Implications of arginine deficiency for growth and organ maturation. Studies on hair, muscle, brain and lymphoid organ maturation

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Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice.

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Abbreviations

A-I, hepatic arginase
A-II, non-hepatic arginase
IEL, intraepithelial lymphocyte
IFNγ, interferon γ
IL, interleukin
LPL, lamina propria lymphocyte
MLN, mesenteric lymph node
NOS, nitric oxide synthase
OTC, ornithine transcarbamoylase
PLN, peripheral lymph node
PP, Peyer’s patch
Spf-ash, sparse-fur and abnormal skin and hair
TNFα, Tumor Necrosis Factor α.
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Abstract

Arginine is required for the synthesis of protein, NO, creatine, agmatine and polyamines and the detoxification of ammonia. Another purported role of arginine is its immunosupportive function. In suckling mammals, arginine is synthesized in the small intestine. The functional relation between intestinal arginine metabolism and lymphocyte maturation and lymphoid organ development was investigated in transgenic mice that express hepatic arginase in their enterocytes. In the highest expressor line, F/A-2, arginine concentration in plasma and tissues is selectively decreased to 30-35 % of controls. B cell numbers were reduced in the peripheral lymphoid organs of these mice. The number and size of Peyer’s patches in the intestine was drastically reduced. Analysis of the bone marrow revealed that arginine deficiency impaired B cell maturation at the transition from the pro- to pre-B cell stage. In F/A-2 serum, immunoglobulin M level is decreased, but a normal B cell proliferative response can be induced. In contrast, the number and phenotype of T cells, found in the intestine, spleen or thymus of F/A-2 transgenic mice is normal. None of these phenotypes were found in NOS- or OTC-deficient Spf-ash mice. The findings demonstrate that a decrease in circulating arginine affects early B cell maturation.
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Introduction

Arginine is a precursor for the synthesis of protein, NO, creatine, agmatine and polyamines and an intermediate in the detoxification of ammonia via the ornithine cycle. Arginine is not considered to be an essential amino acid. In adults, it is produced in the kidney (1, 2) from circulating citrulline (3), which, in turn, is synthesized by enterocytes of the small intestine (4). In adult humans, the endogenous biosynthetic capacity for arginine amounts to approx. 20% of daily expenditure, i.e. is relatively small in comparison with its daily requirement (5). Hence, a dietary supply may become indispensable under conditions of increased demand, such as growth (5) and tissue repair (6), or as a result of decreased dietary supply (7). For this reason, arginine is coined as a conditionally essential amino acid.

An intriguing feature of the nutritional benefit of arginine is its immunosupportive effect, especially under catabolic conditions (8, 9). In studies on wound healing, arginine was already identified as an immunonutrient more than 20 years ago (10, 11). On this basis, it is added to postoperative supplemental formulas at doses as high as 100 g per kg formula (12). However, the molecular mechanism underlying the beneficial effect of arginine on lymphocyte biology has remained unclear.

In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk (13). In this period, the intestine rather than the kidney plays a major role in arginine biosynthesis (14-16). The selective decrease in circulating arginine in neonatal patients who suffer from necrotizing enterocolitis, suggests a similar role of the enterocytes in arginine metabolism in early human development as well (17). On this basis, we developed a transgenic mouse model that suffers from a selective deficiency in arginine (18). In these mice, arginase I (A-I, EC 3.5.3.1) is selectively overexpressed in the enterocytes of the small intestine by coupling the A-I gene to the intestinal Fatty-Acid Binding Protein promoter/enhancer (FABP) (19). Hence, these mice can no longer accumulate arginine in their enterocytes, so that arginine levels decline to 30% of control levels. In the current study, we employed this
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model to dissect the immunomodulatory effect of arginine.

We demonstrate that arginine-deficiency affects early B cell maturation in the bone marrow, but does not affect thymic T-cell development. In secondary lymphoid organs, like spleen and Peyer's patches, the number of B cells is decreased, though resident B cells are capable to proliferate normally upon in vitro stimulation. In addition, the plasma level of IgM is reduced in transgenic animals. These phenotypic abnormalities were not observed in NOS-deficient mice or in Spf-ash mutant mice, which, due to a mutation in the ornithine cycle enzyme OTC (20), suffer from a decreased circulating arginine level resulting from the impaired synthesis of the arginine precursor citrulline. These findings suggest a direct involvement of arginine on B cell maturation.
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Animals

Transgenics. A chimeric construct of the intestinal Fatty Acid Binding Protein promoter/enhancer element and hepatic arginase minigene was used to generate transgenic mice in the FVB strain background (18). The 4.3 kb construct consisted of the -1178 to +28 bp EcoRI-BamHI fragment of the rat intestinal Fatty Acid Binding Protein (FABPi) promoter/enhancer element, the 520 bp PstI-BsrGI fragment of the rat A-I cDNA, containing exons 1 to 4 of rat hepatic arginase and the 2400 bp BsrGI-HinDIII fragment of genomic rat A-I DNA, containing exons 5 to 8 of rat hepatic arginase. The line with the highest expression level of arginase, designated F/A-2 (18), was used in the current investigation.

Spf-ash and NOS1-2-3-deficient mice were of C57/B16 background and purchased from Jackson Laboratories, Bar Harbor, Maine. Litters discovered in the morning were assigned neonatal day 0. The animals were weaned at three weeks of age. Mice were kept under environmentally controlled conditions (lights on at 8:00 a.m., off at 8:00 p.m.; water and rodent chow ad lib; 20-22 °C, 55% humidity). Animal experiments were done in accordance with the guidelines of the local Animal Research Committee.

