Vitamin A Deficiency Increases the In Vivo Development of IL-10–Positive Th2 Cells and Decreases Development of Th1 Cells in Mice1,2

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ABSTRACT Vitamin A deficiency impairs both T helper type 1 (Th1) and type 2 (Th2)-mediated immune responses, although Th2 responses seem to be principally affected. Multiple mechanisms are involved in this immune suppression, but the hypothesis that deficiency affects development of Th1/Th2 memory cell phenotype has not been tested directly in vivo. To do so, lymphocytes from DO11.10 T cell receptor (TCR)-transgenic mice were transferred to vitamin A–deficient or control BALB/c recipients. Recipients were then immunized with the cognate peptide antigen for the TCR-transgenic DO11.10 T cells (OVA323–339). After 2–5 wk, the transferred OVA323–339–specific T cells were identified from draining lymph nodes with the TCR-clonotypic antibody KJ1–26, and their Th1/Th2 phenotype was characterized by intracellular cytokine staining after in vitro stimulation with phorbol myristate acetate and ionomycin. The percentage of CD4+ KJ1–26+ cells positive for interferon (IFN)-γ (8.8 ± 0.73%) and interleukin (IL)-2 (39.5 ± 3.1%) were both lower than the percentages in control mice (11.4 ± 0.67 and 47.0 ± 2.8%, respectively). Thus vitamin A deficiency, at the time of initial antigen exposure, enhances the development of IL-10–producing Th2 T regulatory cells and diminishes the development of Th1 memory cells.

KEY WORDS: • rodent • Th1/Th2 • immunomodulators • transgenic/knockout • vitamin A deficiency

Population-based studies showed that vitamin A supplements decrease the risk of death from infectious diseases (1), presumably by restoring innate and adaptive immune functions compromised by vitamin A deficiency (2). However, some community and clinical studies found that supplements may increase the severity of respiratory infections (3,4) or the risk of vertical transmission of HIV (5). The reasons for these disparities are unclear but highlight the need to understand the mechanism by which vitamin A affects immune function.

Mechanistic studies are difficult to pursue in human subjects, and rodent models have provided valuable insights into the effect of vitamin A deficiency on immune function. For example, vitamin A deficiency decreases T-lymphocyte–mediated antibody responses (6,7), a finding that was replicated in humans (8). These and other studies indicate that immune responses mediated by T-helper type 2 (Th2)4 cells, such as IgG1, IgE, and IgA antibody responses, are impaired by vitamin A deficiency, whereas some aspects of Th1-mediated responses may be enhanced. In particular, production of interferon (IFN)-γ (a Th1 cytokine) on a per cell basis is increased during vitamin A deficiency and can be directly decreased by treatment with retinoic acid, the principal active metabolite of vitamin A (9,10). Because IFN-γ can impede the development of Th2 responses, this may be one mechanism by which vitamin A deficiency decreases T-lymphocyte–mediated antibody responses.

Because Th2 responses are diminished by vitamin A deficiency, it is logical to ask whether the number of Th2 memory cells may be lower in vitamin A–deficient mice. The answer to this question has implications for long-term immunity. For example, if the number of Th2 cells is decreased, this could mean that the memory response would remain impaired even after correction of vitamin A deficiency. Two principal approaches have been taken to address this question. First, lymphocyte populations from vitamin A–deficient and control mice were analyzed for the presence of cells (presumably T lymphocytes) capable of providing help to IgG1-producing B lymphocytes. Such functional studies indicate that vitamin A


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4 Abbreviations used: APC, allophycocyanin; CFA, complete Freund's adjuvant; F, female; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFA, indirect immunofluorescence; IFA, immunofluorescence; IFN, interferon; IL, interleukin; M, male; OVA323–339, peptide comprised of amino acids 323 through 339 of ovalbumin; PE, phycoerythrin; lPEC, pericardial chlorophyll protein; PMA, phorbol myristate acetate; TCR, T cell receptor; T1, T helper type 1; T2, T helper type 2; Treg, T regulatory cell.

