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Vitamin A deficiency alters splenic dendritic cell subsets and increases CD8⁺Gr-1⁺ memory T lymphocytes in C57BL/6J mice¹

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Abstract

Vitamin A-deficient populations have impaired T cell-dependent antibody responses. Dendritic cells (DCs) are the most proficient antigen presenting cells to naïve T cells. In the mouse, CD11b⁺ myeloid DCs stimulate T helper (Th) 2 antibody immune responses, while CD8α⁺ lymphoid DCs stimulate Th1 cell-mediated immune responses. Therefore, we hypothesized that vitamin A-deficient animals would have decreased numbers of myeloid DCs and unaffected numbers of lymphoid DCs. We performed dietary depletion of vitamin A in C57BL/6J male and female mice and used multicolor flow cytometry to quantify immune cell populations of the spleen, with particular focus on DC subpopulations. We show that vitamin A-depleted animals have increased polymorphonuclear neutrophils, lymphoid DCs, and memory CD8⁺ T cells and decreased CD4⁺ T lymphocytes. Therefore, vitamin A deficiency alters splenic DC subpopulations, which may contribute to skewed immune responses of vitamin A-deficient populations.

Keywords

Vitamin A; dendritic cell; antigen-presenting cell; flow cytometry

Introduction

Vitamin A has numerous roles in the immune system. Populations deficient in vitamin A have impaired innate immunity, including loss of mucosal barrier integrity and decreased numbers and/or function of innate immune phagocytic and natural killer cells [1]. The adaptive immune response is also altered by vitamin A deficiency. Lymphocyte homing, immunoglobulin A (IgA) responses in the mucosa, and T-dependent antibody responses are decreased in vitamin A-deficient individuals [1–6]. Vitamin A is also required for the balance of T regulatory (Treg) and T helper (Th) 17 cells in mucosal tissues [7]. Therefore, vitamin A is required in the maintenance of the immune system to adequately respond to danger signals and maintain tolerance to self.

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Dendritic cells (DCs) are the most proficient stimulators of naïve T lymphocytes and link the innate and adaptive arms of the immune system [8,9]. DCs derive from hematopoietic progenitors and reside in peripheral tissues and circulate in blood in an immature state. DC half-lives range from 3 to 9 days [10]. Multiple DC subsets have been distinguished by differential surface protein expression, and these subsets preferentially stimulate varying adaptive immune functions. In the mouse, CD11b⁺ myeloid DCs stimulate Th2 responses, while CD8α⁺ lymphoid DCs stimulate Th1 responses [11,12]. Plasmacytoid DCs are mature DCs that secrete type 1 interferons to augment anti-viral immune responses [13–15]. All mature mouse DC populations appear to arise from one common precursor DC population [16].

Vitamin A plays a role in maintaining DCs, but the reports have been limited primarily to *in vitro* studies. Hengesbach and Hoag (2004) have shown that bone marrow precursors stimulated with GM-CSF differentiate into DCs in the presence of medium containing vitamin A. However, if the medium was depleted of vitamin A or contained a retinoic acid receptor antagonist, the precursors generated increased numbers of neutrophils [17]. *In vivo*, vitamin A-deficient SENCAR mice had increased neutrophils and decreased lymphocyte populations compared to vitamin A-sufficient controls [18]. However, the authors did not characterize the DC populations of these animals. DCs are hematopoietic-derived cells, but also reside and proliferate in secondary lymphoid tissues [19]. In addition, DCs are motile cells traveling between sites of infection and secondary lymphoid tissues. The chemokine receptor for DCs to migrate to intestinal tissues is depressed in vitamin A deficiency [20]. The expression of matrix metalloproteinases (MMPs) are also skewed in DCs of vitamin A deficient origin [21, 22]. The combination of depression of homing receptors and matrix degrading enzymes leads to impaired trafficking of DCs to stimulate immune responses. Therefore, in specific-pathogen free vitamin A-deficient animals, the DC populations may be skewed, even in the absence of an infectious challenge.

Since vitamin A deficiency is known to impair Th2 responses and myeloid DCs stimulate the Th2 response, we hypothesized that vitamin A-deficient (VAD) animals would have decreased myeloid DCs compared to vitamin A-sufficient (VAS) control animals. To address this hypothesis, we used multicolor flow cytometry to quantify the DC populations in the spleen of C57BL/6J mice and compared these populations in animals that were depleted of vitamin A or were vitamin A-sufficient. The combination of surface markers used led to a comprehensive overview of the effect of vitamin A depletion on many cells of the immune system over various degrees of vitamin A deficiency in both male and female mice.

