FIGURE 4. Induction of costimulatory and inhibitory molecules in ICOS-deficient patients. *A*, Induced expression of CD40L, OX40, and 4-1BB. PBMCs from healthy controls (HC) and ICOS-deficient patients (#1 and #2) were stimulated with plate-bound anti-CD3 mAb and anti-CD28 mAb for 48 h and analyzed for CD40L, OX40, and 4-1BB expression by FACS. CD69 expression was monitored as an indicator of cell activation. Numbers indicate percentages of the cell population positive for the indicated Ags among CD4 T cells. A contour plot from one representative control of seven HC is shown for each subset. A dotted line indicates a control staining with isotype-matched Ab. *B*, Summary of frequencies of CD40L, OX40, and 4-1BB in CD4 T cells stimulated as in *A* from HC (*n* = 7) and patients 1 and 2. FACS analysis was performed twice for the patients, and average percentages were plotted. Error bar indicates SD. *C*, Induction of CTLA-4, BTLA, and PD-1. PBMCs were stimulated with plate-bound anti-CD3 mAb and anti-CD28 mAb for 48 h. Cells were stained with Abs to CTLA-4, BTLA, and PD-1 together with anti-CD4 mAb or anti-CD8 mAb. Numbers indicate percentages of cell population positive for indicated Ags among CD4 or CD8 T cells. FACS analysis from one representative control of seven HC is shown for each subset. A dotted line indicates a control staining with isotype-matched Ab. *D*, Pooled data from HC (*n* = 7) and patients (1 and 2). Percentages of CTLA-4$^+$, BTLA$^+$, and PD1$^+$ CD4 T cells among CD4 T cells and that of PD-1$^+$ CD8 T cells among CD8 T cells from HC and ICOS-deficient patients (1 and 2) after CD3/CD28 stimulation are shown. The FACS analysis was conducted three times for the patients, and average percentages were plotted. Error bar indicates SD.

anti-CD28, and the cells were examined at the end of incubation for the expression of TNF/TNFR family proteins (TNFRI (CD120a), TNFRII (CD120b), CD40L, OX40, and 4-1BB) and of CD28 family proteins (CTLA-4, BTLA, and PD1 (CD279)).

The analysis revealed that CD40L expression was induced normally in the patients' CD4 T cells, indicating that the hyper-IgM phenotype observed in patient 2 was not due to defective induction of CD40L.

T cells were fully activated in the patients at the end of CD3/CD28 stimulation, as evidenced by CD69 Ag expression. The levels of OX40, 4-1BB, TNFRI, and TNFRII were normal on the surface of the T cells in the ICOS-deficient patients (Fig. 4, *A* and *B*, and data not shown).

Baseline CD28 expression in CD4 T cells was similar to that of healthy subjects (data not shown). In contrast, the frequency of CTLA-4$^+$ CD4 T cells after CD3/CD28 costimulation was markedly reduced in the patients (Fig. 4*C*). Combined data from seven age-matched controls showed that CTLA-4 was induced in 47.4 ± 4.9% of CD4 T cells. In contrast, induction was observed in 30.1 and 24.6% of CD4 T cells in patients 1 and 2, respectively (Fig. 4, *C* and *D*). Moreover, as seen in the representative plot in Fig. 4*C*, the expression level of CTLA-4 in the CTLA-4$^+$ population was also diminished in the patients.

Induction of BTLA, another inhibitory receptor with similarities to CTLA-4 (5), was then estimated. The average percentage of BTLA$^+$ CD4 T cells was slightly lower in the patients (22.0% for
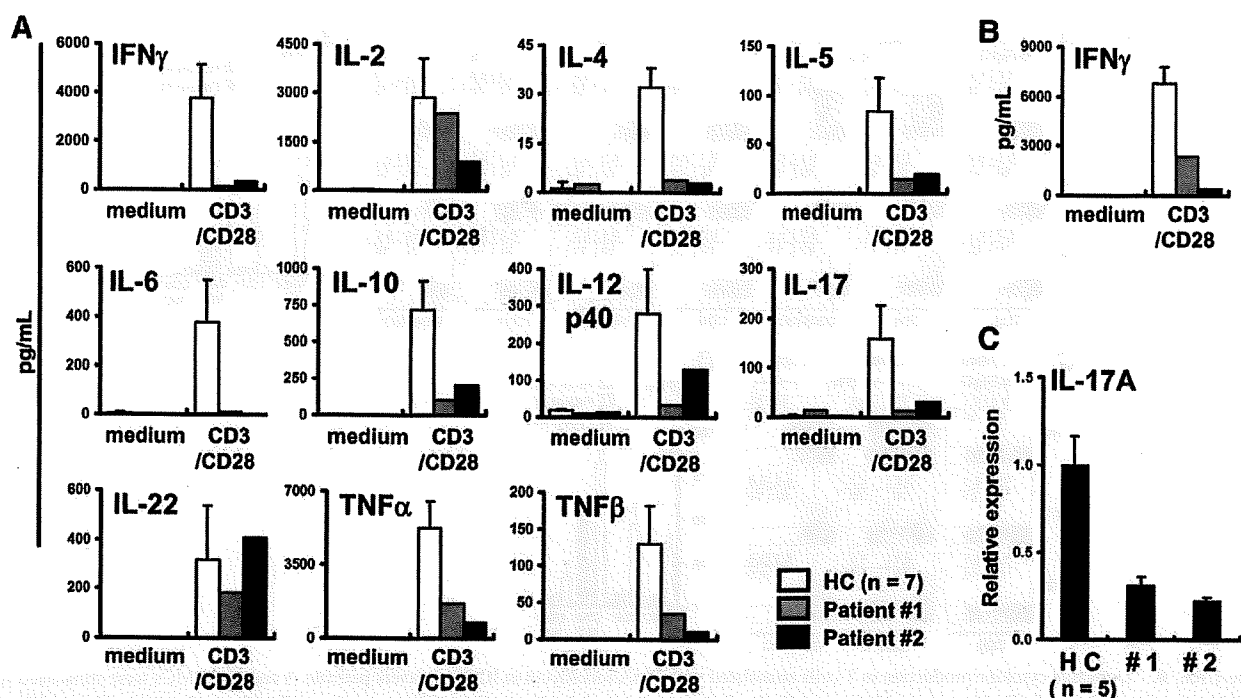
FIGURE 5. Impaired cytokine production in ICOS-deficient patients. *A*, Purified CD4 T cells were stimulated with plate-bound anti-CD3 mAb and anti-CD28 mAb for 24 h or in a medium, and the levels of cytokines in the supernatants were measured by ELISA, FlowCytomix, or both. Error bars for healthy controls (HC, $n = 7$) indicate SD. Experiments were repeated at least twice for the patients, and mean concentrations were plotted. *B*, Purified CD8 T cells were stimulated as in *A*, and the levels of IFN-γ in the supernatants were measured by FlowCytomix. □, HC; ▧, patient 1; ■, patient 2. Error bars for HC ($n = 5$) indicate SD. Experiments were repeated twice for the patients, and mean concentrations were plotted. *C*, IL-17A mRNA expression. The level of IL-17A mRNA was measured in anti-CD3/anti-CD28-stimulated CD4$^+$ T cells by real-time PCR. Relative mRNA level of IL-17A was calculated using GAPDH expression as a reference, and the mean expression level for HC ($n = 5$) was adjusted to 1.0. Error bar indicates SD. IL-17A mRNA expression for the patients was measured three times, and is expressed as mean ± SEM.

patient 1; 21.4% for patient 2) compared with controls (34.0 ± 8.7%, $n = 7$) (Fig. 4, *C* and *D*).

In the patients, the frequency of CD4 T cells bearing PD1, a molecule that plays a critical role in the induction and/or maintenance of T cell tolerance (1), was similar to that in controls (Fig. 4, *C* and *D*). The percentages of PD1$^+$ CD8 T cells, which function as inhibitory T cells (43), were slightly reduced only in patient 2 (29.0%) compared with controls (49.8 ± 9.0%, $n = 7$).

Impaired production of cytokines in ICOS-deficient patients

We next assessed the production of a panel of cytokines by a FlowCytomix bead-based multiplex assay, ELISA, or both, after incubation of CD4 T cells purified to >95% with costimulation of the TCR-CD3 complex via CD28.

In contrast to previous data obtained in other cases of human ICOS deficiency, the production of IFN-γ (Th1 cytokine) and IL-4 and IL-5 (Th2 cytokines) was significantly reduced (Fig. 5*A*) in the patients than in controls (31, 33). Secretion of IL-10 and IL-17 was impaired in the ICOS-deficient patients, in agreement with the previous report (33). To confirm the Th17 defect in the patients, a real-time PCR analysis was used to quantify IL-17A mRNA induction. The results, shown in Fig. 5*C*, demonstrate a significant decrease in relative IL-17A mRNA expression in ICOS-deficient T cells. Furthermore, induction of other cytokines, such as IL-6, IL-12 p40, TNF-α, and TNF-β, in CD4 T cells was impaired to various degrees in the patients (Fig. 5*A*).

Interestingly, the synthesis of the different cytokines was not equally affected in the absence of ICOS: the production of IL-2 was within ±1 SD of normal values, and the IL-22 response was similar to that in controls.

To determine whether the observed defects in effector T cell function can be reproduced by direct activation of intracellular signaling, we examined the capacity of lymphocytes to produce cytokines after PMA/ionomycin stimulation. To that end, intracellular IFN-γ, IL-4, and IL-17 were monitored in PBMCs stimulated with PMA/Ca ionophore by flow cytometry. Fig. 6*A* shows that the CD4 T cells of the patients elicited markedly reduced Th1, Th2, and Th17 cytokine responses.

To corroborate these results, we measured the level of cytokines using a FlowCytomix kit in purified CD4 T cells incubated with PMA and ionomycin for 24 h. A similar trend was noted, as follows: the production of IFN-γ, IL-5, IL-10, TNF-α, and TNF-β was found to be diminished. The capacity of ICOS$^{-/-}$ CD4 T cells to produce IL-2 and IL-6, however, was not markedly impaired (supplemental Fig. 1).[3]