Tissue and blood sampling. Pups were separated from their mother and kept at 37 °C for one hour prior to sacrifice. After decapitation, whole-body blood was collected and centrifuged for 2 minutes at 4 °C. Serum was kept at -70 °C until analysis. Tissue samples were collected, flushed in ice-cold PBS and rapidly frozen in liquid nitrogen. Tissue samples were kept at -70 °C until analysis.

Methods

Cell preparation and culture. Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated as described (21). In short, the small intestine was isolated, trimmed free of fat and mesentery, and flushed with ice-cold PBS to remove fecal content. The small intestine was cut into 1 cm fragments, and incubated for 30 min at 37 °C in calcium-free medium in the presence of 1 mM EDTA and 1 mM DTT, followed by a 30 min incubation in RPMI culture medium, supplemented with 20 U/ml DNase, to remove enterocytes and IELs. For
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subsequent isolation of LPLs, Peyer’s patches were removed, and a cellular suspension was obtained from the remaining intestinal tissue using an automated mechanical tissue desegregation device (Medimachine System, Dako, Denmark). For preparing spleen, thymus and mesenteric lymph node cell suspensions, 40 µm filter cell strainers (Becton/Dickinson Labware, New Jersey, USA) were used. Bone-marrow cell suspensions were obtained by flushing femurs and tibias with Dulbecco’s Modified Eagle medium (DME). Cells were suspended in DME containing 10% Fetal Calf Serum (FCS) (Gibco/BRL, Paisley, Scotland) and counted. Spleen and intestinal cell suspensions were centrifuged in sterile Ficoll (Pharmacia, Uppsala, Sweden). Mononuclear cells were transferred to cold PBS, containing 0.5% bovine serum albumin, 0.3 mmol/l EDTA and 0.01% sodium azide (Sigma) for flow cytometry, or to DME supplemented with 10% FCS for culture.

In vitro stimulation and proliferation. Triplicate cultures of splenic cells containing $10^5$ B cells were started with LPS (E.Coli EH100, 15 µg/ml, a gift from Dr. C. Galanos), goat F(ab’) anti-mouse µ chain (20 µg/ml, Jackson ImmunoResearch), anti-CD40 antibody (clone HM40-3, 4 µg/ml, PharMingen) or IL 4 (500 U/ml, PharMingen), either alone or in combination for the indicated times. ($^3$[H]-Methyl-) Thymidine was added 6 hours before harvesting the cells.

Flow cytometry. The following antibodies were used for detection of lymphocyte surface markers. Monoclonal antibodies to mouse CD3ε, CD4, CD8, CD19, CD21, CD23, CD24, CD25, CD43, integrin αIEL and IgD were either from our collection or purchased from PharMingen or Southern Biotechnology Associates (Birmingham, Alabama). They were used unlabeled or biotin-labeled, followed by staining with appropriately labeled secondary antibodies or streptavidin, or they were used as such, but covalently labeled with an appropriate fluorochrome. Goat F(ab’)2 anti-mouse IgM and goat F(ab’)2 anti-rat IgG were purchased from Caltag Lab (Burlingame, CA). Fluorochromes used were FITC, phycoerythrin (PE), Cy-5, and a tandem dye of Cy-5 and PE. Non-specific binding of goat anti-rat antibodies was blocked by incubation with 5% normal mouse serum. Cells were analyzed by
flow cytometry using a FACSScan® or FACSCalibur flow cytometer, in conjunction with the CellQuest® software (Becton Dickinson, Mountain View, USA).

Histology and immunohistochemistry. For histological analyses of spleen and PPs, tissues were quick-frozen in Tissue-Tek OCT compound and sectioned at 6 μm. Sections were fixed in dehydrated acetone for 10 minutes at room temperature, air dried and incubated with rat anti-mouse antibodies. Polyclonal rabbit anti-mouse IgM was purchased from DAKO, Tilburg, The Netherlands. The following rat antimouse mAbs were used: anti-CD3e (clone KT-3), anti-CD45R (B220), anti-MAdCAM-1 (clone MECA-367) and anti-SER-4 (marginal zone macrophages). After washing in PBS, sections were incubated with the appropriate dilution of the peroxidase-conjugated second step reagent (Jackson ImmunoResearch, San Francisco, CA) in 5 % newborn calf serum and 5 % normal mouse serum in PBS. Double labelings were performed as described (22). In short, acetone-fixed cryostat sections (6 μm thick) were incubated with a rat anti-mouse MAb for 45 min at room temperature. Sections were incubated with a peroxidase-conjugated goat anti-rat Ig (Sigma, St. Louis, MO), followed by blocking of free binding sites with 20 % rat serum for 10 min at room temperature. After staining with 3,3′diaminobenzidine tetrahydrochloride (Sigma) at a concentration of 0.5 mg/ml in the presence of 0.01 % H₂O₂, sections were incubated with biotin-labeled rat anti-mouse monoclonal antibody, followed by an incubation with streptavidin. Alkaline phosphatase activity was visualized using a stock solution of 18.8 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine salt, according to the manufacturer’s instructions (Boehringer, Mannheim, Germany).

