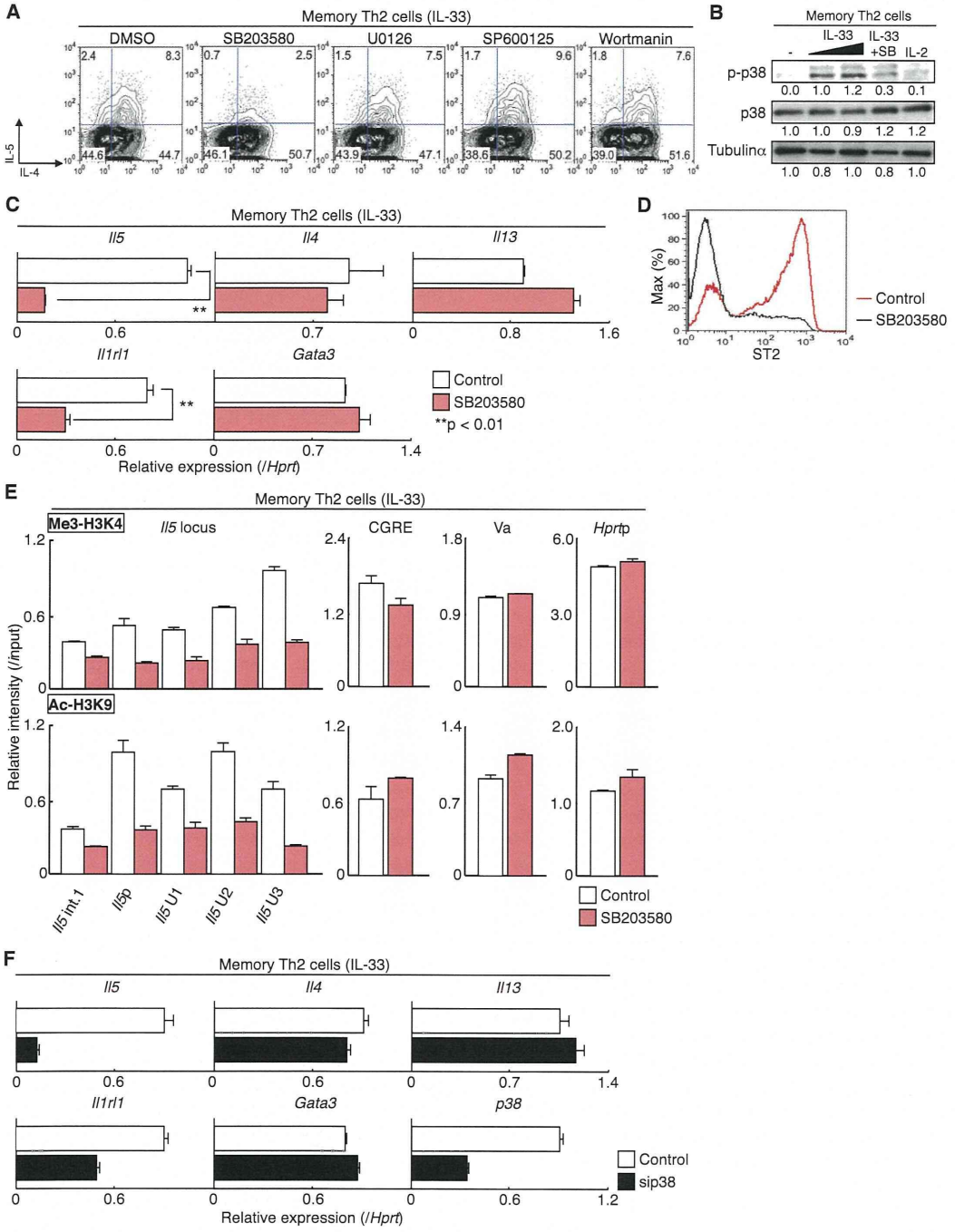

Figure 6. p38 Signaling Is Crucial for IL-33-Dependent IL-5 Induction by Memory Th2 Cells

(A) Intracellular-staining profiles of IL-5 and IL-4 in IL-33-treated memory Th2 cells with the indicated chemical inhibitors.

(B) The phosphorylated p38 (p-p38) and total p38 (p38) in freshly prepared memory Th2 cells stimulated with IL-2 or graded amounts of IL-33 with or without SB203580.

(C) Quantitative RT-PCR analysis of the indicated genes in memory Th2 cells cultured with IL-33 with or without SB203580. Four independent experiments were performed and showed similar results (**$p < 0.01$).

(D) Expression profiles of ST2 on memory Th2 cells treated with IL-33 with or without SB203580.

