Impaired Human Responses to Tetanus Toxoid in Vitamin A-Deficient SCID Mice Reconstituted with Human Peripheral Blood Lymphocytes

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Vitamin A deficiency is associated with increased childhood morbidity and mortality from respiratory and diarrheal diseases. In order to evaluate the effect of vitamin A on human antibody responses, we developed a vitamin A-deficient severe combined immunodeficient (SCID) mouse model. Vitamin A-deficient mice were produced by depriving them of vitamin A at day 7 of gestation. Mice were reconstituted with human peripheral blood lymphocytes (huPBL) from tetanus toxoid immune donors at 6 weeks of age and immunized with tetanus toxoid at 6 and 8 weeks of age. Secondary human antibody responses were determined 10 days later. The geometric mean human anti-tetanus toxoid immunoglobulin G concentrations were 3.75 $\mu\text{g/ml}$ for the deficient mice and 148 $\mu\text{g/ml}$ for controls ($P = 0.0005$). Vitamin A-deficient mice had only a 2.9-fold increase in human anti-tetanus toxoid antibody compared with a 74-fold increase in controls ($P < 0.01$). Supplementation with vitamin A prior to reconstitution restored human antibody responses to normal. These data suggest that vitamin A deficiency impairs human antibody responses. We speculate that impaired responses could increase susceptibility to certain infections. Furthermore, we propose that effects of other nutritional deficiencies on the human immune system could be evaluated in the SCID-huPBL model.

Vitamin A (retinol and its derivatives) is essential to maintain a variety of organ functions such as reproduction, vision, and the integrity of epithelial tissues. Since the beginning of the century, vitamin A has also been known as the “anti-infective” vitamin (21). Recent in vitro studies demonstrate that vitamin A is essential for human and murine B-cell growth and T-cell proliferation (7, 8, 19). In vivo studies have documented both delayed hypersensitivity and impaired antibody responses in vitamin A-deficient mice and rats (24, 34, 40, 41). Analogous controlled human studies are obviously difficult to conduct, although impaired immunity in a vitamin A-deficient population has been reported (38). In addition, community-based trials examining the effect of vitamin A supplementation on childhood survival have produced conflicting results (20, 22, 36, 43, 45, 46). Thus, to further evaluate the effect of vitamin A on human responses, we developed a human reconstituted vitamin A-deficient mouse model.

Mice homozygous for the severe combined immunodeficiency (SCID) gene have an almost complete absence of functional T and B cells due to the lack of somatic rearrangement of either immunoglobulin or T-cell receptor genes (6). SCID mice have recently been successfully reconstituted with either human peripheral blood lymphocytes (SCID-huPBL) or fetal tissues (27, 28). Human reconstituted SCID mice produce substantial amounts of human immunoglobulin and have been utilized as models of human infection (human immunodeficiency and Epstein-Barr viruses), immune mediate diseases, and immune regulation (3, 5, 15, 25, 29, 31). We now report on the establishment of a vitamin A-deficient SCID-huPBL model

and demonstrate impaired human antibody responses to tetanus toxoid immunization.

MATERIALS AND METHODS

Vitamin A-deficient SCID mice. C.B.-17 SCID/SCID mice (Taconic, Inc., Germantown, N.Y.) were bred, and gravid females were randomly assigned to receive either a standard rodent diet with American Institute of Nutrition-76 vitamin mixture containing 4 IU of vitamin A per g (control) or a deficient diet which lacked vitamin A (Zeigler Brothers, Inc., Gardner, Pa.) (Table 1). When pups were weaned at 21 days of age, they were fed either a deficient or a control diet as described above with the fat content reduced from 11 to 5%. For some experiments control SCID mice were obtained from Taconic, Inc., at 4 weeks of age and placed on the control diet. For vitamin A supplementation experiments, vitamin A-deficient mice were provided vitamin A in their drinking water beginning at 4 weeks of age (2 weeks prior to reconstitution with human cells) and supplementation was continued for the duration of the experiment. Water-dispersible vitamin A palmitate beadlets containing 250,000 IU/g (Hoffmann-La Roche, Inc., Nutley, N.J.) were dissolved in sterile water. The supplemented mice consumed an average daily dose of 180 IU of vitamin A calculated on an average water consumption of 2 ml/day.