deficiency decreases the number of such cells, indirectly demon-
strating a reduction in Th2 numbers (11) (12). A limitation of such studies is that increased production of IFN-γ could also have
the same effect as decreased production of Th2 cytokines such as interleukin (IL)-4. Another study used an Elispot method to demonstrate that the number of IL-5 producing cells was decreased by vitamin A deficiency although the number of IFN-γ producing cells was not affected (10). Al-
though the type of Th1/Th2 cytokine was identified in this study, the specific identity of the cells was not established.

In the present study, we addressed the question whether vitamin A deficiency alters the development of Th1 and Th2 memory cells using an adoptive transfer model. Naive T lymphocytes from DO11.10 mice were transferred into vitamin A–deficient and control BALB/c mice. The DO11.10 TCR recognizes amino acids 323–339 of ovalbumin (OVA1323–319) and can be identified using the clonotypic monoclonal antibody KJ1–26 (14). After transfer, recipient mice were immunized with OVA1323–319 in incomplete Freund’s adjuvant (IFA) to stimulate development of memory T lymphocytes. The Th1/Th2 phenotype was determined 2–5 wk after immunization by intracellular cytokine staining. Vitamin A–deficient mice had a higher frequency of IL-10–positive Th2 memory [memory T regulatory (Treg)] cells and a lower frequency of IFN-γ- and IL-2–positive Th1 memory cells than did control mice. A similar difference in supernatant cytokine concentrations was found in splenocyte cultures re-
stimulated with OVA1323–319 peptide.

MATERIALS AND METHODS

Mice. DO11.10 mice were a gift from Casey Weaver, University of Alabama at Birmingham, and were bred in our facility. BALB/c mice were purchased from Charles River. This research was approved by the U.C. Davis Institutional Animal Care and Use Committee.

Diet protocol. Pregnant mice consumed a semipurified vitamin A–deficient diet (88407; Teklad Diets) ad libitum beginning on gestation d 14. The number of pups per litter was balanced within diet groups and so that no litter had >5 pups. Half of the litters in each experiment were then switched to the control diet containing 4000 IU vitamin A as retinyl palmitate (Teklad diet #88406). Litters were weaned at 21 d of age to the same diet fed to the dams. Serum retinol was measured at 7 wk of age in 4 mice per group to determine whether the mice fed the vitamin A–deficient diet had serum retinol that was <0.50 μmol/L and significantly lower than that of the control diet group. This protocol and the diet composition were described previ-
ously (15).

Two experiments with identical protocols were conducted. Intra-
cellular cytokine staining data were combined from the 2 experiments after statistical comparison showed that the results from these experiments were essentially identical. Lymph node and spleen cultures were performed in the second experiment for measurement of cyto-
kine concentrations by ELISA.

Adoptive transfer and immunization. After vitamin A defi-
ciency was confirmed using serum retinol concentration, mice in the 2 diet groups received 5 × 106 CD4+ KJ1–26+ cells by tail vein injection. Splenocytes and lymph node cells from DO11.10 mice were pelleted and analyzed by flow cytometry, as described below, to determine the number of total cells to be injected. The CD4+ KJ1– 
26+ cells were not separated before transfer. Cells were suspended in HBSS. The total injection volume was 0.15 mL.

Three days after transfer, OVA1323–319 peptide (Alpha Diagnostic International) was diluted in PBS and emulsified with incomplete Freund’s adjuvant (IFA; Sigma). The final injection volume was 0.3 mL/mouse and contained 300 μg peptide. Mice were injected s.c. in the left flank and footpad (50 μL per site) at the base of the tail (100 μL) and the back of the neck (100 μL).