(legend continued on next page)

IL-5 expression or production was detected after IL-33 treatment (Figures S7A–S7C). However, IL-33 dramatically enhanced *Il5* expression in $CD45RO^+CD4^+$ T cells from nasal polyps of ECRS patients ($p < 0.01$; Mann-Whitney U test; Figure 7B). Modest enhancement of *Il13* expression was also detected ($p < 0.05$; Mann-Whitney U test). In addition, the expression of *Il1rl1* was enhanced dramatically after IL-33 treatment, which supported the results obtained from analysis of murine memory Th2 cells (Figure 1). In contrast, no obvious effect of IL-33 on the expression of *Il5* or *Il13* was detected in $CD45RO^+CD4^+$ T cells from nasal polyps of NECRS patients. Furthermore, the expression and production of IL-5 and the *Il1rl1* expression that was enhanced by IL-33 in $CD45RO^+CD4^+$ T cells from nasal polyps of ECRS patients were selectively inhibited by SB203580 treatment ($p < 0.01$; Mann-Whitney U test; Figures 7C and 7D). These results indicate that IL-33 specifically induces enhanced expression of *Il1rl1* and IL-5 in $CD45RO^+CD4^+$ T cells from nasal polyps of ECRS patients and that p38 activation is required for IL-33-induced IL-5 augmentation. Thus, IL-33-ST2-p38 signaling might play an important role in the induction of pathogenicity in tissue-infiltrating memory $CD4^+$ T cells in human chronic allergic inflammatory diseases such as ECRS.

DISCUSSION

Herein, we have identified memory Th2 cells as a critical target of IL-33 in the pathogenesis of allergic airway inflammation in both the murine and the human immune systems. IL-33 induced increased expression of ST2 on memory Th2 cells and also enhanced production of IL-5 both in vitro and in vivo. IL-33 exposure induced chromatin remodeling at the *Il5* locus. We found that p38 MAPK was a downstream target of IL-33-ST2 signaling in memory Th2 cells. In vivo, the depletion of IL-33 or ST2 attenuated memory-Th2-cell-mediated allergic responses in the airway. Memory $CD4^+$ T cells in the nasal polyps of ECRS patients showed increased IL-5 expression after stimulation with IL-33, and IL-33 was highly expressed in the chronic inflammatory polyps of ECRS patients. Thus, the IL-33-ST2-p38 pathway could be a potential therapeutic target for treatment of chronic allergic inflammation induced by memory Th2 cells.

IL-33-mediated production of IL-5 and IL-13 in ILC2s depends on the phosphorylation of p38 (Furusawa et al., 2013), and IL-33 increases phosphorylation of p38 in mast cells (Liew et al., 2010). In human T cells, IL-5 production induced by TCR stimulation is dependent on p38 activity (Maneechotesuwan et al., 2007; Mori et al., 1999). Likewise, we found that the p38-mediated signaling pathway is critical for IL-33-induction of IL-5 expression in both murine and human memory T cells. Thus, the p38 MAPK pathway appears to be critical for the production of IL-5 in various types of cells, including memory Th2 cells.

Chromatin remodeling of *Il4* is dependent on the calcineurin-NFAT pathway (Ansel et al., 2006), whereas that of IL-5 and IL-13 appears to be more dependent on NF-κB, AP-1, or other transcription factors rather than NFAT (Guo et al., 2009; Wang et al., 2006). In the current study, we found that the p38 MAPK pathway was activated by IL-33 and was responsible for the induction of chromatin remodeling of the *Il5* locus and thus left the chromatin signature at the *Il4* and *Il13* loci almost unchanged. Therefore, this might represent a connection between the IL-33-p38 MAPK pathway and selective chromatin remodeling of the *Il5* locus.

An important finding from the present study is that IL-33-induced chromatin remodeling at the *Il5* locus in memory Th2 cells occurs independently from TCR stimulation. TCR stimulation is indispensable for the induction of chromatin remodeling at the Th1 and Th2 cell cytokine-encoding loci during differentiation of naive $CD4^+$ T cells into effector Th1 and Th2 cells (Nakayama and Yamashita, 2010). However, we have shown that the cytokine IL-33 alone is capable of selectively inducing chromatin remodeling at the *Il5* locus in memory Th2 cells. These results might indicate that IL-33 possesses a unique ability to induce chromatin remodeling at the *Il5* locus if the IL-33 receptor ST2 is expressed. Indeed, IL-2, IL-7, or IL-25 did not induce chromatin remodeling at the *Il5* locus in memory Th2 cells, although their receptors were expressed on memory Th2 cells. The combination of IL-2 and IL-25 also induced chromatin remodeling at the *Il5* locus in memory Th2 cells, as shown previously in ILC2s (Halim et al., 2012), suggesting that similar mechanisms might operate cytokine-dependent IL-5 induction in memory Th2 cells and ILC2s. In ILC2s, IL-33 is reported to be more potent than IL-25 in inducing IL-13 production and AHR (Barlow et al., 2013). At the *Il5* locus, we detected permissive histone marks accompanied by binding of p300 and pol II in memory Th2 cells stimulated with IL-33. Therefore, the activation of the IL-33-ST2-p38 axis might induce the formation of a chromatin-remodeling complex, including the HAT complex, and recruit it to the *Il5* locus in memory Th2 cells.

We have demonstrated that memory Th2 cells are an important target of IL-33 in the pathogenesis of airway inflammation. We detected increased expression of *Il33* mRNA in the lung after OVA challenge, which is consistent with the notion that tissue damage leads to the release of IL-33 from structural cells, such as epithelial cells and endothelial cells in the lung (Préfontaine et al., 2009; Préfontaine et al., 2010), and that IL-33 is expressed higher in asthmatic patients (Préfontaine et al., 2009). IL-33 secreted around the inflammatory airways might increase ST2 expression and IL-5 production by memory Th2 cells that are either resident in or migrating through the lung tissue. Therefore, IL-33 might enhance allergic airway responses through the induction of our proposed IL-5-producing pathogenic memory Th2 cells in the airway (Endo et al., 2014; Endo et al., 2011; Hegazy et al., 2010; Islam et al., 2011; Upadhyaya et al., 2011; Wang et al., 2010). Although it is already known that ILC2s respond to IL-33 to produce IL-5 in the lung, the depletion of ILC2s in *Rag2*$^{-/-}$ mice by anti-CD90.2 antibody treatment did not affect memory-Th2-cell-dependent airway inflammation.