Materials and Methods

Animals

Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Vitamin A-deficient mice were obtained following an established protocol [23]. Briefly, breeder pairs were allowed to mate and upon indication of pregnancy the female was placed on a vitamin A-deficient AIN-93G diet. The pups were weaned into one of four groups based on diet and gender. Weaned mice were fed *ad libitum* pelletized AIN-93M diets free of vitamin A, or a control diet containing 8 mg retinyl-palmitate/Kg diet. The control diet had added red dye to aid in preventing accidental feeding of incorrect diet. All diets were purchased from Dyets, Inc. (New Bethlehem, PA) and stored frozen in vacuum-sealed bags to maintain freshness. Mice were weighed once per week starting at 3 weeks of age to monitor general animal health. At 6 weeks of age, and every 2 weeks after, serum was obtained from saphenous vein punctures and analyzed for serum retinol. Mice were sacrificed via carbon dioxide asphyxiation at 8, 10, and 12 weeks of age and spleens, livers, and sera were collected.

All animal procedures were performed in accordance with protocols approved by the Michigan State University Animal Care and Use Committee.

Vitamin A analysis

Vitamin A status was assessed using an adapted high performance liquid chromatography (HPLC) protocol [24]. A 75% acetonitrile, 25% water mobile phase was used over a ten minute run time through a Prism RP column (Part #32103-153030) purchased from Thermo Electron Corp (Waltham, MA). A 1 μ M retinyl-acetate (Sigma, St. Louis, MO) internal standard was used to assess retinol concentrations. Retinol was quantified in serum and following saponification of liver retinyl esters. The retinol was extracted into hexane, dried under argon stream, and redissolved in acetonitrile. Liver retinol is expressed as nanomole (nMole) retinol activity equivalents (RAE) per gram of liver.

Tissue Processing

Spleens were processed for flow cytometric analysis as previously described [25]. Briefly, tissues were harvested, weighed and placed in ice cold calcium and magnesium free Hank's Balanced Salts Solution (Lonza, Basel, Switzerland). Tissues were digested into single cell suspensions using enzymatic dissociation medium from Stem Cell Technologies (Vancouver, BC, Canada) per manufacturer's instructions. The digest was filtered, red blood cells lysed, and cell suspensions resuspended in FACS Buffer [0.1% sodium azide (Fisher Scientific, Pittsburgh, PA), 1% fetal bovine serum (Hyclone, Logan, UT), in Dulbecco's phosphate buffered saline, pH 7.4–7.6, sterile filtered].

Flow Cytometry

Spleen cell suspensions were blocked with 5 μ g of anti-FcR γ II/III antibody (2.4G2 hybridoma) on ice for 10 minutes and then incubated with two separate cocktails of monoclonal-antibody (mAb) fluorochrome conjugate reagents. A cocktail of 6 monoclonal antibodies (mAb) was used for DC analysis, as previously described by Duriancik and Hoag [25], and a 3 mAb cocktail was utilized for T lymphocyte analysis [hamster anti-mouse CD3 ϵ -PE (clone 500A2), rat anti-mouse CD4-APC (clone RM4-5), and rat anti-mouse CD8 α -PE-Cy7 (clone 53-6.7)]. All mAb-fluorochrome conjugates were purchased from BD Biosciences (San Jose, CA). Flow cytometry data was collected on a BD Biosciences LSR II flow cytometer and analyzed with FCS Express (version 3) software to identify DC subpopulations [25] and other immune cell populations.

Statistical Analysis

Statistical analysis was performed using SPSS statistics 17. All figures were designed using Prism (GraphPad Software). Three factorial (age, gender, diet) analysis of variance (ANOVA) was performed on spleen and liver weights, liver RAE data, and total spleen cellularity with Bonferroni post-tests. Body weight and serum retinol were analyzed using repeated measures ANOVA at 12 weeks only due to missing data at 8 and 10 week time points. Multiple linear regression analysis was performed to determine the effects of vitamin A (nMole RAE/g liver) depletion on each spleen cell population.