Because CD45RO$^+$ T cells are the major producers of IFN-γ, IL-4, and IL-17 (44) (Fig. 6*A*), we considered the possibility that the impaired cytokine responses were due to the decrease in memory CD4 T cells in the patients. To test this, we stimulated PBMCs with PMA/ionomycin and tested for intracellular IFN-γ, IL-4, or IL-17 in the CD4$^+$CD45RO$^+$ population. Fig. 6, *A* and *B*, shows that memory T cells of the patients produced less IFN-γ than the controls. In the controls, 30% of CD45RO$^+$ memory T cells produced IFN-γ, whereas in the patients, only ~10% of CD45RO$^+$ memory T cells did so. The synthesis of IL-4 and IL-17 in the memory T cell fraction was marginally decreased in the patients,
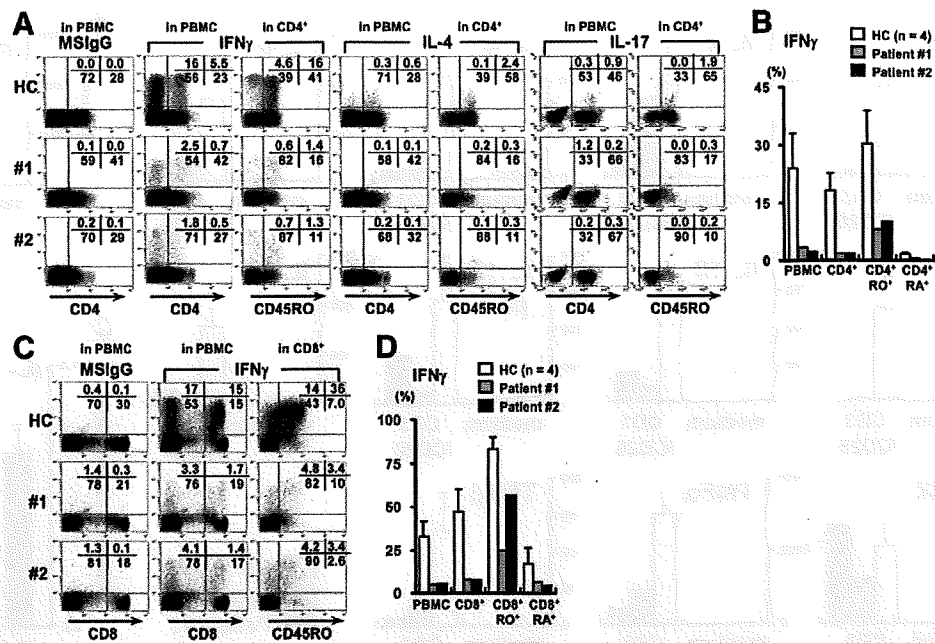
FIGURE 6. Defective cytokine production in T cells stimulated with PMA/ionomycin in ICOS-deficient patients. *A* and *C*, PBMCs were stimulated with PMA and ionomycin for 5 (IL-17) or 8 h (IFN-γ and IL-4). Cells were stained for intracellular IFN-γ, IL-4, and IL-17 together with Abs to CD4 and CD45RO or CD45RA. The same experiment was conducted in CD8 T cells for intracellular detection of IFN-γ. Intracellular staining of each cytokine in CD4 and CD4⁺CD45RO⁺ T cells (*A*) and in CD8 and CD8⁺CD45RO⁺ T cells (*C*) is shown. A representative FACS analysis is illustrated for one of four healthy controls (HC) (for CD4), one of five HC (for CD8), and the patients. *B* and *D*, Pooled data on IFN-γ-producing cells among PBMCs, CD4 T cells, CD4⁺CD45RO⁺ cells, CD4⁺CD45RA⁺ cells, CD8 T cells, and CD8⁺CD45RO⁺ cells, in HC (*n* = 4) and patients 1 and 2. □, HC; ▤, patient 1; ■, patient 2. Error bar for HC indicates SD. The mean percentage obtained from two separate analyses is shown for the patients.

and the decline was not as clear as that observed in IFN-γ production (Fig. 6*A*).

Importantly, the inability to produce IFN-γ does not seem to be restricted to CD4 T cells, because a marked reduction in the IFN-γ response was also evident in the CD4-negative population. To assess effector function of CD8 T cells, we directly measured intracellular IFN-γ in CD8 T cells and a CD8⁺CD45RO⁺ population upon stimulation with PMA/ionomycin. The results displayed in Fig. 6, *C* and *D*, revealed impaired IFN-γ production from CD8 T cells and memory CD8 T cells from the patients. The production of IFN-γ was also significantly reduced in CD8 T cells stimulated through CD3 and CD28 in the patients (Fig. 5*B*).

Mechanism of defective cytokine production in patients: reduced induction of master regulators of Th1, Th2, and Th17 lineage commitment

We next investigated the mechanisms underlying the T cell unresponsiveness in the patients. One potential explanation is that their T cells did not proliferate well or were prone to apoptosis, or both, in the absence of ICOS expression. To examine this possibility, the proportion of cells that underwent PMA/ionophore-induced cell death was assessed by annexin V/7-AAD staining. The results showed that in the patients, this proportion was similar to or rather lower than that in controls. The proliferative capacity of ICOS⁻/⁻ T cells, as assessed by CFSE staining, showed that their T cells proliferated normally or even more vigorously in response to CD3/CD28 cosignal, with significantly more cells with multiple divisions, relative to controls (supplemental Fig. 2).[3]

Although less likely, the absence of the ICOS-ICOS-L interaction during CD3/CD28 costimulation of CD4 T cells may have contributed to impaired cytokine production in the patients. To test the possible contribution of the ICOS signal in cytokine production, we stimulated purified CD4 T cells from healthy controls

(*n* = 5) through CD3/CD28 with or without anti-ICOS-L blocking Ab (45), and measured the level of cytokines in the supernatants. Supplemental Fig. 3, *A–C*,[3] shows that the effect of ICOS blocking is negligible in this cytokine production assay.

Another explanation for the defective production of effector cytokines is that there were fundamental flaws in their development into effector T cell subsets. We therefore investigated the expression of master transcription regulators of Th1, Th2, and Th17 lineage commitments by quantitative real-time PCR.

Purified CD4 T cells were stimulated with PMA/ionomycin for 4 h or anti-CD3/CD28 for 24 h, and the mRNA expression level of T-bet (for the Th1 lineage) (46), GATA3 and MAF (for the Th2 lineage) (47, 48), and RORC/ROR-γt (for the Th17 lineage) (49) was quantified using GAPDH expression as a control, and expressed as relative expression (RE) adjusted for the baseline expression level of healthy controls (*n* = 4), taken as 1.0. We observed reduced PMA/ionophore-driven T-bet induction in ICOS⁻/⁻ CD4 T cells in the patients, and defective induction was more pronounced in patient 2 (Fig. 7*A*). Compared with controls (RE, 16.2 ± 5.3), CD3/CD28-induced T-bet expression was decreased in patient 1 (RE, 7.2), whereas the reduction was less marked in patient 2 (RE, 12.4) (Fig. 7*B*).

GATA-3 induction was detectable after PMA/ionophore stimulation. In the patients, induction of GATA-3 above the baseline level was virtually absent in CD4 T cells (RE, 1.3 for patient 1, and 1.0 for patient 2) (Fig. 7*A*). The RE values of MAF in CD4 T cells in response to PMA/ionomycin and TCR/CD28 in controls were 3.3 ± 1.4 and 4.4 ± 1.0, respectively. In contrast, stimulation-induced MAF expression was virtually absent in both patients (Fig. 7, *A* and *B*).

We also observed that the expression levels of RORC in ICOS-deficient CD4 T cells stimulated with CD3/CD28 or PMA/ionomycin were diminished more than 2-fold (Fig. 7, *A* and *B*).

FIGURE 7. Impaired expression of effector-specific transcription factors for T cell differentiation. Purified CD4 T cells were activated by PMA/ionomycin or incubated in the medium for 4 h (*A*), or were incubated in the presence or absence of anti-CD3/anti-CD28 costimulation for 24 h (*B*). Expression levels of TBX21 (T-bet), GATA-3, MAF, RORC (ROR-γt), and EOMES mRNAs were determined in duplicate by real-time quantitative PCR. Similarly, purified CD8 T cells were activated by PMA/ionomycin or by anti-CD3/anti-CD28; and the expression levels of TBX21 and EOMES were assessed in healthy controls (HC). Patients 1 and 2 (*C*). Levels of each mRNA were expressed as relative expression compared with expression of 18S rRNA, and the mean mRNA expression level in cells from HC cultured in the medium was adjusted to 1.0. □, HC; ▤, patient 1; ■, patient 2. Error bars indicate the SD for HC (*n* = 5). The assay was performed three times for the patients; average and SEM are shown. P/I: PMA/ionomycin.
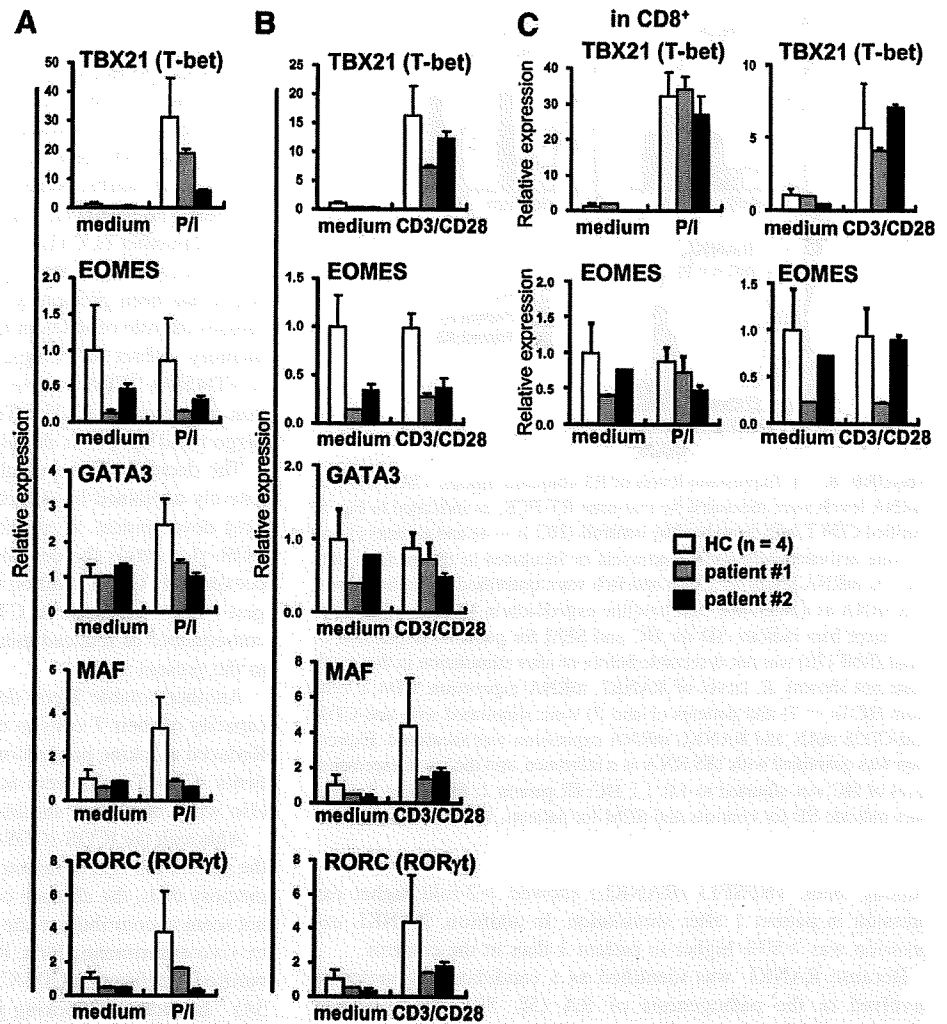
Despite poor IFN-γ production by CD8 T cells, we did not observe low T-bet induction in purified CD8 T cells when stimulated by PMA/ionomycin or by anti-CD3/anti-CD28 mAb (Fig. 7*C*). We then tested the expression of EOMES, a paralog of T-bet and a transcription factor required for CD8 effector function (50, 51). Although induction was negligible, there was a trend toward lower baseline expression of EOMES (0.23 and 0.69 for patients 1 and 2, respectively).

Expression of the E3 ubiquitin ligases that contribute to T cell anergy

Several lines of evidence indicate that E3 ubiquitin ligases (Grail, Cbl-b, and Itch) play important roles in the induction and maintenance of T cell tolerance (52–55). T cell stimulation without costimulation leads to up-regulation of these ligases (56). High expression of these E3 ubiquitin ligases is related to the absence of the expression of the effector-specific transcription factors (56, 57). There has been little research on the ligases in human systems. Because anti-CD3 stimulation did not induce appreciable up-regulation of the ligases, we examined the mRNA level of these molecules at baseline and after PMA/ionophore stimulation using a sensitive real-time PCR assay.