(E) ChIP assays were performed as shown in Figure 2B. Histone modifications at the *Il5* locus in memory Th2 cells treated with IL-33 with or without SB203580 were measured by quantitative RT-PCR analysis.

(F) Effect of silenced *p38* on *Il5* expression in IL-33-cultured memory Th2 cells. Memory Th2 cells were introduced to control *p38* siRNA and cultured with IL-33 for 5 days, and quantitative RT-PCR analysis of the indicated molecules after 4 hr stimulation with immobilized anti-TCRβ is shown.

At least three (A–D) or two (E and F) independent experiments were performed and showed similar results. See also Figure S6.

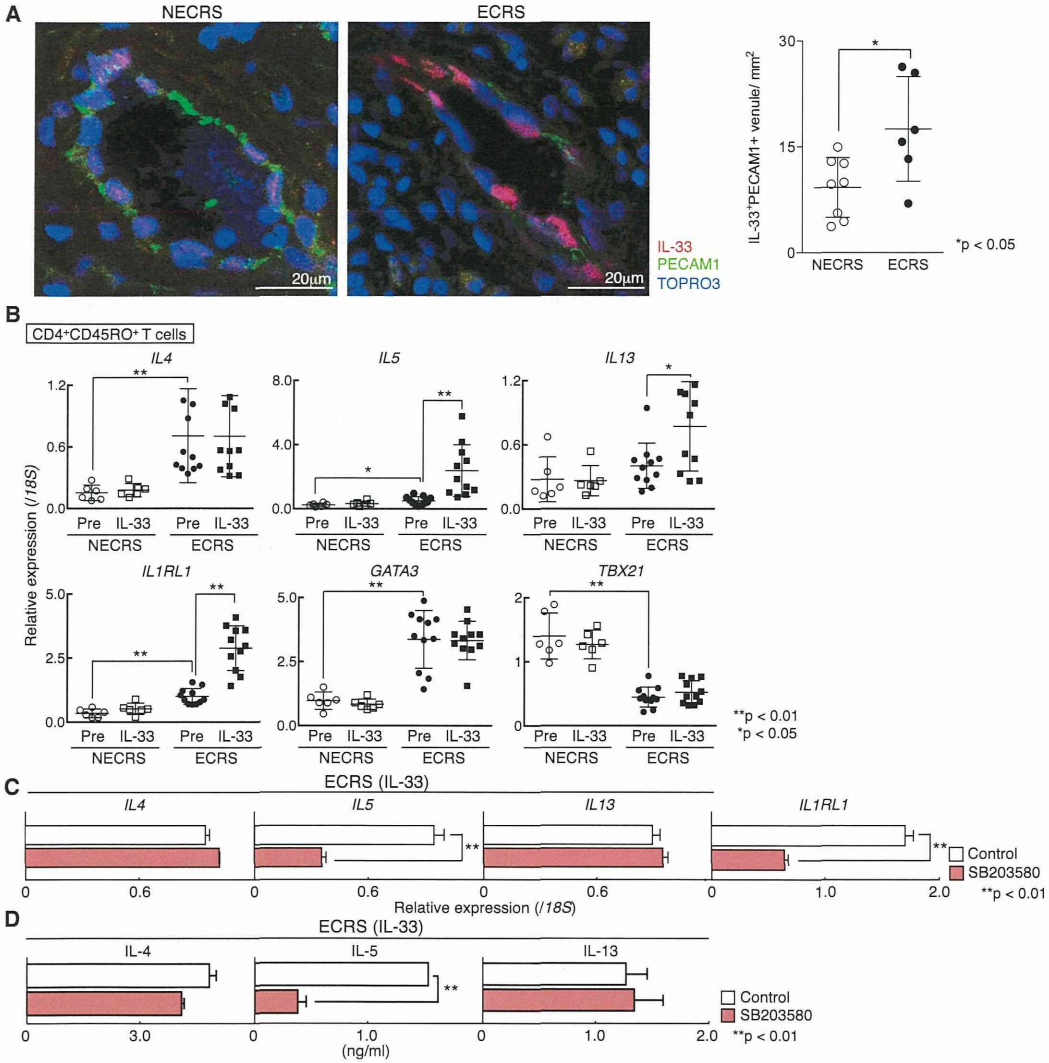

Figure 7. Human IL-33 Enhances IL-5 Production in Memory CD4⁺ T Cells Prepared from Nasal Polyps of ECRS Patients

(A) Immunofluorescent analysis (with staining for IL-33 [red], PECAM [green], and TOPRO3 [blue]) shows a representative section of nasal polyps from NECRS (left) or ECRS (right) patients. The frequency of IL-33⁺PECAM1⁺ cells among PECAM⁺ cells of the nasal polyps from patients is shown (mean ± SD; n = 6 for ECRS and n = 8 for NECRS).

