Cardiorespiratory Fitness Suppresses Age-Related Arterial Stiffening in Healthy Adults: A 2-Year Longitudinal Observational Study

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Cardiorespiratory fitness is negatively associated with arterial stiffness, although it is unclear whether it is associated with prospective arterial stiffness changes. The authors examined cardiorespiratory fitness and arterial stiffness progression in a 2-year follow-up study of 470 healthy men and women aged 26 to 69 years. Peak oxygen uptake ($\dot{V}O_{2peak}$) was measured at baseline using a graded cycle exercise test. Arterial stiffness was assessed using brachial-ankle pulse wave velocity (baPWV) at baseline and after 2 years. Two-year changes in baPWV were significantly higher in patients in the lowest $\dot{V}O_{2peak}$ tertile (28.8±7.6 cm/s) compared with those in the highest $\dot{V}O_{2peak}$ tertile (−1.4±7.5 cm/s) (P=.024) and were inversely correlated with $\dot{V}O_{2peak}$ (r=−.112, P=.015). Stepwise multiple regression analysis revealed that age, glucose, baPWV, $\dot{V}O_{2peak}$, and sex were independent correlates of 2-year changes in baPWV, suggesting that higher cardiorespiratory fitness is associated with age-related arterial stiffening suppression. *J Clin Hypertens (Greenwich).* 2015:1–7. ©2015 Wiley Periodicals, Inc.

Arterial stiffness increases progressively with advancing age, even in healthy individuals.[1] This arterial stiffening is associated with future hypertension[2] and cardiovascular (CV) events[3] and is recognized as a surrogate marker for CV disease. Therefore, the prevention of arterial stiffening is of great clinical importance.

Carotid-femoral pulse wave velocity (cfPWV) is a standard method for assessing aortic stiffness.[4] cfPWV is used in clinical practice mainly in Europe, and in the United States to a lesser extent. Recently, brachial-ankle pulse wave velocity (baPWV) was proposed as an alternative method for assessing arterial stiffness in Asian populations.[5] A major advantage of baPWV is its measurement method, which simply involves wrapping the four extremities in blood pressure (BP) cuffs.[6,7] Moreover, the use of either cfPWV or baPWV is accepted by the Japanese guidelines for the management of hypertension as a tool for assessing subclinical target organ damage.[8] In addition, baPWV has been shown to be associated with an increased risk of total CV events and all-cause mortality,[9,10] as is cfPWV.[11]

Cardiorespiratory fitness (CRF) is independently associated with a lower risk of all-cause mortality and CV events.[12] Thus, previous studies have investigated the relationship between CRF and arterial stiffness as a surrogate marker for CV disease in cross-sectional research and have suggested that higher CRF was associated with lower arterial stiffness.[13–16] To our knowledge, only Ferreira and colleagues[17,18] have reported longitudinal research from adolescence to young adulthood. There is no information regarding middle-aged and elderly populations. In addition, there has been no previous study on whether CRF is associated with the progression of arterial stiffness as assessed by baPWV in longitudinal research.

Previous studies have demonstrated that regular aerobic exercise is effective in preventing and reversing arterial stiffening in healthy adults.[19] Regular aerobic exercise results in higher CRF,[20] and, consequently, CRF may be associated with a lower CV disease risk. Therefore, we hypothesized that higher CRF would be associated with less progression of age-related arterial stiffening in healthy adults. To test our hypothesis, we examined the relationship between CRF and the progression of arterial stiffening with a 2-year follow-up study.

METHODS

Participants

The participants were recruited from the community around the National Institute of Health and Nutrition, Tokyo, Japan, or the Okayama Southern Institute of Health, Okayama Health Foundation, Okayama, Japan. We used data from 470 Japanese adults (128 men and 342 women; mean age, 48.8±9.5 years) selected from among 1125 participants who met the following criteria: (1) they received anthropometric, CRF, exercise habit, physical activity (n=459), and

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arterial stiffness assessments and underwent blood examinations (baseline measurement); (2) they underwent arterial stiffness measurement at 2-year follow-up; and (3) they had no history of stroke, cardiac disease, or chronic renal failure; they were receiving no medical treatment for hypertension, dyslipidemia, or diabetes; they were currently nonsmokers; and they had an ankle-brachial pressure index between 0.9 and 1.3 at both baseline and the follow-up visit (during the observation period). Ethical approval for the study was obtained from the Human Research Committees of the National Institute of Health and Nutrition and Okayama Health Foundation, and it was performed in accordance with the guidelines of the Declaration of Helsinki.

To assess the effects of CRF level on progression of arterial stiffening, the participants were categorized into tertiles based on their peak oxygen uptake ($\dot{V}O_{2peak}$) levels during an incremental cycle exercise test using a cycle ergometer (described below) at baseline for each sex and the following age-specific distributions (20–29, 30–39, 40–49, 50–59, and 60–69 years) (Table I): lowest tertile, unfit; middle tertile, mid-range fitness; and highest tertile, fit.

Arterial stiffness

Participants were observed under quiet resting conditions in the supine position. baPWV and BP were measured with a vascular testing device (form PWV/ABI device; Omron Colin, Kyoto, Japan), according to the method previously described.[7] Bilateral brachial and ankle arterial pressure waveforms were stored for 10 seconds by the extremity cuffs connected to a plethysmographic sensor and an oscillometric pressure sensor wrapped around the participant's arms and ankles. The baPWV was calculated from the distance between the two arterial recording sites divided by the transit time.[21] The coefficient of variation for interobserver reproducibility of baPWV was 4% in our laboratory. The intraclass correlation coefficient was 0.84 (95% confidence interval, 0.78–0.90), and the minimal detectable change with 95% confidence was 371 cm/s for baPWV in a previous study.[22]

Recordings were made in triplicate, and they strictly conformed to American Heart Association guidelines.[23] The mean of the right and left baPWV values were used for analysis.

TABLE I. Age-Specific Distributions for Cardiorespiratory Fitness Based on Peak Oxygen Uptake Tertile

		Peak Oxygen Uptake, mL/kg/min		
	Age, y	Low	Middle	High
Men	20–29 (n=3)	39.5	40.4	44.0
	30–39 (n=38)	28.2–36.6	37.6–44.5	44.6–55.7
	40–49 (n=47)	18.8–30.4	30.5–39.4	39.6–54.3
	50–59 (n=25)	26.8–31.2	31.9–35.4	35.9–47.1
	60–69 (n=15)	22.4–25.6	26.8–34.8	36.1–47.7
Women	20–29 (n=2)	–	37.0	39.6
	30–39 (n=48)	20.6–29.9	30.3–33.9	34.0–48.9
	40–49 (n=100)	17.8–28.2	28.3–32.7	32.8–44.6
	50–59 (n=135)	13.9–25.5	25.6–29.5	29.7–39.8
	60–69 (n=57)	16.9–24.9	25.1–28.8	29.0–37.4

Cardiorespiratory fitness

CRF, which was assessed from the peak oxygen uptake ($\dot{V}O_{2peak}$), was measured by an incremental cycle exercise test using a cycle ergometer (Monark Ergomedic 828E Test Cycle [National Institute of Health and Nutrition], Varberg, Sweden, or Excalibur V2.0 [Okayama Southern Institute of Health], Lode BV, Groningen, The Netherlands), as described previously.[14,24,25] The incremental cycle exercise began at a work load of 30 W to 60 W for women and 60 W to 120 W for men, which was then increased by 15 W/min until the participants could no longer maintain the fixed pedaling frequency (60 rpm). The participants were encouraged during the ergometer test to exercise at the level of maximum intensity. The expired air of participants who were tested at the National Institute of Health and Nutrition was collected over 30-second intervals in Douglas bags. Expired oxygen (O_2) and carbon dioxide (CO_2) gas concentrations were measured by mass spectrometry (Arco-1000; Arco System, Ogaki, Japan), and gas volume was determined using a dry gas meter (DC-5; Shinagawa Seisakusho, Tokyo, Japan). The expired air of participants who were tested at Okayama Southern Institute of Health was collected and the rates of O_2 consumption and CO_2 production were measured breath by breath using a cardiopulmonary gas exchange system (Oxycon Alpha; Mijnhrdt B.V., The Netherlands). The heart rate and rating of perceived exertion[26] were monitored on a minute-by-minute basis during exercise. The highest value of $\dot{V}O_2$ during the exercise test was designated as $\dot{V}O_{2peak}$. The CRF assessment was performed after all other tests.

Anthropometric measures and body composition

Height, weight, and waist circumference were measured and body mass index (BMI) was calculated. Body composition was determined by dual-energy radiography absorptiometry (Hologic QDR-4500; Hologic, Waltham, MA) with participants in the supine position.

Exercise habits

Data on exercise habits were obtained at interviews conducted by well-trained staff using the structured method of the National Nutrition Survey in Japan. The patients were asked if they currently exercised (over 30 minutes per session, two times per week for a duration of 3 months). Participants who answered "yes" were classified as participants with exercise habits (exercised regularly). Participants who answered

"no" were classified as participants without exercise habits.

Physical activity

The duration and intensity of physical activity were evaluated by triaxial accelerometry (Actimarker EW4800; Panasonic Electric Works, Osaka, Japan), as described previously.[27,28] Participants were asked to wear a triaxial accelerometer for 28 days; we used data for 14 days, during which the accelerometer was worn continuously from the time the participant awoke until he or she went to bed. We obtained the duration of daily physical activity corresponding with 1.1 METs to 2.9 METs (light), 3.0 METs to 5.9 METs (moderate), and ≥6.0 METs (vigorous).[29] Time spent in inactivity was defined as the sum of time spent sedentary (<1.1 METs) and the time at which the accelerometer was not worn, which was calculated as 1440 − (daily time spent in light physical activity+moderate+vigorous).

Blood samples

Blood samples were taken from participants following an overnight fast of at least 10 hours. Venous blood withdrawn from the antecubital vein was collected into tubes without additives or EDTA and was immediately centrifuged at 3000 rpm for 20 minutes to obtain serum or plasma. The levels of glucose and glycated hemoglobin (HbA_{1c}) in plasma and total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides in serum were determined.

Statistical Analyses

Data are expressed as mean±standard deviation unless otherwise indicated. The differences across the CRF levels were assessed by one-way analysis of variance or Kruskal-Wallis test. A general linear model was used to analyze the association of the 2-year change in baPWV (Δ: follow-up baPWV − baseline baPWV) across the CRF levels. The 2-year change in baPWV was entered as a dependent variable; the tertile $\dot{V}O_{2peak}$ categories were entered as fixed factors; and baseline age, BMI, body fat, glucose, triglycerides, HDL cholesterol, baPWV, and sex were entered as covariates for adjustment. Bonferroni's test was applied for post hoc pair-wise comparisons. In the general linear model analyses, data were expressed as estimated marginal mean±standard error.

Pearson's correlation coefficients were used to analyze the relationships between the 2-year change in baPWV and the baseline variables (age, height, weight, BMI, waist circumference, body fat, glucose, HbA_{1c}, triglycerides, total cholesterol, HDL cholesterol, systolic BP (SBP) and diastolic BP (DBP), baPWV, and $\dot{V}O_{2peak}$). A stepwise multiple regression analysis was used to determine the influences of baseline variables on the 2-year change in baPWV. $P<.05$ was considered statistically significant. Statistical analyses were performed using SPSS software, version 20.0 (IBM Japan, Tokyo, Japan).

