in LP but requires more time than other reactions. Thus, there are many differences in the functions and/or properties of IgAs, depending on where they are generated in vivo; we would like to identify the common factors in the regulation of IgA CSR itself, because strikingly skewed IgA CSR takes place in GALT and LP but not in other lymphoid tissues in vivo (1–3, 8, 34). In contrast, there is no good experimental setting to induce IgA CSR at high frequency in comparison with other isotypes.

In this paper, we established an efficient system to induce IgA CSR in vitro only using soluble factors and found that RA is as strong an inducer of IgA CSR as TGF- β 1. So far, RA function in IgA production has not been determined completely. Tokuyama et al. (35, 36) demonstrated that RA induced α GLT and post-switch transcription, but the frequency of IgA CSR in individual cells was not estimated. In contrast, other researchers estimated the RA effect in IgA CSR using coculture systems (5, 9, 10), so they could not discriminate the genuine roles of RA in IgA CSR, because coculturing DCs would provide various unidentified signals to B cells. Because RA is also known to be involved in plasma cell differentiation, our result does not exclude the possibility that RA also promotes plasma cell differentiation of IgA-switched cells (37). Furthermore, we found marked synergistic action of RA and TGF- β 1 in IgA CSR. Such synergistic action might occur in vivo, because robust IgA production was observed in mucosal immune tissues, and LP is rich in TGF- β 1, and RA-producing DCs exist in LP (5, 10, 11); however, these data cannot support the previous experimental result, in which TGF- β 1 inhibited the RA effect on IgA production (9, 38). Such a discrepancy comes from differences in the culture conditions used. Mora et al. (9, 38) used a more complicated system in combination with B cells, DCs, and soluble factors. Thus, the exact signaling molecules affecting the functions of TGF- β 1 in RA-treated B cells remain to be identified.

Runx proteins regulate various biological processes. In mammals, three isoforms of Runx, Runx1, Runx2, and Runx3 have been identified. Runx1 is essential for definitive hematopoiesis and angiogenesis (28, 39). Runx2 function is required for osteoblast differentiation and osteogenesis (29, 40). Runx3 plays more widespread roles, especially in the differentiation of many cell types—CD8T cells (41), DCs, Langerhans cells (19), and dorsal root ganglion neurons (42, 43)—and contributes to maintaining the homeostasis of gastrointestinal epithelial cells (27). Interestingly, some functions of the TGF- β superfamily are similar to those of Runx proteins. In TGF- β 1-deficient mice, defective hematopoiesis and vasculogenesis are observed (44). The TGF- β 1 signal is also essential for the differentiation of Langerhans cells and is speculated to be the main cause of the defect in the projection of dorsal root ganglion neurons observed in Runx3-deficient mice. In addition, gastrointestinal epithelial cell hyperplasia in Runx3-deficient mice is caused by reduced sensitivity to TGF- β 1. Additionally, BMP induces differentiation to osteoblasts from mesenchymal stem cells. Thus, functional interactions between Runx proteins and the TGF- β superfamily are speculated. As expected, cooperation between TGF- β 1 signaling and Runx proteins was first discovered in the regulation of IgA