(B) Quantitative RT-PCR analysis of relative expression of the indicated genes in human memory CD4⁺ T cells from nasal polyps (n = 11 for ECRS, n = 6 for NECRS) was performed after 4 hr stimulation with PMA plus ionomycin. Relative expression (normalized to *18S*) with SD is shown. Mean values and SDs are shown.

(C) Human nasal memory CD4⁺ T cells were cultured with IL-33 with or without SB203580 for 5 days. Quantitative RT-PCR analysis of the indicated cytokines in these cells after 4 hr stimulation with PMA plus ionomycin is shown.

(D) ELISA (n = 8 for ECRS and n = 5 for NECRS) of the indicated cytokines secreted by IL-33-cultured memory CD4⁺ T cells stimulated with PMA plus ionomycin for 16 hr. The mean values of triplicate cultures with SDs are shown.

More than five independent experiments in each group were performed and showed similar results (**p < 0.01; *p < 0.05; C and D). Three technical replicates were performed with quantitative RT-PCR and ELISA (B–D). See also Figure S7.

Thus, according to the experimental systems we used, IL-33 appears to act mainly on memory Th2 cells to increase their ability to produce IL-5 and exacerbate eosinophilic inflammation. It remains unknown whether IL-33 induces recruitment of memory Th2 cells to the inflamed lung, given that IL-33 can also act as a chemoattractant for Th2 cells (Komai-Koma et al., 2007).

ECRS is a chronic inflammatory disease characterized by prominent accumulation of eosinophils in the sinuses and nasal polyp tissue (Gevaert et al., 2006). Functionally distinct populations of memory CD4⁺ T cells might be present within the nasal polyps of ECRS and NECRS (Th1 cell type for NECRS and Th2 cell type for ECRS). We detected more IL-33-producing cells in

nasal polyps from ECRS patients than in those from NECRS patients. Consistent with our findings in murine $CD4^+$ T cells, we demonstrated that IL-33 induced augmentation of *IL5* and *IL1RL1* expression in memory $CD4^+$ T cells from ECRS patients. IL-33 induced expression of not only *IL5* but also *IL1RL1*, suggesting that a positive-feedback mechanism resulted in a greater responsiveness to IL-33 in memory $CD4^+$ T cells from ECRS patients. In addition, IL-33-induced IL-5 augmentation also depended on the activation of p38. Our study clearly demonstrates the function and pathophysiological role of IL-33 in ECRS, a chronic inflammatory human disease.

In summary, our study has identified memory Th2 cells as an important target of IL-33 in the pathogenesis of airway inflammation. The p38-mediated signaling pathway is critical for TCR-independent IL-33-induced IL-5 expression in both murine and human memory Th2 cells. Further detailed studies focused on the IL-33-ST2-p38-axis in pathogenic memory Th2 cells might lead to the discovery of potential therapeutic targets for the treatment of chronic allergic diseases.

EXPERIMENTAL PROCEDURES

Mice

The animals used in this study were backcrossed to BALB/c or C57BL/6 mice ten times. Anti-OVA-specific TCR-αβ (DO11.10) transgenic (Tg) mice were provided by Dr. D. Loh (Washington University School of Medicine, St. Louis) (Murphy et al., 1990). *Il33*$^{-/-}$ mice were generated as previously described (Oboki et al., 2010). *Il1rl1*$^{-/-}$ mice were kindly provided by Dr. Andrew N.J. McKenzie (Medical Research Council, Cambridge) (Townsend et al., 2000). Ly5.1 mice were purchased from Sankyo Laboratory. All mice were used at 6–8 weeks old and were maintained under specific-pathogen-free conditions. BALB/c, BALB/c *nu/nu*, and *Rag2*$^{-/-}$ mice were purchased from CLEA Japan. Animal care was conducted in accordance with the guidelines of Chiba University.

The Generation and Culture of Effector and Memory Th2 Cells

Splenic $CD62L^+CD44^-KJ1^+CD4^+$ T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 1 μM) plus antigen-presenting cells (irradiated splenocytes) under Th2-cell-culture conditions (25 U/ml IL-2, 10 U/ml IL-4, anti-IL-12 monoclonal antibody [mAb], and anti-IFN-γ mAb) for 6 days in vitro. The effector Th2 cells (3×10^6) were transferred intravenously into BALB/c *nu/nu* or BALB/c recipient mice. Five weeks after cell transfer, $KJ1^+CD4^+$ T cells in the spleen were purified by autoMACS (Miltenyi Biotec) and cell sorting (BD Aria II) and were then used as memory Th2 cells.

Assessment of Memory Th2 Cell Function In Vivo

Memory Th2 cells were purified by fluorescence-activated cell sorting and transferred (3×10^6/mouse) again into *Il33*$^{+/+}$ or *Il33*$^{-/-}$ mice. The mice were exposed to aerosolized 1% OVA four times on days 1, 3, 9, and 11. For depletion of ILC2s, *Rag2*$^{-/-}$ mice were injected intraperitoneally with anti-CD90.2 (BioX Cell) antibody at a dose of 200 μg per day on days 2, 5, and 9. BAL fluid for the analysis of cytokine production by ELISA was collected 12 hr after the last challenge, and BAL fluid for the assessment of inflammatory cell infiltration was collected on day 13. Intracellular-staining analysis was performed 12 hr after the last inhalation. Lung histology was assessed on day 13. AHR was assessed on day 12.