As shown in Fig. 8, baseline expression of the E3 ubiquitin ligases, with the exception of Grail, was detected in the controls and patients. The mRNA levels of Cbl-b and AIP4/Itch in the steady state were significantly elevated in patient 2 (2.5 and 4.3)

compared with controls (1.0 ± 0.4 and 1.0 ± 0.6, *n* = 4) and patient 1 (1.0 and 1.3).

PMA/ionophore induced up-regulation of Cbl-b and Itch, but not Grail, in normal subjects, whereas the induction of Cbl-b and Itch mRNA above the baseline level was negligible in patient 2 (Fig. 8).

Of particular note was a paradoxical down-regulation of Itch, a regulator of NF-κB activation, upon PMA/ionophore stimulation, which was reproducibly observed only in patient 1.

RANKL induction was augmented in patient 2 with autoimmunity

Patient 1 had an autoimmune manifestation and immunodeficiency, whereas patient 2 had mild psoriasis-like cutaneous lesions and mild skin infections. We therefore attempted to uncover differences in T cell functions between two patients.

To explore the dissimilarities in their immune functions, we assessed mRNA expression levels in negatively selected, >97% pure CD4 T cells by comprehensive mRNA expression analysis using a GeneChip before and after stimulation through CD3/CD28.

The expression of most mRNAs from CD4 T cells poststimulation showed a remarkably high correlation between patients 1 and 2. However, we identified >100 genes that were differently expressed between the patients, and sought to identify the gene(s) that may explain the phenotypic difference from these genes.
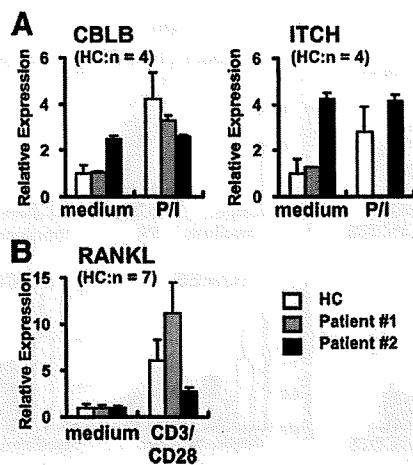
FIGURE 8. *A*, Expression levels of E3 ubiquitin ligases. Cbl-b and Itch mRNA levels were measured by real-time RT-PCR, as indicated in Fig. 5. Purified CD4 T cells from healthy controls (HC; $n = 4$) and patients (1 and 2) were activated with PMA/ionomycin or incubated in the medium only for 4 h. mRNA levels for Cbl-b and Itch were quantified in duplicate with 18S rRNA as a reference, and baseline expression in HC was adjusted to 1.0. Error bars indicate SD for HC and SEM for patients. Expression of Grail (RNF128) was not detectable before or after stimulation in this assay (data not shown). *B*, Level of RANKL mRNA expression. CD4 T cells from HC ($n = 7$) and patients (1 and 2) were stimulated with anti-CD3/anti-CD28 mAb, and RANKL mRNA expression was measured. Expression was quantified with 18S RNA as a reference, and the mean expression level of HC was adjusted to 1.0. □, HC; ▩, patient 1; ■, patient 2. Error bars indicate SD for controls and SEM for patients. P/I: PMA/ionomycin.

Among them, TNFSF11 (RANKL) showed >2-fold higher expression in patient 1 after stimulation. In addition, RANKL expression was >50% higher in patient 1 than in the controls.

Because RANKL was identified as a candidate key molecule involved in the pathogenesis of RA (58, 59), we quantified RANKL mRNA in CD3/CD28-stimulated CD4 T cells by real-time PCR. The result shown in Fig. 8*B* demonstrates that compared with healthy controls, RANKL induction was higher in patient 1, but lower in patient 2.

Discussion

In this study, we describe broad defects in T cell function in two siblings with a novel deficiency of human ICOS. Most of the abnormalities presented in this study have not been reported in humans, and some have not been reported in the murine model of ICOS deficiency.

The marked decline in two T cell subpopulations, memory CD4 T cells and CTLA-4⁺CD45RO⁺ Tregs, can be explained, at least in part, by a recent observation that the ICOS-ICOS-L interaction plays an important role in the expansion and survival of these effector T cells (23, 60).

With regard to CD4 memory T cells, we observed significant reductions in the numbers of both TCMs and TEMs in the steady state, which were not observed in the previously reported cases of human ICOS deficiency (31–33). A reduction in the number of TEMs, but not of TCMs, was demonstrated in ICOS knockout mice by Burmeister et al. (23). TEMs were decreased up to 4-fold in the steady state; the decrease was more pronounced in older mice. TEMs and TCMs display significant and intermediate ICOS expression, respectively (23). Through detailed research on expansion, differentiation, and survival of effector T cells in the absence of ICOS, they suggested that ICOS controls the pool size of effector T cells. These data suggest that both memory subsets may

require ICOS for proliferation and survival in humans. Therefore, a decline in total memory cells may be observed in ICOS-deficient mice over a longer observation period and/or after recurrent infections. Alternatively, it is equally plausible that the ICOS-ICOS-L interaction plays a pivotal role in commitment to memory T cells.

CTLA-4⁺CD45RO⁺ICOS⁺CD4⁺CD25⁺ Tregs, commonly observed in adults, were virtually absent in our ICOS-deficient patients. The reduction was seemingly counterbalanced by an increased number of CTLA-4⁻CD45RO⁻ Tregs. Although the contribution of ICOS to the expansion and maintenance of Tregs as a whole has been previously reported (23, 41), our observation addresses the role of ICOS in the maintenance of an IL-10-producing memory subset of Tregs, but not TGF-β-producing CTLA-4⁻CD45RO⁻ICOS⁻ naive Tregs. Supporting this is the observation that in mice, ICOS⁺ Tregs display a strict propensity to undergo rapid apoptosis in culture unless signaled by ICOS-L (23).

The decrease in the number of CTLA-4⁺ Tregs may be alternatively explained by defective induction of a gene that regulates Treg development. A recent study in mice has demonstrated that ROR-γt controls the development of IL-10-producing Tregs that coexpress ICOS in addition to CCL20 (61). This finding may suggest that the decrease in CTLA-4⁺CD45RO⁺FoxP3⁺ Tregs is a consequence of reduced induction of ROR-γt/RORC, as observed in the present study.

Another notable T cell defect in our patients was the impaired capacity of their T cells to mount Th1, Th2, and Th17 responses. Reduced cytokine production was observed not only when the patients' CD4 T cells were activated by costimulatory signals, but also when they were stimulated by PMA/ionomycin.

Although the ICOS-ICOS-L interaction was important in vivo in the generation and/or maintenance of effector memory and central memory cells, the absence of an ICOS signal through ICOS-L did not seem to contribute to the T cell effector defects observed in our ex vivo experiments. First, ICOS-L expression was not induced in purified T cells upon CD3/CD28 costimulation (supplemental Fig. 3*A*).[3] In addition, blocking potential ICOS-ICOS-L interaction in the controls did not result in decreased cytokine production or in decreased up-regulation of MAF and RORC (supplemental Fig. 3, *B* and *C*).[3]

Additional experiments indicated that there was an abnormality at the level of transcriptional regulation of Th1, Th2, and Th17 polarization, and decreased induction of the master regulators T-bet, GATA-3, MAF, and RORC in the patients. Previous research on mice has shown that ICOS regulates MAF expression and GATA-3 induction (62), and our present study points to an additional role of ICOS in the complete induction of T-bet and RORC.

One major factor contributing to the poor effector T cell responses in the patients could be the decrease in total memory CD4 T cells. This is particularly likely in the case of IL-4 and IL-17 production, because the memory T cells had only mild defect in producing IL-4 and IL-17. Although the CD4⁺CD45RO⁺ T cells in the patients displayed a significantly reduced ability to produce IFN-γ, the decreased response may be explained by pronounced reduction in TEMs in the patients. To determine whether the memory T cell compartment in our patients is functionally defective or intact on a per cell basis, we would need further analysis of various parameters of the T cell effector functions in naive T cells, TCMs, and TEMs.

Nurieva et al. (56) demonstrated that murine ICOS⁻/⁻ CD4 T cells showed defective induction of T-bet, GATA-3, and EOMES in the absence of CD28 costimulation because of up-regulation of E3 ubiquitin ligases: Grail, Cbl-b, and Itch. It is uncertain whether the augmented baseline expression of E3 ubiquitin ligases is relevant to the observed effector T cell dysfunction, because this was

confirmed only in patient 2. It is rather unlikely that the different expression of the E3 ubiquitin ligases contributed to the T cell defects in the patients because augmented induction of these ligases was not detected in the patients.

In contrast to the global impairment in cytokine synthesis, IL-2 production was only marginally affected. Supporting this observation, induction of transcription factors for IL-2 (c-Jun/c-Fos) was normal in the patients (data not shown). Similarly, induction of IL-21 and TGF-β was also unaffected in the CD4 T cells of the patients, although their induction was modest under costimulatory conditions (data not shown). It should be noted that production of IL-22, a Th17 cytokine fundamental for the development of psoriasis, showed normal induction, and that both the patients had psoriatic cutaneous lesions (63). Although whether IL-17A and IL-22 are produced by the same Th17 subset is still unclear (64, 65), our data suggest an IL-22-producing CD4 T cell subset is not functionally impaired in the patients.

Previous studies in mice have shown that ICOS is necessary for optimal CD8 T cell responses (34). ICOS can directly stimulate CD8 T cells (35); and ICOS-Ig-treated mice displayed diminished IFN-γ production by CD8 T cells. Our study has demonstrated that CD8 memory cells are reduced in ICOS deficiency, and that CD8 T cells in the absence of ICOS can mount a very low IFN-γ response, for the first time in humans. ICOS is induced on terminally differentiated CD28$^-$CD8 effector T cells (11), and thus, may play a role in maintaining the CD8 subset. Therefore, a decrease in the number of IFN-γ-producing CD8 T cells could be ascribed to the reduction in CD45RO$^+$ memory CD8 T cells or in CD28$^-$CD8 T cells (data not shown) in our ICOS-deficient patients. IFN-γ production is regulated by T-bet and EOMES cooperatively or redundantly in CD8 T cells (50, 51). T-bet induction was normal when stimulated with PMA/ionomycin or anti-CD3/anti-CD28, whereas a baseline expression of EOMES was decreased in CD8 T cells in the patients. Although this may explain the impaired production in part, further research on the CD8 T cells stimulated with various common γ-chain cytokines would be necessary to assess whether the transcriptional regulation of CD8 effector functions is impaired in the absence of ICOS.

In addition to the reduced numbers of effector T cells, which either potentiate or inhibit T cell responses, the present study demonstrates for the first time an aberrant induction of negative costimulatory molecules on activated T cells in ICOS-deficient patients. CTLA-4 and BTLA are induced upon activation and transmit an inhibitory signal to T cells to regulate the balance between T cell activation, tolerance, and immunopathology (3–5). Costimulatory and coinhibitory molecules are normally induced in the absence of ICOS in mice and humans (23, 31). In our patients, however, induction of CTLA-4 and BTLA following CD3/CD28 signaling was impaired. Although the molecular basis of the defective expression is still not known, this may be ascribed to the decreased memory T cell subset in the patients. At all events, our findings imply that an inhibitory signal to suppress activated T cells could not be appropriately induced in the patients.

Collectively, these data highlight the positive contribution of ICOS to the maintenance of, or commitment to, effector T cells and a subset of Tregs, and the induction of negative costimulatory receptors on activated T cells. The immunodeficiency in our ICOS-deficient patients, although mild, can be understood by the defects in their effector T cell functions as well as in T cell-dependent B cell help, but a reasonable explanation is still required for the development of autoimmunity, RA, IBD, IP, and psoriasis in ICOS-deficient patients.