RESULTS

Table II shows the baseline characteristics of the participants divided by CRF level. There were significant differences in $\dot{V}O_{2peak}$, BMI, body fat, glucose, HbA_{1c}, triglycerides, HDL cholesterol, heart rate, baPWV, exercise habits, and physical activity among the three groups. The Figure highlights the significant association of the 2-year change in baPWV across CRF levels. General linear model analysis revealed that the changes in baPWV during the study period were significantly higher in patients in the low CRF group (unfit) than they were in patients in the high CRF group (fit). An inverse relationship was observed between CRF level and the 2-year changes in baPWV (28.8±7.6, 16.0±6.8, and −1.4±7.5, respectively; P for trend=.029). The 2-year changes in baPWV were significantly higher in the low CRF group than they were in the high CRF group (P=.024). These data indicate that a higher CRF is associated with slower progression of age-related arterial stiffening.

Pearson's correlation coefficients between the 2-year change in baPWV and baseline parameters are as follows: the 2-year change in baPWV correlated with age (r=.133, $P<.01$), glucose level (r=.105, P=.023), HbA_{1c} concentration (r=.095, P=.039), baPWV (r=−.121, $P<.01$), and $\dot{V}O_{2peak}$ (r=−.112, P=.015). These data indicate that CRF is correlated with age-related arterial stiffening.

The stepwise multiple regression analysis revealed that age, glucose level, baPWV, $\dot{V}O_{2peak}$, and sex were independent correlates of 2-year changes in baPWV (Table III). These data indicate that CRF is associated with arterial stiffness, independent of other factors already known to be related to arterial stiffness.

Table IV shows the results of the 2-year change in baPWV according to subgroup. There were no significant differences in the rate of progression of baPWV between the younger (<50 years) and older (>50 years) age groups or between the BMI groups (<25 kg/m^2 and ≥25 kg/m^2). The annual rate of change in baPWV was lower in women than it was in men.

DISCUSSION

In this longitudinal observational study, the 2-year changes in baPWV were higher in patients in the low CRF groups compared with those in the high CRF groups. Moreover, CRF was inversely associated with 2 years of baPWV progression in healthy adults. This association was independent of other confounders. To our knowledge, this is the first study to examine the relationship between CRF and the progression of baPWV in healthy adults in a longitudinal study. These findings suggest that higher CRF is associated with slower progression of age-related arterial stiffening in healthy adults.

TABLE II. Physical Characteristics of Participants by CRF Level

Variable	Overall	CRF Level			*P* Value
		Low	Middle	High	
No.	470	153	161	156	
Peak oxygen uptake, mL/kg/min	31.3±7.0	25.4±4.2	30.9±4.4	37.6±6.2	<.01[a]
Men/women, No.	128/342	41/112	45/116	42/114	NS[b]
Premenopausal women, No. (%)	170 (36)	52 (34)	58 (36)	60 (39)	NS[b]
Age, y	48.8±9.5	49.5±9.4	49.0±9.8	48.1±9.2	NS[a]
BMI, kg/m^2	22.2±2.8	23.1±3.3	22.0±2.5	21.5±2.2	<.01[a]
Body fat, %	25.7±6.7	29.0±6.5	25.6±6.1	22.7±5.9	<.01[a]
Glucose, mg/dL	89.4±9.8	91.4±12.0	87.9±8.3	89.0±8.4	<.01[a]
Glycated hemoglobin, %	5.3±0.5	5.4±0.6	5.3±0.3	5.3±0.3	.03[a]
Triglycerides, mg/dL	85±55	99±66	82±57	76±36	<.01[a]
Total cholesterol, mg/dL	208±34	212±32	206±35	208±36	NS[a]
HDL cholesterol, mg/dL	67±17	63±16	66±15	71±19	<.01[a]
Heart rate, beats per min	61±11	65±10	62±11	59±10	<.01[a]
SBP, mm Hg	116±14	118±15	114±13	117±12	NS[a]
DBP, mm Hg	71±10	72±11	69±10	71±10	NS[a]
baPWV, cm/s	1229±166	1272±197	1214±164	1204±122	<.01[a]
Patients with exercise habits, No. (%)	228 (48.5)	41 (26.8)	77 (47.8)	110 (70.5)	<.01[b]
Jogging	39 (8.3)	4 (2.6)	8 (5.0)	27 (17.3)	–
Swimming	88 (18.7)	16 (10.5)	27 (16.8)	45 (28.8)	–
Dancing	60 (12.8)	14 (9.2)	18 (11.2)	28 (17.9)	–
Stretching	43 (9.1)	10 (6.5)	13 (8.1)	20 (12.8)	–
Resistance	58 (12.3)	15 (9.8)	17 (10.6)	26 (16.7)	–
No.	459	151	160	148	
Daily time spent in physical activity					
Light, min/d	579±113	578±124	577±109	581±105	NS[a]
Moderate, min/d	61±25	52±20	63±26	67±26	<.01[a]
Vigorous, min/d	2.7±7.9	1.6±5.0	1.7±4.4	5.0±11.8	<.01[a]
Inactivity, min/d	798±115	808±124	798±112	787±109	NS[a]

Abbreviations: baPWV, brachial-ankle pulse wave velocity; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure; NS; not significant. Data are presented as mean±standard deviation unless otherwise noted.

[a]Analysis of variance.

[b]Kruskal-Wallis test.

Previous cross-sectional studies indicated that a high level of CRF was associated with arterial stiffness.[13–16] However, little is known about the longitudinal relationships between CRF and the progression of baPWV. Here, we determined the relationship between CRF and the 2-year changes in baPWV. The strengths of the present study were that the CRF levels of all participants were evaluated by maximal exercise testing, and the sample size was relatively large. Similar to previous cross-sectional findings, the present study also showed that the progression of baPWV was significantly related to CRF. Ferreira and colleagues[17,18] showed that longitudinal changes in CRF were inversely and significantly associated with arterial stiffness (distensibility, compliance coefficients, and Young's elastic modulus) in adolescence to young adulthood. In comparison to the studies by Ferreira and coworkers,[17,18] our study not only used a larger cohort of participants but also analyzed middle-aged and older members of the population as well as young adults. In addition, we used baPWV for the arterial stiffness assessment. Therefore, this is the first study to examine the relationship between CRF and the progression of arterial stiffness as assessed by baPWV. We were unable to directly compare Ferreira's results with ours because of the different methods of arterial stiffness assessment. However, our present study indicated a similar tendency and confirmed that CRF is inversely associated with baPWV progression in middle-aged and older populations. Our results suggest that maintaining greater CRF would generate a protective effect on the progression of age-related arterial stiffening in middle-aged and elderly populations.

BP is the most important determinant of arterial stiffening. Determination of baPWV has been the subject of many cross-sectional studies, but few studies have examined the rate of progression of baPWV, especially in healthy adults. Thus, it is unclear whether BP changes or baseline BP correlates with changes in baPWV in healthy adults. Although the baseline SBP was highly correlated with the baseline baPWV (r=.72) in our study, we did not find a significant correlation

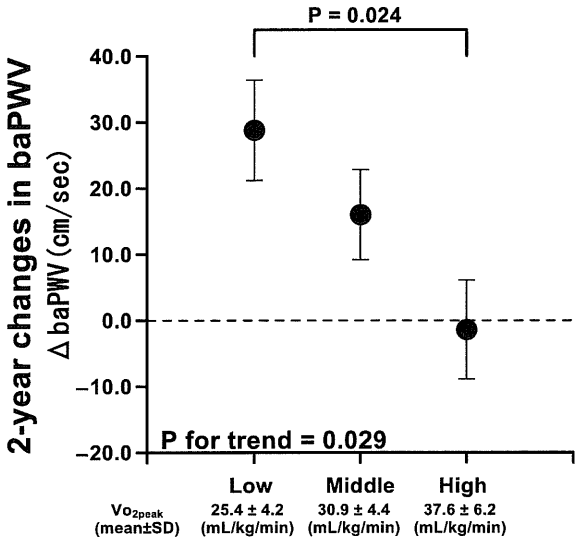

FIGURE. Estimated marginal mean±standard error of the 2-year changes in brachial-ankle pulse wave velocity (baPWV) grouped by peak oxygen uptake ($\dot{V}O_{2peak}$) tertiles in healthy adults. Data are adjusted for baseline age, body mass index, body fat, glucose, triglycerides, high-density lipoprotein cholesterol, baPWV, and sex.

between the changes in baPWV and the baseline SBP, which is consistent with a previous report by Wildman and colleagues.[30] These findings may indicate a strong and independent association between BP levels and arterial stiffening. Several previous studies have indicated that SBP changes or baseline BPs correlate with changes in PWV. However, the SBP of participants in these studies was higher than that in our study participants. SBP is the main longitudinal determinant of PWV. We think that a normal SBP value (<120 mm Hg) may not affect PWV trajectories, while an SBP value >120 mm Hg may affect PWV.[31] Since our study participants were very healthy (no history of stroke, cardiac disease, or chronic renal failure; received no medical treatment for hypertension, dyslipidemia, or diabetes; were currently nonsmokers; and had an ankle-brachial pressure index of 0.9–1.3), the SBP was lower than that in previous studies. On the other hand, the $\dot{V}O_{2peak}$ was correlated with both the baseline baPWV and the changes in baPWV. Therefore, we think that CRF is one of the relevant factors affecting baPWV.

It is well established that the annual rate of change in PWV accelerates with advancing age[31] and BMI.[30] In the present study, however, there were no significant differences in the rate of arterial stiffness progression as assessed by baPWV in the younger (<50 year) and older (>50 year) groups or in the BMI <25 kg/m^2 and BMI ≥25 kg/m^2 groups. Because we extracted a population of apparently healthy participants who had no atherogenic or metabolic disorders, there might have been no differences between these groups. On the other hand, the annual rate of change in baPWV was lower in women than in men, which is consistent with a previous report by Tomiyama and colleagues.[32]

Our study showed that CRF was inversely associated with increased baPWV. One possible reason for this finding could be that glucose, HbA_{1c}, and body fat were lower in people with high CRF (Table II). Hyperglycemia and obesity have been shown to be associated with arteriopathy.[33] A previous study suggested that glucose or HbA_{1c} and body fat had a negative correlation with PWV.[34,35] However, in multiple linear regression and general linear model analyses that included these factors, the $\dot{V}O_{2peak}$ was independently related to the progression of arterial stiffness as assessed by baPWV. Therefore, maintaining a higher CRF may directly influence the progression of baPWV. The endurance-trained state is associated with an elevated overall content of elastin, reduced calcium content,[36] reduced formation of advanced glycation end products and collagen cross-linking in the arterial wall,[37] and improved endothelial function.[38,39] Thus, habitual exercise and a higher fitness level are thought to decrease arterial stiffness by preventing arterial remodeling and dysfunction. Moreover, CRF heritability in sedentary people ranged from 25% to 65%,[40] and some individuals can perform very little physical activity and still possess a high $\dot{V}O_{2peak}$. It is possible that this study's findings are also affected by genetic factors. Therefore, additional studies are required to further investigate this topic in longer observational or interventional studies.

STUDY STRENGTHS AND LIMITATIONS

Our findings have several important implications. First, the present study showed that a higher level of CRF was associated with lower levels of arterial stiffness progression in healthy adults. The baPWV is a risk factor

TABLE III. Stepwise Multiple Regression Analyses Showing Association Between 2-Year Changes in baPWV and Baseline Variables

	Nonstandardized Coefficient	95% CI	Standardized Coefficient	*P* Value
2-year changes in baPWV				
Age, y	1.712	0.73–2.69	0.181	<.01
Glucose, mg/dL	1.053	0.21–1.90	0.115	.015
baPWV, cm/s	−0.151	−0.21 to −0.10	−0.281	<.01
Peak oxygen uptake, mL/kg/min	−2.187	−3.59 to −0.78	−0.172	<.01
Sex, male=0; female=1	−37.783	−59.2 to −16.4	−0.188	<.01

Abbreviations: baPWV, brachial-ankle pulse wave velocity; CI, confidence interval.