Quantitative Real-Time PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen). cDNA was synthesized with an oligo (dT) primer and Superscript II RT (Invitrogen). Quantitative real-time PCR was performed with the ABI PRISM 7500 Sequence Detection System as described previously (Endo et al., 2011). Primers and TaqMan probes were purchased from Applied Biosystems. Primers and Roche Universal probes were purchased from Sigma and Roche, respectively. Gene expression was normalized with the *Hprt* mRNA signal or the *18S* ribosomal RNA signal.

siRNA Analysis of Gene Targeting

siRNA was introduced into memory Th2 cells by electroporation with a mouse T cell Nucleofector Kit and Nucleofector I (Amaxa). Memory Th2 cells were transfected with 675 pmol of control random siRNA or siRNA for *p38* (Applied Biosystems) and cultured for 5 days with IL-33.

Nasal Polyp Mononuclear Cells and Homogenate Preparation

Nasal polyp mononuclear cells (NPMCs) were obtained as previously described (Yamamoto et al., 2007). In brief, freshly obtained nasal polyps were immediately minced and incubated in RPMI 1640 medium containing 1 mg/ml collagenase, 0.5 mg/ml hyaluronidase, and 0.2 mg/ml DNase I (Sigma-Aldrich). After incubation, NPMCs were obtained by the Ficoll-Hypaque technique. A volume of 1 ml of PBS was added for every 100 mg of tissue and was supplemented with aprotinin and leupeptin (Roche).

Statistical Analysis

Data were analyzed with GraphPad Prism software (version 6). Comparisons of two groups were calculated with non-parametric Mann-Whitney U tests. Differences with p values below 0.05 or 0.01 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.01.016.

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Effects of vitamin D supplements on influenza A illness during the 2009 H1N1 pandemic: a randomized controlled trial

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In a prior randomized trial, we found that the incidence of influenza A was less in the vitamin D3 group than among those on placebo, but the total incidence of either influenza A or B did not differ between groups. In this trial, the incidence of influenza A or B was less in the vitamin D3 group than in the placebo group only during the first half of the study. To elucidate whether vitamin D3 has preventive actions against influenza A, we conducted another trial during the 2009 pandemic of the H1N1 subtype of influenza A. Students (n = 247) of a Japanese high school were randomly assigned to receive vitamin D3 supplements (n = 148; 2000 IU per day) or a placebo (n = 99) in a double-blind study for 2 months. The primary outcome was incidence of influenza A diagnosed by a rapid influenza diagnostic test by medical doctors. Influenza A was equally likely in the vitamin D3 group (20/148: 13.5%) compared with the placebo group (12/99: 12.1%). By *post hoc* analysis, influenza A occurred significantly less in the vitamin D3 group (2/148: 1.4%) compared with the placebo group (8/99: 8.1%) (risk ratio, 0.17; 95% confidence interval, 0.04 to 0.77; P = 0.009) in the first month. However, during the second month, the vitamin D3 group experienced more events and effectively caught up with the placebo group. Vitamin D3 supplementation did not lower the overall incidence of influenza A during the 2009 H1N1 pandemic. A *post hoc* analysis suggests that the initial benefit during the first month of treatment was lost during the second month.

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1. Introduction

A novel influenza A (H1N1) virus of swine-origin emerged in April 2009.[1] This influenza expanded from North America to Europe, Asia, and the southern hemisphere, which prompted the World Health Organization to declare it a "pandemic of influenza: phase 6." In an analysis of hospitalized patients, most subjects infected by the 2009 pandemic influenza (H1N1) virus recovered without complications, but certain patients, particularly patients with chronic diseases, obese adults, young children, and pregnant/postpartum women, had severe and prolonged infections.[2] In the southern hemisphere (*i.e.*, Australia and New Zealand), the pandemic influenza accelerated markedly in June, reaching a peak within 4 to 6 weeks, and then declined after mid-July,[3,4] which was earlier than the traditional peak of seasonal influenza. Accordingly, the peak in the northern hemisphere, including Japan, was predicted to occur from October to November, which is earlier than the traditional peak of seasonal influenza.

During the preceding influenza season, from 2008 to 2009, we showed that vitamin D supplementation significantly reduced the incidence of influenza A in school children by means of a randomized, double-blind, placebo-controlled trial (RCT).[5] However, we also noted a non-significant excess of influenza B in the vitamin D3 group (39/167: 23.3%) compared to the placebo group (28/167: 16.8%).[5] It was difficult to explain why vitamin D supplementation was effective only in preventing influenza A but not in influenza B. In Japan, influenza A is usually prevalent in the early period of the influenza season (*i.e.*, January), whereas the incidence of influenza B increases later in the influenza season (*i.e.*, March). In a *post hoc* analysis of this trial, we found that the incidence of influenza A or B was less in the vitamin D3 group than in the placebo group only during the first half of the study. Moreover, another randomized trial from Finland showed that vitamin D supplementation similarly prevented the incidence of acute respiratory tract infections during the first 6 weeks of use.[6] Therefore, we hypothesized that vitamin D supplementation may have delayed the susceptible period to influenza, whether type A or type B. To test this hypothesis, we conducted another RCT comparing vitamin D3 supplements with placebo in high school students during the 2009 influenza A (H1N1) pandemic.