Most studies have depicted ICOS as a positive costimulator in the immune reaction. For example, research in ICOS-deficient

mice suggests that ICOS is critically involved in autoimmune development and allogeneic reactions (21, 25–29). There are, however, some results indicating that abrogation of the ICOS-ICOS-L interaction aggravates the disease process. For example, in some initial studies on ICOS-null mice, experimental autoimmune encephalomyelitis was unexpectedly exacerbated and allergen-dependent airway sensitivity was augmented (14, 66, 67). What is the role of ICOS in autoimmune development?

Burmeister et al. (23) reported that ICOS supports the expansion and survival of Th1 or Th2 responder cells, Th17 cells, and FoxP3$^+$ regulatory effector cells. They hypothesized that the absence of ICOS function in a particular mouse model would result in a phenotype reflecting a deficiency of the dominant effector T cell type. Thus, blockade of the ICOS-ICOS-L interaction could mainly affect Treg subsets and lead to the development of autoimmune disorders.

Our observations in human ICOS deficiency may fit the concept of ICOS as an agonist molecule. In our ICOS-deficient patients, defects in T cell function leading to termination of the activated T cell response may have been dominant.

Another question remains, as we observed a wide range of autoimmune diseases in patient 1, but not in patient 2. Although phenotypic variation in siblings with the same mutation is not uncommon in human genetic disorders, there might have been some contributing factor(s).

First, our analysis showed paradoxical down-regulation of Itch expression after PMA/ionophore stimulation in patient 1. Because Itch induction is important in the control of NF-κB activation (55), an activation signal in the absence of CD28 costimulation may have led to continuous inflammation in patient 1.

Second, although the T cell immune functions and stimulation-induced mRNA expression pattern of CD4 T cells were strikingly similar between the siblings, we found that the T cells of patient 1 showed exaggerated induction of RANKL expression and poorer production of IFN-γ. Previous studies demonstrated that T cells, which contribute to the development of RA and IBD, were characterized by poor IFN-γ production, production of inflammatory cytokines including IL-17A and TNF, and RANKL expression (59). Although IL-17A induction was not increased, the augmented RANKL expression and poor IFN-γ production in T cells may have contributed to the autoimmune disease progression. Characterization of RANKL-expressing IL-17A-negative T cells requires further investigation.

Third, we surmised that a major infectious episode may have upset the subtle balance between effector T cells and Tregs in our patients. In fact, patient 1 developed a series of autoimmune disorders after a severe bacterial infection.

Finally, the reason for the apparent discrepancy in T cell functions between the ICOS-deficient patients presented in this study and the ICOS deficiency described in previous reports (31–33) is elusive. One possibility that explains the difference in cytokine production in our patients and ICOS deficiency in previous reports could be different stimulation condition (dose of mAb and incubation period). However, it is unlikely because effective cytokine synthesis from the T cells was not observed in our patients even with an increased dose of anti-CD28 mAb and with longer time periods (supplemental Fig. 4).[3] This indicates the presence of other intrinsic factor(s).

Another possibility is the difference in the mutation site of the ICOS gene. Our patients harbored a homozygous single-base deletion at codon 285 located in the extracellular domain, whereas other ICOS deficiencies have homozygous deletion in exons 2 and 3 of the ICOS gene (33). In addition to defective ICOS expression,

this mutation may result in the expression of a 120-aa ICOS protein that affects immune function, for example, by binding to ICOS-L on B cells, monocytes, or a subset of T cells. Despite extensive investigation, however, we have been unable to demonstrate the expression of a truncated ICOS protein in our patients' lymphocytes.

In summary, the present study on T cell functions in two novel ICOS-deficient patients has shown that the interaction between ICOS and ICOS-L is critical for the development and maintenance of multiple types of effector cells and Tregs, and that the defects are at least in part due to diminished memory T cells and/or impaired induction of master regulators. Collectively, the results of our study highlight a major role of ICOS as a coordinator of T cell immune responses and T cell maintenance.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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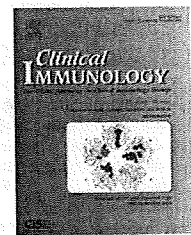
Potentiation of TLR9 responses for human naïve B-cell growth through RP105 signaling

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Abstract   Toll-like receptor 9 (TLR9) signals induce important pathways in the early defense against microbial pathogens. Although TLR9 signaling can activate memory B cells directly, efficient naïve B cell responses seem to require additional, but as yet unidentified, signals. We explored the effects of RP105 (CD180) on CpG DNA-activated naïve and memory B cells from normal controls and patients with common variable immunodeficiency (CVID). RP105 dramatically enhanced CpG DNA-induced proliferation/survival by naïve B cells but not by memory B cells. This enhancement was mediated by TLR9 upregulation induced by RP105, leading to Akt activation and sustained NF-κB activation. CpG DNA-activated CVID B cells showed enhancement of proliferation/survival by RP105 and produced specific IgM antibody to Streptococcus pneumoniae polysaccharides in response to interleukin-21 stimulation. Thus, RP105 strongly affects expansion of the naïve B-cell pool, and suggests that the putative RP105 ligand (s) upon cytokine stimulation facilitates antibody-mediated acute pathogen clearance.
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Introduction

Innate immune responses are initiated through various pathogen receptors, including Toll-like receptors (TLRs) and NOD-like receptors (NLR). TLRs recognize various

microbial components and initiate signaling cascades not only by evoking innate immune responses [1], but also by potentiating subsequent adaptive immune responses. An important consequence of TLR mediated signaling is polyclonal activation of B cells leading to the production of germline-encoded, poly-reactive antibodies that are believed to be an essential part of the first line defense against systemic bacterial and viral infections [2]. Human B cells express TLR1 and TLR6-10 and the expression predominate TLR9 and TLR10 [3,4]. While naïve human B cells express TLR9 at low levels, memory B cells express TLR9 at constitutively higher levels [5]. The CpG motif of bacterial

DNA, the ligand of TLR9, plays an important role in early immunoglobulin (Ig) responses against pathogens. CpG DNA-activated B cells proliferate, produce cytokines, and become high secretors of immune globulins [6–8]. This ligand has been shown to be a most potent inducer of generation of plasma cells not only from memory and germinal center B cells, but also from naïve B cells, although to a lesser extent [9].

RP105 (CD180), originally discovered as a murine B cell surface molecule, protects B cells from irradiation-induced apoptosis [10]. RP105 is considered to be B cell specific and is physically associated with MD-1, a surface molecule that is necessary for cell surface expression of RP105 in human B cells [11]. The observation that B cells from RP105-deficient mice exhibit impaired lipopolysaccharide (LPS)-driven pro-liferative responses suggests that RP105 signals cooperate with TLR4 in LPS-induced B cell activation [12], and that putative RP105 ligand(s) act as potentiators of polyclonal B cell activation. Although TLR9 expression is high in B cells, the effect of RP105 on TLR9 responses in human B cells is unknown.

To address this point, we examined the role of RP105 signals on CpG DNA-activated human naïve and memory B cell responses. We found that CpG DNA-activated human naïve B cells, obtained either from normal individuals or from patients with common variable immunodeficiency (CVID), underwent marked expansion in vitro by RP105 signals and, if co-cultured with interleukin (IL)-21, produced IgM, which is specific to Streptococcus pneumoniae polysaccharide.

Methods

Patient population

Three Japanese females with well-documented CVID were included in this study after informed consent was obtained. Mean age was 40 years, ranging from 16 to 42 years with a median age at the onset of 18 years. All three patients presented with severe hypogammaglobulinemia, normal numbers of circulating B cells (4.9±2.1%) and absence of memory B cells (CD27⁻ naïve B cells 92.1±3.2% of total B cells). All received regular infusions of intravenous immunoglobulin.

Antibodies and reagents

Anti-CD27 monoclonal antibody (mAb) (8H5, IgG1) was provided by Dr. T. Morimoto (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Anti-RP105 mAb (MHR73, IgG1) was described previously [10]. FITC-conjugated anti-CD20 mAb (anti-CD20-FITC), PE-conjugated anti-CD38 mAb (anti-CD38-PE), PE-conjugated streptavidin, anti-mouse IgG-FITC and anti-IgD-FITC were obtained from DAKO Japan (Tokyo, Japan); anti-CD40 mAb (G28-5; IgG1) from American Type Culture Collection (Manassas, VA); PerCP-conjugated anti-CD20 mAb (anti-CD20-PerCP) and goat anti-mouse Ig-FITC from Becton Dickinson (San Jose, CA); and anti-TLR9-PE from eBioscience (San Diego, CA). Staphylococcus aureus Cowan strain (SAC) and Propinium iodide (PI) were obtained from Sigma Chemical (Perth, Australia); IL-21 from Biosource (Camarillo, CA) and the proteasome inhibitor, MG132 from Calbiochem (San Diego, CA). The CpG oligodeoxynucleotide

2006 with the sequence 5′ TCG TCG TTT TGT CGT TTT GTC GTT 3′ was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Cell preparation and culture

Human B cells were purified from peripheral blood of healthy adult volunteers, from the three CVID patients and from normal cord blood using the human B cell enrichment cocktail, RosetteSep (IgG1: StemCell Technologies, Vancouver, Canada), which uses antibodies that are bound as bispecific antibody complexes directed against common cell surface antigens on human hematopoietic cells (CD2, CD3, CD16, CD36, CD56) and glycophorin A on red blood cells. The isolated B cells did not express activation makers, and their purity exceeded 90%. The residual cells contained with <1% of CD3⁺ T cells, <2% of CD56⁺ NK cells, <1% of CD14⁺ monocytes and <7% of dendritic cells (CD123⁺ plasmacytoid dendritic cells and CD11c⁺ myeloid dendritic cells). CD20⁺ CD27⁻ or CD20⁺ CD27⁺ B cells were separated using magnetic micro-beads coated with anti-CD27 mAb (Miltenyi Biotec., Bergisch Gladbach, Germany). The sorted CD27⁻ naïve B cells contained <3% of CD27⁺ B cells.

Purified human total, naïve or memory B cells were cultured in RPMI-1640 containing 10% fetal calf serum (Hyclone, Logan, UT) using 96-well round-bottom plates (Nunc, Roskide, Denmark) in the presence of various stimuli at a final cell density of 5×10^5/mL in a volume of 200 μL per well for 2 to 14 days at 37 °C in a humidified atmosphere with 5% CO_2.

Flow cytometric analysis

Double or triple-color analyses of B cell surface molecules were performed by FACScalibur (Becton Dickinson). The antibody-coated cells were gated on living cells by cell size and granularity, and then counted by flow cytometry.

B cell proliferation

After 72 h of culture in the presence of medium alone, SAC, anti-CD40 mAb and CpG DNA with or without anti-RP105 mAb or Mouse IgG1 (Zymed, South San Francisco, CA) as an isotype control, the purified B cells were pulsed with 0.5 μCi [³H]-thymidine, incubated for additional 15 h, and harvested by an automatic cell harvester (Packard, Meriden, CT). [³H]-thymidine incorporation was measured by a liquid scintillation analyzer (Packard).

Assessment of cell survival

Purified human B cells were cultured in the presence of medium alone, anti-CD40 mAb and CpG DNA, anti-RP105 mAb, CpG DNA plus anti-RP105 mAb or Mouse IgG1 as an isotype control for 7 or 14 days. Dead and viable cells were discriminated by staining with PI, and evaluated by flow cytometry.