TABLE IV. Two-Year Changes in baPWV According to Subgroup

Subgroup	Two-Year Changes in baPWV, cm/s			
	Crude	*P* Value	Adjusted	*P* Value
Age,[a] y				
<50 (n=238)	12.1±5.4	NS	9.5±6.2	NS
≥50 (n=232)	16.8±6.3		19.5±6.3	
Sex[b]				
Men (n=128)	20.6±8.7	NS	34.3±9.7	.03
Women (n=342)	12.1±4.6		6.9±5.1	
BMI[c], kg/m^2				
<25 (n=404)	13.3±4.3	NS	13.3±4.3	NS
≥25 (n=66)	21.1±12.5		21.2±10.9	

Abbreviations: BMI, body mass index; NS, not significant. [a]Adjusted for sex, body fat, glucose, total cholesterol, systolic blood pressure (SBP), and brachial-ankle pulse wave velocity (baPWV). [b]Adjusted for age, body fat, glucose, total cholesterol, SBP, and baPWV. [c]Adjusted for age, glucose, total cholesterol, SBP, baPWV, and sex. Bold value indicates significance.

for total CV events and all-cause mortality.[9,10] The maintenance of a higher level of CRF may have a protective effect against CV events by attenuating age-related arterial stiffening. Therefore, the maintenance of a higher level of CRF may be important for primary prevention strategies. Second, the annual rate of change in baPWV in the intermediate CRF level group was 8.1 cm/s per year (16.1 cm/s per 2 years). Compared with previous cross-sectional findings of arterial stiffening with aging,[32,41] our data are similar, which adds to its validity.

There are several potential limitations to the present study. First, CRF was measured only once at baseline. It is possible that the participants' CRF levels changed during the observation period. During our 2-year observational study, we observed that a higher level of CRF was associated with lower levels of arterial stiffness progression in healthy adults. However, the field would benefit from more longitudinal research to determine the cause-effect relationships between CRF and arterial stiffness progression. Second, depending on which clinic the participants attended, two different methods to analyze O_2 consumption and CO_2 production were used. However, a previous study reported that O_2 consumption and CO_2 production attained from the incremental cycle test were similar for the two different methods.[42] Third, we used baPWV to assess arterial stiffness; this method has been popularized primarily in Asian countries for over 15 years. However, the cfPWV is considered the gold-standard method for assessing aortic stiffness. Numerous studies have showed that baPWV performs comparatively well to cfPWV in identifying vascular damage.[5,7,43] Therefore, we believe that baPWV provides qualitatively similar information to that derived from the aortic pulse wave velocity. Measuring baPWV is a simple but powerful technique that can be easily applied in clinical practice. Fourth, arterial stiffness undergoes major changes during the menstrual cycle. Since the present study did not control for the menstrual cycle, it is possible that our findings were affected by this. However, the number of premenopausal women included in our study was relatively low (36%), and there were no significant differences in the number of premenopausal women among the three groups. Therefore, we think that there was a relatively small effect on the progression of baPWV.

CONCLUSIONS

This was the first prospective study to indicate that CRF is inversely associated with age-related arterial stiffening. This association was independent of some confounders, specifically age and glucose. Therefore, CRF may be an effective measure for preventing age-related arterial stiffening.

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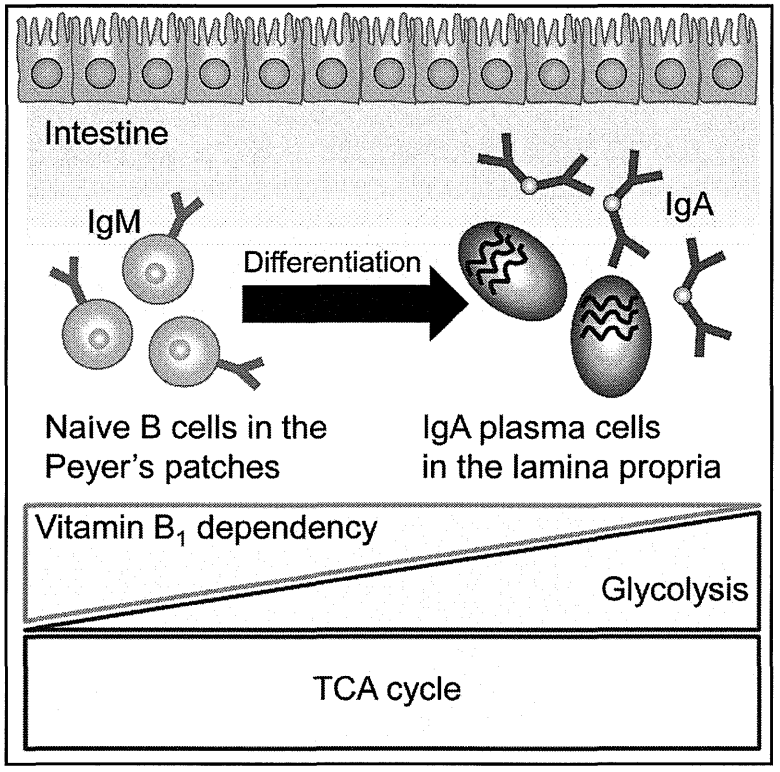
Mode of Bioenergetic Metabolism during B Cell Differentiation in the Intestine Determines the Distinct Requirement for Vitamin B_1

Graphical Abstract



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In Brief

Kunisawa et al. associate differentiation of intestinal naive B cells into IgA antibody-producing plasma cells with changes in energy metabolism and dependence on dietary vitamin B_1. Depletion of dietary vitamin B_1 decreases naive B cells preferentially, affecting induction of IgA antibody responses against oral antigen.

Highlights

- Naive B cells and IgA^+ PCs use non-glycolytic- and glycolysis-TCA axis, respectively
- Vitamin B_1 depletion impairs TCA cycle activity
- Vitamin B_1 depletion decreases naive B cells without affecting IgA^+ PCs
- Vitamin B_1 depletion impairs initiation of antigen-specific antibody responses

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Mode of Bioenergetic Metabolism during B Cell Differentiation in the Intestine Determines the Distinct Requirement for Vitamin B_1

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SUMMARY

Bioenergetic metabolism varies during cell differentiation, but details of B cell metabolism remain unclear. Here, we show the metabolic changes during B cell differentiation in the intestine, where B cells differentiate into IgA$^+$ plasma cells (PCs). Naive B cells in the Peyer's patches (PPs) and IgA$^+$ PCs in the intestinal lamina propria (iLP) both used the tricarboxylic acid (TCA) cycle, but only IgA$^+$ PCs underwent glycolysis. These metabolic differences reflected their dependencies on vitamin B_1, an essential cofactor for the TCA cycle. Indeed, the diminished activity of the TCA cycle after dietary vitamin B_1 depletion decreased the number of naive B cells in PPs without affecting IgA$^+$ PCs in the iLP. The maintenance of naive B cells by dietary vitamin B_1 was required to induce—but not maintain—intestinal IgA responses against oral antigens. These findings reveal the diet-mediated maintenance of B cell immunometabolism in organized and diffuse intestinal tissues.

INTRODUCTION

In general, cells are capable of breaking down large molecules (e.g., proteins, lipids, and sugars) into small ones (e.g., amino acids, fatty acids, and monosaccharides) and of constructing large molecules from small units. In the catabolism of sugars, fats, and proteins, their metabolites are incorporated into the tricarboxylic acid (TCA) cycle, a series of biochemical reactions leading ultimately to the generation of ATP. Unlike the catabolic pathways of proteins and lipids, glycolysis itself also generates ATP. The regulation of cellular metabolism by achieving a balance between catabolism and anabolism is a key to the maintenance of appropriate cellular responses and function.

Recently, much attention has been paid to the concept of "immunometabolism." Immunometabolism comprises two components: immune responses mediated by whole-body metabolism (e.g., obesity and diabetes) (Mathis and Shoelson, 2011) and various metabolic pathways within immune cells (Pearce and Pearce, 2013). Accumulating evidence regarding immune cells has revealed that their metabolism changes during development, activation, and differentiation, as has been well documented in T cells (Pearce et al., 2013; Wang and Green, 2012). In general, T cell activation (e.g., via T cell receptor and

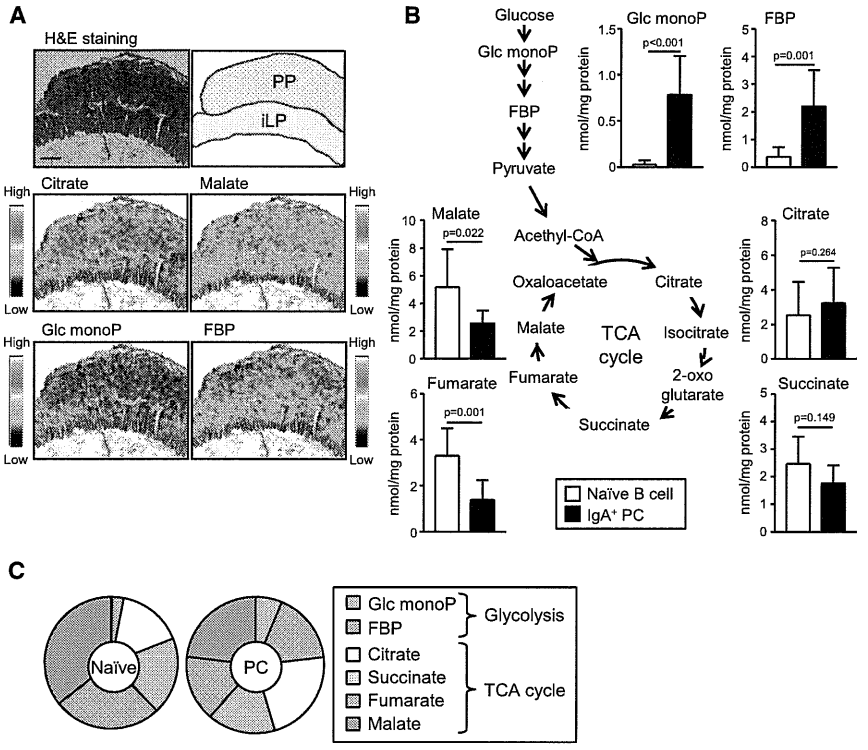

Figure 1. Metabolites Generated in Naive B Cells in PPs and IgA$^+$ PCs in the iLP

(A) Small intestinal tissues, including PPs and iLP, underwent H&E staining and MALDI-IMS for the detection of citrate, malate, glucose monophosphate (Glc monoP), and fructose-bi-phosphate (FBP). The results shown are representative of three independent experiments. Scale bars indicate 300 μm.

(B and C) Naive B cells and IgA$^+$ PCs were purified from PPs and iLP, respectively. CE-MS quantification of metabolites from glycolysis (Glc monoP and FBP) and the TCA cycle (citrate, succinate, fumarate, and malate) in purified naive B cells and IgA$^+$ PCs (B). Data are given as means ± 1 SD (n = 11). (C) The relative amounts of glycolysis- (Glc monoP and FBP) and TCA-cycle-intermediate metabolites (citrate, succinate, fumarate, and malate) in naive B cells (Naive) and IgA$^+$ PCs (PC). The data shown are averages (n = 4) and are representative of three independent experiments.