Quantification of serum immunoglobulin levels. Serum was collected from 1 to 6 week-old wild-type and homozygous F/A-2 transgenic littermates. IgG1, IgG2A, IgG2B, IgG3, IgM and IgA levels were each quantified using ELISA-based assays according to the manufacturer’s instructions (Southern Biotechnology Associates, Birmingham, AL, USA). 96-Wells ELISA plates were prepared by overnight coating with goat anti-mouse Ig. Plates were blocked for 1 hour at room
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temperature with 1 % BSA in PBS, except for IgA, which was blocked with 3 % non-fat milk powder in PBS. Incubation with serum dilutions in PBS containing 0.05 % Tween-20, pH 7.4 and specific horseradish peroxidase-conjugated, isotype-specific anti-mouse immunoglobulins was for 1 hour at room temperature. Staining was performed using OPD substrate in the presence of H₂O₂. The optical density was read at 405 nm on a microplate reader.

Statistics. Biochemical data on immunoglobulins were tested with a two-way repeated measure analysis of variance (ANOVA; factors age and genotype). Results were considered significantly different at p<0.05. Counts on Peyer's patches were tested with a Poisson distribution.
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Results

Impaired development of Peyer’s patches in arginine-deficient transgenic mice.

The phenotype of homozygous F/A-2 transgenic mice (F/A-2+/+) was described in detail elsewhere (18). The intestinal arginase activity caused a decrease in circulating plasma arginine concentration to 80 μM, as opposed to approx. 250 μM in wild-type suckling and 150 μM in adult mice (18).

A striking abnormality in the F/A-2+/+ mice was the macroscopical absence of Peyer’s patches (PP). Whole mount immunohistochemical staining for intestinal expression of VCAM-1 as a parameter for early PP development (23) revealed that F/A-2+/+ mice developed a normal number of PP anlagen in the first postnatal week (18). However, subsequent development of PP appeared to be critically dependent on arginine availability, because PP remained underdeveloped in the first 4-6 neonatal weeks in F/A-2+/+ mice, whereas neonatal arginine injections restored the development of PP back to normal (18). Fig. 1 shows an occasionally found PP in an F/A-2+/+ small intestine at the age of 3 weeks. These PPs hardly protruded from the serosa, which made them difficult to identify macroscopically. In normal mice, PPs consist of large B cell follicles (Fig. 1A) and smaller T-cell-rich areas on the outside of the follicles (Fig. 1C). The rudimental PPs in F/A-2+/+ mice showed hypoplasia of the B- and T-cell areas, though B cell follicles were still identifiable (Fig. 1B and D). Both control and F/A-2 B cells were positive for membrane-bound IgM (mIgM) (Fig. 1E and F). The interaction between integrin α4β7 and the mucosal vascular addressin MAdCAM-1 is essential for lymphocyte homing to the PPs (24). MAdCAM-1 was normally expressed on high-endothelial venules within the PPs in both control and F/A-2+/+ mice (Fig. 1G, H). PPs became macroscopically identifiable in F/A-2+/+ mice at 6-7 weeks of age, in parallel with the gradual decline in the difference of circulating arginine between control and transgenic mice, demonstrating that the development of PPs in F/A-2+/+ mice was temporarily suspended, but not abolished.
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Fig. 1 Wild-type and F/A-2⁺⁺ Peyer’s patches stained for the presence of B220, IgM, CD3e, and MAdCAM. Histological examination of small intestines occasionally reveals a small Peyer’s patch (PP) in ND21 F/A-2⁺⁺ animals. These PPs (left column) are reduced in size and do not protrude from the serosal surface, compared with PPs from wild-type littermates (right column). Nevertheless, immunohistochemical staining for B220- and IgM-positive B cells (panels A,B, and C,D, respectively)), CD3⁺ T-cells (panels E,F) revealed the presence of segregated T- and B cell areas in F/A-2⁺⁺ PPs. Endothelial cells in sinuses lining the PPs normally express MAdCAM-1 (panels G, H). Sections were counterstained with haematoxylin. Bar is 200 μm.
T-cell populations in central and peripheral lymphoid organs

Arginine has been reported to alter T-cell function in vitro (25). Therefore, we investigated the development and phenotype of T lymphocytes in F/A-2\(^{+/+}\) transgenic mice in more detail. T cells were isolated from thymus, spleen, cervical lymph nodes, mesenteric lymph nodes, and intestine. In the F/A-2\(^{+/+}\) thymus, absolute cell numbers were decreased, but proportional to the decreased body mass of the transgenic mice (18). The fraction of CD3\(\varepsilon\)-positive cells and the distribution of CD4,CD8 double-negative, CD4,CD8 double-positive, CD4 single-positive and CD8 single-positive cells showed no major differences between transgenic and wild-type mice at ND21 (Table 1 and Fig. 2, upper panel). These results indicate that maturation and selection of transgenic thymocytes is not hampered by arginine deficiency.

<table>
<thead>
<tr>
<th>thymocytes</th>
<th>% of cells</th>
<th>fraction of CD4(^+)CD8(^-)(%)</th>
</tr>
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<tr>
<td>CD3(^+)CD4(^+)</td>
<td>11.2</td>
<td>18.1</td>
</tr>
<tr>
<td>CD3(^+)CD8(^+)</td>
<td>4.4</td>
<td>6.8</td>
</tr>
<tr>
<td>CD4(^+)CD8(^+)</td>
<td>82.4</td>
<td>75.7</td>
</tr>
<tr>
<td>CD4(^+)CD8(^-)</td>
<td>11.6</td>
<td>15.8</td>
</tr>
<tr>
<td>CD4(^+)CD8(^-)</td>
<td>0.84</td>
<td>1.3</td>
</tr>
<tr>
<td>CD4(^-)CD8(^+)</td>
<td>1.8</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 1: expression of T-cell surface markers on thymocytes of wild-type or F/A-2\(^{+/+}\) mice at 4 weeks of age. Thymocytes from either wild-type or F/A-2\(^{+/+}\) mice, analyzed by flow cytometry for the indicated markers. The percentage of CD4 or CD8 single positive or double negative T cells, compared to CD4 CD8 double positive T cells is similar, indicating that F/A-2\(^{+/+}\) thymocytes mature normally. Percentages shown are representative of three analyses.