Cell culture and intracellular cytokine staining. Two to 5 weeks after immunization, the spleen and 6 draining lymph nodes (brachial,
cells with a memory phenotype (CD62L−) was determined by a fluorescence-activated cell sorter. The cutoff level for CD62L− cells was determined on each day using the predominant CD62L− population from the CD4+KJ1−26− cells from the lymphocyte gate were then identified as double-positive cells (B, E). The percentages of such double-positive cells positive for IL-10, IL-2, IL-4, IL-10, and IFN-γ (F) were then enumerated.

Cell culture and cytokine ELISA. Lymph node and spleen cells were cultured at 2 × 10^6 cells/mL in Russ-10 media containing either 6 mg/L OVA_{323-339} or an equal volume of diluent. Cultures were then placed in wells of a 48-well (0.5 mL) plate (Nunc) and cultured at 37°C in 5% humidified CO₂ for 7 d. Individual wells were harvested at 2, 4, 5, and 7 d. Data from d 4 and 5 were analyzed together as a single day. Cells were pelleted by centrifugation at 200 × g at 4°C for 10 min. The supernatant was frozen for later analysis. IL-2, IL-4, IL-10, and IFN-γ were measured in cell culture supernatants by capture ELISA using antibodies, purified standards, and protocols suggested by the manufacturer (Pharmingen). The limit of detection of the 4 ELISAs was 0.03 μg/L.

Statistical analysis. Statistical analysis was performed with the SigmaStat program (Jandel Scientific). A two-tailed P-value of 0.05 was used to determine statistical significance unless otherwise indicated. Serum retinol concentrations were compared between diet groups by 2-way ANOVA to control for gender, and then by Student’s t-test because gender was not a significant factor. Body weights were measured weekly, and weights for males (M) and females (F) were compared separately between diet groups by 2-way repeated-measures ANOVA to control for changes in weight over time.

The percentage of CD4+KJ1−26− cells in lymph nodes, the percentage of cells producing cytokines, and the concentration of cytokines from lymph node cultures were compared by two-way ANOVA with mice categorized by time after immunization and gender. Gender was a significant factor for predicting the percentage of CD4+KJ1−26− cells in lymph nodes; thus, diet groups were compared separately for males and females by 2-way ANOVA controlling for time after immunization. Gender was not a significant predictor for cytokine-positive cells and cytokine concentrations in lymph node cultures. Thus, these variables were compared between diet groups using 2-way ANOVA to control for time after immunization. Cytokine concentrations from lymph node cultures were compared between diet groups only on days when an antigen-specific response occurred. When the OVA_{323-339}-treated culture had a significantly higher cytokine concentration (P < 0.05 by one-tailed t-test) than the unstimulated culture from the same mouse, the response was considered antigen specific.

Data from antigen-stimulated and unstimulated splenocyte cultures were available from 24 mice (deficient, n = 6 F, 6 M; control diet n = 6 F, 6 M) from the second experiment. Equal numbers of observations were available in each diet group on all culture days (d 2, 4/5 and 7) for OVA_{323-339}-stimulated as well as unstimulated cultures, for both males and females. Because of this balance, 3-way ANOVA could be used to compare cytokine concentrations among these groupings. The initial analysis simultaneously compared males vs. females, days in culture, and stimulated vs. unstimulated cultures. Cytokine concentrations did not differ between males and females; thus, the data from males and females were analyzed together to determine the effects of diet, days in culture, and antigen-specific stimulation.

Backwards, stepwise regression analysis was used to identify significant predictors of the percentage of cytokine-producing CD4+KJ1−26− cells in lymph nodes using the categorical variables “gender” (female = 1, male = 0), “vitamin A deficiency” (control diet = 0, deficient diet = 1) and “experiment” (first experiment = 1, second experiment = 2), as well as the continuous variable “days postimmunization.”

Results of 2- and 3-way ANOVAs are presented as means ± SE. Summary statistics and the results of t-tests and l-way ANOVA are presented in the text and figures as means ± SD.