See also Figure S1.

CD28) is associated with increased glucose uptake and glycolysis (Fox et al., 2005; Frauwirth et al., 2002). In addition, among effector T cells, Th1, Th2, and Th17 cells utilize glycolytic pathways, but regulatory T (T_{reg}) cells use lipid oxidation (Michalek et al., 2011). Similarly, lipid oxidation and mitochondrial respiratory capacity are important factors in the development of memory CD8$^+$ T cells (Pearce et al., 2009; van der Windt et al., 2012, 2013). In addition to T cells, other immune cells (e.g., macrophages and dendritic cells) demonstrate various features of immunometabolism to exert their immune functions (Everts et al., 2014; O'Neill and Hardie, 2013); however, little is known about bioenergetic metabolism in B cells, especially terminally differentiated antibody-producing plasma cells (PCs).

In this study, we focused on the intestinal immune system. Unlike that in other immune compartments (e.g., spleen, lymph nodes, lung, and skin), immunologic activation in the intestine occurs spontaneously due to continuous stimulation from intestinal environmental antigens (e.g., commensal bacteria and dietary materials) (Hooper et al., 2012; Spencer and Belkaid, 2012). Therefore, terminally differentiated IgA$^+$ PCs are abundantly present in the intestinal lamina propria (iLP) (Cerutti et al., 2011; Pabst, 2012). Another important immunologic site in the intestine is Peyer's patches (PPs). PPs are the primary gut-associated lymphoid tissue, where naive B cells predominate and where unique immunologic crosstalk by cytokines (e.g., IL-4 and transforming growth factor-β [TGF-β]) and cell-cell interactions (e.g., CD40L) induce immunoglobulin class switching from IgM to IgA to initiate intestinal IgA responses against intestinal antigen (Kunisawa et al., 2012b). These immunologic features allow us to compare in vivo energy metabolism between naive B cells in the PPs and terminally differentiated IgA$^+$ PCs in the iLP. Here, we identified changes in immunometabolism during B cell differentiation into IgA$^+$ PCs in the intestine. Furthermore, we showed that the metabolic differences between naive B cells in the PPs and IgA$^+$ PCs in the iLP were associated with distinct dependencies on vitamin B_1, an essential cofactor for the TCA cycle. The requirement for dietary vitamin B_1 was pivotal for the induction of intestinal IgA responses against oral vaccine.

RESULTS

Naive B Cells in the PPs and IgA$^+$ PCs in the iLP Produce Different Metabolites

We initially used matrix-assisted laser desorption-ionization imaging-mass spectrometry (MALDI–IMS) to visualize the in vivo distribution of metabolites in the intestine (Sugiura et al., 2011). Signals associated with citrate and malate, representative metabolites generated through the TCA cycle, were similar between PPs and iLP, whereas metabolic intermediates of glycolysis (e.g., glucose mono-phosphate [Glc monoP] and fructose bis-phosphate [FBP]) were detected preferentially in iLP (Figure 1A).

Because PPs and iLP contain various types of immune cells, we next aimed to measure the metabolites in purified cells ex vivo. We purified naive B cells and IgA$^+$ PCs from the PPs and iLP, respectively, and used capillary electrophoresis-mass spectrometry (CE-MS) metabolomics to comprehensively measure their metabolites (Sugiura et al., 2011). Consistent with in vivo MALDI-IMS results, the amounts of Glc monoP and FBP were higher in IgA$^+$ PCs than in naive B cells (Figure 1B). In line with this, concentrations of metabolites representative of the early phase of the TCA cycle (e.g., citrate and succinate) were identical between naive B cells and IgA$^+$ PCs, but those indicative of the

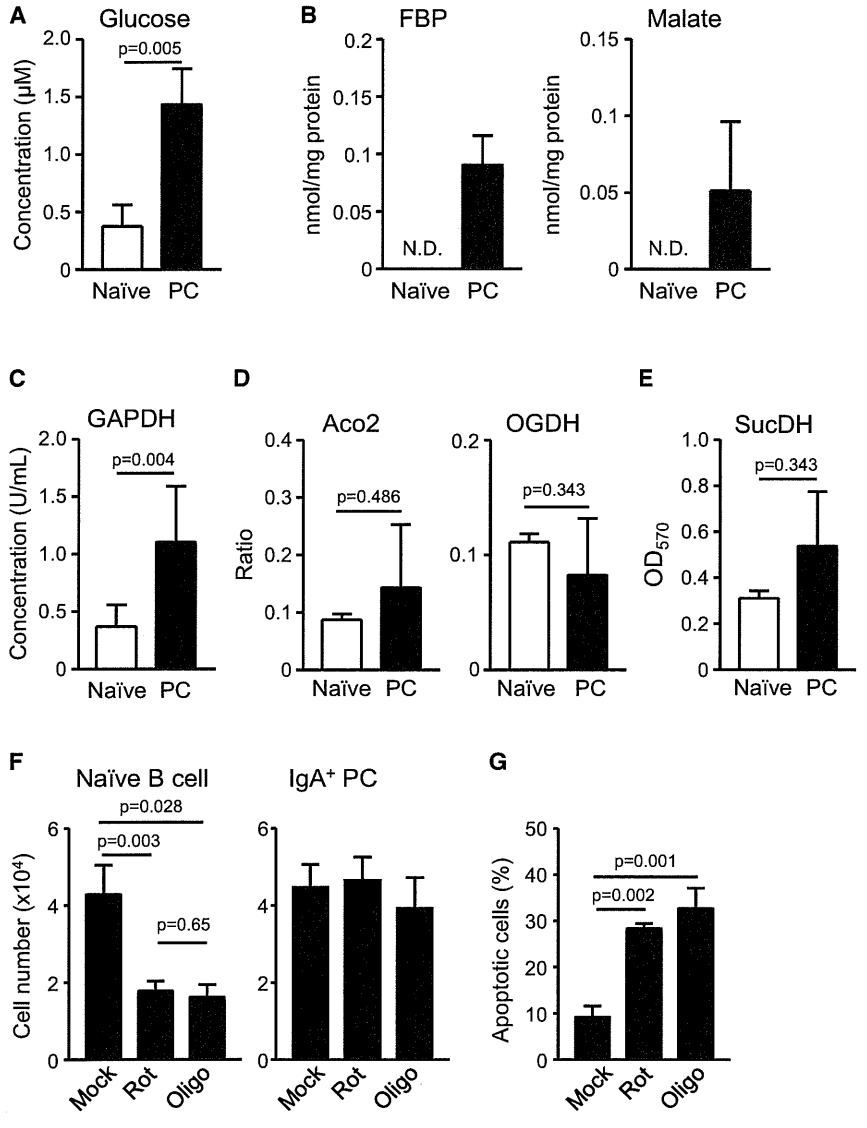

Figure 2. Different Metabolic Pathways between Naive B Cells and IgA$^+$ PCs in the Intestine

(A) Naive B cells and IgA$^+$ PCs were purified from PPs and iLP, respectively, for the analysis of glucose uptake (n = 8).

(B) Purified naive B cells and in vitro-differentiated IgA$^+$ cells were treated with ^{13}C6-glucose and used for the measurement of ^{13}C-labeled FBP and malate (n = 3).

(C–E) Naive B cells and IgA$^+$ PCs were purified from PPs and iLP, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity (C) (n = 8), expression of mRNAs encoding aconitase 2 (Aco2) and 2-oxoglutarate dehydrogenase (OGDH) (D) (n = 4), and succinate dehydrogenase activity (E) (n = 4) were compared between naive B cells (naive) and IgA$^+$ PCs (PC). Data are given as means ± 1 SD and are representative of at least two independent experiments.

(F and G) Purified naive B [left in (F) and (G)] and IgA$^+$ PCs [right in (F)] were treated with vehicle only (mock), rotenone (Rot), or oligomycin (Oligo) for 20 hr, after which cells were counted (F) or evaluated as apoptosis (G) (n = 4). Data are given as means ± 1 SD and are representative of three independent experiments.

See also Figure S2.

late phase of the TCA cycle (e.g., fumarate and malate) were higher in naive B cells than in IgA$^+$ PCs (Figure 1B). Therefore, the composition of metabolites from glycolysis and the TCA cycle differed between naive B cells and IgA$^+$ PCs (Figure 1C).

We next examined whether this metabolic profile in B cells was specific to intestinal B cells or a general phenomenon independent of anatomic localization. To this end, we measured metabolites in naive B cells, CD93$^+$ CD138$^+$ short-lived PCs, and CD93$^-$ CD138$^+$ long-lived PCs derived from the spleen. Like naive B cells and IgA$^+$ PCs in the intestine, these splenic populations showed identical levels of metabolites from the TCA cycle (Figure S1), but none of the splenic cells had detectable levels of glycolytic metabolites (e.g., Glc monoP and FBP). Therefore, the generation of glycolytic metabolites was unique to in the intestinal IgA$^+$ PCs and may reflect features of their unique immunologic environment, such as continuous exposure to commensal bacteria and consequent spontaneous production of cytokines.

Naive B Cells and IgA$^+$ PCs in the Intestine Have Distinct Metabolic Pathways

Our findings obtained from MALDI-IMS and CE-MS analyses suggested that glycolysis was used preferentially by IgA$^+$ PCs in the iLP but not by naive B cells in the PPs. This idea is supported by a result showing that the efficacy of glucose uptake was higher in IgA$^+$ PCs than in naive B cells (Figure 2A). Because the pool size of each metabolite does not correlate with flux through metabolic pathways, we performed metabolic-pathway tracing analysis using a stable isotope tracer, ^{13}C-labeled glucose. Consistent with our current findings, naive B cells lacked detectable signal derived from ^{13}C-labeled glucose due to their low efficacy of glucose uptake (Figure 2B). In contrast, in vitro differentiated IgA$^+$ PCs showed signals from ^{13}C-labeled FBP and malate (Figure 2B).

Pyruvate, a glycolytic metabolite, leads to either lactate, which does not enter the TCA cycle, or to acetyl-CoA, which subsequently enters the TCA cycle. In this context, we detected a weak lactate-associated signal in both the PPs and iLP (Figure S2A). In addition, pathway tracing using ^{13}C-labeled glucose demonstrated that ^{13}C-glucose was predominantly metabolized into glutamate through the TCA cycle (Figure S2B), suggesting that the pyruvate generated by IgA$^+$ PCs through glycolysis enters the TCA cycle rather than forms lactate.

In agreement with these findings, the enzymatic activity of glyceraldehyde 3-phosphate dehydrogenase, a key enzyme in glycolysis, was higher in IgA$^+$ PCs than in naive B cells (Figure 2C).