Peripheral and mesenteric lymph nodes were easily found in F/A-2\(^{+/+}\) mice. The relative number of CD3\(^+\), CD4\(^+\) and CD8\(^+\)-T cells in those lymph nodes was not different from those in normal mice (Fig. 2, lower panel). Similarly, the relative number of CD3\(^+\), CD4\(^+\) and CD8\(^+\)-T cells present in transgenic intestine was not different from wild-type intestine (Fig. 3A-C). Also, the relative number of intestinal IEL, expressing the gut-homing integrin \(\alpha_{\text{IEL}}\), was not affected (Fig. 3D).
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Fig 2. Flowcytometric analysis of T lymphocytes from thymus, spleen and lymph nodes of 3 weeks old normal and F/A-2^{-/-} mice. Cells were stained with antibodies to CD4 and CD8, and the percentage of CD4-CD8-double-negative, CD4-CD8-double-positive, CD4-single-positive and CD8-single-positive cells is indicated.
However, the absolute number of T cells that could be recovered from the FA-2\(^{-/-}\) intestinal LPL was lower than that isolated from wild-type intestine (not shown) due to the reduced size of the animals and the PPs.

In the spleen of F/A-2\(^{-/-}\) transgenic mice, CD4-positive and CD8-positive T cells were readily found at three weeks of age, with a normal distribution. (Fig 2, middle panel). However, in line with the reduced intestinal T-cell content, the absolute number of T cells was lower than control mice, also when corrected for the reduced body weight of the transgenic mice (not shown).

Reduced numbers of B cells in the intestine of arginine-deficient mice
To investigate the presence of lymphocyte populations expressing the pan B cell marker CD19 and the MHCII antigen I-A, intestinal lymphocytes were isolated, purified free of enterocytes and IELs, and analyzed (Fig. 3E and F). As the transgenic PPs are too small to be identified macroscopically, these B cell rich structures were not excised, but included in the lymphocyte fractions of both wild-type and F/A-2\(^{-/-}\) mice. Figs 3 E and F show an almost complete lack of CD19\(^+\) and I-A\(^+\) B cells in the small intestines of F/A-2\(^{-/-}\) mice during the first four weeks of development. At ND 21, the lymphocyte population in the small intestine of F/A-2\(^{-/-}\) mice contained 5 times fewer CD19\(^+\) and I-A\(^+\) B cells than that of wild-type mice (Table 2). The B cells in the LPL fraction are all located in PPs, as CD19\(^+\) or I-A\(^+\) cells could not be recovered after excision of PPs from wild-type small intestine prior to homogenization (not shown). Thus, the difference in CD19 and I-A staining between wild-type and F/A-2\(^{-/-}\) mice corroborates the macroscopical observation of the virtual absence of PP in F/A-2\(^{-/-}\) mice.

In order to establish whether the effect of arginine on PP development is mediated by the arginine product nitric oxide (NO), we investigated mice carrying null mutations of either of the three isoforms of the NOS gene (26-28). At three weeks of age, a normal PP development was observed in nNOS-, iNOS-, and eNOS-deficient mice, compared to their appropriate controls (18). In addition, B cell numbers were not decreased in intestinal lymphocyte populations isolated from NOS-deficient mice (Table 2).
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Fig. 3 F/A-2−/− small intestines contain a normal number of T cells, but a reduced number of B cells. The percentage of intestinal lymphocytes, including those from PPs that express the T-cell markers CD3, 4, 8, and αEL (Panels A-D) as well as the B cell markers CD19 and MHC-II (Panels E–F), was determined in wild-type (black squares), and F/A-2−/− (gray diamonds), by flow cytometry. The percentage of cells that express T-cell markers is similar in both groups, but the percentage that expresses B cell markers is reduced. The reduction in B cells in F/A-2−/− mice can be fully ascribed to the lack of PPs, as no CD19 and I-A positive cells are found after removal of the PPs (see text).

In OTC-deficient Spf-ash mice, arginine levels are decreased to approx. 120 μM (29, 30), that is, to slightly higher levels than those observed in F/A-2+/+ mice. However, in contrast to F/A-2 mice, Spf-ash mice suffer not only from hypoargininemia, but also from hyperammonemia and moderately to severely reduced levels of nearly all other amino acids (30). Nevertheless, Spf-ash mice displayed normal PPs and intestinal B cell numbers (Table 2). These observations
show that mucosal arginine deficiency affects PP and B cell development via a mechanism that is selectively dependent on arginine availability.

<table>
<thead>
<tr>
<th>%</th>
<th>WT (FVB)</th>
<th>F/A-2+/+</th>
<th>WT (C57BL/6) spf-ash</th>
<th>iNOS+/−</th>
<th>nNOS+/−</th>
<th>eNOS+/−</th>
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<tbody>
<tr>
<td>CD19</td>
<td>17.7 ± 5.6 2.7 ± 0.8 *</td>
<td>12.9 ± 3.7 14.8 ± 2.2 21.3 ± 5.5 12.5 ± 2.5 23.5 ± 7.5</td>
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<tr>
<td>I-A</td>
<td>20.7 ± 5.2 4.0 ± 1.5 *</td>
<td>13.8 ± 3.3 17.3 ± 1.9 24.7 ± 5.5 13.0 ± 3.0 25.5 ± 7.5</td>
<td></td>
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*Table 2: expression of B cell surface markers on intestinal lymphocytes of various mouse strains at 3 weeks of age. The percentage of B cells is selectively decreased in F/A-2+/+ mice, but not in spf-ash, or NOS deficient lines, compared to their strain-matched wild types. Data given are the means ± SEM of two separate analyses. Asterisks indicate significant differences from wild type (P < 0.05).