RESULTS

Confirmation of vitamin A deficiency using serum retinol. Serum retinol was measured at 7 wk of age (n = 2 M and 2 F/diet group in each experiment). In both experiments, serum retinol was higher in control mice (1.15 ± 0.49 and 0.91 ± 0.37 μmol/L) than in mice fed the vitamin A–deficient diet (0.36 ± 0.11 μmol/L, P = 0.020; 0.30 ± 0.071 μmol/L, P = 0.018, respectively).

Body weights of vitamin A–deficient and control mice. Body weights were compared by diet for the 45 mice in the 2 experiments (including 5 unimmunized controls). In the first experiment (deficient diet; n = 4 M, 6 F; control diet; n = 3 M, 5 F), body weights were compared at 8, 9, 10 and 11 wk of age and mice were killed for analysis beginning at 11 wk of age. Males did not differ (P = 0.41) on the basis of diet, but there was an interaction of diet with age (P = 0.011) reflecting the fact that male mice fed the deficient diet began the study slightly heavier than control mice (mean of 24.3 vs. 23.4 g) but ended the study slightly lighter. Body weights did not differ in females due to diet (P = 0.68). In the second adoptive transfer study (deficient diet; n = 6 M, 8 F; control diet; n = 7
M, 6 F) body weights were compared at 5, 7, 8, 9, and 10 wk of age. Mice were killed beginning at 10 wk of age for analysis. Males did not differ (P = 0.81) on the basis of diet, but there was an interaction of diet with age (P = 0.013), again reflecting the fact that male mice fed the deficient diet began the study slightly heavier than control mice (mean of 22.2 vs. 20.7 g) but ended the study slightly lighter (24.6 vs. 25.5 g). Body weights did not differ in female mice due to diet (P = 0.062), although the deficient females were slightly heavier than the control mice at all time points.

Percentages of CD4+KJ1–26+ donor cells in draining lymph nodes. After adoptive transfer, CD4+KJ1–26+ cells accounted for 3.2 ± 0.8% of total CD4+ cells in the lymph nodes of unimmunized mice fed the vitamin A–deficient diet (n = 2 M, 3 F). After immunization, the percentage of CD4+KJ1–26+ cells decreased from 3.2 ± 1.2% (n = 9 F, 9 M) during wk 3 to 1.9 ± 1.0% (n = 6 F, 6 M) during wk 4 and remained stable at 1.6 ± 0.8% during wk 5 (n = 7 F, 3 M) (P = 0.002, wk 3 vs. 5; P = 0.015, wk 3 vs. 4). The percentages did not differ on the basis of diet, but male recipients had higher percentages of CD4+KJ1–26+ cells than females at all time points: 3.5 vs. 2.5% in wk 3, 2.3 vs. 1.4% in wk 4 and 1.9 vs. 1.3% in wk 5 (P = 0.019 for gender comparison by 2-way ANOVA, comparing weeks and gender). Staining with anti-CD62L demonstrated that the mean percentage of CD4+. KJ1–26+ cells with a memory phenotype (CD62Lhi) was 73.5 ± 5.4% after immunization (n = 11 mice).

Intracellular cytokine staining of CD4+KJ1–26+ donor cells from draining lymph nodes. The mean percentages of cytokine-positive cells in unimmunized mice (n = 2 M, 3 F; vitamin A–deficient diet) were as follows: IL-4, 0.27 ± 0.40%; IFN-γ, 0.81 ± 0.32%; IL-10, 0.20 ± 0.30%; and IL-2, 22.9 ± 7.4%.

Vitamin A deficiency increased the percentage of memory T lymphocytes positive for the Th2 cytokine IL-10 (P = 0.003) (Fig. 2A). The overall percentage of IL-10 positive cells was 100% higher in vitamin A–deficient mice (3.49 ± 0.41%) than in control mice (1.74 ± 0.37%). This difference occurred during wk 3 (90% higher), wk 4 (170%), and wk 5 (70%) after immunization. This positive association of the percentage IL-10–positive cells with vitamin A deficiency was also present in the multiple regression analysis (Table 1). The overall percentage of IL-4–positive cells was quite low throughout the experiment, and the diet groups did not differ (P = 0.27; Fig. 2B, Table 1).