Reconstitution and immunization of SCID mice. huPBL obtained from leukopacks (discarded leukocytes from platelet donation) were fractionated on Ficoll-Hypaque gradients. Donors who had received tetanus toxoid immunization within 5 years prior to donation were selected. Vitamin A-deficient and control SCID mice were reconstituted at 6 weeks of age with 5×10^7 to 7×10^7 huPBL intraperitoneally. Mice were immunized intraperitoneally with 100 μg of tetanus toxoid (Massachusetts Public Health Laboratory, Jamaica Plain, Mass.) on the day of reconstitution and 18 days later. This schedule of reconstitution and immunization of mice was chosen on the basis of previous murine studies documenting low serum vitamin A concentrations in deficient mice without growth impairment until 11 weeks of age (41). For the vitamin A supplementation experiments, tetanus toxoid at a lower dose (10 μg per dose) was used. We chose a lower immunization dose for these experiments in order to conserve our supply of tetanus toxoid after documenting that the lower dose of 10 μg produced measurable antibody responses. Serum was obtained before and 10 days following the second immunization to assess immune response.

Assays. Vitamin A and vitamin E concentrations were determined simultaneously by high-pressure liquid chromatography on serum or liver homogenates (13). Vitamin A concentrations were assigned values in micrograms per deciliter, with 1 $\mu\text{g/dl} = 0.035 \mu\text{mol/liter}$. The lower limit of sensitivity of the vitamin A assay was 2 $\mu\text{g/dl}$ (0.07 $\mu\text{mol/liter}$), and values below detectable limits were

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TABLE 1. Mouse diet^a

Ingredient	Concn (% [wt/wt])
Sugar.....	50
Casein (vitamin-free).....	20
Corn starch.....	15
Corn oil ^b	5
Cellulose.....	5
AIN-76 mineral mixture.....	3.5
AIN-76 vitamin mixture ^c	1.0
DL-Methionine.....	0.3
Choline bitartrate.....	0.2

^a Modification of American Institute of Nutrition (AIN)-76A diet for rodents.

^b Breeder diet contained 11% corn oil and 44% sugar.

^c Control diet contained 4.02 IU of vitamin A per g in the form of retinyl acetate (1 IU of vitamin A = 0.3 µg of preformed retinol). The vitamin A-deficient diet contained no vitamin A.

assigned a value of 2 µg/dl. Human total immunoglobulin G (IgG), anti-tetanus toxoid IgG, and anti-diphtheria toxoid IgG antibody concentrations were determined by enzyme-linked immunosorbent assay (ELISA), as previously described (2, 39). Mouse IgG was measured by ELISA, using goat anti-mouse IgG capture antibody (Tago, Inc., Burlingame, Calif.) and goat anti-mouse IgG alkaline phosphatase conjugate (Tago).

Analysis. Data analysis was performed with the PROPHET system, a national computer system sponsored by the Chem/Biological Information Handling Program of the National Institutes of Health. Antibody concentrations were transformed to logarithms to normalize distributions for statistical analyses. Geometric mean antibody concentrations of total human IgG and antigen-specific human IgG of the control and vitamin A-deficient mice were compared by using the two-tailed *t* test or the Mann-Whitney rank sum test for nonparametric data.

RESULTS

Establishment of vitamin A deficiency in SCID mice. We first determined the effect of eliminating vitamin A from the diet of SCID mice at different ages. We compared initiating deficient diets for pregnant females at 7 to 10 days of gestation, at 15 days of gestation, and 9 days after birth. After weaning, the pups were fed deficient diets, and serum was collected to assess vitamin A status at 6 to 10 weeks of age.

Initiation of deficient diets 9 days after birth did not result in significantly lower serum vitamin A levels compared with controls at 6 weeks of age (data not shown). However, initiating the deficient diet at 15 days of gestation resulted in a pooled mean serum level of 9 µg/dl (*n* = 3) compared with 30 µg/dl (*n* = 3) in control mice at 8 weeks of age. Initiation of the deficient diet at 7 to 10 days of gestation resulted in pooled mean serum levels of 14 (*n* = 7), 4 (*n* = 3), and 2 (*n* = 11) µg/dl at 6, 8, and 10 weeks of age, respectively, compared with 23 (*n* = 7), 28 (*n* = 3), and 25 (*n* = 6) µg/dl for controls at the same time points.