Preparation of nuclear and cytosolic extracts

Cultured B cells (1×10^6 cells) were suspended in solution A (10 mM Hepes-NaOH, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride) and incubated for 15 min at 4 °C. After addition of

250 μL of 10% Nonidet P-40, the cell suspension was incubated for 30 min at 4 °C and centrifuged. The supernatant was saved as the cytosolic extract. The pellet was resuspended in solution C (50 mM Hepes-NaOH, 50 mM KCl, 300 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol) and kept at 4 °C for 30 min. After centrifugation, the supernatant was saved as the nuclear extract.

Immunoblot analysis

Nuclear or cytosolic extracts prepared from cultured B cells were separated by 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes and incubated with rabbit polyclonal antibodies to human I kappa B kinase α (IκBα) (Santa Cruz Biotech., Santa Cruz CA), β-actin (Cell Signaling Technology, Danvers, MA), NF-κB RelA (p65), p50 (Santa Cruz Biotech.) or Phospho-Akt (Ser473) (Cell Signaling Technology). Membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega Corporation, WI) or horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences, Buckinghamshire, UK). Protein bands were visualized by the alkaline phosphatase color reaction or by the ECL system (Amersham Biosciences).

Enzyme-linked immunosorbent assay (ELISA) to measure Igs

Purified human B cells were cultured for 7 days. The supernatants were added to 96-well flat-bottom plates (Nunc) coated with goat anti-human Igs (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After overnight incubation at 4 °C, alkaline phosphatase-conjugated goat anti-human IgG, IgA or IgM (Sigma chemical) was added. Color detection was performed with 3-[cyclohexylamino]-1-propanesulfonic acid buffer containing p-Nitrophenyl phosphate (Sigma chemical). No cross-reactions between IgG, IgA and IgM were observed.

Quantitative real-time PCR

Total RNA was extracted from purified B cells cultured for 2 days with various stimuli. First-strand cDNA copies were synthesized by using random hexamers as primer (Applied Biosystems, Foster city, CA) and Improm-II reverse transcription system (Promega, Madison, WI). Quantitative real-time PCR was performed on an ABI Prism 7000 sequence detector (Applied Biosystems) and TaqMan reagents (Applied Biosystems), according to the manufacturer's instructions. The identification numbers of TaqMan MGB primer/probe sets for
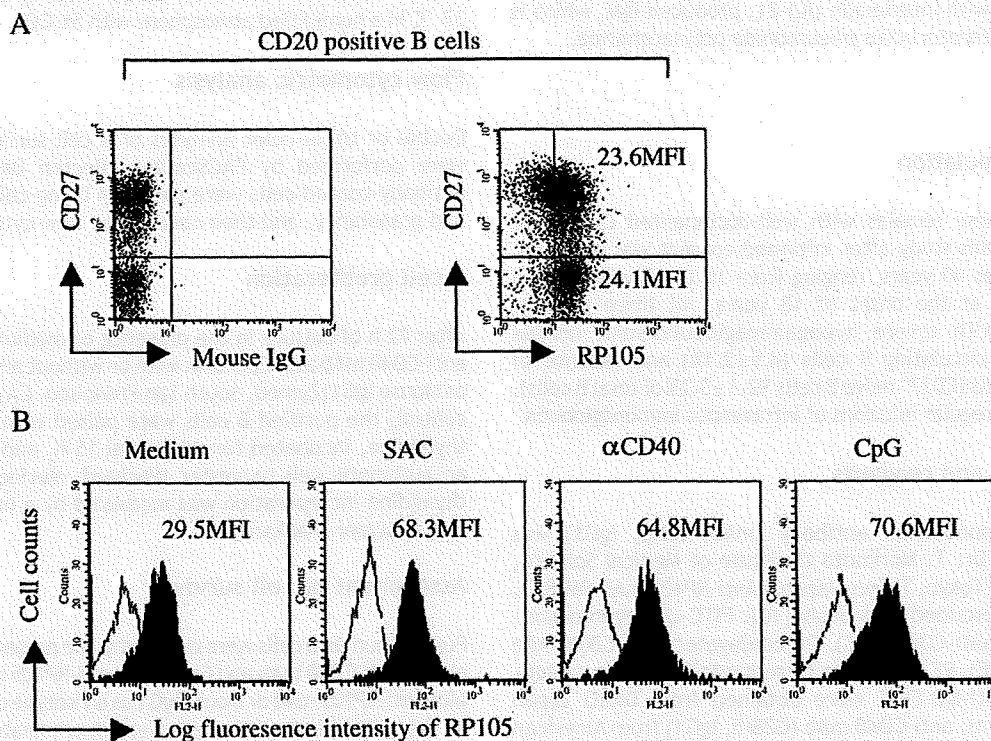

Figure 1 RP105 expression on resting and stimulated B cells. (A) Human peripheral resting B cells were stained with anti-RP105 mAb followed by goat anti-mouse Ig-FITC or mouse IgG-FITC as the isotype control, and anti-CD20-PerCP and anti-CD27-biotin followed by streptavidin-PE. Three-color analysis was conducted by gating on CD20 positive B cells. (B) B cells were cultured in the presence of medium alone, SAC (0.01%), anti-CD40 mAb (1 μg/mL), or CpG DNA (1 μg/mL) for 2 days. The cultured cells were stained with anti-CD20-PerCP or anti-RP105 mAb-biotin followed by streptavidin-PE. Flow cytometric analysis was conducted by gating on CD20 positive B cells. Histogram data indicating fluorescence intensity are displayed with PE for anti-RP105 (filled-in area) and isotype control (open area). The results depicted are representative of three independent experiments.

B-lymphocyte-induced maturation protein-1 (Blimp-1), activation-induced cytidine deaminase (AID) and β_2 microglobulin (β_2MG) were as follows: Hs00153357_ml (Blimp-1), Hs00221068_ml (AID), and Hs99999907_ml (β_2MG).

Specific IgG or IgM antibodies against pneumococcal polysaccharides

S. pneumoniae polysaccharide-specific (SP-specific) IgG or IgM was measured by ELISA. The protocol was identical to the Ig assay with the following exceptions. The cell culture supernatants were applied to 96-well flat bottom plates coated with 0.5 μg/mL of Pneumovax (Banyu Co., Tokyo, Japan), which is composed of 23 polysaccharide types. The concentration of SP-specific IgG or IgM was expressed as units per mL using a standard curve obtained from absorbance values of serial dilutions of pooled serum of healthy donors. The amounts of SP-specific Ig in pooled serum were

arbitrarily set at 20,000 U/mL. The lower detection limit of this assay was 4 U/mL.

Statistical analysis

Statistical significance between two groups was determined by unpaired Student's t-test (StatMate 3). Cell sizes were analyzed by Mann-Whitney U analysis (StatMate 3). Differences were considered significant when p values were less than 0.01.

Results

RP105 expression in human B cells

The expression of RP105 by human B cell subpopulations was examined at resting state and following activation. At resting
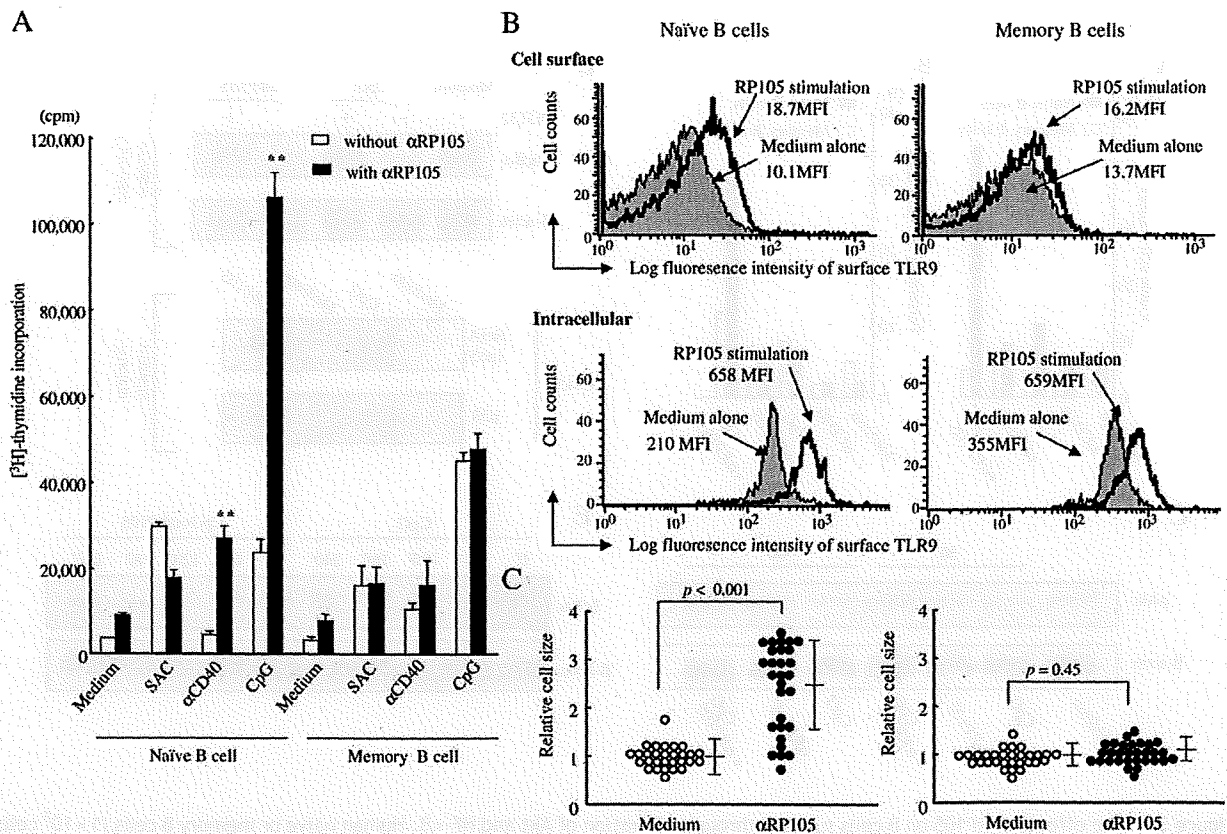

Figure 2 RP105 cross-linkage enhances proliferation, TLR9 expression and growth. (A) Naïve or memory B cells were cultured for 72 h with or without anti-RP105 mAb (1 μg/mL) in the presence of medium alone, SAC (0.01%), anti-CD40 mAb (1 μg/mL) or CpG DNA (1 μg/mL). Proliferation assays were performed in triplicate, and results are shown as mean ± SD. The results depicted are representative of five independent experiments. **indicates significant difference between cultures with or without anti-RP105 mAb at $p<0.001$. (B) Naïve B cells were cultured for 24 h in the presence of medium alone or anti-RP105 mAb (1 μg/mL). The surface and intracellular TLR9 expression of cultured cells was measured by flow cytometry by staining with anti-CD20-FITC and anti-TLR9-PE. Flow cytometric analysis was conducted by gating on CD20 positive B cells. Histogram plots shown are B cells cultured in medium alone (closed area) and with anti-RP105 mAb (open area). The data shown are representative of three independent experiments. (C) Naïve B cells (left panel) and memory B cells (right panel) were cultured for 7 days with or without anti-RP105 mAb. The cultured cells were cytospun and stained with May-Giemsa staining. The size of individual cells was measured. The size of cells stimulated with anti-RP105 mAb was shown as fold change compared with the average cell size cultured with medium alone and results are shown as mean ± SD.

state, both CD20+ CD27− naïve and CD20+ CD27+ memory B cells expressed RP105 at similar levels (Fig. 1A). Purified human B cells stimulated with SAC, anti-CD40 mAb, or CpG DNA showed a twofold increase in RP105 expression (Fig. 1B). Although we evaluated that the RP105 surface expression of naïve and memory B cells in the presence of medium, SAC, anti-CD40 mAb or CpG DNA, no difference of RP105 surface expression was observed between naïve and memory B cells (data not shown).