Selective defects of B cell subpopulations in other peripheral lymphoid organs

Peripheral and mesenteric lymph nodes were relatively unaffected in F/A-2+/+ mice. In 3 weeks-old F/A-2+/+ mice, the fraction of lymph node B lymphocytes was reduced from 9% wild-type mice, to 5% in the transgenic mice, with slightly more immature phenotypes (not shown). These differences were most pronounced in 3-weeks old mice and least in adult mice, with 4-weeks old animals being intermediate (a reduction from 13% in 4 weeks-old wild types to 8% in transgenics).

Spleen size was severely reduced in suckling F/A-2+/+ mice (18). As a result, the total number of splenic cells in F/A-2+/+ mice was 10 times less than in controls (Table 3). In order to investigate splenic architecture under these conditions, spleens from 3-week old mice were analyzed immunohistochemically (Fig 4 A-L). In the white pulp of wild-type mice, a wide cuff of B cells, expressing the B cell specific splice variant of CD45 (B220) (Fig. 4A), IgM (Fig. 4C) and, to a lesser extent, IgD (Fig. 4E), surrounded the T-cell-rich periarteriolar lymphatic sheath (PALS) (Fig. 4K). In the transgenic spleen, the area of B220+ or IgM+ B cells surrounding the PALS was approx. 3-fold reduced in diameter, compared to that in wild-type spleen (Fig. 4B, D). Splenic sections of 3-week old mice were also incubated with anti-B220 and anti SER-4 (marginal zone macrophage marker, Fig. 4G and H), anti-B220 and anti-MadCAM-1 (Fig. 4I and J), or anti-CD3 and anti-SER-4 (Fig. 4K and L). Anti-MadCAM-1 antibodies label the sinus-lining cells.
of the marginal zone. B cell follicles were clearly identified in wild-type mice as B220-positive (Fig. 4G and I), or CD3-negative (Fig. 4K), areas within the SER-4 (Fig. 4G and K), or MadCAM-1 (Fig. 4I) -positive marginal zone. In F/A-2^+/+ mice, B cell follicles were virtually absent (Fig. 4H, J and L). Thus, these data suggest that in F/A-2^+/+ mice, the splenic architecture is not disturbed, though the decreased number of B cells in the transgenics impairs a normal B cell follicle formation.

The differentiation and maturational stage of the F/A-2^+/+ splenic B cells was further assessed by flow cytometry (Fig. 5). As already indicated by the immunohistochemical analyses, the fraction of B cells in transgenic spleen was reduced to 65% of that in wild-type controls at 3 (Fig. 5A), and 4 weeks (not shown) of age. The fraction of B cells expressing very low levels of CD21 (receptor for mouse complement factor C3), high levels of mIgM, but no IgD, are recent immigrants from the bone marrow, and are designated type 1 transitional B cells (31). In transgenics, this fraction is modestly decreased to 31% of the total B cells population in the spleen, compared to 38% in wild-type (Fig. 5B). The fraction of B cells, that expresses high levels of both IgM and CD21 and is positive for IgD (Type II transitional B cells, (31)), is increased to a similar extent, indicating a shift from Type I to Type II transitional B cells in the transgenic spleen (Fig. 5B). The population of mature, resting B cells, that express low levels of IgM, high levels of IgD and intermediate levels of CD21, is similar in F/A-2^+/+ and wild-type spleen (Fig. 5B and C). This maturation profile could still be observed at 4 weeks of age (not shown).

<table>
<thead>
<tr>
<th>age (ND)</th>
<th>genotype (x10^6 cells)</th>
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<tr>
<td></td>
<td>WT</td>
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<tr>
<td>21</td>
<td>spleen</td>
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<tr>
<td>21</td>
<td>bone marrow</td>
</tr>
<tr>
<td>28</td>
<td></td>
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<td>ad</td>
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Table 3: Reduced recovery of spleen and bone marrow lymphocytes from 3-weeks old, 4-weeks old, and adult mice.
Fig. 4 Immunohistochemical analysis of expression of B220, IgM, IgD in the spleen of 3-weeks old wild-type and F/A-2\(^{+/+}\) mice. Serial sections of wild-type (left panels) and F/A-2\(^{+/+}\) (right panels) were stained for the expression of B220 (panels A,B; G,H; I,J), IgM (panels C,D) or IgD (panels E,F). The B220- and IgM-positive B cell layer surrounding the PALS is reduced to a narrow rim of cells. Splenic marginal zones were visualized by staining marginal zone macrophages &sialoadhesin-specific mAb SER-4)(G,H and K,L; red), and sinus-lining cells in the marginal zone expressing MadCAM-1 (MECA-
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In the same sections, B220 positive cells are stained blue. Follicles of B220 positive blue staining B cells (G-J) within the borders of the marginal zones (*) are easily distinguishable in wild-types, but are lacking in F/A-2+/+. Serial sections were also stained for CD3 (KT3) and the SER-4 antigen (K,L; both red). B cell follicles are marked by CD3-negative areas within the SER-4 positive marginal zone. B220 positive B cells migrating through the PALS (black arrow in G) to form follicles are lacking in F/A-2++. Bar is 100 μm.