To assess total body vitamin A status, we determined the concentration of vitamin A in the livers of five SCID mice deprived of vitamin A beginning at 7 to 10 days of gestation compared with five controls. Mice were sacrificed at 10 weeks of age, and their livers were surgically removed. The mean (± standard deviation) vitamin A content of liver homogenates for deficient mice was 0.53 (± 0.51) µg/g (wet weight) compared with 2.29 (± 0.56) µg/g (wet weight) for controls (*P* = 0.001).

Thus, mice fed deficient diets beginning at 7 to 10 days of gestation had reduced serum levels by 6 weeks of age and total body vitamin A deficiency documented by 10 weeks of age. We therefore chose this schedule to produce vitamin A-deficient SCID mice to be reconstituted with human cells.

Effect of vitamin A deficiency on tetanus toxoid response. We concurrently reconstituted vitamin A-deficient and control SCID mice with 5×10^7 to 7×10^7 huPBL at 6 weeks of age

in three separate experiments. Each mouse received huPBL from a single human donor, and in each of the three experiments, equal numbers of vitamin A-deficient and control mice were reconstituted with cells from the same human donor. All mice received tetanus toxoid immunization at the time of cell transfer since we confirmed the observation of others that immunization at the time of reconstitution was necessary to elicit reliable responses 2 weeks later (data not shown) (26). Mice were then immunized with tetanus toxoid on day 18 postreconstitution, and antibody responses were assessed 10 days later. Since the mice were reconstituted with huPBL from tetanus toxoid immune donors, secondary antibody responses were evaluated in the mice.

The geometric mean serum vitamin A levels were significantly lower at 6, 8, and 10 weeks of age for the mice fed deficient diets compared with controls (Table 2). At 6 weeks of age, the deficient mice had a geometric mean serum vitamin A level that was 36% that of controls (9 versus 25 µg/dl; *P* < 0.01). By the time of immunization at 8 weeks of age, the deficient group had a geometric mean serum vitamin A level that was 8% that of controls (2 versus 24 µg/dl; *P* < 0.05). Geometric mean serum concentrations of vitamin E, a nutrient provided in equal concentrations in deficient and control diets, were not significantly different between the two groups (Table 2). In addition, each mouse was weighed weekly as a measurement of overall nutritional status. At 10 weeks of age the mean weights (± standard deviations) were not significantly different between the deficient and control groups. The deficient mice had a mean weight of 23.0 (± 3.6) compared with 20.5 (± 2.9) g for controls (*P* = 0.093).

We then assessed the effect of vitamin A deficiency on the secondary human immune response to tetanus toxoid immunization. Following reconstitution and immunization at 6 weeks of age, the geometric mean IgG anti-tetanus toxoid antibody concentrations at 8 weeks of age prior to the second immunization were not significantly different for the two groups (Table 2). In contrast, at 10 weeks of age following the second immunization, the geometric mean human IgG anti-tetanus toxoid antibody concentration was significantly lower for the deficient group: 3.75 µg/ml compared with 148 µg/ml for controls (*P* = 0.0005). The geometric mean antibody fold increase for tetanus toxoid was 2.9 in the vitamin A-deficient group compared with 74 in the controls (*P* < 0.01) (Fig. 1).

To demonstrate that an active human antibody response to tetanus toxoid immunization occurred, we determined antibody concentrations to another protein antigen, diphtheria toxoid. Since the mice had not been immunized with diphtheria toxoid at the time of and following huPBL reconstitution, an active antibody response to this antigen would not be expected. The concentrations of human IgG anti-diphtheria toxoid antibody increased only slightly over time, with geometric mean antibody fold increases of 1.1 and 1.6 for the deficient and control groups, respectively (Fig. 2). The geometric mean human IgG anti-diphtheria toxoid antibody concentrations were not significantly different between the two groups either before or following tetanus toxoid immunization (Table 2). Thus, vitamin A deficiency impaired the active secondary response to tetanus toxoid immunization, whereas passive acquisition of antigen-specific antibody was not affected.