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2. Results and discussion

2.1. Participants

2.1.1. Characteristics. A total of 895 students were asked to participate in this study at their own discretion during September–October 2009 *via* a letter and an assembly at the school; 105 did not meet the inclusion criteria (69 had already been infected by influenza A after May 2009, 35 had underlying diseases such as bone fracture or asthma and had taken medicine, and 1 had serious allergies). Among the remaining 790 students, 543 chose not to participate (no reason was specified), and 247 volunteered to participate; 148 were randomly assigned to take vitamin D3 and 99 to take placebo for 2 months starting October 18 and ending December 17, 2009 (Fig. 1). The two randomly assigned groups did not differ according to the sex or year in the school (Table 1).

2.1.2. Follow-up. All 247 students were followed until the end of the study without any loss to follow-up. Six students from the vitamin D group and four from the placebo group declined to participate shortly after randomization; although reasons were not specified; it was not due to the presence of clear adverse events. Adherence to vitamin D supplements or placebo was evaluated on the basis of the daily logs; 99% of students reported taking the supplement daily, as directed, and 1% forgot to take the study supplement once per week on average. Consistent with an intention-to-treat analysis, data from all 247 participants were included in the analysis.

2.2. Primary outcome

2.2.1. RIDT-positive influenza A in total. Rapid influenza diagnostic test (RIDT)-positive influenza A occurred in 32 students (13.0%) over the 2 month period. No students were infected by influenza B or infected more than once with RIDT-positive influenza A during the study period.

2.2.2. RIDT-positive influenza A in the vitamin D3 group and in the placebo group. Overall, RIDT-positive influenza A occurred in 20/148 (13.5%) students in the vitamin D3 group compared with 12/99 (12.1%) in the placebo group, with no significant difference between groups (risk ratio (RR), 1.11, 95% confidence interval (CI), 0.57–2.18, $P = 0.75$) (Table 2).

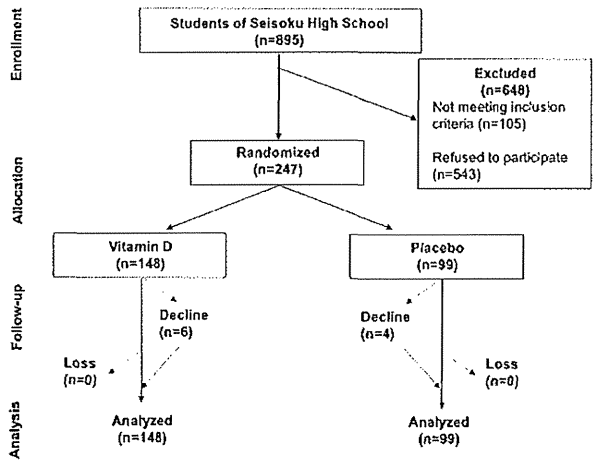

Fig. 1 Participant flow.

Table 1 School year by a randomly assigned group

	Vitamin D3	Placebo	Total
Male – *n*, (%)	98 (67)	64 (65)	163
School year – *n*, (%)			
Freshman	55 (37)	36 (36)	91
Sophomore	45 (30)	32 (32)	77
Senior	48 (33)	31 (32)	78
Total	148	99	246

2.2.3. RIDT-positive influenza A during the first month. When the comparison was limited to the first month, RIDT-positive influenza A occurred significantly less in the vitamin D3 group (2/148: 1.4%) compared with the placebo group (8/99: 8.1%) (RR 0.17; 95% CI, 0.04–0.77, $P = 0.009$) (Table 2).

2.2.4. Discussion in the primary outcome. In Tokyo, prevalence of influenza pandemic peaked during a week from October 29 to November 4, which was included in the first month of this study period: October 18th to December 17th. We found that vitamin D3 supplementation did not decrease the overall incidence of RIDT-positive influenza A. However, the RCT also suggests that short-term (*i.e.*, 1 month) use of vitamin D3 dietary supplementation may temporarily decrease the incidence of influenza A during an influenza pandemic. In a previous article, both low and high levels of cord blood 25-hydroxyvitamin D (25OHD) were associated with increased aeroallergen sensitization.[7] Therefore, we hypothesized that effects of vitamin D supplementation were U-shaped: the risk of influenza increases in both lower and higher 25OHD levels, but the risk decreases in optimal 25OHD levels, there is a possibility that vitamin D reduced the incidence of infection in the first month whereas vitamin D increased that in the second month, resulting in "caught up" with those in the placebo group (Fig. 2), thereby yielding no net difference between groups. In our prior RCT using 1200 IU of vitamin D3, we found that the incidence of influenza A was lower in the vitamin D3 group than in the placebo group.[5] However, in a *post hoc* analysis of this earlier RCT, influenza A or B was observed in 11 children in the vitamin D3 group (1200 IU per day: 60% dose of this study) *vs.* 26 children in the placebo group during the first 1.5–2.0 months of the 3.5 to 4 month trial ($P = 0.009$). This result differs from that over the course of the entire trial, where incident influenza A or B was observed in 57 (33.5%) of the vitamin D3 group *vs.* 59 (34.1%) of the placebo group ($P = 0.91$). Thus, both the current RCT and our prior RCT show a similar trend – namely, that vitamin D3 may be preventive to influenza in an initial phase, but that the incidence of influenza in the vitamin D3 group then seems to catch up with that in the placebo group during subsequent weeks. Although it would be easy to dismiss one such result as due to chance, we now have found this pattern in two similar Japanese RCTs. Outside Japan, Laaksi *et al.* conducted a RCT of vitamin D3 (400 IU) *versus* placebo among 164 military conscripts with a primary outcome of acute respiratory