Follicular B cells are CD23-positive. Fig 5D shows that the fraction of IgM-positive, CD23-positive cells was not changed in the spleen of F/A-2+/+ mice. Together, these results suggest a reduced output of B cells from the marrow of F/A-2+/+ mice resulting in a slow fill of peripheral lymphoid organs, in particular the spleen. The B cells that arrive in the spleen, undergo a normal maturation program, and the architecture of the spleen is not grossly disturbed.

**B cell development in F/A-2+/+ mice is hampered at the pro- to pre-B cell transition.**

The consistently decreased B cell content in peripheral organs prompted us to examine the early B cell maturation in the bone marrow of F/A-2+/+ mice in more detail. The total number of cells, recovered from bone marrow was reduced in 3-week old transgenic mice, mostly due to a reduced size of the bones in these mice. After 4 weeks of age, and in adults, normal numbers of bone marrow B cells were recovered from F/A-2+/+ mice (Table 3). We studied the differentiation stage of B lymphocytes using staining protocols based on the studies of Hardy et al (32), which allowed the identification of precursor B cells in pro-, pre- and immature B cells. In 3 and 4 -weeks-old mice, a substantial reduction of the fraction of lymphoid cells was seen in F/A-2+/+ mice (Fig. 6A and B, top panels), and the differentiation profile of the bone-marrow B cells was perturbed. Cells of the B lineage were identified with antibodies to B220.
Fig 5. Flowcytometric analysis of splenic B cells of 3 weeks old normal and F/A-2⁺/⁺ mice. Spleen cells were incubated with combinations of antibodies to IgM, IgD, CD45 (B220), CD21, and CD23. The percentage of the indicated subpopulations are shown, as fraction of the total number of cells (panels A), or as fraction of IgM-positive, B220-positive cells (panels B, C and D). The small squares show schematically the analyzed subpopulations: M, mature; T1, transitional type I; T2, transitional type II, Fol, follicular B cells.
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Fig. 6. Flowcytometric analysis of bone marrow B-lineage cells of 3 and 4 weeks old normal and F/A-2++ mice. Panel A: 3 weeks old mice, panel B, 4-week old mice. B-lineage lymphocytes are contained in the red windows of the upper panels. Cells in this window were analyzed for the expression of IgM and CD45 (B220) (middle panels), and the B220-positive, IgM-negative cells were analyzed for the expression of CD24 and CD43 (S7) (lower panels). The percentage of the indicated subpopulations are shown, as fraction of the total number of cells (upper panels), as fraction of the cells in the B-lineage cell window (middle panels), and as fraction of the B220-positive, IgM-negative cells in the lower panels. The ratio of pre- to pro-B cells is also given in the lower panels. Panel B: 4 weeks old mice. Cells were analyzed as in A), except that for the analysis of the B220-positive, IgM-negative cells antibodies to CD25 and CD43 were used. The small square shows schematically the analyzed subpopulations: M, mature B cells; T1, transitional type I B cells; I, immature B cells; P, pre- and pro-B cells; O, other cell types.
Membrane-IgM-negative, B220-positive cells were further characterized by labeling them with antibodies to CD43 and CD24 (Fig. 6 A, middle and lower panels), or with antibodies to CD43 and CD25 (Fig. 6B, middle and lower panels). The middle panel of Fig. 6A clearly shows that F/A-2^{+/-} mice had a reduced pool of
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pre- and pro-B cells of less than 50% of wild-type controls. This reduction was mainly due to a severe reduction in the fraction of pre-B cells (Figs 6A and B, lower panels). At 3, as well as 4 weeks of age, the ratio of pre- to pro-B cells was reduced from 3.9 and 2.5, respectively, in wild-type mice to 0.7 and 0.8, respectively, in F/A-2<sup>+/+</sup> animals (Figs 6A and B, lower panels). The subsequent differentiation stages were also affected: immature B cells (low levels of mIgM), type I transitional B cells (high levels of mIgM) and mature, recirculating B cells (lower levels of mIgM, mIgD-positive and high levels of CD45) were all severely reduced down to 10-25% of wild-type fractions. In concert, the fraction of non-B cells was doubled (Figs. 6A and B, middle panels).

Together, we conclude that B cell development in the bone marrow F/A-2<sup>+/+</sup> mice is impaired at the transition from the pro- to the pre-B cell stage. Hence, a reduced number of B cells leave the bone marrow and enter the periphery. The B cell maturation in the secondary lymphoid organs, however, is not grossly affected.

Decreased serum immunoglobulin M production in F/A-2<sup>+/+</sup> transgenic mice.