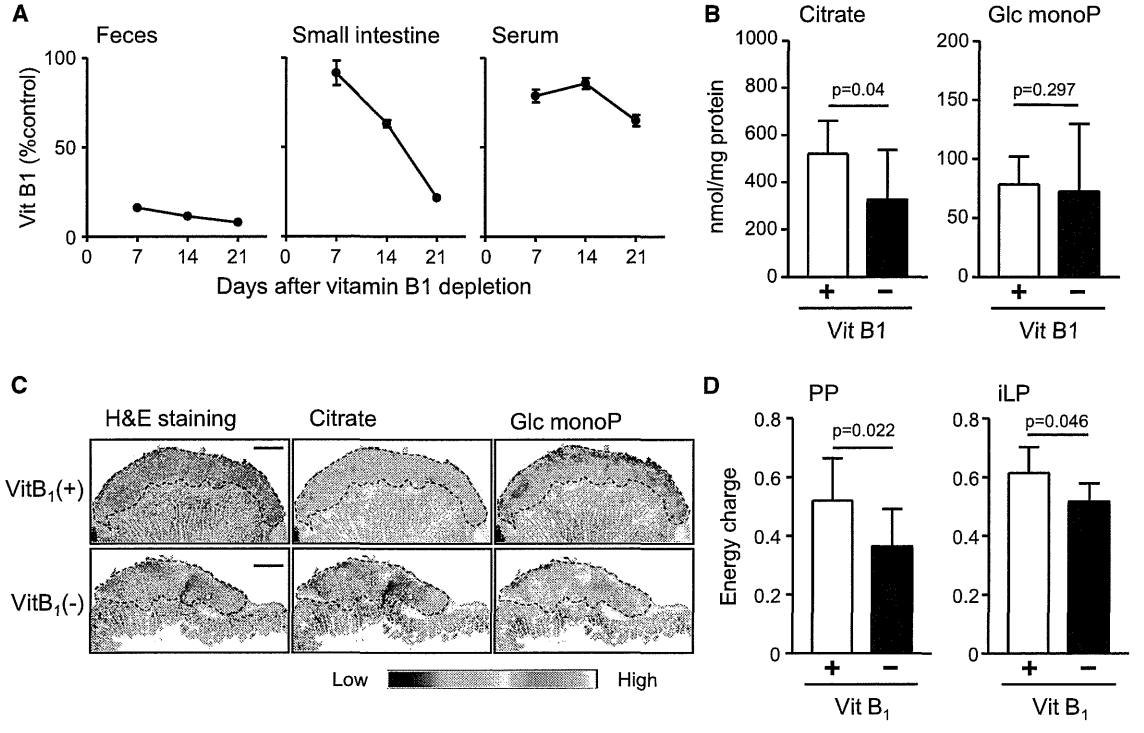

Figure 3. Impaired TCA Cycle by Depletion of Dietary Vitamin B_1

(A) Mice were maintained on vitamin B_1(+) or vitamin B_1(−) diet. The concentration of vitamin B_1 in feces, small intestine, and serum was measured on days 7, 14, and 21. The data represent the means ± 1 SD (n = 3 to 8).

(B–D) After maintenance of mice on vitamin B_1(+) or vitamin B_1(−) diet for 21 days, CE–MS (B) and MALDI-IMS (C) of intestinal tissues were performed to measure citrate and Glc monoP (B and C). Scale bars indicate 300 μm. Energy charge in the PPs and iLP was calculated from the amounts of ATP, ADP, and AMP (D). The data represent means ± 1 SD (n = 8) and are representative of three independent experiments.

In contrast to those of the glycolytic pathway, the expression of key enzymes related to the TCA cycle (aconitase 2 [Aco2], 2-oxoglutarate dehydrogenase [OGDH], and succinate dehydrogenase) was similar between naive B cells and IgA^+ PCs (Figures 2D and 2E).

Our current findings collectively suggest that IgA^+ PCs in the iLP obtain energy (ATP) from both glycolysis and the TCA cycle, whereas naive B cells in the PPs preferentially use the TCA cycle, initiated by the non-glycolytic pathway, for energy generation. Therefore, we considered it possible that inhibition of the TCA cycle would have different effects on naive B cells and IgA^+ PCs. To test this hypothesis, we used rotenone to inhibit mitochondrial respiratory chain complex I and thus impair ATP generation via the TCA cycle (Barrientos and Moraes, 1999). In vitro treatment with rotenone decreased the number of naive B cells but not of IgA^+ PCs (Figure 2F). Under this condition, cells did not proliferate; therefore, the reduction in the number of naive B cells was due to increased cell death. Indeed, rotenone treatment resulted in increased numbers of annexin V^+ apoptotic cells (Figure 2G). Similarly, the number of naive B cells but not of IgA^+ PCs was selectively reduced with an increase in apoptotic cells when ATP generation from the TCA cycle was inhibited by treating cells with oligomycin, an inhibitor of mitochondrial ATPase (Chappell and Greville, 1961) (Figures 2F and 2G). These findings collectively imply that naive B cells obtain ATP predominantly through the TCA cycle, whereas IgA^+ PCs use both glycolysis and the TCA cycle to generate ATP.

Depletion of Dietary Vitamin B_1 Inhibits the TCA Cycle without Affecting Glycolysis In Vivo

Our in vitro analysis indicated that inhibition of the TCA cycle resulted in the selective reduction of naive B cells. To confirm the effect of TCA cycle inhibition in vivo, we focused on vitamin B_1, an essential cofactor that is specifically required for the enzymatic activity of OGDH and pyruvate dehydrogenase, essential enzymes in the TCA cycle (Frank et al., 2007). Because vitamin B_1 is synthesized in bacteria, protozoans, fungi, and plants but not in mammals, mammalian species must obtain vitamin B_1 from their diets (Webb et al., 2007). This feature allowed us to examine the in vivo effects of vitamin B_1-mediated maintenance of the TCA cycle in mice fed a vitamin B_1-deficient diet (vitamin B_1[−] mice). Depletion of dietary vitamin B_1 was associated with a rapid decrease in fecal vitamin B_1 levels and a consequent gradual reduction of those in the small intestine (Figure 3A). In contrast to the intestine, serum vitamin B_1 levels tended to be maintained but ultimately showed a statistically significant decrease after 21 days of dietary vitamin B_1 depletion (Figure 3A; control: 503.3 ± 50.6; vitamin B_1[−]: 318.0 ± 17.7; p = 0.029).

As expected, intestinal cells of vitamin B_1(−) mice showed selective impairment of the TCA cycle. Indeed, CE-MS and

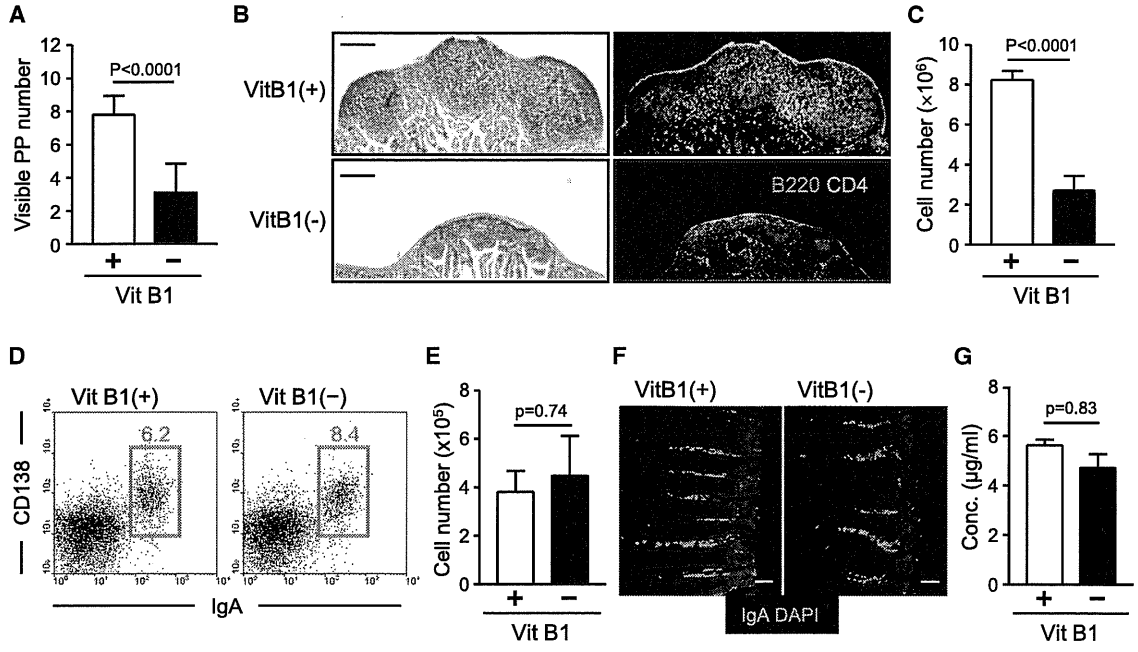

Figure 4. Reduction of Naive B Cells and Lymphoid Tissues in the PPs but Normal Numbers and Function of IgA$^+$ PCs in the iLP after Depletion of Dietary Vitamin B_1

Mice were maintained on vitamin (Vit) B_1(+) or Vit B_1(−) diet for 21 days.

(A) The number of visible PPs was examined. The data are given as means ± 1 SD (n = 8).

(B) H&E staining (left) and immunostaining of PPs with B220 and CD4 were performed for histologic analyses. Scale bars indicate 300 μm.

(C) Naive B cells in the PPs were counted. The data are given as means ± 1 SD (n = 8).

(D and E) Mononuclear cells were isolated from the iLP for analysis of the frequency (D) and number (E) of IgA$^+$ PCs. The data are given as means ± 1 SD (n = 7).

(F) The distribution of IgA$^+$ PCs in the iLP was examined by fluorescence microscopy. Scale bars indicate 100 μm.

(G) Fecal extracts were obtained for the measurement of IgA amounts by ELISA. The data are given as means ± 1 SD (n = 7).

See also Figures S3, S4, and S5.

MALDI-IMS revealed decreases in TCA cycle metabolites (e.g., citrate) with little effect on glycolytic metabolites (e.g., Glc monoP) in the intestine of vitamin B_1(−) mice (Figures 3B and 3C). These effects were associated with a decreased energy charge, an indicator of cellular energy status that is calculated from the concentrations of ATP, ADP, and AMP (Atkinson, 1968) in the vitamin B_1(−) mice (Figure 3D).

The Impaired TCA Cycle due to Vitamin B_1 Deficiency Leads to Decreased Numbers of Naive B Cells and Consequent Atrophy of PPs with Little Effect on IgA$^+$ PCs in the iLP

We typically saw seven to ten PPs in the small intestine of mice fed a vitamin-B_1-sufficient diet (vitamin B_1[+] mice), whereas few (if any) PPs were discernible in vitamin B_1(−) mice; therefore the number of visible PPs was decreased in vitamin B_1(−) mice (Figure 4A). In addition, the reduction in the size and number of PPs in vitamin B_1(−) mice was accompanied by decreases in the size of B cell follicles and in the number of naive B cells with little effect on T cells (Figures 4B, 4C, and S3A). We next examined the effect of dietary vitamin B_1 depletion on bone marrow cells, revealing the reduction of pre/pro-B cells (Figure S3B). Therefore, the recruitment of naive B cells from bone marrow was reduced, which explained, at least partly, the decrease in the number of naive B cells in the periphery.

As occurred with PPs, other lymphoid tissues including spleen and mesenteric lymph nodes were smaller in vitamin B_1(−) than in control mice (Figure S4). We then investigated whether the effect of vitamin B_1 depletion was reversible. To address this issue, vitamin B_1(−) diet was replaced with vitamin B_1(+) diet after a 21-day depletion of dietary vitamin B_1. PPs, spleen, and mesenteric lymph nodes all recovered in size by 14 days after the switch to the vitamin B_1(+) diet (Figure S4), suggesting that the lymphoid atrophy induced by vitamin B_1 deficiency was reversible.