Vitamin A deficiency decreased the percentage of memory T lymphocytes positive for the Th1 cytokines IL-2 and IFN-γ. The overall percentage of IL-2–positive cells in the vitamin A–deficient mice (39.5 ± 3.1%) tended to be lower (16%) than in control mice (47.0 ± 2.8%; Fig. 2C) (P = 0.081). However, a significant, negative association was found between vitamin A deficiency and the percentage of IL-2–positive cells when multiple regression analysis was used to control for variation due to experiment (1st vs. 2nd experiment) and time after immunization (Table 1). The overall percentage of IFN-γ–positive cells in the vitamin A–deficient mice (8.8 ± 0.73%) was 23% lower than in control mice (11.4 ± 0.67%; P = 0.013) (Fig. 2D). This negative association of vitamin A deficiency with the percentage of IFN-γ–positive cells was also seen by multiple regression analysis (Table 1).

Cytokine concentrations after antigenic stimulation of draining lymph node cells. Draining lymph node cells not needed for flow cytometric analysis were used to assess antigen-specific cytokine production by ELISA (n = 10 control and 8 deficient mice). Unstimulated control cultures were included when cells were available (n = 5). An antigen-specific response did not occur for IL-4 but was present for IL-2 on d 2 and for IFN-γ and IL-10 on both d 4/5 and 7. Cytokine concentrations did not differ between the vitamin A–deficient and control groups (data not shown).

Cytokine concentrations after antigenic stimulation of spleen cells. Concentrations of the Th2 cytokines IL-4 and IL-10 were both higher in antigen-stimulated splenocyte cultures from vitamin A–deficient mice than in cultures from control mice, whereas concentrations of the Th1 cytokines IL-2 and IFN-γ were both lower (Fig. 3). Although IL-10 concentrations did not differ by diet group when all days were compared (P = 0.41), within-day comparisons showed that the IL-10 concentration on d 4/5 was 19% higher in cultures from deficient mice than in cultures from control mice (P = 0.043) (Fig. 3A). The IL-4 concentration on d 2 was higher in cultures from the deficient mice than from the control mice (P < 0.001) (Fig. 3B) but subsequent days did not differ. On both d 2 (P = 0.01) and d 4/5 (P < 0.001), IL-2 concentrations were lower in cultures from vitamin A–deficient mice than in those from control mice (Fig. 3C), whereas IFN-γ concentrations were lower in cultures from deficient mice than from control mice (P < 0.001) on all days (Fig. 3D).

DISCUSSION

The purpose of this study was to determine whether vitamin A deficiency at the time of immunization affects the development of Th1 and Th2 memory cells. The DO11.10 adoptive-transfer model was used, in which naïve OVA323–339–specific T lymphocytes are transferred to recipient mice in numbers sufficient to allow monitoring of T-lymphocyte de-
development, but not at levels high enough to produce an “unusual” abundance of responding cells in lymph nodes draining the site of OVA_{323-339} immunization (18–20). This model allows the direct enumeration of responding Th1 and Th2 cells using the clonotypic KJ1–26 monoclonal antibody and intracellular cytokine staining. IFA was used so as to not to strongly bias the cytokine response in a Th1 or Th2 direction. IFA induces memory T cells that produce both Th1 and Th2 cytokines, although markedly less IFN-γ than complete Freund’s adjuvant (CFA), which contains mycobacterial cell wall components (21–23). However, some studies suggested that IFA produces a Th2 bias (i.e., inducing Th2 cytokines rather than simply failing to induce IFN-γ). For example, when IFA and CFA were compared using intraperitoneal immunization with multiple antigens in different strains of mice, IFA typically induced IL-5 but little IFN-γ, whereas CFA had the opposite effect (24). Interestingly, the use of the OVA_{323-339} peptide in BALB/c mice produced a balanced IL-5/IFN-γ response in that study. Thus, it is likely that the immunization protocol used in this study did not strongly favor either a Th1 or Th2 response.