Promotion of proliferation and cellular enlargement of naïve B cells by cross-linking RP105

We investigated the difference, if any, between human naïve and memory B cell proliferation in response to RP105. Although RP105 cross-linkage alone did not significantly affect B cell proliferation, it increased the proliferation of naïve B cells synergistically with anti-CD40 mAb or CpG DNA, but not with SAC (Fig. 2A). In contrast, anti-RP105 mAb did

not enhance proliferation of similarly stimulated memory B cells (Fig. 2A). Notably, surface and intracellular expression of TLR9 by naïve and memory B cells was upregulated by anti-RP105 mAb (Fig. 2B). In contrast to the surface TLR9 expression, the intracellular TLR9 expression was lower in naïve B cells than that in memory B cells at resting state and the expression was significantly increased in naïve B cells compared with memory B cells by RP105 cross-linkage (Fig. 2B). We next measured the size of individual naïve and memory B cells microscopically after staining with May-Giemsa. The majority of naïve B cells but not memory B cells were enlarged following stimulation with anti-RP105 mAb alone (Fig. 2C).

RP105 cross-linking enhances survival of B cells and activation of Akt and NF-κB

The addition of anti-RP105 mAb to CpG DNA-stimulated naïve B cells cultured for 7 days dramatically increased the number
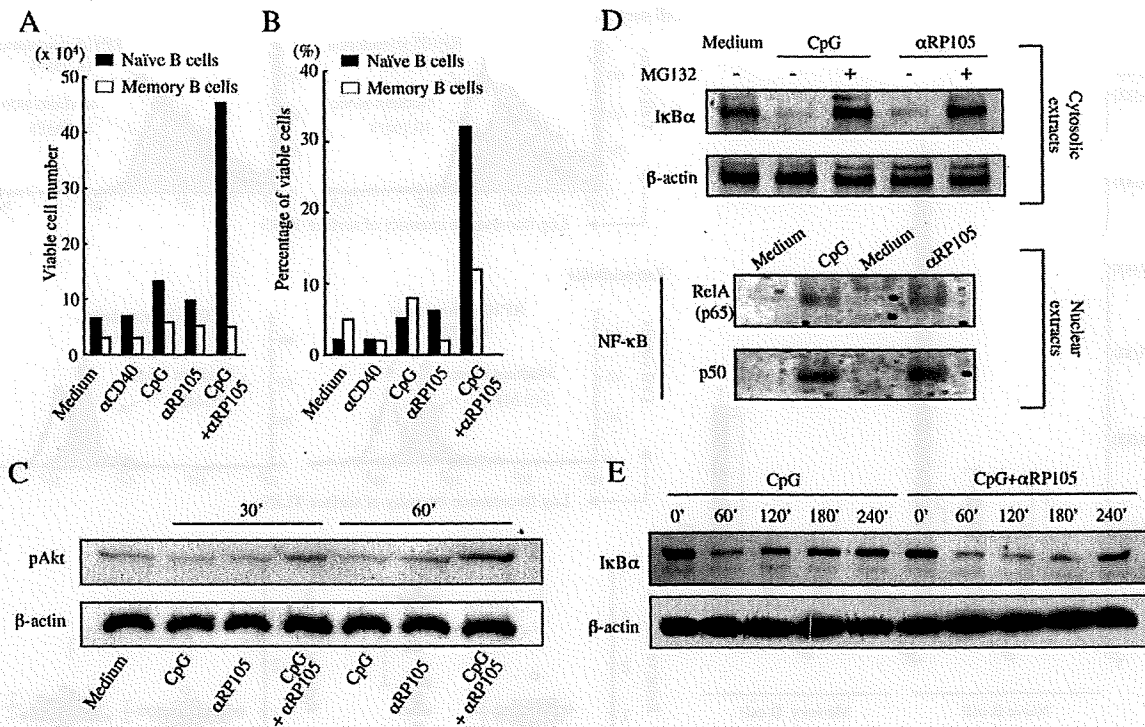

Figure 3   The effects of RP105 on B cells survival through activation of Akt and NF-κB. Human naïve or memory B cells (1×10⁵ cells) were cultured in the presence of medium alone, anti-CD40 mAb (1 µg/mL), CpG DNA (1 µg/mL), anti-RP105 mAb (1 µg/mL), or CpG DNA plus anti-RP105 mAb for 7 days and the number of viable cells determined (A) or for 14 days when the percentage of viable cells was determined (B). Cultured cells were stained with PI. Viable cell numbers were estimated by flow cytometry as: [viable cell number]=[cultured cell number]×[percentage of viable cells]. The results depicted are representative of four independent experiments. (C) Human B cells were stimulated with medium alone, CpG DNA (1 µg/mL), anti-RP105 mAb (1 µg/mL), or CpG DNA plus anti-RP105 mAb for 30 or 60 min. Whole cell lysates were analyzed by Western blotting with antibodies to Phospho-Akt and β-actin. A similar result was obtained in another experiment. (D) Human B cells were pre-treated with or without MG132 (40 µM), a proteasome inhibitor, for 2 h and then stimulated with CpG DNA (1 µg/mL) or anti-RP105 mAb (1 µg/mL) for 1 h. The cytosolic extracts were analyzed by immunoblotting with antibodies to IκBα and β-actin (upper panel). The nuclear extracts were analyzed by immunoblotting with antibodies to RelA (p65) and p50 (lower panel). (E) Human B cells were stimulated with CpG DNA (1 µg/mL) or CpG DNA plus anti-RP105 mAb (1 µg/mL) for 0, 60, 120, 180, or 240 min. Whole cell lysates were analyzed by Western blotting with antibodies to IκBα and β-actin. The data shown are representative of those from three independent experiments.

of viable cells in culture. (Fig. 3A). The percentage of viable naïve B cells, activated with CpG DNA for 14 days, was significantly increased in the presence of anti-RP105 mAb (Fig. 3B). In contrast, cross-linkage of RP105 did not increase the viability of CpG DNA-stimulated memory B cells (Fig. 3B).

Because the activation of Akt plays a role in cell growth and survival [13], we next examined Akt phosphorylation in activated B cells by Western blotting. As shown in Fig. 3C, B cells cultured for 30 or 60 min in the presence of both anti-RP105 mAb and CpG DNA increased Akt phosphorylation, while CpG DNA or anti-RP105 mAb alone failed to induce Akt

phospholylation (Fig. 3C). Activation of NF-κB prolongs cell survival through inhibiting apoptosis [14]. We therefore examined NF-κB activation by assessing the quantity of cytoplasmic IκBα, the inhibitor of NF-κB activation, and by estimating nuclear translocation of RelA (p65) and p50. As shown in Fig. 3D (upper panel), IκBα was degraded in response to stimulation with either CpG DNA or anti-RP105 mAb; this degradation was inhibited by the proteasome inhibitor, MG132. The degradation of IκBα was paralleled by the translocation of p65 and p50 to the nucleus (Fig. 3D, lower panel). IκBα levels were restored to normal within120
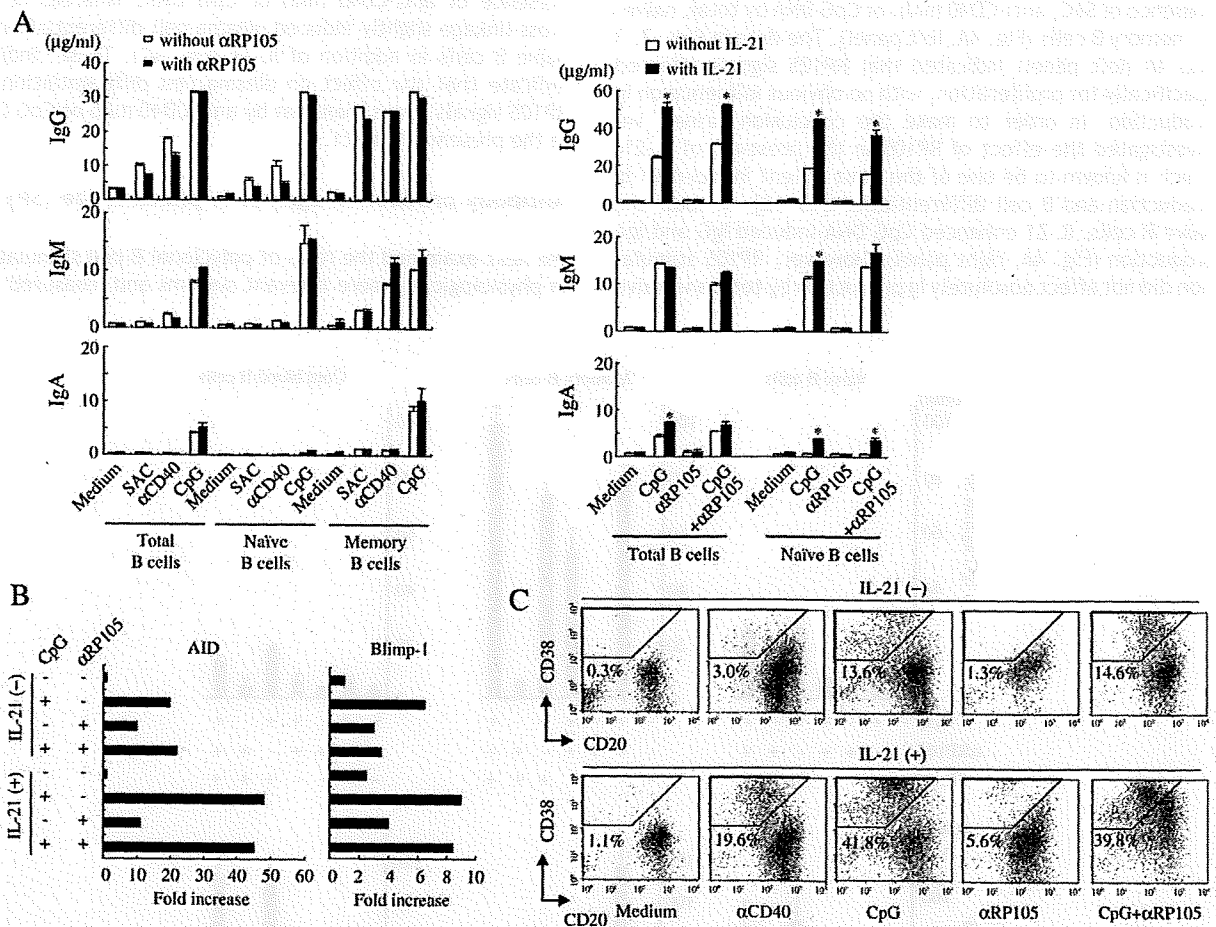

Figure 4   The effects of RP105 cross-linking on Ig production and plasma cell differentiation in B cells. (A) Purified total, naïve, and memory B cells were cultured with or without anti-RP105 mAb (1 μg/mL) in the presence of SAC (0.01%), anti-CD40 mAb (1 μg/mL), or CpG DNA (1 μg/mL) for 7 days (left panel). Total and naïve B cells were cultured with or without IL-21 (25 ng/mL) in the presence of medium alone, CpG DNA, anti-RP105 mAb, or CpG DNA plus anti-RP105 mAb (right panel). IgG, IgM, or IgA concentrations in culture supernatants were measured by ELISA. The results depicted are representative of four independent experiments. * indicates significant difference between cultures with or without IL-21 at $p<0.01$. (B) Purified peripheral B cells were cultured with medium alone, CpG DNA (1 μg/mL), anti-RP105 mAb (1 μg/mL), or CpG DNA plus anti-RP105 mAb with or without IL-21 (25 ng/mL) for 3 days. mRNA was isolated and Blimp-1 and AID expression was determined by quantitative real-time PCR. Data were normalized to β2MG mRNA and shown as fold-change compared with samples incubated with medium alone. Data are representative of results from two similar experiments. (C) Purified naïve B cells were cultured in the presence of medium alone, anti-CD40 mAb (1 μg/mL), CpG DNA (1 μg/mL), anti-RP105 mAb (1 μg/mL), or CpG DNA plus anti-RP105 mAb with or without IL-21 (25 ng/mL) for 7 days. Cultured cells were stained with anti-CD20-FITC and anti-CD38-PE. The fraction of CD38^{high} cells, which is the upper polygonal gate on the chart, was considered to be the plasma cell population. The results depicted are representative of three independent experiments.

min following CpG DNA stimulation. In contrast, costimulation of B cells with anti-RP105 mAb and CpG DNA resulted in reduced levels of IκBα for up to 180 min (Fig. 3E), demonstrating that RP105 and TLR9 signals acted synergistically on NF-κB activation.