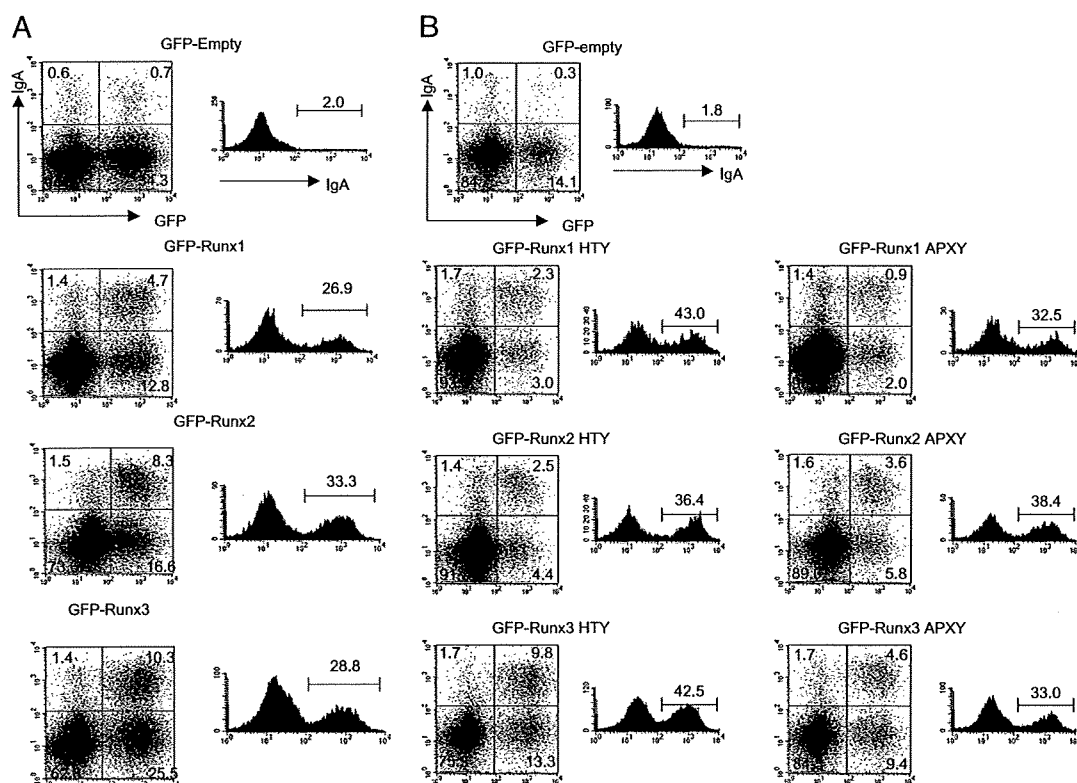


FIGURE 6. Reintroduction of Runx1, Runx2, or Runx3 proteins restores the defect in IgA class switching. *A*, IgA CSR and Runx protein expressions were analyzed by flow cytometry for surface expressions of IgA and GFP after culturing for 5 d (left column). The respective relative numbers are indicated as percentages of individual boxes. *A*, Histogram of IgA expression within GFP⁺ cells (right column). Numbers refer to the percentages of IgA-positive cells in all GFP⁺ cells. *B*, Almost the same experimental results as described in *A*, except using mutant Runx proteins: Runx-HTY mutant loses the ability to bind Smad; Runx-APXY mutant loses the ability to bind TAZ. Numbers in quadrants indicate the percentage of indicated regions, and those above bracketed lines indicate the percentage of IgA-positive cells in GFP⁺ cells. Data are representative of three independent experiments.

CSR in B cells. TGF- β 1 responsive elements in the promoter region of α GLT include Smad binding sites and Runx binding sites in close vicinity (15). All three Runx proteins physically interact with Smad2/3 proteins and act on the TGF- β 1 signaling pathway. Thus, they cooperatively stimulate the induction of α GLT, demonstrated by reporter assays. Among three Runx proteins, it is believed that Runx3 plays a central role in IgA CSR, because Runx3 is predominantly induced by TGF- β 1 in B cells (15). This finding has been confirmed by many investigators (16–18), and this concept was further extended to another Smad-dependent signal, BMP. It has been clearly demonstrated that Runx2 functions synergistically with Smad1 and Smad5 to induce bone-specific genes upon BMP signaling (14, 45). Accumulating genetic evidence indicates the functional importance of Runx2 in BMP signaling; however, there is no direct evidence of the involvement of Runx proteins in IgA CSR. To answer this question, Runx3-deficient mice were analyzed, but it was indicated that Runx3 is dispensable for *in vivo* IgA production (19). We also confirmed their results by estimating the levels of IgA in sera and feces. Next, we further examined whether Runx proteins are indeed dispensable for IgA production by assessing the effect on IgA in Runx2 and Runx3 double-deficient mice. In this case, we found a significant reduction in IgA production in sera and feces. In comparison with mice deficient in TGF- β 1 pathways or April, BAFF, and TACI pathways, the levels of IgA in sera and feces were specifically low in Runx2^{-/-}Runx3^{-/-} mice. These data further indicate that other pathways will work *in vivo* to induce IgA CSR. Indeed, we have demonstrated that RA is a strong in-

ducer of IgA CSR *in vitro* and synergistically activated IgA CSR with TGF- β 1. Our data further indicate that Runx proteins are possibly required for both pathways; therefore, Runx proteins play a key role in IgA CSR by integrating various signals to induce α GLT. Interestingly, the expression of Runx3 was significantly reduced in iNOS-deficient B cells; thus, the defect in IgA CSR observed in iNOS^{-/-} B cells could be partly explained by the loss of Runx3 expression in B cells. Using specific agonists and antagonists, the involvement of RAR α and RAR β in IgA CSR is suggested (Supplemental Fig. 3); however, we could not elucidate the exact functions of Runx proteins in RA- and TGF- β 1-dependent pathways for IgA CSR. Future studies should help define the exact functional links among Runxs, Smads, and RARs under various immunological conditions *in vivo*.

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Disclosures

The authors have no financial conflicts of interest.

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