Results

Mouse Characteristics

The VAD altered the growth kinetics of the mice (Figure 1). Male and female mice gained weight through 11 weeks of age and maintained weight from 11 to 12 weeks. There was a significant effect of diet from 8 weeks through 11 weeks of age. Gender had a significant effect on weight from 3 weeks through 9 weeks, with an exception from 7 to 8 weeks of age. The

weight, diet, and gender interaction was significant starting at 9 weeks of age and continued through 12 weeks of age. Diet and gender both significantly affected the weight of the animals, with VAD animal weight significantly lower than VAS animal weight, and as expected, female animal weight was significantly lower than male animal weight.

As expected, livers of male mice weighed significantly more than livers of female mice due to body size, but diet and age had no effect on liver weight of the mice (Table I). Serum retinol significantly differed from previous measurement within a group only from 6 to 8 weeks of age (Figure 2A), indicating that release of vitamin A from liver stores could no longer adequately supply retinol to the circulation past 8 weeks of age. Diet, gender, and the diet-gender interaction significantly affected serum retinol. Male animals on control vitamin A-sufficient diet had higher serum retinol values than females, but lower liver RAE. Despite other factors, diet, age, and gender each significantly affected liver RAE (Figure 2B). As expected, the interaction of age and diet on liver RAE was significant, indicating that time consuming the vitamin A-deficient diet affected liver stores. However, surprisingly, the interaction of diet and gender was significant indicating that consuming a VAD diet affects male and female mice differently.

Liver retinyl esters are mobilized to maintain consistent serum retinol levels. However, serum retinol was severely depleted by 8 weeks of age and was not further depleted with extended intake of vitamin A-deficient diet (Fig 2B). Therefore, the most accurate assessment of vitamin A status was the analysis of liver stores of retinyl esters.

Spleens of VAD animals weighed significantly more than spleens from VAS animals, regardless of age or gender (Table II). There was a diet-independent significant interaction of age and gender for spleen weight, because as males aged the spleen weight decreased, but as females aged the spleen weight increased. The total cellularity of the spleen was unaffected by vitamin A depletion (Figure 3). The age and gender of the animal significantly affected the total spleen cell counts. In addition, the interaction of age and diet and age and gender significantly affected the cell counts.

Multiple linear regression analyses were performed on each cell population identified in the spleen (Figures 4, 5, and 6). Multiple linear regression was performed so that changes in immune parameters could be attributed to liver RAE, and the effects of confounding variables, age and gender, could be removed. The total spleen cells were not different between VAD and VAS groups; therefore any difference in percent cell populations would also be reflected in cell population total numbers. Thus, only percent of each cell type is shown. The liver RAE was log transformed and the logarithmic regression line was interpolated. Vitamin A status was defined as log liver RAE > 2.4 as VAS, 1.7–2.4 as marginal VAD, and < 1.7 as severe VAD. The table insets indicate the β coefficients and their p values as well as the R^2 value and the ANOVA significance of the model which was adjusted for age and gender variables. Significant and strong associations were defined as β coefficients and R^2 values greater than 0.4 and p-values less than 0.05.

Myeloid DCs were only slightly decreased by VAD. Even though the model and the β coefficient were highly significant (both $p=0.00$), the β coefficient for liver RAE was small at 0.32 (Figure 4A). Therefore, liver RAE was not a strong predictor of myeloid DC percentage in the spleen. Lymphoid DCs significantly increased as severity of VAD increased (Figure 4B; $p=0.00$). The β coefficient for liver RAE and lymphoid DCs was -0.63 . Therefore, liver RAE was a strong predictor of spleen lymphoid DC percent, and severely VAD animals had an approximate 50% increase in lymphoid DC compared to VAS animals. Since lymphoid and myeloid DCs preferentially stimulate Th1 and Th2 responses, respectively, we also assessed the lymphoid to myeloid DC ratio, as this would be assumed to alter the Th1/Th2 bias in

immune responses. The ratio of lymphoid DCs to myeloid DC significantly increased as severity of VAD increased with a β coefficient of -0.68 (Figure 4C). Liver RAE was a strong predictor of the lymphoid to myeloid DC ratio in the spleen and had the strongest β coefficient of all regression analyses performed. This indicates that the lower the liver RAE level progresses, the more extreme the perturbation in lymphoid to myeloid DC ratio became. It also indicates that marginal vitamin A deficiency does not have as severe an effect on this DC ratio, and that there is not a cut-off level of liver RAE (or serum retinol) at which a DC subpopulation is lost altogether. Rather, DC myeloid and lymphoid subpopulations change gradually over time as the severity of VAD progresses. Unlike myeloid and lymphoid DC, liver RAE was not a strong predictor of plasmacytoid or preDC percentages in the spleen. Therefore, plasmacytoid and preDCs were unaffected by VAD (Figures 4D and 4E).