Table 2 Risk ratio of rapid influenza diagnostic test (RIDT)-positive influenza and influenza-like illness by the randomly assigned group

	Total 2 month period (Oct 18th to Dec 17th)				The first month (Oct 18th to Nov 17th)			
	Vitamin D	Placebo			Vitamin D	Placebo		
	Case/total (%)	Case/total (%)	RR (95% CI)	*P*-value	Case/total (%)	Case/total (%)	RR (95% CI)	*P*-value
RIDT-positive influenza	20/148 (13.5)	12/99 (12.1)	1.11 (0.57, 2.18)	0.75	2/148 (1.4)	8/99 (8.1)	0.17 (0.04, 0.77)	0.009
Influenza-like illness[a]	32/148 (21.6)	17/99 (17.2)	1.26 (0.72, 2.14)	0.39	11/148 (7.4)	11/99 (11.1)	0.67 (0.30, 1.48)	0.32

[a] Influenza-like illness includes both RIDT-positive influenza and RIDT-negative but clinically relevant to influenza-like illness.

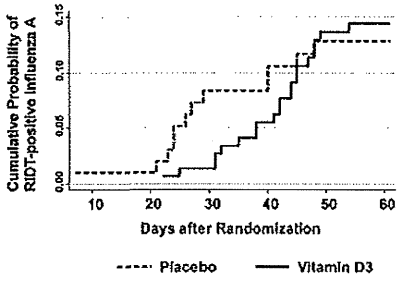

Fig. 2 Probability of the rapid influenza diagnostic test (RIDT)-positive influenza according to a randomly assigned group.

tract infection.[6] The authors reported that there was an effect during the first 6 weeks of the study, with a mean (SD) of 0.7 (0.2) days of absence in the intervention group and 1.4 (2.6) days of absence in the placebo group, but that there tended to be no difference between the two groups after the first 6 weeks. These results are similar to our two Japanese RCTs.

2.3. Secondary outcome

2.3.1. Influenza-like illness. Influenza-like illness, including both RIDT-positive and -negative results, occurred in 49 students (19.8%). The incidence of influenza-like illness did not differ between groups over the 2 month study period, with 32/148 (21.6%) of participants affected in the vitamin D3 group and 17/99 (17.2%) of participants affected in the placebo group (RR 1.26, 95% CI, 0.74–2.14, $P = 0.39$) (Table 2). Even when the study period was limited to the first month of intervention, the incidence of influenza-like illness was not significantly different between the vitamin D3 group (11/148: 7.4%) and the placebo group (11/99: 11.1%) (RR 0.67, 95% CI, 0.30–1.48, $P = 0.32$).

2.3.2. School absence. Frequencies and duration of school absence did not differ between groups (Table 3).

2.3.3. Influenza-related symptoms. Frequency of subjective symptoms reported by students on daily logs (*e.g.*, fever including influenza-like illness, runny nose, cough, sore throat, and arthralgia) did not differ between groups (Table 3).

2.3.4. Discussion in secondary outcomes. In contrast to the primary outcome (RIDT-positive influenza A), the incidence of influenza-like illness, school absence, and influenza-related symptoms was not significantly different between the two groups (Fig. 3). In our prior RCT, RIDT-negative influenza-like illness, nonspecific febrile disease, pneumonia, admissions to hospital, and days absent from school also were not different between the vitamin D3 and placebo groups. However there are still insufficient numbers of participants in this and previous studies to conclude that these secondary outcomes did not differ between the two groups. Since the completion of these Japanese RCTs in 2009, several RCTs of vitamin D supplementation to prevent acute respiratory infection have been published around the world, and they have yielded conflicting results. For children's pneumonia in Kabul, a single high-dose oral vitamin D3 supplementation to young children along with antibiotic treatment for pneumonia reduced the occurrence of repeat episodes of pneumonia,[8] whereas quarterly bolus doses of oral vitamin D3 supplementation were not an effective intervention to reduce the incidence of pneumonia in infants.[9] Among adults whose mean baseline 25OHD levels were approximately 30 ng mL^{-1}, there was no difference in the incidence or severity of upper respiratory infection between the vitamin D and placebo groups.[10,11] In contrast, among Mongolian children whose median 25OHD levels were 7 ng mL^{-1}, vitamin D supplementation significantly reduced the risk of

Table 3 School absence and influenza-related symptoms, by the randomly assigned group

	Vitamin D3	Placebo	*P*-value[g]
Absent students: number[a] (%)	68 (46)	38 (38)	0.24
Absent days: mean ± SD	1.7 ± 2.5	1.1 ± 1.9	0.14
Fever: number[b] (%)	52 (35)	34 (34)	0.85
Runny nose: number[c] (%)	23 (16)	17 (17)	0.73
Coughing: number[d] (%)	25 (17)	21 (21)	0.39
Sore throat: number[e] (%)	19 (13)	16 (16)	0.46
Arthralgia: number[f] (%)	13 (9)	10 (10)	0.73

[a] Number of students absent at least one day, as reported by the school. [b] Number of students who had 'fever more than 37.0' in the log file at least one day. [c] Number of students who checked 'runny nose' in the log file at least one day. [d] Number of students who checked 'cough' in the log file at least one day. [e] Number of students who checked 'sore throat' in the log file at least one day. [f] Number of students who checked 'Arthralgia' in the log file at least one day. [g] Chi-square test was used for all comparisons except absent days, where the Mann–Whitney test was used.