The reduced number of B cells in the spleen and gut of F/A-2<sup>+/+</sup> mice should have a functional impact on immunoglobulin production. Before weaning, IgG isotypes, but not IgM, are actively taken up by the intestinal epithelium from the mother’s milk by specific Fc-receptors on the brush border of the intestinal epithelium (33). Fig. 7 shows serum immunoglobulin levels in wild-type and transgenic mice from birth through adulthood. IgG concentrations in serum of suckling transgenic mice were not different from those in wild type mice, indicating that the intestinal uptake mechanism was not affected in the transgenics. In contrast, the IgM level was significantly lower (p<0.01) in suckling transgenic mice, while IgA was not yet detectable. After weaning, IgG2a and IgG2b levels increased towards a similar adult level in both wild-type and F/A-2<sup>+/+</sup> mice. The concentration of IgM also increased, but remained at a significantly lower level in transgenics than in wild-type mice (p<0.05). The levels of IgA and IgG1 tended to increase at a slower pace in transgenic mice, but the differences were not significant.
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Fig. 7 Serum immunoglobulin levels in wild-type and F/A-2⁺/+ mice. Serum immunoglobulin levels in wild-type (gray diamonds), and F/A-2⁺/+ (black squares) increase with a similar time course after weaning, but IgM levels are depressed to 30% of control in the F/A-2⁺/+ serum (p<0.05). Furthermore, IgG1 levels do not increase after weaning in the F/A transgenics. For each measurement, serum of three mice was pooled. Values are ± SEM and based on triplicates in two independent analyses.

B cells of F/A-2⁺/+ mice show normal proliferative responses

In order to establish whether the hampered development of B cells is due to a defective proliferative capacity, we stimulated primary B cell cultures in vitro (Fig. 8). Lymphocytes, isolated from the spleen of 3 week-old wild-type and transgenic mice were stimulated for 2 and 3 days with the polyclonal B cell activator LPS. A comparable proliferative response was seen in B cells, isolated from either F/A-2⁺/+ or wild-type mice. Also in experiments where B cells were stimulated with a goat F(ab')2 anti-μ antibody, no differences in proliferation were found (Fig. 8).
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Fig. 8. In vitro stimulation of splenic B cells of F/A-2 mice elicits a normal proliferation. Splenic lymphocytes (1*10^6 cells per well) of 4 weeks old mice were started with goat F(ab') anti-mouse μ chain (αIgM), LPS, IL 4, or anti-CD40 antibody, either alone or in combination for the indicated times. Cells isolated from either wild-type or F/A-2^{+/-} spleen displayed a similar proliferative response upon stimulation. Data shown are the means ± SEM of three measurement sessions, each with 3 cultures per condition.

Stimulation of B lymphocytes by T-helper cells in vivo is dependent on the binding of CD40 on the B cell. Culture of B cells in the presence of anti-CD40 antibodies and IL 4 normally results in a strong proliferative response and isotype switching (34). Therefore, B cells were also stimulated with anti-CD40 antibody alone, with IL 4 alone, or anti-CD40 antibody together with IL 4. Fig. 8 shows that none of these challenges revealed a defective transgenic B cell proliferation, compared to wild-type responses. The analysis of culture supernatants for the presence of IgG1 and IgE antibodies revealed normal levels both in cultures of F/A-2^{+/-}-derived B cells and in cultures of control B cells (data not shown). These data demonstrate that, despite the observed retardation in B cell maturation, transgenic B cells are
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fully capable of mounting appropriate responses both to T-cell-independent (LPS, anti-\mu) and T-cell-dependent (CD40/IL-4) stimuli.
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Discussion
At present, limited information is available on how nutrients influence the development of lymphoid organs or immune function. Arginine was surmised to be beneficial for immune system responses (35), but the reported effects are assorted, ranging from an increased production of polyamines (36), via amelioration of tumor growth (37, 38) and wound healing (10, 11), to a direct effect on T-cell gene expression (25). Here, we show that an arginase-mediated selective arginine deficiency results in an obstruction of B cell development in the bone marrow. This is accompanied by a pronounced reduction in the number of peripheral B cells and the macroscopic absence of Peyer's patches. Development of T cells in the thymus appears normal, although the size of the thymus is reduced in proportion to the general growth retardation of arginine-deficient mice (18). However, the number of T cells in the spleen is more reduced than expected. This is not an unusual finding in the spleen of mice with a severe distortion of B cell development (M.C.L., unpublished observations). Peripheral and mesenteric lymph nodes are affected to a much lesser extent. The severity of the phenotype depends on the level of arginase expression in the enterocytes, with hemizygous F/A-2 mice showing an intermediate phenotype. Furthermore, daily arginine injections reverse the lymphocyte phenotype of F/A-2+/+ mice (18). The syndrome begins to mitigate after weaning, that is, when intestinal synthesis of arginine ceases and the arginase-expressing enterocytes only catabolize circulating arginine. For these reasons, we believe that this phenotype develops as a result of a selective arginine deficiency.

How can we explain the phenotype? Strikingly, only precursor B lymphocytes appear to be affected. Later steps in development, i.e. immature, transitional and mature B cells in the bone marrow, and the spleen were all reduced in number, but not disproportional. This suggests an unaltered capacity of pre-B cells to mature, once they escape the early maturation block. Further, lack of PP development is associated with B cell deficiency (39, 40). We found no gross defect in T-cell maturation in the thymus, or aberrant T-cell populations in peripheral lymphoid organs. Moreover, T-cell-deficient mice develop normal PPs (41, 42). It therefore does not seem far-fetched to assume that the defective generation of B-
lymphocytes forms the basis of the phenotype. In concert, B cells isolated from peripheral lymphoid organs were able to mount a normal proliferative response upon B cell-specific stimulation, demonstrating that the effect of arginine deficiency on B cell development is restricted to the bone-marrow compartment.