We next examined IgA$^+$ PCs in the iLP of vitamin B_1(−) mice. Unlike naive B cells in the PPs, IgA B220$^-$ PCs in the iLP of vitamin B_1(−) mice were unchanged in frequency and absolute cell number when compared with those of vitamin B_1(+) mice (Figures 4D and 4E). Fluorescent microscopic analysis further confirmed that IgA$^+$ PCs were distributed normally in the iLP of vitamin B_1(−) mice (Figure 4F). We then examined whether these PCs were functionally normal and produced IgA in the absence of vitamin B_1 in vivo. The amount of total IgA in the feces was comparable between vitamin B_1(+) and vitamin B_1(−) mice (Figure 4G). These findings indicated a change in B cell homeostasis in the vitamin B_1-deficient condition. To address this issue, we performed a BrdU uptake assay, showing that the number of BrdU$^+$ IgA$^+$ cells decreased rapidly in the vitamin B_1(−) mice when compared with the vitamin B_1(+) mice (Figure S5),

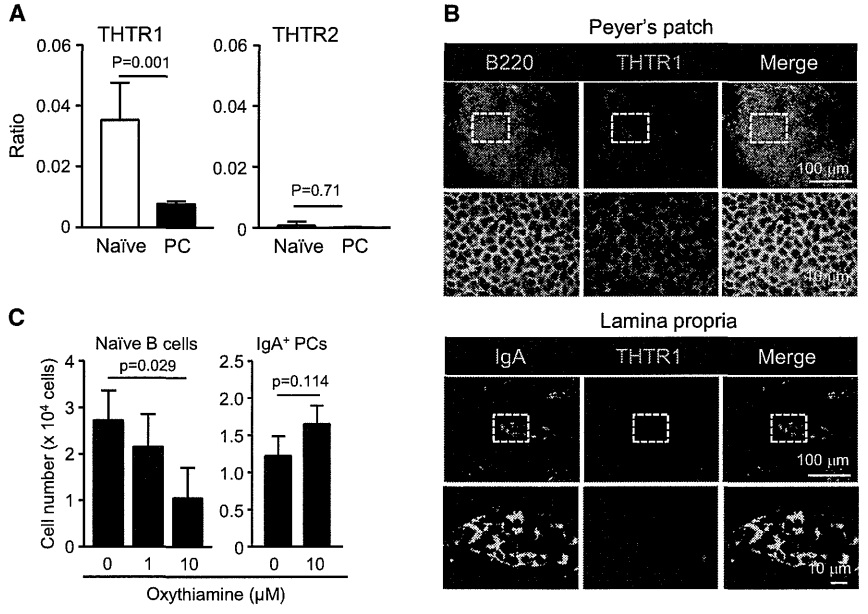

Figure 5. Vitamin B_1 Transporter Expression on Naive B cells in the PPs and on IgA$^+$ PCs in the iLP

(A) qRT-PCR was performed to measure the gene expression of THTR1 and THTR2 in the naive B cells in the PPs (naive) and IgA$^+$ PCs in the iLP (PC). Data are given as means ± 1 SD (n = 7).

(B) PPs were immunostained for B220 and THTR1; iLP was stained with IgA and THTR1. The data shown are representative of three independent experiments.

(C and D) Naive B cells or IgA$^+$ PCs were cultured with different concentrations of oxythiamine for 3 days. Cell numbers of both populations (C) and viability of naive B cells (D) were then determined. Data are given as means ± 1 SD (n = 4) and are representative of two independent experiments.

(E) Naive B cells labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with different concentrations of oxythiamine in the presence of LPS for 3 days. The CFSE-associated signal was determined by flow cytometry. Data are given as means ± 1 SD (n = 4) and are representative of two independent experiments.

presumably due to the decreased supply of BrdU$^+$ IgA$^+$ cells from the PPs. These findings collectively indicate that the dependency on vitamin B_1 differed between naive B cells and IgA$^+$ PCs and that, unlike naive B cells, IgA PCs in the iLP were sufficient in number and function to produce IgA in the absence of vitamin B_1.

The Expression Levels of Vitamin B_1 Transporters in B cells and IgA$^+$ PCs Are Consistent with Their Vitamin B_1 Dependency

The differing dependencies of naive B cells and IgA$^+$ PCs on vitamin B_1 led us to examine the expression of vitamin B_1 transporters, which mediate the active transport of vitamin B_1 into the intracellular compartment. Regarding the vitamin B_1 transporters thiamine transporter 1 and 2 (THTR1 and THTR2) (Dutta et al., 1999; Rajgopal et al., 2001), qPCR analysis showed that THTR1, but not THTR2, was expressed in both naive B cells and IgA$^+$ PCs and that the THTR1 level was higher in the naive B cells than in IgA PCs (Figure 5A). Immunostaining further confirmed that the majority of the population of naive B cells expressed THTR1, whereas only occasional IgA$^+$ PCs expressed this transporter (Figure 5B). These findings suggest that distinct vitamin B_1 dependencies of naive B cells and IgA$^+$ PCs were consistent with their expression levels of vitamin B_1 transporters, especially THTR1.

These findings led us to examine whether vitamin B_1 depletion directly affected B cells. To address this issue, we performed in vitro analysis using purified B cells. Treatment with vitamin B_1 antagonist (oxythiamine) decreased the number and viability of naive B cells, whereas cell numbers of IgA$^+$ PCs were unchanged (Figures 5C and 5D). We also found that oxythiamine inhibited LPS-induced proliferation of purified naive B cells (Figure 5E). Therefore, although we cannot exclude the possible involvement of other cells, vitamin B_1 depletion has a direct effect on the survival and proliferation of B cells.

Vitamin B_1-Dependent Maintenance of Naive B Cells Is Required for the Induction but Not Maintenance of Intestinal IgA Responses against Orally Immunized Antigen

We wondered whether the different dependencies on vitamin B_1 during B cell differentiation in the intestine affected the induction of antigen-specific IgA responses against orally administered antigen. In this experiment, groups of mice were maintained on the control (vitamin B_1[+]) diet throughout the 31-day experimental period (controls), on the vitamin B_1(+) diet for the first 10 days but on the vitamin B_1(−) diet for the remaining 21 days, or on the vitamin B_1(−) diet for the first 21 days and on the vitamin B_1(+) diet for the remaining 10 days (Figure 6A). All groups received oral immunization with cholera toxin (CT) on days 14 and 21, and feces and mononuclear cells were collected on day 31 for measurement of IgA antibody production against the B subunit of cholera toxin (CTB) by ELISA and of IgA-antibody forming cells (AFCs) by ELISPOT assays (Figure 6A).

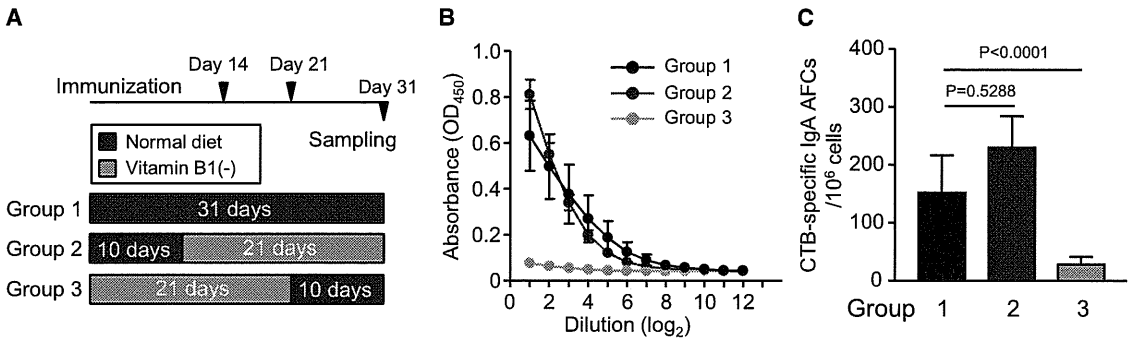

Figure 6. Vitamin B_1-Mediated Maintenance of Naive B Cells in the PPs Is Required for the Induction Phase but Not the Effector Phase of the Intestinal IgA Response against Oral Antigen

(A) The experimental schedule is summarized. Mice were maintained on the vitamin B_1(+) diet throughout the 31-day experimental period (group 1); for 10 days on the vitamin B_1(+) diet and then on the vitamin B_1(−) diet for 21 days (group 2); or on the vitamin B_1(−) diet for 21 days and then on the vitamin B_1(+) diet for 10 days (group 3). All groups underwent oral immunization with 10 μg CT on days 14 and 21.

(B and C) On day 21, ELISA (B) and ELISPOT assays (C) were performed to assess CT subunit B (CTB)-specific IgA production and the number of IgA antibody-forming cells (AFCs), respectively. The data are given as means ± 1 SD (n = 6) and are representative of three independent experiments.

See also Figure S6.

After oral immunization with CT, mice maintained on the vitamin B_1(+) diet throughout the 31-day experiment showed high levels of CTB-specific fecal IgA production as well as increased numbers of CTB-specific IgA AFCs in the iLP (Figures 6B and 6C). Mice that received the control diet followed by the deficient diet had normal numbers of naive B cells in the PPs during the oral immunization period (days 14 and 21) but decreased numbers on day 31. In addition, the CTB-specific fecal IgA and IgA AFCs in the iLP of these mice were similar to those of the controls (Figures 6B and 6C). In contrast, mice fed the deficient diet followed by the control diet showed reduced numbers of naive B cells during the oral immunization period and correspondingly decreased levels of CTB-specific fecal IgA production and IgA AFCs in the iLP (Figures 6B and 6C). Similarly, antigen-specific IgG production was decreased in the serum of vitamin B1(−) mice when they underwent intraperitoneal immunization with ovalbumin plus alum (Figure S6). Taken together, these results indicate that vitamin B_1-mediated maintenance of naive B cells is required during immunization—but not thereafter—for efficient antibody responses against orally or systemically immunized antigens.

DISCUSSION

In this study, we showed the different patterns of energy metabolism between naive B cells and terminally differentiated IgA^+ PCs. Our study revealed that IgA^+ PCs in the iLP showed higher expression of glycolysis-related metabolites than did naive B cells in the PPs. It had been reported that B cell activation by crosslinking through B cell receptors (BCRs) induced a metabolic shift to the glycolytic pathway in a phosphatidylinositol 3 kinase- and protein kinase Cβ-dependent manner; this shift reportedly was associated with the upregulation of GLUT1 and the activation of hexokinase and phosphofructokinase, key enzymes in glycolysis (Blair et al., 2012; Doughty et al., 2006; Moon et al., 2011). Similar to that of BCRs, IL-4- and toll-like receptor (TLR)-mediated stimulation likewise induced metabolic changes in B cells (Dufort et al., 2007; Haimovich et al., 2010). Because BCRs, TLRs, and IL-4 are prerequisite molecules for the differentiation of B cells into IgA^+ PCs (Kunisawa et al., 2012b), class switching from IgM to IgA likely is associated with the metabolic shift toward preferential use of the glycolytic pathway, allowing IgA^+ PCs to respond to the increased energy demand associated with antibody production. Unlike intestinal IgA^+ PCs, PCs in the systemic immune compartment (e.g., spleen) contained few glycolytic metabolites. This difference may be explained by the unique immunologic environment in the intestine, where there is continuous exposure to bacterial products (e.g., LPS) and consequent simultaneous production of cytokines.