To minimize the effect of antigen-presenting cells and bystander cells on the assessment of Th1/Th2 phenotype after immunization, we chose to stimulate T-cell cytokine production directly using PMA and ionomycin. This approach was taken because vitamin A has multiple effects on antigen-presenting cell development and function. For example, vitamin A deficiency can increase IL-12 production by antigen-presenting cells and IFN-γ production by T cells (10). On the other hand, retinoic acid treatment during in vitro development of dendritic cells from peripheral blood monocytes can also result in the development of IL-12–producing antigen-presenting cells that promote Th1 development (25). Using PMA and ionomycin to stimulate cytokine production directly bypassed these potentially confounding effects on assessment of Th1/Th2 phenotype. Many stimuli could have been used to stimulate T-cell cytokine production directly. We evaluated anti-CD3 plus anti-CD28 antibodies in addition to PMA plus ionomycin and chose the latter because anti-CD3/anti-CD28 stimulation interfered with identification of CD4^{+}KJ1–26^{+} cells by flow cytometry, presumably due to internalization of the TCR complex after antibody binding.

Immunization with the OVA_{323-339} peptide transformed naïve DO11.10 T lymphocytes into memory cells, as shown by CD62L staining and Th1/Th2 cytokine profiles. We chose to immunize with the peptide not only to provide a potent antigenic stimulus for the adoptively transferred transgenic T lymphocytes, but also to minimize the response by host lymphocytes to other epitopes that might occur had we used intact ovalbumin protein as the antigen. The percentage of CD4^{+}KJ1–26^{+} cells expressing Th1/Th2 cytokines from unimmunized mice after adoptive transfer was very low and increased after immunization, as expected with the development of a memory cell phenotype. The percentage of cells positive for the Th1 cytokine IFN-γ was ~10\%, although the percentage of IL-2–positive cells was much higher. Higher percentages of IFN-γ–positive cells (20–40\% of CD4^{+}KJ1–26^{+} cells) occurred in our laboratory after 2 immunizations using CFA followed by IFA, or using alum, or after in vitro stimulation with IL-12 treatment (60\%), but the lower percentage response seen here was reproducible and appears to represent a robust memory response to a single immunization. Previous work comparing ovalbumin protein to OVA_{323-339} immunization in BALB/c mice showed similar T-cell cytokine recall responses (24), supporting our conclusion that a peptide immunization produced a recall response similar to that induced by intact protein. The percentage of cells positive for the Th2 cytokine IL-4 was quite low throughout the study. Similar percentages were found using 2 immunizations, or after oral immunization with cholera toxin as an adjuvant (data not shown).

**TABLE 1**

Association of vitamin A deficiency with the percentage of cytokine-producing CD4^{+}-KJ1-26^{+} T lymphocytes isolated from draining lymph nodes of OVA_{323-339}-immunized mice as assessed by backward, stepwise multiple regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL-4 R^2 = 0.456</th>
<th>IFN-γ R^2 = 0.238</th>
<th>IL-2 R^2 = 0.871</th>
<th>IL-10 R^2 = 0.423</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A deficiency</td>
<td>β</td>
<td>P</td>
<td>β</td>
<td>P</td>
</tr>
<tr>
<td>Experiment</td>
<td>-0.135</td>
<td>0.034</td>
<td>-0.179</td>
<td>0.005</td>
</tr>
<tr>
<td>Days postimmunization</td>
<td>0.369</td>
<td>0.014</td>
<td>0.600</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1 Correlation coefficients (R^2), standardized regression coefficients (β), and P-values (P) are shown for equations predicting the percentage of cytokine-positive cells.