Some, but not all spleen lymphocyte populations were altered by VAD (Figure 5). B lymphocytes ($B220^+/MHC-II^{variable}$), $CD3^+/CD4^-/CD8^-$ lymphocytes, and total $CD3^+/CD8^+$ T lymphocytes were unaffected by vitamin A deficiency (Figure 5A, B, and D). Liver RAE was not a strong predictor of these lymphocyte populations. However, $CD3^+/CD4^+$ T lymphocytes were decreased as severity of VAD increased (Figure 5C). The β coefficient of liver RAE and $CD4^+$ T cells was 0.62 and therefore classified as a strong predictor of $CD4^+$ T cell percents in the spleen. Although total $CD3^+/CD8^+$ lymphocyte percentage was not correlated with liver RAE (Figure 5D), interestingly memory $CD8^+$ T lymphocytes ($Gr-1^+/CD8^+$) were increased as severity of VAD increased (Figure 5E). The β coefficient of liver RAE and memory $CD8^+$ T cells was a strong predictor at -0.62 . Taken together, it appears that release of new naïve T lymphocytes from primary lymphoid organs decreases as VAD progresses, and that memory populations are preferentially maintained to prevent loss of the benefit of existing adaptive immunity.

As hypothesized and previously reported by others, polymorphonuclear neutrophils (PMN) percentage increased as severity of VAD increased (Figure 6). The β coefficient of liver RAE and PMN was -0.44 and classified as a moderate predictor.

Discussion

The data presented here indicate the C57BL/6J animals depleted of vitamin A have skewed splenic DC subpopulations that could contribute to the observed Th1 bias in vitamin A-deficient populations. Vitamin A-deficient populations have impaired Th2-dependent antibody immune responses, and enhanced Th1 cell-mediated immune responses [4,26,27]. In the mouse, $CD11b^+$ myeloid DCs stimulate Th2 responses, while $CD8\alpha^+$ lymphoid DCs stimulate Th1 responses [11,12]. Previously, *in vitro* work has established that vitamin A is important for the differentiation of bone marrow progenitor cells into myeloid DCs and blocking vitamin A signaling leads to greater numbers of neutrophils [17]. We hypothesized that myeloid DCs would be decreased in the spleens of vitamin A-deficient mice. Interestingly, our data indicate that *in vivo*, lymphoid DCs are increased in VAD mice and myeloid DCs are only modestly affected. The skewed DC proportion was contradictory to our original hypothesis of decreased myeloid DCs and unaffected lymphoid DCs. However, our data demonstrating an alteration in lymphoid to myeloid DC ratio in VAD could directly contribute to insufficient Th2 responses associated with VAD.

Careful consideration of all data together in the paper provides some mechanistic clues to the origin of the lymphoid to myeloid DC skewing we observed in the spleens of VAD animals. $CD8^+$ lymphoid DC derive from preDC seeded from the bone marrow, whereas myeloid DC derive primarily from blood monocyte precursors [28]. We did not observe significant changes in spleen preDC percentages, indicating that the bone marrow development and release of preDC from the bone marrow to the periphery is not altered by vitamin A deficiency. It is

therefore the final maturation of preDC to the mature CD8⁺ lymphoid DC phenotype that is altered by vitamin A deficiency. So, either preDC to CD8⁺ lymphoid DC maturation in the spleen is enhanced, or alternatively, CD8⁺ lymphoid DC proliferation is increased in the spleen in the context of vitamin A deficiency.