Effects of RP105 signals on Ig production, and plasma cell differentiation

We next investigated the effect of anti-RP105 mAb on Ig synthesis by B cells. In contrast to its effect on B cell proliferation, enlargement of cell size and survival, RP105 signals did not enhance IgG, IgM, or IgA production in the presence of SAC, anti-CD40 mAb, or CpG DNA by total, naïve, or memory B cells (Fig. 4A, left panel). The data in Figs. 2, 3 and 4A (left panel) indicated that RP105 signals affected specifically for proliferation, with no obvious influence on Ig production. In order to make the conclusion firmer, we investigated the effect of RP105 in the presence of IL-21, which is known to be one of the most potent inducers of Ig production and B cell differentiation [15–18]. In total and naïve B cells, IL-21 enhanced CpG DNA-induced IgG and IgA production (Fig. 4A, right panel). However, RP105 stimulation did not affect absolutely Ig production by total and naïve

B cells, even in the presence of IL-21 (Fig. 4A, right panel). In support of these findings, RP105 cross-linkage had no additional effect on the expression of AID or Blimp-1 mRNAs, which were maximally upregulated in response to CpG DNA stimulation (Fig. 4B). The addition of IL-21 consistently enhanced CpG DNA-induced AID and Blimp-1 expression (Fig. 4B). This increase of AID and Blimp-1 expression was not further enhanced by the addition of anti-RP105 mAb (Fig. 4B). RP105 cross-linkage alone did not induce B cell differentiation into CD38^{high} plasma cells and did not enhance plasma cell differentiation in the presence of CpG DNA (Fig. 4C). The addition of IL-21 markedly enhanced plasma cell differentiation of naïve B cells in the presence of anti-CD40 mAb or CpG DNA, whereas RP105 cross-linkage slightly induced plasma cell differentiation of naïve B cells in addition of IL-21 (Fig. 4C). These findings indicate that the effect on plasma cell differentiation by RP105 signals is less than that by anti-CD40 mAb or CpG DNA in the presence of IL-21.

Antibody production against *S. pneumoniae* (SP)

We next examined the roles of polyclonal B cell stimulation in physiologically more relevant systems and measured the
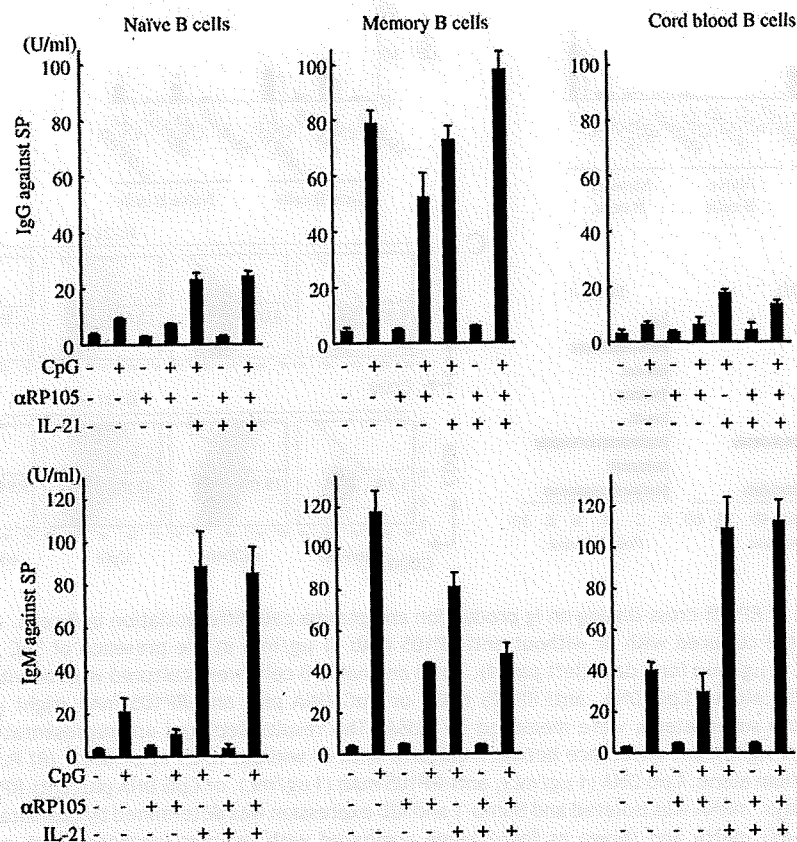

Figure 5 The effect of RP105 signaling on *Streptococcus pneumoniae*-specific antibody production by B cells. Purified adult naïve or memory B cells or cord blood B cells were cultured in the presence of medium alone, CpG DNA (1 μg/mL), anti-RP105 mAb (1 μg/mL), or CpG DNA plus anti-RP105 mAb with or without IL-21 (25 ng/mL) for 7 days. The concentration of SP-specific IgG or IgM in culture supernatants was measured by ELISA. The results depicted are representative of four independent experiments.

production of germline-encoded, polyreactive antibodies, which is specific to *S. pneumoniae* polysaccharide (SP-specific antibodies) by purified human naïve and memory B cells. CpG DNA induced the production of SP-specific IgG and IgM in memory and, to a lesser extent, naïve B cells (Fig. 5). As expected, IL-21 enhanced CpG DNA-induced production of SP-specific antibodies by naïve B cells with greater effect on

IgM than IgG antibody production (Fig. 5), whereas SP-specific antibody production by memory B cells was not affected by IL-21 (Fig. 5). RP105 alone or in conjunction with other stimuli did not affect SP-specific IgG/IgM production by naïve or memory B cells (Fig. 5). These results confirmed that RP105 signals did not contribute to the production of Igs including germline-encoded antibodies, which is specific to
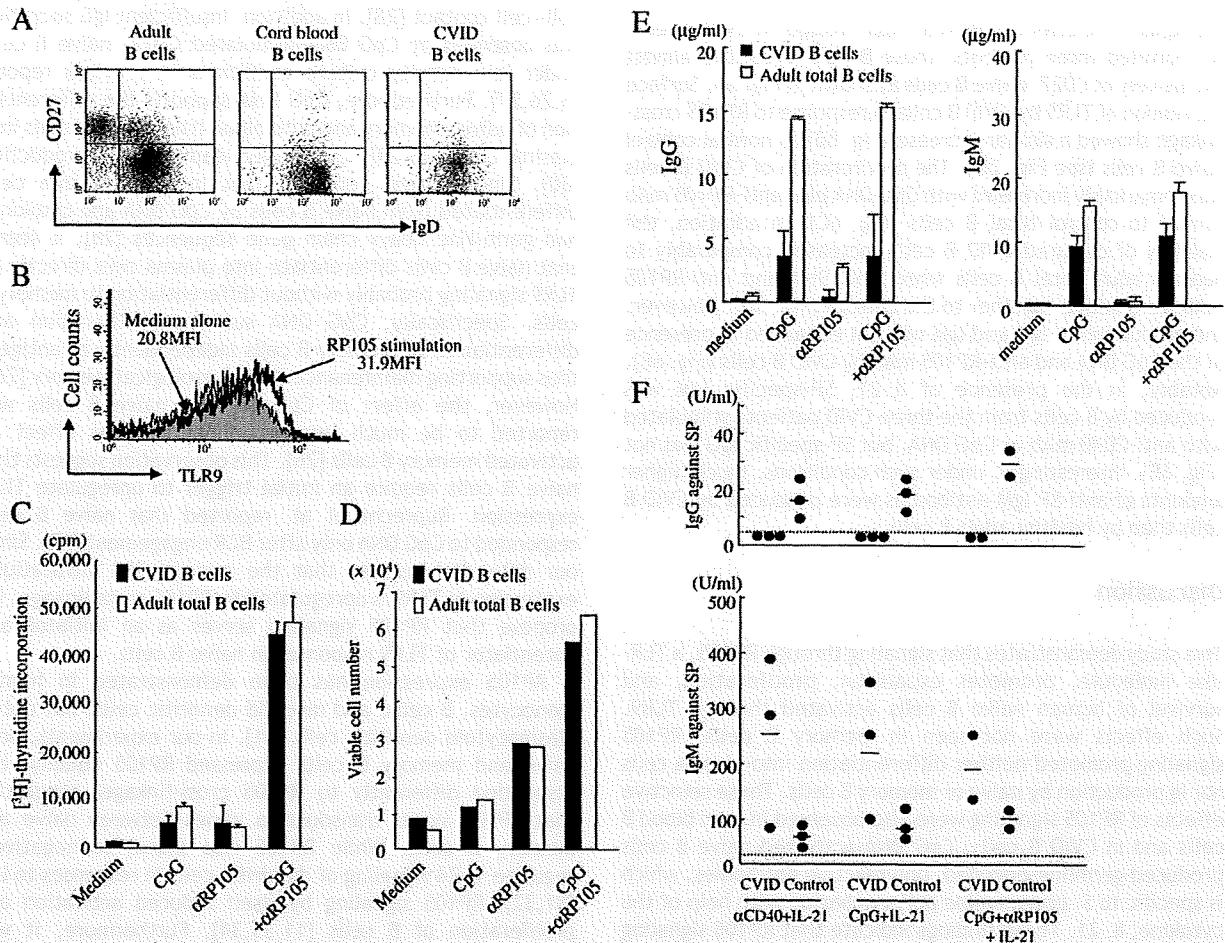

Figure 6 Characteristics of CVID B cells. (A) Mononuclear cells isolated from normal adult blood, cord blood, or peripheral blood of three CVID patients were stained with anti-IgD-FITC, anti-CD20-PerCP and anti-CD27-biotin followed by streptavidin-PE. Three-color analysis was performed by gating on CD20 positive B cells. (B) CVID B cell surface expression of TLR9. CVID B cells were cultured in the presence of medium alone or anti-RP105 mAb (1 µg/mL) for 24 h. The cultured cells were stained with anti-CD20-FITC and anti-TLR9-PE. Flow cytometric analysis was conducted by gating on CD20 positive B cells. Histogram plots shown are B cells cultured in medium alone (filled-in area) and in anti-RP105 mAb (open area) (see Fig. 2B for normal control). The data shown are one experiment for each of three CVID patients. (C) CVID B cells or control B cells were cultured in the presence of medium alone, CpG DNA (1 µg/mL), anti-RP105 mAb (1 µg/mL), or CpG DNA plus anti-RP105 mAb for 72 h. Proliferation assays were performed in triplicate, and results are shown as mean ± SD. The data shown are one experiment for each of three CVID patients. (D) CVID B cells or control B cells (1 × 10⁵ cells) were cultured in the presence of medium alone, CpG DNA, anti-RP105 mAb, or CpG DNA plus anti-RP105 mAb for 7 days. Cultured cells were stained with PI and viable cell numbers were estimated by flow cytometry as: [viable cell number] = [cultured cell number] × [percentage of viable cells]. Similar results were obtained in a duplicate experiment. (E) CVID B cells or normal adult B cells were cultured in the presence of medium alone, CpG DNA, anti-RP105 mAb, or CpG DNA plus anti-RP105 mAb. IgG or IgM concentrations in culture supernatants were measured by ELISA. The results depicted are representative of three independent experiments. (F) CVID B cells or normal adult B cells were cultured in the presence of anti-CD40 mAb (1 µg/mL) plus IL-21 (25 ng/mL), CpG DNA plus IL-21, or CpG DNA plus anti-RP105 mAb plus IL-21 for 7 days. The concentration of SP-specific IgG or IgM in culture supernatants was measured by ELISA. The dashed line shows the lower detection limit of this assay.