**FIGURE 3** The effect of vitamin A deficiency on the concentration of the Th2 cytokines IL-10 (A) and IL-4 (B) and the Th1 cytokines IL-2 (C) and IFN-γ (D) in supernatants from splenocyte cultures from vitamin A–deficient and control mice following adoptive transfer of lymphocytes from DO11.10 mice and immunization with OVA_{323-339} peptide. Cells were restimulated with OVA_{323-339} peptide (OVA) or were treated with a vehicle control (no OVA). Diet treatments were significantly different for all cytokines on the days indicated by asterisks (*). Values are means ± SE, n = 12 deficient and 12 control.
shown), although never as high as that after in vitro stimulation with IL-4 (30%). The percentage of IL-10–positive cells increased from wk 3 to 5 after immunization, as might be expected for a regulatory cytokine that can dampen T-cell–mediated immune responses (26).

The mice consuming the vitamin A–deficient diet in this study presented biochemical evidence of deficiency by the time of immunization, as indicated by low serum retinol levels, but they were not so deficient that body weight was affected. Although the rate of weight gain in vitamin A–deficient males was lower than in males fed the control diet, the potential confounding effect of inanition on immune function was avoided in this study because significant weight differences were not present at the time of immunization.

A principal finding of this study was that vitamin A deficiency doubles the frequency of antigen-specific, IL-10–producing CD4+ T cells. The magnitude of the difference between the diet groups increased over time and may represent a relative increase in abundance of T cells, Th2 or Treg, capable of downregulating immune responses (26). This is a novel observation and may represent a mechanism by which vitamin A deficiency could diminish the immune response to infectious or inflammatory diseases. This could include Th2-mediated responses, which are diminished by vitamin A deficiency (2), as well as Th1-mediated immune responses, which have received less attention, such as cytotoxic T-lymphocyte and delayed-type hypersensitivity responses (27–29).

Research on regulatory T cells is in its infancy in many regards, and even the identification of which T cells are “regulatory T cells,” i.e., induced vs. naturally occurring Treg cells, Tr1 cells, or Th3 cells, is still a point of contention (26,30,31). Thus, it is not clear whether the IL-10–positive cells identified in this study fall into this category of regulatory T cells. Clearly, some CD4+ T-cell types with regulatory activity act by producing IL-10 that downregulates immune responses. Thus, it will be of interest to determine whether the IL-10–positive cells identified here do indeed play a regulatory role.

How vitamin A deficiency affects the development of IL-10–producing T cells is not clear. The development of Treg cells, which produce IL-10 and little IL-2 (as seen in the present study), is facilitated by an early exposure to IL-10. However, previous work demonstrated lower antigen-specific IL-10 production in immunized, vitamin A–deficient mice (10). In addition, work from this laboratory showed no effect of retinoic acid treatment on IL-10 production during development of DO11.10 memory T cells cultured in vitro (16). Further characterization of these cells is required to clarify their role in immune suppression caused by vitamin A deficiency.

Vitamin A–deficient mice had 23% fewer IFN-γ–positive and 16% fewer IL-2–positive cells in draining lymph nodes than did mice consuming the control diet with adequate vitamin A. These differences indicate that vitamin A deficiency decreased development of Th1 memory cells, although the magnitude of the decrease was not large. Previous work using an Elispot method to enumerate IFN-γ–positive cells in vitamin A–deficient and control mice after immunization showed no difference between the diet groups, but the variability of the Elispot method (based on reported SE from that study) was such that a 20% difference would not have been detected (10). In vitro studies from our laboratory (16) with naïve DO11.10 T cells indicated increased Th2 development with 9-cis but not all-trans retinoic acid, although other groups reported increased Th2 development with all-trans retinoic acid using similar methods (32,33). The present findings suggest that in vitro methods may not adequately recapitulate the in vivo effects of vitamin A deficiency on Th1/Th2 development. For example, direct addition of retinoic acid to cell culture may by-pass the normal regulation of retinoic acid production that exists in vivo (34), thereby producing a temporary excess in the in vitro studies. Thus, in vitro treatment with retinoids may be more representative of a condition of vitamin A excess vs. deficiency, rather than adequacy vs. deficiency. This question could be addressed by assessing the effect of high-level dietary vitamin A on Th1/Th2 development using the DO11.10 adoptive-transfer model.