The data presented is limited by the lack of analysis of VAD and VAS antigen-presenting function and expression of T cell co-stimulatory proteins. However, our laboratory has previously documented changes in antigen-presentation and T cell co-stimulatory markers of VAD bulk antigen presenting cells and bone marrow-derived DCs, respectively [17,29]. Without exogenous cytokines, IFN- γ production was increased and IL-4 was unchanged in primary cultures B10.PL transgenic T cells with DMSO compared to cultures with 10 nmol/L all-*trans* retinoic acid (atRA) added [29]. Furthermore, atRA treated antigen-presenting cells induced increased T cell production of IL-4 compared to DMSO treated antigen-presenting cells in co-culture experiments [29]. Unfortunately, the authors did not characterize the production of IFN- γ in these co-culture experiments. In addition, murine vitamin A deficiency leads to constitutive IL-12 production [30] and lymphoid DCs are the major producers of IL-12 [31]. Therefore, our data presented documents a DC subpopulation alteration in VAD mice that is consistent with other published mechanistic studies. Upon maturation, DCs up-regulate the expression of T cell co-stimulatory proteins including CD80, CD86, and MHC-II. Bone marrow-derived cultures of DCs express variable percent positive and mean fluorescence intensity (MFI) for CD80, CD86, and MHC-II with atRA, DMSO, characterized serum or charcoal dextran-filtered serum [17]. Despite conflicting data on the T cell co-stimulatory marker expression of *in vitro*-derived DCs, VAD skews antigen-presentation from Th2 to Th1 immune response bias.

We used the dietary depletion protocol of Smith et al (1987) to obtain VAD animals [23]. We monitored feed consumption (data not shown) and body weights (Figure 1) during the study to determine the point of inanition which would lead to protein-energy malnutrition (PEM). Detection of inanition was established to be a loss of greater than 10% of body weight. Interestingly, depletion of vitamin A in female mice had slower kinetics than depletion of male mice (Figure 2). Few male mice reached the 12 week time point without losing 10% of their body weight, hence only four VAD males at 12 weeks of age were analyzed. Therefore, the data presented represent various degrees of vitamin A deficiency without the confounding factor of PEM.

Our data are in agreement with many of the spleen immune cell percentages of vitamin A-deficient populations reported previously by others, including B and T lymphocytes [23,27, 32]. However, the dramatic increase in neutrophils reported in VAD SENCAR mice contradicts the mild increase observed in our mice [Figure 6, [18]]. In conjunction with the large increase in neutrophils, VAD SENCAR mice also had decreased lymphocyte populations [18]. Therefore, the 14 week dietary depletion of vitamin A may have resulted in PEM and/or correspondingly increased glucocorticoids. Lymphocytes decrease and neutrophils increase in response to PEM and increased levels of glucocorticoids [33–35]. Chronic vitamin A deficiency leads to PEM, which can complicate study of vitamin A deficiency if not properly controlled for.

In agreement with previous reports, we show that B lymphocyte and CD8⁺ T lymphocyte numbers were unaffected and CD4⁺ T lymphocyte numbers were decreased in vitamin A-deficient mice. However, we can not distinguish CD4⁺ T cells as Th1, Th2, or Treg using our multicolor flow cytometric analysis. Surprisingly, our gating strategy identified a population of CD8⁺ T lymphocytes that expressed Gr-1 were increased in VAD. These CD8 α ⁺/Gr-1⁺ cells were previously characterized as memory CD8⁺ T lymphocytes [36,37]. Vitamin A-deficient populations have an increased reliance on memory cell immune responses and decreased

reliance on naïve cell immune responses [32,38,39]. Memory T cells, as well as natural killer (NK) and NKT cells, are maintained by the growth factor interleukin- (IL) 15. Hepatic and pancreatic stellate cells, as well as DCs, produce IL-15 [40–42]. Hepatic stellate cells and DCs can also store and metabolize vitamin A [3]. Although vitamin A did not significantly alter IL-15 cytokine production *in vitro*, the dual function of vitamin A metabolism and production of IL-15 by DCs and liver stellate cells leads to a possible inter-relationship that would be expected to selectively enhance memory T cell reliance during VAD [43].

Our data indicate, for the first time, that *in vivo* spleen DC populations are altered by vitamin A deficiency. Vitamin A deficiency significantly skews DC subset proportions in favor of Th1 responses, leading to the down-regulation of Th2 responses. The altered DC proportions provide mechanistic evidence for a role of DCs in altered immune responses of vitamin A-deficient populations. Further work should expand the DC analysis of vitamin A-deficient populations to other tissues and newly described DC subpopulations. The DC populations of the mesenteric lymph nodes and Peyer's patches should be assessed during vitamin A deficiency. The effects of vitamin A deficiency on newly identified DC populations such as CD103⁺ DCs should also be assessed due to the roles of vitamin A and CD103⁺ DCs in the Treg and Th17 immune cell balance in the gastrointestinal tract [7,44–46].

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Figure 1. Body weights of C57BL/6J mice consuming VAS and VAD diet

VAS animals are indicated by filled symbols and VAD animals are indicated by open symbols, male mice are indicated by squares (■) while female mice are indicated by triangles (▼).