S. *pneumoniae* polysaccharide. In contrast, IL-21 is involved in the production of both SP-specific IgM and IgG antibody by naïve B cells. This observation is supported by the finding that IL-21 enhanced SP-specific IgM production by CpG DNA-stimulated cord blood B cells (Fig. 5), which are almost entirely CD27- B cells (Fig. 6A).

Characteristics of common variable immunodeficiency (CVID) B cells

To explore the effect of RP105 cross-linkage on CVID B cells, we enrolled three patients whose B cells consisted almost exclusively of CD27- naïve B cells as shown in Fig. 6A. Surface expression of TLR9 by CVID B cells in response to RP105 cross-linkage showed a similar increase (Fig. 6B) as normal control naïve B cells (see Fig. 2B). The proliferation of CVID B cells was remarkably increased with CpG DNA plus anti-RP105 mAb similar to control total B cells (Fig. 6C). In addition, the viability of cultured CVID B cells increased comparably to healthy adult total B cells when CpG DNA and anti-RP105 mAb were added together to the culture (Fig. 6D). However, the production of IgG and IgM was not enhanced in presence of the CpG DNA and anti-RP105 mAb by CVID B cells (Fig. 6E). Notably, in the presence of IL-21, SP-specific IgM was produced by B cells from the three CVID patients stimulated with anti-CD40 mAb or CpG DNA, but SP-specific IgG was not (Fig. 6F). Interestingly, under such conditions, much higher amounts of anti-SP IgM antibodies were produced by CVID B cells than by healthy adult B cells.

Discussion

This study demonstrates that signaling through RP105, a TLR-like molecule, promotes expansion, proliferation, and survival of human naïve B cells activated through TLR9. Such effects were not seen in memory B cells. RP105 signaling promoted neither differentiation into plasma cells nor Ig production by naïve or memory B cells. These selective effects of RP105 signaling were also observed in cord blood B cells and in CVID B cells. CpG DNA-activated-naïve B cells produced germline-encoded, polyreactive antibodies, which is specific to S. *pneumoniae* polysaccharide with help of the cytokine, IL-21. These findings indicate that RP105 signaling potentiates naïve B cell responses to TLR9 signals, thereby contributing potentially to innate host defense against microbes. Lastly, we also observed that RP105 signals enhanced the proliferation of naïve B cells through CD40 signaling. This finding is consistent with the report that signaling through RP105 in combination with CD40 ligand decreased the time taken to enter division for naïve and memory B cells, and it had a more potent effect on naïve B cells than on memory B cells [19]. Although CD40 ligand expression was not confined in activated T cells [20], this observation suggested that RP105 could be involved in T cell-dependent antibody responses of naïve B cells.

TLR9, expressed by human B cells and plasmacytoid dendritic cells, recognizes unmethylated CpG DNA [21]. The majority of TLR9 is present within the endosomal compartment, though a recent study showed that TLR9 appears on the surface of B cells during activation [22]. It has been reported that TLR9 signaling promotes cell cycle entry and

survival [23] and induces class switch recombination in B cells [24]. In addition, Bing et al. showed that CpG DNA initiates germline immunoglobulin heavy chain constant region Cγ1, Cγ2 and Cγ3 gene transcription in total and naïve B cells in cooperation with IL-10 [25]. In contrast, previous reports showed that CpG DNA alone did not produce IgG by naïve B cells [5,26,27]. The reason of the discrepancies may be due to B-cell concentrations in the culture. Huggins et al. reported that activation and proliferation of CpG DNA-activated CD27- naïve B cells required high-density cell–cell contact [28]. In addition, insufficient IgG secretion was observed by CpG DNA-stimulated CD27- naïve B cells under low density culture conditions in previous reports [5,26,27]. Furthermore, CpG DNA supports the differentiation of naïve, memory and cord blood transitional B cells into plasma cells [28,29], eventually leading to Ig production [30]. Since it was reported that most of plasma cells differentiated from naïve B cells by CpG DNA and cytokines had germ-line heavy chain gene sequences [28], it seems that naïve B cells differentiate into plasma cells directly by TLR9 signaling probably without differentiation to memory B cells. Specifically, CpG DNA sustains proliferation and differentiation of memory B cells independently of antigen, thus supporting maintenance of the serological memory [26]. However, the effect of CpG DNA on naïve B cells was reported to be much less prominent than its effect on activated memory B cells [26]. This observation suggests that naïve B cells require an initial trigger to upregulate TLR9 expression. Ruprecht et al. reported that naïve B cells responded to CpG DNA only after BCR engagement [27]. Since our data demonstrate that the surface and intracellular expression of TLR9 is upregulated by RP105 cross-linkage, we propose that RP105 signaling serves as an initiator and potentiator of TLR9 responses in naïve B cells.

RP105 expression has been demonstrated in human monocytes, B cells, and myeloid dendritic cells, but not in plasmacytoid dendritic cells [31]. In our experiments, both naïve and memory B cells expressed RP105 equally, but responded differently to RP105 cross-linkage, suggesting that RP105 signal transduction varies between naïve and memory B cells. While RP105 was shown to negatively regulate TLR4 signaling of dendritic cells in responses to LPS [31,32], RP105 signaling by itself induced activation and proliferation of B cells [11,33,34]. Furthermore, it was reported that RP105 signaling cooperated with TLR4 signaling in human B cell activation and proliferation [12]. We have shown here that RP105 signaling synergizes with TLR9 signaling in proliferation and survival of human naïve B cells, whereas we observed no synergistic growth-promoting effects of RP105 signaling on B cell proliferation stimulated with the TLR5 ligand flagellin (data not shown). The augmenting effect of RP105 signals might not be specific to TLR9.

Our data suggest several mechanisms by which RP105 signals may synergize with CpG DNA to enhance naïve B-cell expansion. Our observation that TLR9 induced activation of Akt was enhanced by RP105 cross-linkage (Fig. 3C) is in agreement with the recent observation that B lymphocyte stimulators, such as BAFF, induce B-cell growth and survival through the activation of the phosphatidylinositol 3-kinase (PI3-k)/Akt pathway [13]. Enhanced Akt activation could directly promote growth and survival of naïve B cells by

RP105/TLR9 co-stimulation. Our second observation that RP105 and TLR9 co-signaling sustained the activation of NF-κB (Fig. 3E) is relevant in view of the previously reported findings that NF-κB is a downstream target of Akt [14,35]. In B cells, RP105 signals seem to activate the pathways involving Lyn/CD19/Vav complexes and PI3-k [36,37]. Given that Akt and NF-κB activation was reduced in Vav1$^{-/-}$ Vav2$^{-/-}$ double knock out B cells, RP105 signals used Vav1 and Vav2 to activate NF-κB and Akt, which is well known as the target of PI3-k [38]. In addition, B cells lacking p110 delta subunit of PI3-k were reported to exhibit severe reduction in IκB degradation that was efficiently induced upon RP105 cross-linkage in wild-type B cells [39]. Therefore, RP105 signals activate Akt and NF-κB via PI3-k pathway. We hypothesize that the Akt-NF-κB pathway is instrumental in the RP105-mediated enhanced proliferation and prolonged survival of naïve human B cells. This interpretation is supported by the recent observation that antigen-induced proliferation by lymphocytes depends on NF-κB activation [40], which prolongs cell survival through inhibition of apoptosis [14].

Notably, our findings show that naïve B cells have much greater responses of proliferation and survival to RP105 and TLR9 signals than memory B cells. We suggest on two reasons for the difference of responses between naïve and memory B cells. First, the enhancement of intracellular expression of TLR9 in naïve B cells and memory B cells after stimulating with anti-RP105 mAb is different. The intracellular TLR9 expression was lower in naïve B cells than that in memory B cells at resting state (Fig. 2B). Since the expression was significantly increased in naïve B cells compared with memory B cells by RP105 cross-linkage (Fig. 2B), RP105 signaling may synergistically affect the proliferation with TLR9 signaling especially in naïve B cells more robustly than that in memory B cells. Second, we speculate that the signals emanated upon RP105 cross-linkage might be different in naïve and memory B cells.

Unexpectedly, RP105 signals do not play a role in Ig production, which seem to be controlled by IL-21, a strong inducer of human B cell differentiation and Ig production [15–17,41–43]. IL-21 is generated not only by CD4$^+$ T cells, but also by NKT cells [44]. Our data show that CpG DNA-stimulated naïve B cells differentiated into plasma cells and produced immunoglobulins including SP-specific antibody in the presence of IL-21. However, even with IL-21 present, RP105 did not enhance Ig production, indicating that RP105 signals are not involved in Ig synthesis. It is intriguing that both RP105 and IL-21 act on naïve B cells rather than on memory B cells and synergize with TLR9 signals despite having individually distinct roles: while RP105 enhances naïve B-cell growth and survival, IL-21 induces naïve B-cell differentiation into plasma cells and Ig production.

Finally, we investigated the effect of cross-linking RP105 on peripheral blood B cells isolated from three classic CVID patients. CVID B cells, although almost exclusively naïve and CD27$^-$, increased TLR9 expression in response to RP105 stimulation. In addition, CVID B cells showed increased viability and proliferation when co-cultured with CpG DNA and anti-RP105 mAb. Most importantly, CVID B cells activated by CpG DNA or by anti-CD40 mAb produced IgM but not IgG anti-SP antibody at high titers in response to IL-21. CVID is a heterogeneous disorder characterized by

hypogammaglobulinemia and reduced numbers of memory B cells but normal numbers of naïve B cells [45,46]. Affected patients tend to present with milder clinical symptoms and develop less invasive bacterial disease when compared with X-linked agammaglobulinemia patients who exhibit absence of circulating B cells and fail to produce antigen-specific antibody. Since antibody-producing memory B cells, both switched and unswitched, play an important role in the defense against systemic bacterial infections [47], our observation that naïve B cells can produce germline-encoded SP-specific IgM antibody is relevant and suggests that CVID B cells are capable of contributing to the elimination of invasive pneumococcal infections.

Although neither endogenous nor exogenous ligands for RP105 have been identified to-date [32], it has been speculated that RP105-specific ligands might be derived from pathogens or may consist of molecules generated by cells involved in innate immunity [11]. Our findings support the possibility that RP105 and its ligand(s) are instrumental for the proliferation, growth and survival by naïve B cells. The identification of RP105-specific ligand(s) will not only improve our understandings of naïve B-cell function in primary immune responses, but may in addition lead to the development of novel therapeutic strategies to improve rapid clearance of pathogen from the circulation.

Acknowledgment

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