When cultures from draining lymph nodes were restimulated with peptide antigen, IFN-γ and IL-2 concentrations did not differ, even though the frequencies of IFN-γ–producing CD4+KJ1–26+ cells differed between the diet groups. However, the IL-2 concentration in the deficient cultures at 2 d was 21% lower than in the control cultures. This value is similar to the difference seen with intracellular cytokine staining; however, given the SD in these ELISA data, a much larger sample size (n = 64) would be required to identify a 20% difference as significant (assuming power = 0.80 and P-value = 0.05 for Student’s t test). A similarly large sample size would be required to detect a 23% difference in IFN-γ concentrations. Cytokines produced by non-CD4+KJ1–26+ cells would also be detected by ELISA, but not by flow cytometry, and may also be a factor in explaining the different findings with the 2 methods.

Splenocyte cultures from vitamin A–deficient mice in the present study had lower antigen-specific IFN-γ and IL-2 concentrations and higher IL-4 and IL-10 concentrations than did cultures from control mice. When data from all 3 postimmunization days were considered, the IFN-γ and IL-2 concentrations in cultures from the deficient mice were 41 and 60% of the levels in the control cultures, whereas the concentration of IL-4 was 181% of control levels (using data from d 2). These deficits for IFN-γ and IL-2 are greater than the 20% lower frequency of IFN-γ– and IL-2–positive CD4+KJ1–26+ cells in lymph nodes from deficient mice, but the direction of the difference is the same. It is possible that the differences in splenocyte cultures reflect even greater differences in frequencies of cytokine-producing CD4+KJ1–26+ cells than in draining lymph nodes. Higher IL-10 concentrations occurred in splenocyte cultures from deficient mice on 1 of 3 d in culture, which is consistent with the higher frequency of IL-10–producing CD4+KJ1–26+ cells in lymph nodes from deficient mice, although the magnitude of the difference is smaller.

Earlier studies showed that vitamin A deficiency increases antigen-specific production of IFN-γ in antigen-restimulated lymphocyte cultures on an absolute and per cell basis (10,35). The higher rate of IFN-γ was diminished by the addition of exogenous vitamin A. In contrast, in the present study, vitamin A deficiency decreased the production of IFN-γ in antigen-restimulated splenocyte cultures. One possible explanation for this phenomenon is that the use of 10% FBS in the culture media in the present study produced a less Th1–biased environment in vitro than in previous studies using serum-free medium, due to the presence of vitamin A or other nutrients or hormones in FBS that support lymphocyte and antigen-presenting cell function. Differences between the DO11.10 adoptive transfer system and previous methods may also be involved. For example, previous studies immunized with intact proteins or infectious agents, whereas the present study used a peptide. Because intact proteins require processing by antigen-presenting cells before presentation by major histocompatibility complex molecules, and these processes are affected by vitamin A (36), initial antigen presentation to naïve T lymphocytes could have been less affected by vitamin A deficiency in the present study.
study than in previous studies. In addition, the present study used IFA as the adjuvant, whereas previous studies have used adjuvants such as CFA, which has greater Th1-inducing activity (23). Use of such an adjuvant may interact with vitamin A deficiency to affect Th1/Th2 development.

In summary, our data demonstrate that vitamin A deficiency at the time of initial antigen exposure significantly enhanced the development of IL-10–producing Th2 or Treg cells while diminishing the development of Th1 memory cells. This observation adds to our knowledge of how vitamin A deficiency affects Th1/Th2-mediated immune responses and suggests a novel mechanism by which vitamin A deficiency may diminish such responses. Such decreases in immune function may contribute to the increased risk of death from infectious diseases that is a hallmark of human vitamin A deficiency.

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LITERATURE CITED