Effect of vitamin A deficiency on total IgG concentrations. We determined total human IgG concentrations to evaluate the effect of vitamin A deficiency on immune reconstitution. Both control and vitamin A-deficient mice had human IgG serum concentrations of between 0.1 and 10 mg/ml by 2 weeks following cell transfer (Fig. 3). The geometric mean total human IgG antibody concentrations were significantly lower at

TABLE 2. Serum vitamin A, vitamin E, and immunoglobulin concentrations in vitamin A-deficient and control SCID-huPBL mice^a

Mouse group tested	Geometric mean concn (mean $\pm$ SD)				
	Vitamin A (μ g/dl)	Vitamin E (μ g/dl)	Anti-tetanus toxoid IgG (μ g/dl)	Anti-diphtheria toxoid IgG (μ g/dl)	Total IgG (μ g/ml)
6 wk of age					
Deficient ($n = 11$)	8.48 (10.5 $\pm$ 5.3)	515 (541 $\pm$ 50)	ND ^b	ND	ND
Control ($n = 11$)	24.5 (24.9 $\pm$ 5.0)	511 (516 $\pm$ 76)	ND	ND	ND
<i>P</i> value ^c	<0.01	NS			
8 wk of age					
Deficient ($n = 11$)	2.00 (2.00 $\pm$ 0)	495 (500 $\pm$ 81)	1.33 (4.14 $\pm$ 6.55)	0.10 (0.11 $\pm$ 0.06)	1,100 (1,380 $\pm$ 848)
Control ($n = 11$)	23.7 (24.0 $\pm$ 3.7)	426 (428 $\pm$ 45)	2.06 (7.85 $\pm$ 11.7)	0.08 (0.09 $\pm$ 0.05)	1,950 (2,010 $\pm$ 505)
<i>P</i> value	<0.05	NS	NS	NS	0.043
10 wk of age					
Deficient ($n = 11$)	2.00 (2.00 $\pm$ 0)	408 (416 $\pm$ 90)	3.75 (29.7 $\pm$ 68)	0.11 (0.20 $\pm$ 0.35)	1,660 (2,190 $\pm$ 1,730)
Control ($n = 11$)	23.9 (24.2 $\pm$ 4.1)	328 (332 $\pm$ 56)	148 (415 $\pm$ 491)	0.13 (0.17 $\pm$ 0.16)	3,240 (3,660 $\pm$ 2,050)
<i>P</i> value	<0.01	NS	0.0005	NS	0.03

^a Mice were reconstituted with huPBL at 6 weeks of age and immunized with tetanus toxoid at 6 and 8 weeks of age, and human antibody responses were measured at 8 and 10 weeks of age.

^b ND, not done.

^c Normally distributed data were compared by two-tailed *t* test, and non-normally distributed data were compared by Mann-Whitney rank sum test. NS, not significant.

1,100 μ g/ml in the deficient group compared with 1,950 μ g/ml for controls at 8 weeks of age ($P = 0.043$) (Table 2). By 10 weeks of age, the deficient group had a geometric mean IgG of 1,660 μ g/ml compared with 3,240 μ g/ml for controls ($P = 0.03$). Though the concentrations of total human IgG were significantly lower for deficient mice at both time points, the geometric mean fold increases of 1.5 and 1.7 were similar for the two groups. We also assessed mouse IgG concentrations to determine if the SCID mice were "leaky," since some SCID mice can develop B- and T-cell function over time. Fifteen of the 22 mice were examined for murine IgG, and all had less than 10 μ g of murine IgG per ml in their sera.

Effect of vitamin A supplementation on tetanus toxoid response and total IgG concentrations. Vitamin A supplementation experiments were conducted to confirm that impaired

responses to tetanus toxoid immunization were solely due to vitamin A deficiency. We first determined the necessary dose and schedule of vitamin A supplementation to achieve normal serum vitamin A concentrations by the time of reconstitution. Forty-five international units of vitamin A per day, beginning 4 days prior to reconstitution, failed to restore serum levels to normal by the time of reconstitution. However, when 180 IU of vitamin A was provided daily to deficient mice for 2 weeks prior to reconstitution, geometric mean serum levels became equivalent to those of controls at 6 and 10 weeks of age (Table 3).