B cells develop in the marrow from hemopoietic precursor cells, and can be divided in several differentiation stages that are characterized by surface phenotype (32, 43). Using the staining protocols of Hardy et al. (32), we found that the relative number of pro-B cells was normal in suckling F/A-2+/+ mice, although the absolute numbers were reduced due to the substantially smaller size of the long bones of the transgenic mice in comparison to the control animals. The population of pre-B cells was severely reduced. What can be the reasons for this developmental block? Early B cell progenitors require cell-to-cell contacts and growth factors, like c-kit ligand and IL7, for further development into pre-B cells (44). The transition of pro- to pre-B cells is marked by the successful completion of the rearrangement process at the heavy chain locus (45). This allows the formation of a premature B cell receptor (BCR) complex with the heavy chain and two surrogate light chains. The constitutive signal that is generated by the expression of this receptor and an IL-7-dependent signal collaborate in the induction of a rapid expansion of the pre-B cell pool (46). The study of mouse strains with induced mutations has revealed several factors that are necessary for the transition of the pro- to pre-B cell stage and the subsequent expansion of the pre-B cell pool. As could be expected, most factors influence the signal transduction pathways initiated by the premature BCR complex or by IL-7. Limited or no expansion of the pre-B cell pool is seen in mice lacking i) components of the BCR complex (e.g. the cytoplasmic domain of the Ig-α chain (47) or the Ig-β chain (48)(signal transduction effectors of the BCR), λ5 (49)(a component of the surrogate light chain of the premature BCR)), ii) components of the signal transduction pathway of the BCR (e.g. syk (50), BLNK (51), vav (52) and PI3K (53)(all signaling intermediates of the BCR)), iii) IL-7 (54) or components of the IL-7 receptor (e.g. IL-7Rα (46), the common γ-chain (55)), or Jak3 (56), and iv) transcriptional activators of components of the BCR or IL-7/IL-7
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receptor system (e.g. E2A (57), Pax-5 (58), EBF (59), Sox-4 (60), mel-18 (61) or C/EBP/β (62)).

Because we assume that an arginine-dependent metabolic mechanism involved in the transition of pro- to pre-B cells is hampered, we do not expect a fully penetrant phenotype, and therefore factors that are known to cause a complete block can be candidate targets as well. The few pre-B cells that escape the block, develop normally. Furthermore, peripheral B cells can be readily induced to differentiate or proliferate by a variety of stimuli mimicking T-cell-dependent and -independent activation. These observations make a B cell autologous defect (i.e. defects in the signaling pathway originating from the (premature) BCR) unlikely.

IL-7 plays a role in both the development of T and B cells. IL-7 deficiency (54, 63), or deletions of components of the IL-7 receptor (46, 54-56) cause a strong reduction in the cellularity of the thymus, with disturbances in the early precursor populations, but with a normal distribution of CD4- or CD8-double-negative, -double-positive, and -single-positive cells (63). Further, a severe deficiency of thymic and peripheral γδ T cells is found (64). We think that the reduced number of thymocytes found in the F/A-2+/+ mice is due to the smaller body size of these mice, but a final conclusion awaits a careful analysis of the precursor T-cell populations. Mice with deletions of transcriptional activators often display a broader phenotype (PAX-5 (58), Sox-4 (60), E2A (57), mel-18 (61)) than that seen in our transgenic mice. We therefore hypothesize that the disturbance in the development of B cells is either caused by a specific defect of genes expressed at the transition of pro- to pre-B cells (e.g. λ5 or V-pre-B, forming the surrogate light chain, or the IL-7 receptor α-chain), or by a bone marrow-specific defect of stroma cells that produce factors interacting with the IL-7 receptor α-chain, i.e IL-7 or thymic stromal lymphopoietin (65).

Mice that lack B cells, suffer from a deficient development of PPs, follicle-associated epithelium and M-cells (39). The near-absence of B cells in the F/A-2+/+ small intestine may therefore suffice to explain the rudimentary development of PPs in these mice. In agreement with this hypothesis, a normal number of VCAM-1-
positive PP anlagen was present in F/A-2^{+/+} intestine in the first postnatal week (18). The follicle-associated epithelial cells and the M-cells, that is, the specialized cells on the luminal surface of the PP dome that are involved in tunneling pathogens from the lumen into the basal surface, also develop under the influence of B-, and not T-lymphocytes (66). Accordingly, the follicle-associated epithelial domes on rudimentary PPs of F/A-2^{+/+} intestines were reduced in size and the density of M-cells in these domes was less (unpublished observations). However, the PP phenotype may also result from local interference of arginine deficiency with signal-transduction pathways. Recently, it has been reported that transgenic expression of IL-7 in the enterocytes of IL-7-knockout mice reestablished PP development (67), implying that IL-7 biosynthesis in arginine-deficient mice might not only be compromised in bone marrow, but also in enterocytes. VCAM-1-positive PP anlagen do not form in absolute IL-7 deficiency, but, as mentioned before, we do not expect a full penetrance of the deficiency in F/A-2^{+/+} neonates. At present, solving the mechanism underlying the deficient development of PPs in arginine-deficient mice therefore seems more complex than addressing the pre- to pro-B cells obstruction in the bone marrow.

Several reports have suggested that the amino acid arginine is a potential immunostimulant (35, 68), but the molecular and cellular mechanism via which arginine modulates lymphocyte biology and exerts its beneficial effect on mucosal defense, has remained obscure. Under normal conditions, arginine deficiency may not readily occur. However, under certain conditions of malnutrition, as they occur due to a poor diet or old age, a deficiency may occur. It is interesting in this regard, that at older age, bone marrow stromal cells have an impaired ability to support B cell poiesis, most likely due to an impaired release of IL-7 (69). The current study demonstrates an unambiguous effect of arginine deficiency on early B cell development. This finding should allow us to identify the site of action of arginine and to assess the prospects of arginine to become a bona fide immunonutrient.
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