Weight is significantly different from previous week ($p < 0.05$) for weeks 3–11 within each diet and gender group. #, indicates a significant time*gender interaction ($p < 0.05$) at weeks 3–7 and 8 to 9. \$, indicates a significant time*diet interaction ($p < 0.05$) at weeks 8–11. &, indicates a significant time*diet*gender interaction at weeks 9–12 ($p < 0.05$). The data represents 10 animals per group, except VAD males ($n = 4$).

Figure 2. Vitamin A status of C57BL/6J mice

VAS animals are indicated by filled symbols and VAD animals are indicated by open symbols. Male mice are indicated by squares (■) and female mice are indicated by triangles (▼). *A*, serum retinol analyzed with repeated measures ANOVA. *, serum retinol is significantly different from previous week ($p < 0.05$). Diet, gender, and the diet*gender interaction are significant ($p < 0.05$). The data represents 2–6 animals per group for serum retinol analysis. *B*, liver retinol activity equivalents analyzed with 3-factorial ANOVA. Diet, gender, age*diet, and diet*gender are significant ($p < 0.05$). The data represents 10 animals per group, except week 12 VAD males ($n = 4$) for liver RAE analysis.

Figure 3. Total spleen cells per animal of C57BL/6J mice

Filled bars are vitamin A-sufficient animals, open bars are vitamin A-deficient animals. * indicates significant differences ($p < 0.05$). Week 8 is significantly different than week 10, but week 8 and 10 are not significantly different than week 12, regardless of gender and diet. Male and female mice are significantly different regardless of time and diet. The diet*age and age*gender interactions are significant as well. The data represents 10 animals per group, except week 12 VAD males ($n=4$).

Figure 4. Effects of depleted liver RAE on spleen DC populations

A, myeloid DCs. B, lymphoid DCs. C, ratio of myeloid to lymphoid DCs. D, plasmacytoid DCs. E, preDCs. Table insets indicate significant β coefficients, R^2 values, and significance of model.

Figure 5. Effects of depleted liver RAE on spleen lymphocyte populations
A, B cells. B, CD3⁺/CD4⁺/CD8⁻ T cells. C, CD4 T cell. D, total CD8 T cells. E, Gr-1⁺/CD8⁺ memory T cells. Table insets indicate significant β coefficients, R^2 values, and significance of model.

Figure 6. Effects of depleted liver RAE on spleen PMN

Table inset indicates significant β coefficients, R^2 values, and significance of model.

Table I
Liver weight of VAS and VAD male and female animals

Week 8 (n=10 mice per group), week 10 (n=10 mice per group), week 12 VAS female and VAD female (n=10 mice per group), week 12 VAS male (n=8) and week 12 VAD male (n=4 mice).

	Week 8		Week 10		Week 12	
	VAS	VAD	VAS	VAD	VAS	VAD
Male *	0.91 +/- 0.05 ^a	0.81 +/- 0.05	0.88 +/- 0.04	0.85 +/- 0.04	0.93 +/- 0.02	0.98 +/- 0.03
Female	0.75 +/- 0.02	0.68 +/- 0.05	0.75 +/- 0.05	0.82 +/- 0.02	0.78 +/- 0.05	0.72 +/- 0.05

^aValues are the mean +/- standard error of the mean in grams.
* Statistical significance (p<0.05). Male livers weighed significantly more than female livers, regardless of age or diet.

Table II
Spleen weight of VAS and VAD male and female animals

Week 8 (n=10 mice per group), week 10 (n=10 mice per group), week 12 VAS female and VAD female (n=10 mice per group), week 12 VAS male (n=8) and week 12 VAD male (n=4 mice).

	Week 8		Week 10		Week 12	
	VAS*	VAD	VAS*	VAD	VAS*	VAD
Male	0.07 +/- 0.01 ^a	0.08 +/- 0.01	0.06 +/- 0.00	0.07 +/- 0.01	0.06 +/- 0.00	0.07 +/- 0.01
Female	0.06 +/- 0.00	0.07 +/- 0.01	0.07 +/- 0.01	0.08 +/- 0.01	0.07 +/- 0.01	0.09 +/- 0.01

^aValues are the mean +/- standard error of the mean in grams.
* Statistical significance (p<0.05). Spleens of VAD animals weighed significantly more than spleens of VAS animals, regardless of age or gender.