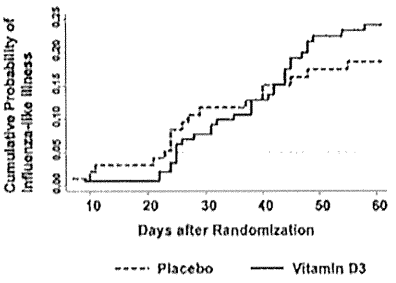

Fig. 3 Probability of influenza-like illness according to a randomly assigned group.

acute respiratory infection in winter.[12] These divergent results probably reflect the heterogeneity of outcomes but also of study populations and interventions, with large differences in subjects' ages, baseline vitamin D status, vitamin D dosing regimens, study duration, and season. The role of differences in genetic background is unknown, but also merits investigation.

2.4. Safety

The supplements were well-tolerated, without any reported adverse events.

2.5. Study limitations

The study has several limitations. First, since we could not predict the differential effects of vitamin D supplementation between the early and late phase prior to starting the trial and since we did not want to reduce the participant rate by painful blood sampling, we did not measure serum levels of 25OHD, which probably explains why vitamin D supplements reduce a risk of acute respiratory infection in one population,[12] but have no effect in another.[11] Without knowing the vitamin D status of participants during the course of the current study, we are reluctant to speculate on the mechanism of how vitamin D3 supplementation may delay susceptibility to RIDT-positive influenza A. Second, when we separated the first half from the whole study period, the event numbers were small. The similarity of results in two previous RCTs,[5,6] and this RCT does, however, support a true biological finding. Third, this trial was performed at a single Japanese high school during the 2009 H1N1 pandemic and not in a more diverse population during a more typical influenza season, a study design that reduces generalizability. It may be that particular individuals (*e.g.*, adolescents and young adults) and highly pathogenic subtypes of influenza A (*e.g.*, H1N1) are less likely to benefit from interventions such as vitamin D3 supplementation. Fourth, the investigators did not perform RITD directly or consult medical records, which are shortcomings of this study. Fifth, since we made a protocol of this trial in early phase of the pandemic and we could not predict the incidence of pandemic influenza A H1N1 among high school students between middle of October and middle of December, the incidence of RIDT-positive influenza A was 13% which was far less than 25% we had expected. So, the sample size was too small to conclude that vitamin D supplementation may not lower overall incidence of influenza A during the 2009 H1N1 pandemic. Sixth, we did not measure individual UVB/sun exposure per day and diet as parts of the questionnaire.

3. Experimental

3.1. Study design

3.1.1. Randomized, double-blind, placebo-controlled, parallel-group trial. We conducted a randomized, double-blind, placebo-controlled, parallel-group trial at Seisoku High School in Minato-ku (35° N), Tokyo, Japan, over a 2 month period, from October 18, 2009, to December 17, 2009, during the high season of pandemic influenza A H1N1 in Japan. The study protocol was reviewed and approved by the institutional review board of Seisoku High School. The entire process of study design and protocol, data monitoring, and analyses was performed at the Division of Molecular Epidemiology, Jikei University School of Medicine; there was no industry support or involvement in the study. The safety review board consisted of two physicians from Jikei University Hospital who are not co-authors of this study. Both vitamin D3 and placebo were purchased from Zenyaku Co., Ltd. (Otsuka, Bunkyo-ku, Tokyo, Japan) as a dietary supplement. This trial was registered at UMIN Clinical Trials Registry as UMIN000002532.

3.1.2. Study population, eligibility, and consent. Because the first outbreak of the influenza pandemic among high school students occurred during May and June in Japan, we planned to target only high school students in this study. The background, aims, methods, and possible risks/benefits of this study were explained to 895 Seisoku High School students aged 15 to 18 years and their parents, first by a letter and then *via* talks and communication by the first author (M.U.) at the school. Participants were asked to start taking the study supplements from October 18 and to continue taking them until December 17. Exclusion criteria were: (1) students who had already been infected with an influenza-like illness after May 2009; (2) those who had a history of urinary tract stones or diseases of calcium/bone metabolism; (3) those who had a bone fracture; (4) those who were already taking vitamin D supplements or activated vitamin D; (5) those who had asthma, as asthma may be an exacerbating factor in the pandemic influenza; and (6) those who had serious allergies, in order to avoid severe reactions to ingredients in the study supplement. Parents and students were asked to provide written informed consent. Study participation was completely voluntary.

3.1.3. Randomization, blinding, and intervention. We (M.U. and H.M) used a central computerized procedure to randomly assign students in permutated blocks of five to receive either vitamin D3 or placebo in a 3 : 2 ratio. Parents were provided with one numbered bottle containing 450 capsules. One capsule contained 400 IU of vitamin D3 or placebo; active and placebo capsules were identical in appearance. Participants were asked to take five capsules daily (total 2000 IU vitamin D3 or placebo); all five capsules could be taken at the same time or divided into two daily doses. The tolerable upper intake is currently set at 2000 IU per day by the Japanese Ministry of