Antibody responses to tetanus toxoid immunization of mice fed deficient diets beginning early in gestation and then supplemented with vitamin A were compared with those of control mice (Table 3). Responses of supplemented mice were equivalent to those of controls, with a geometric mean human IgG

FIG. 1. Human anti-tetanus toxoid IgG antibody concentrations in vitamin A-deficient and control SCID-huPBL mice. Mice were immunized with tetanus toxoid at the time of reconstitution (6 weeks of age) and at 8 weeks of age. Antibody concentrations were measured at 8 and 10 weeks of age. Closed circles, vitamin A-deficient mice; open circles, control mice. Bars, geometric means $\pm$ standard errors of the mean.

FIG. 2. Human anti-diphtheria toxoid IgG antibody concentrations in vitamin A-deficient and control SCID-huPBL mice at 8 and 10 weeks of age. Closed circles, vitamin A-deficient mice; open circles, control mice. Bars, geometric means $\pm$ standard errors of the mean.

FIG. 3. Total human IgG concentrations in vitamin A-deficient and control SCID-huPBL mice at 8 and 10 weeks of age. Closed circles, vitamin A-deficient mice; open circles, control mice. Bars, geometric means $\pm$ standard errors of the mean.

anti-tetanus toxoid antibody concentration of 428 $\mu\text{g/ml}$ in the supplemented mice compared with 159 $\mu\text{g/ml}$ for the control mice ($P > 0.05$). Vitamin A supplementation of deficient mice also restored immune reconstitution, with the two groups having similar human IgG concentrations at 10 weeks of age (2,390 versus 2,880 $\mu\text{g/ml}$; $P > 0.05$).

DISCUSSION

We established a vitamin A-deficient SCID mouse model by eliminating vitamin A from the diet of gravid females. Mice deprived of vitamin A beginning at days 7 to 10 of gestation had geometric mean serum levels that were 36% those of controls at 6 weeks of age, declining to levels that were 8% those of controls by 8 and 10 weeks of age. Our results in SCID mice confirm the published observation that deprivation of vitamin A during gestation is required to produce vitamin A-deficient mice (41). We demonstrated that the low serum vitamin A levels reflected total body vitamin A depletion by documenting reduced vitamin A content in the livers of the deficient mice.

After establishing a nutritional deficiency in the SCID mice, we examined the effects of vitamin A deficiency on the human immune response. Secondary antibody responses were evalu-

ated following huPBL reconstitution from tetanus toxoid immune donors and immunization of SCID mice with tetanus toxoid. Deficient mice had a significantly lower antibody response to tetanus toxoid immunization, with only a 2.9-fold increase in their human IgG anti-tetanus toxoid concentration compared with a 74-fold increase in control mice following the second immunization. To confirm that this dramatic difference was due to the effect of vitamin A deficiency, supplementation experiments were performed. Vitamin A supplementation of deficient mice before huPBL reconstitution restored the human IgG anti-tetanus toxoid response to normal. In addition, vitamin A deficiency also affected immune reconstitution in the SCID mice, with significantly lower total human IgG concentrations at 8 and 10 weeks of age compared with controls. Vitamin A supplementation restored total human IgG concentrations to control levels. Thus, the reconstituted SCID-huPBL mouse model documented that total human IgG production and secondary antigen-specific human antibody responses are significantly diminished in vitamin A deficiency.

An association of vitamin A deficiency and impaired human immunity has been suggested by several clinical studies. An initial longitudinal observational study found a significantly higher mortality rate in children with vitamin A deficiency compared with nondeficient children (44). Subsequent community-based vitamin A supplementation trials in areas where vitamin A deficiency is endemic documented decreased childhood mortality in children supplemented with vitamin A versus nonsupplemented children (22, 36, 43, 46). Furthermore, a hospital-based randomized double-blind trial in South Africa documented reduced morbidity and mortality from complications of acute measles in children treated with vitamin A compared with nonsupplemented children (23). Significant increases in measles IgG antibody and total lymphocyte number were also found with vitamin A supplementation during measles infection (16). In addition, a recent prospective study suggests that maternal vitamin A deficiency contributes to higher rates of human immunodeficiency virus transmission from mother to child, possibly through decreased production of maternal antibody (37). The results of these clinical trials, though compelling, are potentially confounded by additional nutritional variables such as protein energy malnutrition or other micronutrient deficiencies.