B cell differentiation into PCs is accompanied by increases in the sizes of cells, ER, and secretory organelles to accommodate massive secretion of antibody (Manz and Radbruch, 2002), a process that requires large amounts of amino acids, lipids, and sugar and increased demand for ATP. Therefore, it is reasonable that IgA^+ PCs exploit a pathway that combines glycolysis and the TCA cycle to obtain large amounts of ATP and amino acids for the efficient production of IgA. Glucose-initiated energy generation is essential for antibody production, according to a recent study that has demonstrated that B cell-specific deletion of GLUT1 results in impaired antibody production in vivo (Caro-Maldonado et al., 2014). In contrast to PCs, naive B cells use the TCA cycle in isolation from glycolysis. In this regard, it previously was reported that B cells use glucose-independent glutamine metabolism via the TCA cycle for their survival (Le et al., 2012). Together with our current finding that glucose was metabolized into glutamate through the TCA cycle, the cited study may explain our current findings of increased levels of the glutamine-derived TCA cycle metabolites, fumarate, and malate in naive B cells.

Recent studies have disclosed several examples of vitamin-mediated immune regulation, including lymphocyte trafficking

by vitamins A and D (Mora et al., 2008); T cell differentiation (Hall et al., 2011), innate lymphoid cell function (Spencer et al., 2014), and mast cell activation (Kurashima et al., 2014) by vitamin A; and regulatory T cell survival by vitamin B_9 (Kinoshita et al., 2012; Kunisawa et al., 2012a). In the current study, we extend our understanding of vitamin-mediated immune regulation by showing the immunologic association between vitamin B_1 and B cell immunometabolism in the intestine. The metabolic changes during B cell differentiation into IgA^+ PCs were accompanied by decreased expression of THTR-1 and loss of the dependency on vitamin B_1. A previous study demonstrated that treatment with high glucose concentrations decreased the expression of THTR-1 in proximal tubular epithelial cells via the downregulation of specificity protein 1, a transcription factor of THTR1 (Larkin et al., 2012). Therefore, the high glucose uptake of IgA^+ PCs may induce the downregulation of THTR1 and thus independence from vitamin B_1.

Vitamin B_1 deficiency is rare in developed countries but still characterizes chronic alcohol abuse and severe malnutrition (Zahr et al., 2011). In addition, some dietary preservatives (such as sulfites) (Stammati et al., 1992) and some kinds of plants, raw fish, shellfish, and bacteria produce thiaminases that degrade vitamin B_1 (Murata, 1982). Furthermore, vitamin B_1 uptake is reduced by thyroid hormone and diabetes (Larkin et al., 2012). Therefore, aspects that alter the intestinal environment, such as diet and commensal bacteria, as well as host intrinsic factors that affect the absorption and cellular uptake of vitamin B_1, affect host immune responses through the regulation of immunometabolism.

EXPERIMENTAL PROCEDURES

Mice

Female Balb/c mice (age, 7 weeks) were purchased from Japan Clea. Vitamin B_1(−) and control diets composed of chemically defined materials were purchased from Oriental Yeast (Kunisawa et al., 2012a). All animals were maintained in the experimental animal facilities of the University of Tokyo and National Institute of Biomedical Innovation. The experiments were approved by the Animal Care and Use Committees of both institutes and were conducted in accordance with their guidelines.

Lymphocyte Isolation

Lymphocytes were isolated from the spleen, bone marrow, PPs, and iLP of mice as previously described (Kunisawa et al., 2013). Briefly, PPs were stirred in 1.6 mg/ml collagenase (Wako) to obtain a single-cell suspension. To isolate lymphocytes from the iLP, PPs were removed from the intestinal tissue, and the remaining intestinal tissue was rinsed in RPMI 1640 medium, cut into 2-cm pieces, and stirred for 20 min in RPMI 1640 medium containing 1 mM EDTA and 2% fetal calf serum. The tissue pieces then were stirred for 15 min three times in 0.8 mg/ml collagenase, and the dissociated cells underwent centrifugation through a discontinuous Percoll (GE Healthcare) gradient. Lymphocytes were isolated at the interface between the 40% and 75% Percoll layers.

Flow Cytometry and Cell Sorting

A standard protocol was used for cell staining and subsequent flow cytometric analysis (Kunisawa et al., 2013). Briefly, cells were incubated with 5 μg/ml anti-CD16/32 antibody (Ab) (Fc Block, Biolegend) for 5 min and stained with fluorescently labeled Abs specific for B220, CD3, CD93, CD138, IgM (Biolegend), and IgA (BD Biosciences) for 30 min at 4°C. The Viaprobe reagent (BD Biosciences) was used to discriminate between dead and live cells. Apoptotic cells were detected by using the Annexin V Apoptosis Detection kit according to the manufacturer's instructions (Biolegend). For BrdU incorporation, mice were injected intraperitoneally with 1 mg BrdU (Sigma-Aldrich) in PBS as previously described (Kunisawa et al., 2007, 2013). At the indicated times, mononuclear cells were isolated from the small intestine and stained with fluorescent IgA-specific antibodies. BrdU incorporation was detected with a BrdU Flow kit according to the manufacturer's instructions (BD Biosciences). Flow cytometric analysis and cell sorting were performed by using FACSCanto II and FACSAria III (BD Biosciences) instruments, respectively.

Histologic Analysis

Immunohistochemical analysis and H&E staining were performed as previously described (Kurashima et al., 2014; Obata et al., 2013). Briefly, small intestine was fixed in 4% paraformaldehyde for 15 hr at 4°C and washed and treated in 20% sucrose for 12 hr at 4°C. The tissues were embedded in OCT compound (Sakura Finetechnical). Cryostat sections (7 μm) were preblocked with an anti-CD16/CD32 Ab (Biolegend) for 15 min at room temperature and stained with fluorescent-conjugated Abs specific for B220 (Biolegend) or IgA (BD Biosciences). Slides were counterstained by using DAPI (Sigma-Aldrich). For the detection of THTR1, cryostat sections (7 μm) were fixed with cold acetone for 1 min without paraformaldehyde fixation. THTR1 was detected by using a rabbit anti-SLC19A2 (THTR1) polyclonal Ab (Atlas Antibodies) and a Cy3-conjugated donkey anti-rabbit IgG Ab (Jackson Immunoresearch Laboratories). For the detection of CD4, a biotin-conjugated anti-CD4 antibody (BD Biosciences) and a TSA-Direct kit (Perkin-Elmer) were used according to the method previously described (Obata et al., 2013). The specimens were analyzed by using a fluorescence microscope (model BZ-9000, Keyence).

MALDI-IMS

MALDI-IMS and CE-MS were performed as previously reported (Kunisawa et al., 2015; Sugiura et al., 2011). Briefly, frozen tissues were sectioned to a thickness of 8 μm by using a cryostat (CM 3050, Leica). The frozen sections were thaw mounted onto indium-tin-oxide (ITO)-coated glass slides (Bruker Daltonics). MALDI-IMS was performed by using 9-aminoacridine as the matrix (10 mg/ml, dissolved in 80% ethanol). MALDI imaging was performed by using an MALDI-TOF mass spectrometer (UltraFlextreme, Bruker Daltonics) equipped with an Nd:YAG laser. Data were acquired in the negative reflectron mode with raster scanning by a pitch distance of 50 μm. Image reconstruction was performed by using FlexImaging 4.1 software (Bruker Daltonics).

CE-MS

CE-MS was performed as previously reported (Sugiura et al., 2011). Frozen tissues or purified cells were homogenized in methanol (500 μl) by using a manual homogenizer (Finger Masher [AM79330]; Sarstedt), followed by the addition of an equal volume of chloroform and 0.4 times the volume of Milli-Q water. After centrifugation (3 cycles of 60 s each at 4,000 rpm), the aqueous phase was ultrafiltered by using an ultrafiltration tube (Ultrafree-MC, UFC3 LCC NB; Human Metabolome Technologies), and the filtrates were dried. The dried residues were resuspended in 50 μl of Milli-Q water and were used for CE-MS. All CE-MS experiments were performed by using an Agilent CE System equipped with an air pressure pump, an Agilent 6520 Accurate Q-Tof mass spectrometer, an Agilent 1200 series isocratic high-performance LC pump, 7100 CE-system, a G1603A Agilent CE-MS adaptor kit, and a G1607A Agilent CE-MS sprayer kit (Agilent Technologies).

Metabolic-Pathway Tracing Analysis Using In Vitro Differentiated IgA^+ Cells

For the differentiation of naive B cells into IgA^+ cells, purified naive B cells were cultured with interleukin-4 (IL-4), IL-5, lipopolysaccharide (LPS), and TGF-β for 4 days and then additionally cultured with IL-6 for 3 days (Gohda et al., 2008). These in vitro differentiated IgA^+ cells and freshly isolated naive B cells were cultured with 2 mg/ml of ^{13}C-labeled glucose (ISOTEC, Sigma-Aldrich) in RPMI1640 medium (plus 10% FCS and 2-mercaptoethanol, no pyruvate or antibiotics) for 1 hr. After being washed with PBS twice, the cells were used for the CE-MS analysis.

Measurement of Vitamin B_1 Concentration

Vitamin B_1 concentration was measured by using VitaFast Vitamin B_1 (thiamine) (r-Biopharm). Briefly, sample extract was added to *Lactobacillus*

fermentum in vitamin B_1(–) medium. The growth of *L. fermentum* is dependent on the vitamin B_1 concentration of the medium. The vitamin B_1 concentration of the sample was determined by referring to a standard curve.

Measurement of Glucose Uptake, GAPDH, and Succinate Dehydrogenase Activity

To measure glucose uptake and enzymatic activity of GAPDH and succinate dehydrogenase, we used the 2-Deoxyglucose Uptake Measurement Kit (Cosmobio), KDalert GAPDH Assay Kit (Life Technologies), and MTT Cell Proliferation Assay kit (Cayman Chemical) according to the manufacturers' protocols.

Drug Treatment

Purified naive B cells or IgA^+ PCs (5×10^4 cells) were cultured with 3 μM oligomycin (Sigma-Aldrich) or 5 μM rotenone (Sigma-Aldrich) for 20 hr. For the oxythiamine treatment, purified naive B cells or IgA^+ PCs (1×10^5 cells) were cultured with oxythiamine for 3 days. Live cells were counted by using the trypan blue exclusion assay. For the proliferation assay, purified naive B cells were stained with 2 μM of carboxyfluorescein succinimidyl ester (Life Technologies) and then were cultured in the presence of various concentrations of oxythiamine and 10 μg/ml of LPS (O127:B8; Sigma-Aldrich) for 3 days.

Quantitative and Conventional RT-PCR

To measure mRNA expression, qRT-PCR was performed (Kurashima et al., 2014). Briefly, total RNA was isolated by using TRIzol reagent (Invitrogen), and cDNA was synthesized by using Powerscript reverse transcriptase (BD Biosciences). RT-PCR was performed by using LightCycler 480 (Roche Diagnostics) with the SYBR Green system. The oligonucleotide primers used in this study were obtained from Hokkaido System Science and include those for OGDH (forward primer, 5′-ACTGTGCCTGGTTGGAGAATCCC-3′; reverse primer, 5′-ACTGCACCGCCAAGTGGTCC-3′), Aco2 (forward primer, 5′-GCG CAGGGCCAAGGACATAAA-3′; reverse primer, 5′-GGGGGTGTGCGAATCA GTGCC-3′), THTR1 (forward primer, 5′-CGACAAGAACTTGACCGAGA-3′; reverse primer, 5′-AAGGAACACGGGAAACAGC-3′), THTR2 (forward primer, 5′-CATTGTTGGGTGAATTGGACT-3′; reverse primer, 5′-GAGTTGCTCGGTG GAGTTCT-3′), and cyclophilin (forward primer, 5′-GACGAAGGTAGCCAGT CACAAG-3′; reverse primer, 5′-AATCAGGCCTGTGGAATGTGAG-3′).

Immunization and Detection of Antibody Responses by ELISA and ELISPOT Assays

For oral immunization on days 14 and 21, mice were given sodium bicarbonate solution to neutralize stomach acid (Kunisawa et al., 2013, 2014); 30 min later, the mice were orally immunized with 10 μg of CT (List Biological Laboratories). At 10 days after the final immunization, fecal samples and mononuclear cells were collected for measurement of CTB-specific antibody responses by using ELISA and ELISPOT assays, respectively, as previously described (Kunisawa et al., 2013). For systemic immunization, mice were maintained on vitamin B_1(+) or vitamin B_1(–) diet. On day 22, mice were injected intraperitoneally with 100 μg of ovalbumin (OVA) plus alum (Life Technologies) and serum samples were collected on day 28 for the measurement of OVA-specific IgG by ELISA.

Statistics

Results were compared by using the nonparametric Mann-Whitney U test and two-tailed unpaired t test (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.063.

AUTHOR CONTRIBUTIONS

J.K., Y. Sugiura, M. Suematsu, and H.K. conducted research and wrote the manuscript. J.K., T.W., R.N., T.N., E.H., Y. Suzuki, H.S., and S.S. performed immunological experiments and analyzed data, and Y. Sugiura, K.H., and M. Setou performed metabolic analyses.

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Vitamin-Mediated Immune Regulation in the Development of Inflammatory Diseases

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Abstract: Mucosal tissues and especially the intestine are constantly exposed to abundant non-self materials yet simultaneously establish immune homeostasis to prevent excessive inflammatory responses. The maintenance of intestinal homeostasis is achieved by a harmonized immune network mediated by endogenous factors (e.g., cytokines and chemokines) and exogenous factors (e.g., commensal bacteria and dietary matter). Specifically, vitamins from such exogenous sources function immunologically in the control of homeostatic immune responses; thus, their deficient or excessive intake is associated with the development of inflammatory diseases. The focus of this review is the immunologic functions of vitamins B3, B9, A, and D in the regulation and development of inflammation.

Keywords: Diet, inflammation, mast cells, mucosal immunology, regulatory T cells, vitamins

INTRODUCTION

The physiologic role of the intestine is digestion and absorption of nutrients. This process is at least partly mediated by commensal bacteria. Thus, intestinal tissues are constantly exposed to abundant non-self materials from diet and commensal bacteria. The peripheral immune system recognizes and responds to non-self to eliminate or exclude them, but the intestinal immune system is uniquely tolerant to non-self in order to prevent unnecessary inflammation and hypersensitivity. Indeed, the intestinal immune system is comprised of versatile immune cells with regulatory functions (e.g., regulatory dendritic cells and macrophages, IgA-producing plasma cells, regulatory T (Treg) cells, and IL-10-producing Type 1 regulatory T (Tr1) cells) [1]. Furthermore, the disruption of immunologic tolerance in the intestine leads to the development of intestinal inflammation and allergy [2]. The dynamic nature of the intestinal immune system is controlled by not only host-derived factors (e.g., cytokines and chemokines) but also gut environmental factors (e.g., commensal bacteria and diet); thus, immunologic crosstalk is a critical factor in the development of inflammatory diseases [3].

Vitamins are vital compounds that are classified as water-soluble (e.g., vitamins B and C) or hydrophobic (e.g., vitamins A, D, E and K). Most vitamins cannot be synthesized by mammals; therefore, they must be obtained from the diet, commensal bacteria, or both. Vitamins and their metabolites maintain homeostasis through various functions, including immunologic regulation and cell growth and differentiation. Furthermore, vitamin deficiency is associated with increased susceptibility to immune diseases [4]. Recent studies have illustrated specific functions of vitamins in the regulation of host immune responses and their involvement in the development of inflammatory diseases. In this review, we describe these recent findings on the immunologic functions of vitamins B3, B9, A, and D and their association with inflammatory responses.

Regulation of Intestinal Immune Responses by B Vitamins

The vitamin B-complex contains thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pantothenic acid (vitamin B5), pyridoxine (vitamin B6), biotin, folic acid (vitamin B9), and cobalamins (vitamin B12), all of which contribute to energy metabolism. In addition to the general importance of the vitamin B-complex, some B vitamins have specific immune regulatory functions.

Niacin (vitamin B3) is present in many foods, including meat, fish, milk, eggs, and green vegetables; it likely has immunoregulatory functions because severe niacin deficiency leads to the development of pellagra, which is characterized by diarrhea and inflammation in the intestine and skin [5]. Furthermore, a receptor of niacin, Gpr109a, is expressed on intestinal epithelial cells and immune cells, such as macrophages and dendritic cells [6]. Gpr109a also plays an important role in the control of intestinal inflammation because its activation promotes the expression of IL-10 in intestinal dendritic cells and macrophages. Additionally, Gpr109a-mediated signaling induces the differentiation of IL-10-producing Tr1 cells and Treg cells. Consistently, mice lacking Gpr109a have fewer intestinal

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Treg cells and IL-10-producing T cells; consequently, they are highly susceptible to intestinal inflammation [7]. Because of such immunoregulatory functions, niacin analogue is a good candidate as an anti-inflammatory drug.

Folate (vitamin B9) is essential for nucleic acid and protein synthesis [8]. Immunologically, folate deficiency induces the inhibition of $CD8^+$ T cell and NK cell activities, leading to decreased resistance to infection [9]. Interestingly, folate receptor 4 (FR4), one of the folate receptors, is highly expressed in Treg cells [10], which allowed us to examine folate function in the regulation of Treg cells. We found that folate plays an important role in the maintenance but not induction of Treg cells [11]. Indeed, naïve T cells could differentiate into Treg cells in the absence of folate; however, the differentiated Treg cells failed to survive with decreased expression of anti-apoptotic molecules (e.g., Bcl-2) [11]. Consequently, the decrease of intestinal Treg cells under conditions of insufficient folate leads to increased susceptibility to intestinal inflammation (Fig. **1**) [12].

Vitamin A Controls Skin and Intestinal Inflammatory Responses

Vitamin A is a hydrophobic vitamin and critical for the maintenance of various physiologic functions (e.g. vision, cell differentiation, and reproduction). Specifically for immunologic function, the vitamin A metabolite retinoic acid (RA) regulates cell trafficking toward the intestine by inducing gut-homing molecules, including $\alpha4\beta7$ integrin and chemokine receptor CCR9 on lymphocytes [13]. In addition to this gut-homing imprinting function, at steady state in the intestine, RA mediates the preferential differentiation of naïve T cells into Treg cells [14-16]. Moreover, RA controls the survival and immunologic function of $\alpha\beta$T cells (including the Th1, Th2, Th17, and cytotoxic T cell subsets), $\gamma\delta$T cells, and type 3 innate lymphoid cells (ILC3) [17]. ILC3-mediated IL-22 production subsequently plays a role in the repair of epithelial cells, production of antimicrobial agents, and attenuation of intestinal inflammation [18]. Thus, RA is a homeostatic vitamin especially in the intestinal immunity.

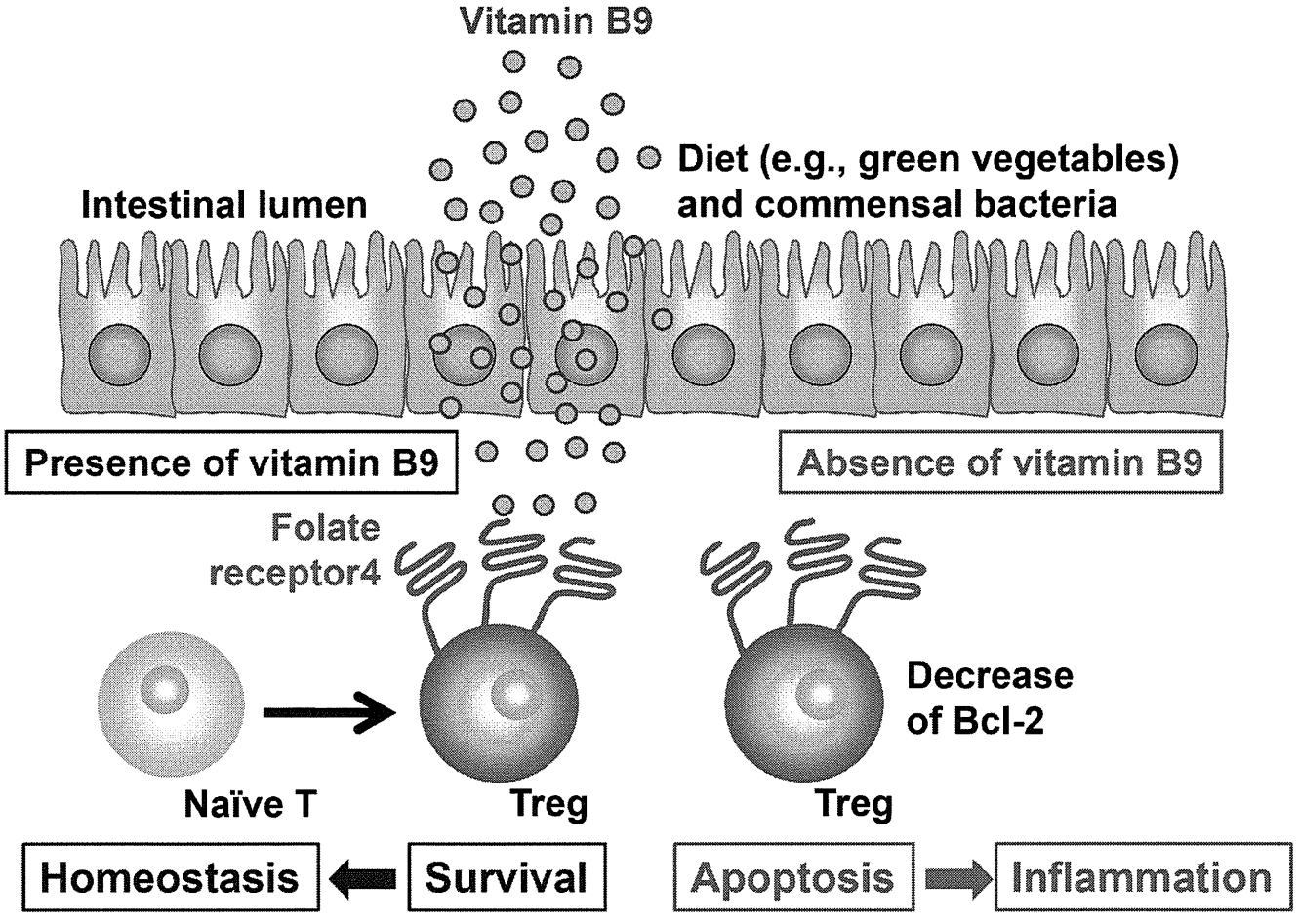

Fig. (1). Vitamin B9 plays a pivotal role in maintenance but not induction of regulatory T (Treg) cells in the intestine. When naïve T cells differentiate into Treg cells, they obtain the expression of vitamin B9 receptor, folate receptor 4 (FR4). The vitamin B9-FR4 axis is essential for their survival and thus participates in the maintenance of immunological homeostasis. In the absence of vitamin B9, differentiated Treg cells lack the expression of anti-apoptotic molecules (e.g., Bcl-2), leading to the induction of their apoptosis. The reduction of Treg cells is coincident with the disruption of immunological homeostasis, which consequently results in the development of intestinal inflammation.