Murine studies have documented impaired primary responses to protein antigens in vitamin A-deficient mice (14, 40, 41). Diminished primary and secondary antibody responses to

TABLE 3. Serum vitamin A and immunoglobulin concentrations in vitamin A-supplemented^a and control SCID-huPBL mice

Mouse group tested	Geometric mean concn (mean $\pm$ SD) ^b		
	Vitamin A ($\mu\text{g/dl}$)	Anti-tetanus toxoid IgG ($\mu\text{g/ml}$)	Total IgG ($\mu\text{g/ml}$)
6 wk of age			
Supplemented ($n = 6$)	31.3 (32.3 $\pm$ 9.9)	ND	ND
Controls ($n = 3$)	26.0 (26.0 $\pm$ 1.0)	ND	ND
<i>P</i> value	NS ^c		
10 wk of age			
Supplemented ($n = 6$)	26.3 (26.5 $\pm$ 3.9)	428 (484 $\pm$ 256)	2,390 (3,130 $\pm$ 1,680)
Controls ($n = 3$)	22.1 (23 $\pm$ 8.2)	159 (258 $\pm$ 198)	2,880 (3,700 $\pm$ 2,700)
<i>P</i> value	NS	NS	NS

^a Vitamin A-deficient mice were supplemented with vitamin A starting at 4 weeks of age and continuing through 10 weeks of age.

^b All mice were reconstituted with huPBL at 6 weeks of age and immunized at 6 and 8 weeks of age with tetanus toxoid, and human antibody responses were measured at 10 weeks of age. ND, not done.

^c NS, not significant.

tetanus toxoid immunization and impaired antibody responses to *Streptococcus pneumoniae* and *Neisseria meningitidis* capsular polysaccharides have also been shown in rats (24, 32–34).

In vitro studies of human lymphoblastoid B cells have shown decreased cell survival when cells were cultured in vitamin A-free or serum-free medium (8). Fresh serum (which contains vitamin A) or vitamin A-containing medium restored the growth of human B cells in culture. Distinct effects of four endogenous metabolites of vitamin A (retinol) on the growth of a human promyelocytic leukemia cell line, HL-60, have been delineated (18). In addition, murine experiments have demonstrated that T-cell activation and early proliferation require the presence of retinol or one of its metabolites (19).

Thus, human and murine in vitro data demonstrate that vitamin A has effects on both B and T cells. Direct effects of vitamin A deficiency on B- and/or T-cell proliferation may be responsible for the decreased anti-tetanus toxoid antibody production we have demonstrated in this study. Alternatively, the difference may have been due to the effect of vitamin A deficiency on the reconstitution of the SCID mice and not lymphocyte function. However, the magnitude of the effect of vitamin A deficiency on the active response to tetanus toxoid (40-fold) versus the effect on total IgG (2-fold) suggests that active responses were affected beyond the differences in reconstitution noted between the two groups. Other studies suggest a dysregulation of cytokine secretion in vitamin A deficiency. T helper (Th) cell subpopulations secrete distinct lymphokines which affect the growth and differentiation of B cells and regulate immunoglobulin isotype production (30, 35, 42). Excess Th1 and decreased Th2 responses have been described for vitamin A-deficient mice with overproduction of gamma interferon (IFN- γ) and reduced secretion of interleukins 2, 4, and 5 (9–12). Though the overproduction of IFN- γ seen in vitamin A-deficient mice may involve a dysregulation of Th cell subsets, another potential source of IFN- γ are natural killer (NK) cells. NK cells downregulate antibody response following immunization and inhibit polyclonally induced B-cell proliferation through IFN- γ secretion (1, 4). We have recently shown in the SCID-huPBL mouse model that human NK cells downregulate total human IgG2 immunoglobulin (3). We speculate that NK cells may also be involved in the impaired human IgG anti-tetanus toxoid and total immunoglobulin antibody production documented in vitamin A-deficient SCID-huPBL mice. Future studies will be conducted to address the mechanism of vitamin A deficiency on human immune responses. Specifically, the effect of vitamin A deficiency on NK activity could be initially evaluated in the SCID mouse model as SCID mice possess NK cells (17).

In summary, vitamin A deficiency impairs human antibody responses to tetanus toxoid immunization. We speculate that the overproduction of IFN- γ from either NK or T cells results in downregulation of antigen-specific antibody and total immunoglobulin production. Furthermore, we propose that effects of other nutritional deficiencies on the human immune system could be evaluated in the SCID-huPBL model.

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